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ROZPRAWA DOKTORSKA

Poprawa hydrolizy osadów ściekowych w celu intensyfikacji fermentacji metanowej

**Improving the hydrolysis of sewage sludge
to enhance anaerobic digestion**

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1. Wykaz publikacji wchodzących w skład dysertacji

Podstawą prezentowanej pracy doktorskiej jest seria publikacji tworzących jednotematyczny cykl prac dotyczących poprawy hydrolizy osadów ściekowych w celu intensyfikacji fermentacji metanowej. Treść rozprawy przygotowana została na podstawie publikacji naukowych cytowanych poniżej, zgodnie z prezentowaną kolejnością:

PUBLIKACJA 1: Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A., Sewage sludge pretreatment: current status and future prospects: Environmental Science and Pollution Research, 2023, 30, 88313-88330. IF₂₀₂₂= 5,8; MNiSW₂₀₂₂= 100

PUBLIKACJA 2: Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A., Cellulolytic Fungi from Sewage Sludge and Their Potential to Improve Methane Fermentation, Biomass Conversion and Biorefinery, <https://doi.org/10.1007/s13399-026-07082-1>. IF₂₀₂₆= 4,1; MNiSW₂₀₂₆= 70

PUBLIKACJA 3: Ćwierniewicz-Wojciechowska M., Wolny A., Jurczyk S., Cema G., Ziemińska-Buczyńska A., Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia, w trakcie recenzji

PUBLIKACJA 4: Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A., Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych: Współczesne problemy ochrony środowiska i energetyki, 2024, 101-110, Gliwice, Politechnika Śląska. ISBN 978-964116-6-2. MNiSW₂₀₂₄= 20

PUBLIKACJA 5: Ćwierniewicz-Wojciechowska M., Wolny A., Cema G., Ziemińska-Buczyńska A., Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and Microbial Community Effects, w trakcie recenzji

2. Alfabetyczny spis skrótów i akronimów

carb. – węgiel aktywny

CE – enzymy komercyjne

CMC – karboksymetyloceluloza

ChZT – chemiczne zapotrzebowanie na tlen (ang. Chemical Oxygen Demand, COD)

EPS – (ang. Extracellular Polymeric Substances) polimery zewnątrzkomórkowe

IE – enzymy izolowane

IM – enzymy immobilizowane z wykorzystaniem nieorganicznego nośnika, węgla aktywnego

LKT – lotne kwasy tłuszczowe (ang. Volatile Fatty Acids)

NGS – (ang. Next Generation Sequencing) Sekwencjonowanie Następnej Generacji

NIM – enzymy nieimmobilizowane

N-NH₄ – azot amonowy

SS – (ang. Sewage sludge) osad ściekowy

PN – białka

PS – węglowodany

TN – (ang. Total Nitrogen) azot całkowity

WWTP – (ang. Wastewater Treatment Plant) oczyszczalnia ścieków

WAS – (ang. Waste Activated Sludge) osad nadmierny

WKF – wydzielona komora fermentacyjna

Streszczenie

Oczyszczanie ścieków stanowi złożony proces technologiczny, którego celem jest usuwanie zanieczyszczeń fizycznych, chemicznych oraz biologicznych ze ścieków. Skuteczna realizacja tego procesu umożliwia bezpieczne odprowadzanie ścieków oczyszczonych do odbiorników wodnych lub do środowiska gruntowego, zgodnie z obowiązującymi wymaganiami legislacyjnymi.

W trakcie oczyszczania, obok ścieków oczyszczonych, powstają osady ściekowe, które ze względu na swój skład oraz ilość wymagają odpowiedniego ustabilizowania i racjonalnego zagospodarowania. Procesy związane z ich przetwarzaniem, obejmujące m.in. odwadnianie, higienizację, transport oraz unieszkodliwianie, generują istotne koszty eksploatacyjne dla oczyszczalni ścieków.

Ustabilizowane osady ściekowe mogą być wykorzystywane w różnych celach, takich jak nawożenie roślin nieprzeznaczonych do spożycia przez ludzi i zwierzęta, rekultywacja terenów zdegradowanych, kompostowanie, bądź składowanie. Do ich stabilizacji stosuje się metody biologiczne, chemiczne oraz termiczne, przy czym najczęściej wykorzystywane są metody biologiczne, w szczególności fermentacja metanowa. Należy jednak podkreślić, że efektywność fermentacji beztlenowej nie zawsze gwarantuje uzyskanie parametrów osadu zgodnych z wymaganiami określonymi w Obwieszczeniu Ministra Klimatu i Środowiska z dnia 18 listopada 2022 r.

W związku z powyższym coraz częściej stosuje się wstępną obróbkę osadów ściekowych przed procesem fermentacji. Zabieg ten intensyfikuje przebieg hydrolizy oraz całego procesu fermentacji, przyczyniając się do zwiększenia efektywności stabilizacji, poprawy jakości i ilości uzyskiwanego pofermentu, a także wpływa na wydajność i skład produkowanego biogazu.

Na efektywność stabilizacji osadów ściekowych istotny wpływ ma ich skład, w szczególności zawartość trudno biodegradowalnych substancji organicznych. Do tej grupy należy m.in. celuloza, czyli polisacharyd stanowiący podstawowy element strukturalny ścian komórkowych roślin, a zarazem jeden z najpowszechniej występujących polimerów w środowisku. Celuloza stanowi również istotny składnik materii organicznej dopływającej do systemów kanalizacyjnych, głównie na skutek wprowadzania do nich materiałów higienicznych. Jej obecność w ściekach oraz osadach ściekowych wpływa bezpośrednio na przebieg procesów technologicznych w oczyszczalniach oraz na efektywność gospodarki osadowej.

W związku z koniecznością efektywnego zagospodarowania osadów ściekowych oraz ograniczania kosztów eksploatacyjnych oczyszczalni ścieków, prowadzone są intensywne badania nad metodami umożliwiającymi jednoczesną intensyfikację procesów stabilizacji osadów ściekowych oraz optymalizację ekonomiczną tych działań.

Niniejsza praca doktorska przedstawia działania umożliwiające efektywną obróbkę osadów ściekowych, bez dodatkowego obciążania środowiska substancjami chemicznymi, poprzez wykorzystanie celulolitycznych mikroorganizmów bytujących w osadach ściekowych. Praca doktorska obejmuje pięć publikacji, jedną przeglądową i cztery badawcze, opisujące wpływ działania autochtonicznych celulolitycznych mikroorganizmów i produkowanych przez nie enzymów na efektywność degradacji celulozy i produkcji metanu. Realizowane badania poprzedzone zostały szczegółowym przeglądem literatury (**Publikacja 1**), w którym analizowane były metody wspomagania fermentacji metanowej pod kątem usprawnienia hydrolizy osadów i samej produkcji metanu. W przeglądzie przedstawiono wpływ chemicznej, fizycznej i biologicznej obróbki na hydrolizę osadów ściekowych i efektywność każdej z metod w odniesieniu do oznaczenia takich produktów hydrolizy, jak lotne kwasy tłuszczowe (LKT), chemiczne zapotrzebowanie na tlen (ChZT) i potencjał metanowy. Ponadto, opisane zostały inhibitory procesu i substancje, które w znaczący sposób wpływają na efektywność fermentacji metanowej. **Publikacja 1** wykazała w szczególności niewielką liczbę badań związanych z optymalizacją wykorzystania biologicznych metod obróbki osadów ściekowych jako metod intensyfikacji fermentacji metanowej i etapu hydrolizy, dając podstawy do postawienia hipotezy badań laboratoryjnych. Przegląd teoretyczny tematu pozwolił na wyodrębnienie słabo zbadanych metod obróbki wstępnej osadów ściekowych, określanych jako biologiczne, a w szczególności takich, w których udział miałyby mikroorganizmy autochtoniczne i produkowane przez nie enzymy. Przegląd literatury pozwolił również na zaplanowanie badań pod kątem wypełnienia luki badawczej, z uwzględnieniem potrzeb i możliwości przemysłu.

W **publikacji 2** opisana została izolacja grzybów celulolitycznych z osadu ściekowego. Wyizolowane zostały grzyby zdolne do wzrostu w warunkach tlenowych i beztlenowych, z założeniem możliwości wykorzystania ich na różnych etapach gospodarowania osadami ściekowymi. Wyizolowano 12 szczepów grzybów celulolitycznych. Siedem z nich było zdolnych do wzrostu w warunkach tlenowych i beztlenowych. Za pomocą sekwencjonowania Sangera regionu ITS1, zidentyfikowanych

zostało 5 szczepów. Wszystkie z wyizolowanych grzybów należały do rodzaju *Aspergillus*. Określono, że wyizolowane grzyby były zdolne do degradacji czystej celulozy. Na podstawie aktywności enzymatycznej i efektywności degradacji czystej celulozy, do testów degradacji celulozy w osadzie ściekowym i testu określającego potencjał metanowy wybrany został jeden szczep, oznaczony jako G3 (*Aspergillus terreus* S011).

Znacząca większość badań opisywanych w literaturze wykorzystuje obróbkę wstępną, prowadzoną przed komorą fermentacji metanowej. W badaniach opisanych w **Publikacji 2**, biologiczna obróbka zastosowana była jako forma wspomagania procesu prowadzonego już w trakcie testu na potencjał metanowy (AMPTS- ang. Automatic Methane Potential Test). Testowany osad ściekowy zaszczepiony został strzępkami wyizolowanego grzyba G3 (*Aspergillus terreus* S011). W badaniach wykorzystany został mikroorganizm zamiast samych produkowanych przez niego enzymów, zakładając intensyfikację obróbki i uproszczenie metody wstępnej obróbki osadów, w przypadku potencjału zastosowania w przemyśle. Wprowadzenie wyizolowanego podczas badań autochtonicznego grzyba celulolitycznego z rodzaju *Aspergillus* pozwoliło na uzyskanie wzrostu produkcji biogazu o 11% w odniesieniu do osadu ściekowego niepoddawanej obróbce. Dodatkowo, uzyskano 6% wzrost degradacji celulozy, w porównaniu do próbki kontrolnej. Grzyby uznaje się za najbardziej efektywne, spośród wszystkich mikroorganizmów celulolitycznych. W związku z tym, badania opisane w tej publikacji stanowiły punkt wyjścia dalszych prac badawczych, rozwiniętych w **Publikacjach 3, 4 i 5**.

Kolejne zaplanowane w ramach rozprawy doktorskiej badania zakładały wykorzystanie autochtonicznych bakterii celulolitycznych, wyizolowanych z osadu ściekowego, zwiększających szybkość hydrolizy osadów ściekowych. Podczas badań jednak izolacja czystych kultur bakteryjnych nie była możliwa. Niemożliwe było również przechowywanie wyizolowanych mikroorganizmów, jako że testowane metody zabezpieczania materiału prowadziły do utraty właściwości celulolitycznych wyizolowanych bakterii. Przetestowane zostało mrożenie w glicerynie, liofilizacja i pasażowanie na skosach przechowywanych w temperaturze 4°C. W każdym z analizowanych przypadków, wyizolowane bakterie traciły właściwości celulolityczne. Wyniki te wskazały kierunek badań w stronę mieszanej hodowli o potencjale celulolitycznym, czyli bakterii wyizolowanych z osadu ściekowego, które były zdolne do wzrostu i namnażania w syntetycznej pożywce, w której jedynym źródłem węgla była karboksymetyloceluloza. Mieszana kultura autochtonicznych bakterii o potencjale

celulolitycznym została scharakteryzowana pod kątem aktywności enzymatycznej FPA (ang. Filter Paper Assay) i CMC (ang. Carboxymethyl Cellulose) oraz zdolności do degradacji celulozy czystej i celulozy obecnej w osadzie ściekowym.

Badania porównawcze długotrwałego kontaktu enzymów z osadem przedstawione zostały w **Publikacji 3**, w której również weryfikowany był wpływ działania enzymatycznej obróbki osadów ściekowych na stopień degradacji celulozy i potencjału metanowego. Z mieszanej hodowli bakterii autochtonicznych o potencjale celulolitycznym wyizolowane zostały enzymy związane z powierzchnią komórek. Izolacja enzymów wykonana została za pomocą kombinacji metod termiczno-mechanicznych, selekcionowanych pod kątem łatwości ich wykorzystania w oczyszczalni ścieków. Celem zabezpieczenia i zwiększenia możliwości późniejszego użycia, wyizolowane enzymy zostały unieruchomione na nieorganicznym nośniku, jakim był węgiel aktywny. W **Publikacji 3** opisane zostało wykorzystanie enzymów produkowanych przez autochtoniczne bakterie celulolityczne. Podobnie jak w przypadku oddziaływania grzybów, oznaczona została aktywność enzymatyczna mieszanej hodowli bakterii celulolitycznych i efektywność degradacji czystej celulozy. Określony został również potencjał metanowy osadów ściekowych, inokulowanych izolowanymi enzymami, zarówno w formie immobilizowanej, jak i nieimmobilizowanej. Działanie enzymów izolowanych z uzyskanej w tej pracy hodowli zostało porównane z działaniem komercyjnej mieszanki enzymów celulolitycznych Cellic CTec2 (Novozymes, Dania). Na podstawie przeprowadzonych badań stwierdzono wzrost efektywności degradacji celulozy o 27% w próbkach inokulum i substratu (osadu fermentującego z dodatkiem świeżego osadu ściekowego) poddanych obróbce izolowanymi enzymami nieimmobilizowanymi oraz o 66% w przypadku osadu fermentowanego, niezasilanego świeżą partią osadu nadmiernego i wstępnego. Dodatkowo, uzyskano intensyfikację produkcji metanu o 21,7% w przypadku osadów inokulowanych izolowanymi enzymami nieimmobilizowanymi i o 18,9% w przypadku osadów inokulowanych izolowanymi enzymami immobilizowanymi. W przypadku enzymów komercyjnych obserwowano wzrost produkcji metanu o 42,5% i 27%, odpowiednio dla enzymów nieimmobilizowanych i immobilizowanych.

W **Publikacji 4** przedstawiono krótkotrwały wpływ działania obróbki enzymatycznej z wykorzystaniem enzymów izolowanych z pozyskanej hodowli bakterii i komercyjnych enzymów, nieimmobilizowanych i immobilizowanych na nieorganicznym nośniku, jakim był węgiel aktywny. W trakcie testu opisanego w **Publikacji 4** weryfikowany był wpływ dozowania enzymów izolowanych i komercyjnych na stężenie lotnych kwasów

tłuszczowych (LKT), chemicznego zapotrzebowania na tlen (ChZT) i polimerów zewnątrzkomórkowych (EPS, ang. Extracellular Polymeric Substances). Przeprowadzony test pozwolił na zaobserwowanie zmian stężenia oznaczanych parametrów w pierwszych godzinach od inokulacji osadu enzymami. W próbkach osadu ściekowego, inokulowanych izolowanymi nieimmobilizowanymi i immobilizowanymi enzymami uzyskano wzrost produkcji LKT odpowiednio o 25% i 128%. Zaobserwowano również wzrost stężenia ChZT o 272% i 73%. W przypadku inokulacji osadów ściekowych komercyjnymi enzymami nieimmobilizowanymi i immobilizowanymi, zaobserwowano wzrost stężenia LKT o 1856% i 175%. Stężenie ChZT całkowite w próbkach inokulowanych komercyjnymi nieimmobilizowanymi i immobilizowanymi enzymami wzrosło odpowiednio o 1792% i 670%.

W **Publikacji 5** przedstawiono wpływ działania inokulacji osadów ściekowych izolowanymi i komercyjnymi enzymami immobilizowanymi i nieimmobilizowanymi na parametry fizykochemiczne osadu ściekowego podczas 15-dniowego testu określającego potencjał metanowy badanych osadów. Dodatkowo, weryfikowany był również wpływ dodatku enzymatycznej obróbki osadów ściekowych na zbiorowisko mikroorganizmów bytujących w osadzie. Na podstawie przeprowadzonych badań określono, że wybrany rodzaj obróbki i zastosowane stężenie enzymów nie wpływa w znaczącym statystycznie stopniu na skład jakościowo-ilościowy biocenozy mikroorganizmów osadu ściekowego. Porównanie wyników fizykochemicznych opisanych w **Publikacji 4** i **Publikacji 5** pozwoliło na zaobserwowanie, że wykorzystanie enzymów izolowanych i komercyjnych, zarówno immobilizowanych, jak i nieimmobilizowanych pozwala na uzyskanie takich samych parametrów osadu i ilości produkowanego metanu w krótszym czasie, niż w przypadku osadów niepoddawanych obróbce. W **Publikacji 5** dodatkowo określono korelacje pomiędzy parametrami fizykochemicznymi a obfitością występowania najbardziej licznych gatunków bakterii, wskazując na silne zależności pomiędzy występowaniem poszczególnych mikroorganizmów i poziomem badanych parametrów. Jednocześnie wykazano, że unieruchomienie enzymów miało większe znaczenie, niż rodzaj zastosowanego enzymu. W sytuacji, kiedy obserwowano negatywną korelację przy wykorzystaniu enzymów nieimmobilizowanych, zastosowanie ich immobilizowanych odpowiedników wpływało na wystąpienie pozytywnej korelacji pomiędzy obfitością występowania danego gatunku i oznaczanego parametru.

Abstract

Wastewater treatment is a complex technological process aimed at removing physical, chemical, and biological contaminants from wastewater. Effective implementation of this process enables the safe discharge of treated wastewater into water bodies or the ground, in accordance with applicable environmental requirements. During treatment, in addition to treated wastewater, sewage sludge is produced, which, due to its composition and volume, requires appropriate and rational management. The processes involved in their treatment, including dewatering, sanitization, transport, and disposal, generate significant operating costs for wastewater treatment plants.

Stabilized sewage sludge can be used for various purposes, such as fertilizing plants not intended for human or animal consumption, the reclamation of degraded land, composting, or landfilling. Biological, chemical, and thermal methods are used for its stabilization,

with biological methods- particularly anaerobic digestion- being the most commonly employed. It should be emphasized, however, that the effectiveness of anaerobic fermentation does not always guarantee that the sludge parameters will meet the requirements specified in the Announcement of the Minister of Climate and Environment dated November 18, 2022. Consequently, pretreatment of sewage sludge prior to the fermentation process is increasingly being employed. This treatment intensifies the course of hydrolysis and the entire fermentation process, contributing to increased stabilization efficiency, improved quality and quantity of the resulting digestate, and also affects the yield and composition of the biogas produced. The effectiveness of sewage sludge stabilization is significantly influenced by its composition, particularly the content of poorly biodegradable organic substances.

This group includes, among others, cellulose, a polysaccharide that is the primary structural component of plant cell walls and one of the most common polymers in the environment. Cellulose is also a significant component of the organic matter entering sewer systems, mainly due to the use of hygiene products by humans. Its presence in wastewater and sewage sludge directly affects the course of technological processes in treatment plants and the efficiency of sludge management. Given the need for effective sewage sludge management and the reduction of operating costs, intensive research is being conducted on methods that enable both the intensification of stabilization processes and the economic optimization of these activities.

The following doctoral dissertation presents methods for the effective treatment of sewage sludge without additional environmental burden from chemicals, through the use of cellulolytic microorganisms present in sewage sludge. The doctoral dissertation comprises five publications- one review and four research papers, describing the impact of autochthonous cellulolytic microorganisms and the enzymes they produce on the efficiency of cellulose degradation and methane production. The research was preceded by a detailed literature review (**Publication 1**), which analyzed methods for enhancing methanogenic fermentation with a view to improving sludge hydrolysis and methane production itself. The review presented the impact of chemical, physical, and biological treatment on sewage sludge hydrolysis and the effectiveness of each method in terms of the determination of hydrolysis products such as volatile fatty acids (VFA), chemical oxygen demand (COD), and methane potential. Furthermore, inhibitors of the process and substances that significantly affect the efficiency of methanogenic fermentation are described. **Publication 1** highlighted, in particular, the limited number of studies related to optimizing the use of biological sewage sludge treatment methods as means to intensify methanogenic fermentation and the hydrolysis stage. A theoretical review of the topic allowed for the identification of poorly studied methods of preliminary sewage sludge treatment, referred to as biological, and in particular those involving autochthonous microorganisms and the enzymes they produce. The literature review also enabled the planning of research aimed at filling this research gap, taking into account the needs and capabilities of the industry.

Publication 2 describes the isolation of cellulolytic fungi from sewage sludge. Fungi capable of growing under aerobic and anaerobic conditions were isolated, with the aim of utilizing them at various stages of sewage sludge management. Twelve strains of cellulolytic fungi were isolated. Seven of them were capable of growing under aerobic and anaerobic conditions. Five strains were identified using Sanger sequencing of the ITS1 region. All of the isolated fungi belonged to the genus *Aspergillus*. It was determined that the isolated fungi were capable of degrading pure cellulose. Based on enzymatic activity and the efficiency of pure cellulose degradation, one strain, designated G3 (*Aspergillus terreus* S011), was selected for cellulose degradation tests in sewage sludge and for the methane potential test.

The vast majority of studies described in the literature utilize pretreatment conducted prior to the methane fermentation chamber. In the studies described in **Publication 2**, biological treatment was applied as a form of support for the process already underway

during the Automatic Methane Potential Test (AMPTS). The tested sewage sludge was inoculated with mycelium of the isolated fungus G3 (*Aspergillus terreus* S011). The study utilized the microorganism itself rather than the enzymes it produces, with the aim of intensifying the treatment and simplifying the sludge pretreatment method, in view of its potential industrial application. The introduction of an autochthonous cellulolytic fungus of the genus *Aspergillus*, isolated during the study, resulted in an 11% increase in biogas production compared to untreated sewage sludge. Additionally, a 6% increase in cellulose degradation was observed compared to the control sample. Fungi are considered the most effective of all cellulolytic microorganisms. Consequently, the research described in this publication served as the starting point for further studies, which were expanded upon in **Publications 3, 4, and 5**.

Further research planned as part of the doctoral dissertation involved the use of indigenous cellulolytic bacteria, isolated from sewage sludge, to increase the rate of sewage sludge hydrolysis. However, during the research, it was not possible to isolate pure bacterial cultures. It was also impossible to store the isolated microorganisms, as the tested methods led to the loss of the cellulolytic properties of the isolated bacteria. Freezing in glycerin, freeze-drying, and slant inoculation were tested. In each of the tested cases, the isolated bacteria lost their cellulolytic properties. These results pointed the research toward mixed cultures with cellulolytic potential, i.e., bacteria isolated from sewage sludge that were capable of growing and multiplying in a synthetic medium where the sole carbon source was carboxymethylcellulose. A mixed culture of indigenous bacteria with cellulolytic potential was characterized in terms of enzymatic activity using the Filter Paper Assay (FPA) and carboxymethyl cellulose (CMC), as well as their ability to degrade pure cellulose and cellulose present in sewage sludge.

Comparative studies of long-term enzyme contact with sludge are presented in **Publication 3**, which also examined the effect of enzymatic treatment of sewage sludge on the degree of cellulose degradation and methane potential.

Bound enzymes were isolated from a mixed culture of indigenous bacteria with cellulolytic potential. Enzyme isolation was performed using thermomechanical methods that could be easily implemented in a wastewater treatment plant. To preserve and enhance their usability, the isolated enzymes were immobilized on an inorganic carrier- activated carbon. **Publication 3** describes the use of enzymes produced by indigenous cellulolytic bacteria.

As in the case of fungal activity, the enzymatic activity of a mixed culture of cellulolytic bacteria and the degradation efficiency of pure cellulose were determined.

The methane potential of sludge inoculated with isolated enzymes was also determined, both in immobilized and non-immobilized forms. The performance of the isolated enzymes was compared with that of the commercial cellulolytic enzyme mixture Cellic CTec2 (Novozymes, Denmark).

Based on the conducted studies, a 27% increase in cellulose degradation was observed in inoculum and substrate samples (fermenting sludge supplemented with fresh sludge) treated with isolated non-immobilized enzymes, and a 66% increase in the case of fermented sludge not fed with a fresh batch of excess and primary sludge. Additionally, methane production was intensified by 21.7% in sludges inoculated with isolated non-immobilized enzymes and by 18.9% in sludges inoculated with isolated immobilized enzymes. For commercial enzymes, an increase in methane production of 42.5% and 27% was observed for non-immobilized and immobilized enzymes, respectively.

Publication 4 presents the short-term effects of enzymatic treatment using isolated and commercial enzymes, both non-immobilized and immobilized on an inorganic carrier, namely activated carbon. During the test described in **Publication 4**, the effect of dosing isolated and commercial enzymes on the concentration of volatile fatty acids, chemical oxygen demand (COD), and extracellular polymeric substances (EPS) was evaluated. The test allowed for the observation of changes in the concentrations of the measured parameters during the first hours following the inoculation of the sludge with enzymes.

In sewage sludge samples inoculated with isolated non-immobilized and immobilized enzymes, an increase in VFA production of 25% and 128%, respectively, was observed. An increase in COD concentration of 272% and 73% was also observed. In the case of inoculation of sludge with commercial non-immobilized and immobilized enzymes, an increase in VFA concentration of 1856% and 175% was observed. The total COD concentration in samples inoculated with commercial non-immobilized and immobilized enzymes increased by 1,792% and 670%, respectively.

Publication 5 presents the effect of inoculating sewage sludge with isolated and commercial immobilized and non-immobilized enzymes on the physicochemical parameters of sewage sludge during a 15-day test determining the methane potential of the tested sludges. Additionally, the effect of enzymatic treatment of sewage sludge on the microbial community residing in the sludge was also verified. Based on the conducted

studies, it was determined that the selected type of treatment and the applied enzyme concentration do not significantly affect the composition and microbial community of the sewage sludge. A comparison of the physicochemical results described in **Publication 4** and **Publication 5** revealed that the use of isolated and commercial enzymes, both immobilized and non-immobilized, allows for the same sludge parameters and methane production rates to be achieved in a shorter time than in the case of untreated sludge. **Publication 5** further identified correlations between physicochemical parameters and the abundance of the most common bacterial species, indicating strong relationships between the presence of specific microorganisms and the concentration of the parameters studied. At the same time, it was demonstrated that enzyme immobilization was more significant than the type of enzyme used. In situations where a negative correlation was observed with non-immobilized enzymes, the use of their immobilized counterparts resulted in a positive correlation between the abundance of a given species and the measured parameter.

3. Wprowadzenie

Oczyszczanie ścieków prowadzi do powstawania osadów ściekowych, które stanowią około 1 - 3% całkowitej objętości ścieków dopływających do oczyszczalni. Do 2023 roku według danych Głównego Urzędu Statystycznego ilość powstających osadów ściekowych rosła z roku na rok. W 2023 roku tendencja wzrostowa zatrzymała się i zaobserwowano spadek ilości produkowanych osadów o około 4% w stosunku do roku poprzedniego (Wojciechowska i wsp., 2023). Wzrost w poprzednich latach związany był bezpośrednio ze stale rosnącą urbanizacją i rozbudową systemów kanalizacyjnych, wpływającymi na zwiększającą się ilość ścieków dopływających do oczyszczalni komunalnych (Ding i wsp., 2021). Stabilizacja ilości produkowanych osadów ściekowych po 2023 roku wynika ze wzrostu liczby oczyszczalni ścieków w Polsce, dysponujących infrastrukturą pozwalającą na termiczną przeróbkę powstałych osadów ściekowych. Obecnie 11 oczyszczalni w Polsce posiada instalację do termicznej obróbki osadów, a wydajność ich działania pozwala na spalanie około 25% masy powstających osadów.

Skład osadów, efektywność ich obróbki oraz możliwość i sposób późniejszego ich wykorzystania związany jest bezpośrednio ze składem ścieków dopływających do oczyszczalni. Ponadto wiąże się również z zastosowaną technologią oczyszczania ścieków stanem technicznym urządzeń oraz uprzemysłowienia obszaru (Pilarska i wsp., 2024). Na generowaną ilość ustabilizowanych osadów ściekowych wpływa: 1) ilość ścieków przyjmowanych przez oczyszczalnię, 2) stosowane technologie, 3) skład ścieków. Zarówno ilość, jak i jakość osadów ściekowych determinują bezpośrednio koszty, jakie oczyszczalnie muszą ponosić w związku z koniecznością ich zagospodarowania.

Osady ściekowe stanowią problem ze względu na konieczność ich obróbki, mającej na celu ich stabilizację oraz zmniejszenie objętości. W tym celu, poddaje się je takim procesom jak fermentacja beztlenowa, odwadnianie, suszenie czy spalanie (Cieślik i wsp., 2015). Osady ściekowe są siedliskiem wielu mikroorganizmów patogennych, przez co niewłaściwe zarządzanie może prowadzić do uwalniania odorów, rozprzestrzeniania zanieczyszczeń i chorób (Przydatek i Wota, 2020).

Do stabilizacji osadów ściekowych wykorzystywane są metody biologiczne (np. fermentacja metanowa, kompostowanie), chemiczne oraz termiczne (np. spalanie, piroliza) (Hoang i wsp., 2022). Zarówno fermentacja beztlenowa, jak i procesy termicznej obróbki mogą być wykorzystywane do produkcji energii elektrycznej i ciepłej,

ale różni je produkt powstający po procesie stabilizacji (Cao i Pawłowski, 2012). W Polsce najczęściej stosowanym sposobem stabilizacji jest fermentacja metanowa (Królik i wsp., 2019). Jest to proces, podczas którego oprócz ustabilizowanych osadów powstaje również biogaz. Fermentacja metanowa jest procesem mikrobiologicznym, podczas którego degradowane są wielkocząsteczkowe związki organiczne, do prostych związków rozpuszczalnych w wodzie, z wydzieleniem metanu, dwutlenku węgla i śladowych ilości innych gazów, takich jak wodór, i siarkowodór. Pozostały po fermentacji osad może być wykorzystany na przykład rolniczo jako nawóz dla roślin nieprzeznaczonych do spożycia przez ludzi i zwierzęta (Ziemiński i Frąć, 2012). Proces prowadzony jest w czterech równocześnie zachodzących etapach, czyli hydrolizie, kwasogenezie, octanogenezie i metanogenezie. Hydroliza jest etapem najdłuższym i najważniejszym dla kolejnych etapów fermentacji. Podczas etapu hydrolizy rozkładane są wielkocząsteczkowe związki organiczne, takie jak białka, węglowodany i tłuszcze, odpowiednio do aminokwasów, cukrów prostych i długocząsteczkowych kwasów tłuszczowych. Powstałe związki rozkładane są dalej do lotnych kwasów tłuszczowych i alkoholi, które w octanogenezie zostają przekształcone do octanów. W ostatnim etapie mikroorganizmy metanogenne wykorzystują produkty octanogenezy do produkcji metanu (Miyamoto i wsp., 1997).

Jak wcześniej wspomniano, na efektywność stabilizacji osadów ściekowych wpływa m.in. ich skład, a dokładniej ilość trudno rozkładalnych substancji organicznych. Efektywna ich degradacja podczas etapu hydrolizy wpływa na poprawę wydajności produkcji metanu, odwadniania, zmniejszenia ilości substancji stałych czy eliminację patogenów. W konsekwencji determinuje to możliwość dalszego zagospodarowania osadów (European Commission, Wood i wsp., 2022). Jednym z głównych związków występujących w osadach ściekowych, istotnie wpływających na efektywność fermentacji metanowej jest celuloza, czyli trudno rozkładalny polimer (Liu i Smith, 2022). Celuloza w osadach ściekowych pochodzi głównie z papierów toaletowych i innych artykułów higienicznych (Khan i wsp., 2022). Sam papier toaletowy stanowi około 25 - 30% całkowitego ładunku ChZT w dopływie (Ruiken i wsp., 2013). Są to jednak szacunkowe założenia, ponieważ jego zawartość w ściekach zależy od stopnia urbanizacji terenu czy uprzemysłowienia. W USA jedna osoba wprowadza do ścieków 18 ± 8 kg papieru toaletowego w ciągu roku. W Europie te wartości szacuje się na 11 ± 7 kg/rok (Palmieri i wsp., 2019). Ze względu na trudność w rozkładzie osadów, jak i generowane

ich ilości, sugeruje się, aby obróbka osadów prowadzona była z uwzględnieniem obecności celulozy (Reijken i wsp., 2018).

Celuloza obecna w osadach ściekowych może być zagospodarowana na dwa sposoby: odzyskiwana lub degradowana (Čater i wsp., 2014; Wiśniowska i Kowalczyk, 2022; Liu i wsp., 2022; Djordjevićová i wsp., 2024). W pierwszym przypadku konieczne jest dostosowanie infrastruktury oczyszczalni ścieków do jak najbardziej efektywnego odzysku, co wiąże się z nakładami finansowymi, zarówno pod kątem inwestycyjnym, jak i eksploatacyjnym (Palmieri i wsp., 2019). Intensyfikacja degradacji celulozy może być dla oczyszczalni ścieków bardziej opłacalna w dłuższej perspektywie, ze względu na to, że związek ten stanowi potencjalne źródło węgla do produkcji biogazu (Kang i wsp., 2014). To jednak determinuje konieczność wprowadzenia metod dodatkowej obróbki osadów ściekowych, która pozwoli na jak najbardziej efektywną degradację celulozy do prostszych związków organicznych.

W zależności od możliwości i potrzeb przedsiębiorstwa, oczyszczalnie ścieków wykorzystują różne metody obróbki wstępnej osadów ściekowych, rozumianych jako wspomaganie procesu. Zalicza się do nich mechaniczne, chemiczne i biologiczne metody (Khanh Nguyen i wsp., 2021; Mitraka i wsp., 2022; Waseem i wsp., 2025). W oczyszczalniach ścieków najczęściej wykorzystywane są metody mechaniczne, czyli na przykład ściskanie, rozdrabnianie, odwadnianie. Wynika to z wysokiej efektywności, łatwości zastosowania i niskiej szkodliwości w odniesieniu do środowiska. Wykorzystanie tych metod posiada niestety również wady, do których zalicza się wysokie koszty inwestycyjne i później eksploatacyjne, czy przygotowanie odpowiednio dużej przestrzeni w zakładzie na implementację urządzeń (Carrere i wsp., 2016). Rzadziej, jednak wciąż efektywnie wykorzystywane są metody chemiczne lub termochemiczne. Te zakładają stosowanie kwasów, zasad lub cieczy jonowych do degradacji wielkocząsteczkowych związków organicznych w podwyższonej temperaturze (Li i wsp., 2010; Dai i wsp., 2018; Haykir i wsp., 2023). Efektywność działania tych metod uznawana jest za zadowalającą, jednak koszty, niszczący wpływ na urządzenia i szkodliwy wpływ na środowisko oraz mikroorganizmy pracujące w oczyszczalni, przewyższają zalety takiego rozwiązania (Neumann i wsp., 2016). Ostatnia grupa metod służących do obróbki osadów ściekowych to metody biologiczne. Wykorzystanie tych metod stanowi szczególnie korzystne rozwiązanie z punktu widzenia ekologii i efektywności procesowej. Mikroorganizmy biorące udział w fermentacji osadów ściekowych są mikroorganizmami autochtonicznymi, czyli należącymi do tego środowiska,

permanentnie kolonizującymi daną niszę. Naturalnie adaptują się do danego środowiska i odgrywają kluczowe role w procesach prowadzonych w miejscu występowania (Dhall i wsp., 2012). Ich naturalna zdolność do funkcjonowania w trudnym środowisku, jakim są osady ściekowe, stanowi potencjał do wykorzystania ich celem intensyfikacji już prowadzonych przez nie procesów. Z drugiej jednak strony, wprowadzenie biologicznej obróbki osadów ściekowych do skali półprzemysłowej lub przemysłowej może stanowić trudność (Zhen i wsp., 2017). Wynika to z długiego czasu obróbki i kosztów związanych z hodowlą mikroorganizmów lub pozyskaniem ich enzymów. Każda z wymienionych powyżej metod może być stosowana samodzielnie lub w połączeniu z inną metodą, co może zarówno poprawiać jak i limitować obróbkę osadów ściekowych.

Niezwykle ważną kwestią podczas wyboru metod obróbki osadów ściekowych do fermentacji metanowej jest również ich wpływ na społeczność mikroorganizmów. Jak wspomniano, fermentacja metanowa jest procesem mikrobiologicznym, prowadzonym przez zbiorowisko mikroorganizmów, funkcjonujących w nim w równowadze. Równowaga ta może zostać łatwo zakłócona zaburzając tym samym prowadzone przez nie procesy (Vitéz i wsp., 2020). To skutkować może obniżoną efektywnością któregośkolwiek z etapów fermentacji metanowej, wpływając tym samym na ilość i jakość produkowanego biogazu lub spowodować całkowitą inhibicję metanogenezy (Yang i wsp., 2019; Yin i wsp., 2020).

Działania prowadzone w ramach opisywanej rozprawy doktorskiej zakładają wykorzystanie biologicznych metod obróbki osadów ściekowych, z pominięciem etapu wstępnej obróbki, który w praktyce może trwać nawet kilka tygodni. Zamiast tego, weryfikowany był wpływ obróbki osadu z wykorzystaniem mikroorganizmów celulolitycznych i produkowanych przez nie enzymów na osad pobrany z wydzielonych komór fermentacyjnych (WKF), podczas procesu fermentacji metanowej.

Mikroorganizmy celulolityczne przyczyniają się nie tylko do degradacji celulozy, ale często biorą również udział w degradacji kompleksu lignocelulozowego (Nugraha i wsp., 2021). Enzymatyczna obróbka osadów ściekowych z wykorzystaniem celulaz wpływa pozytywnie na zwiększenie rozpuszczalności osadów, obniżenie zawartości suchej masy organicznej, a w konsekwencji na zwiększenie objętości produkowanego biogazu (Rashed i wsp., 2010; Agabo-García i wsp., 2019; Wang i wsp., 2020). Ze względu jednak na niską stabilność, krótki okres przechowywania i wysoką wrażliwość na warunki procesowe, wykorzystanie celulaz w przemyśle jest ograniczone. Determinuje to konieczność zabezpieczenia enzymów, pozwalając na zachowanie

wyciekaniu enzymu do środowiska (Imam i wsp., 2017). Uwzględniając konieczność ograniczenia kosztów i stosowania jak najprostszej metody, dobrym rozwiązaniem może być immobilizacja odwracalna, jaką jest adsorpcja. Ten rodzaj immobilizacji wykorzystuje interakcje jonowe pomiędzy enzymami i nośnikiem (Mohamad i wsp., 2015). Adsorpcja celulaz na nośniku pozwala na zwiększenie ich stabilności, wydłużenie czasu przechowywania w temperaturze 4°C, czy możliwość wielokrotnego wykorzystania (Sun i wsp., 2024; Hadadi i Habibi, 2025). Możliwa jest adsorpcja celulaz na różnego rodzaju nośnikach. Ponownie jednak biorąc pod uwagę koszty, łatwość, a dodatkowo możliwość wykorzystania odpadu jako nośnik, na uwagę zasługuje węgiel aktywny. Immobilizacja celulaz na węglu aktywnym pozwala na zwiększenie stabilności i możliwości ponownego wykorzystania enzymów w procesach przemysłowych. Węgiel aktywny posiada dużą powierzchnię, wysoką porowatość i jest stabilny chemicznie (Olkiewicz i wsp., 2025). Dodatkowo, sam węgiel aktywny może być pozyskany z materiałów poddanych pirolizie (Li i wsp., 2020; Bolan i wsp., 2023; Winarsa i wsp., 2024).

Zastosowanie węgla aktywnego do intensyfikacji degradacji celulozy podczas fermentacji metanowej może mieć dodatkowe zastosowanie. W przypadku, kiedy immobilizacja enzymu na nośniku nie jest na tyle efektywna, aby osadzić go na całej powierzchni nośnika, wolne miejsca mogą odgrywać dodatkową rolę w samym procesie. Obecność węgla aktywnego w procesie fermentacji metanowej wpływa na poprawę funkcjonowania zbiorowiska mikroorganizmów, w szczególności metanogenów, co prowadzi do zwiększenia objętości produkowanego biogazu (Yang i wsp., 2017; Wu i wsp., 2022).

Przedstawione zagadnienia stanowiły podstawę badań realizowanych w ramach niniejszej rozprawy doktorskiej. W wyniku realizacji pracy doktorskiej powstała praca przeglądowa (**Publikacja 1**) oraz 4 publikacje opisujące wyniki badań (**Publikacja 2, Publikacja 3, Publikacja 4, Publikacja 5**). Poniżej przedstawione zostały szczegółowe cele, których realizacja umożliwiła odpowiedź na hipotezę badawczą postawioną w ramach prezentowanej rozprawy doktorskiej. Ponadto, poniżej przedstawione zostały również najważniejsze osiągnięcia, opublikowane w wyżej wspomnianych publikacjach.

4. Cel pracy

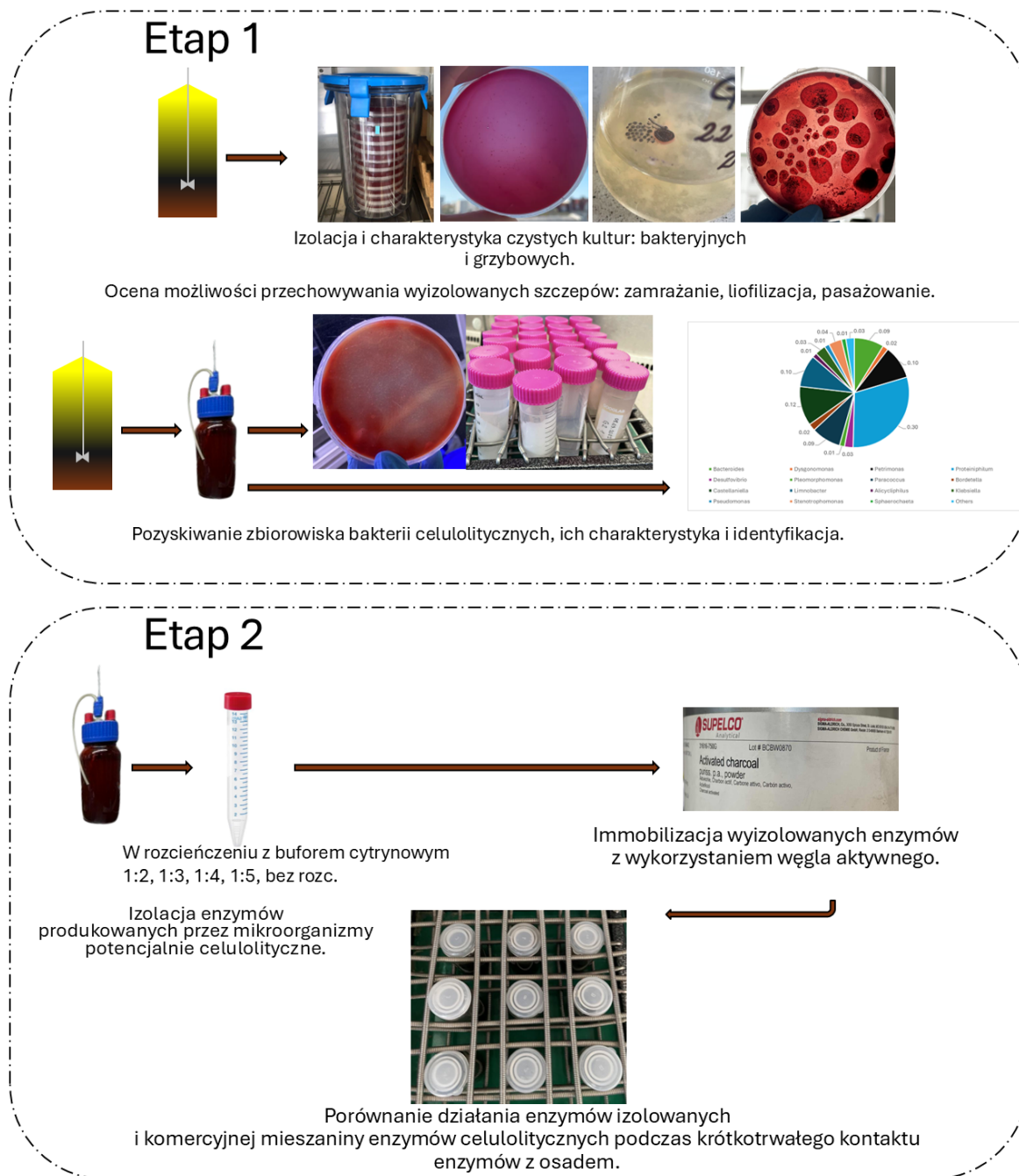
Celem pracy doktorskiej była ocena możliwości wykorzystania autochtonicznych mikroorganizmów celulolitycznych jako biologicznej metody obróbki wstępnej osadów ściekowych. Zastosowanie tej metody ukierunkowane było na intensyfikację degradacji celulozy oraz zwiększenie produkcji biogazu w procesie fermentacji metanowej.

W ramach pracy zrealizowano następujące cele cząstkowe:

- Izolacja celulolitycznych mikroorganizmów, zdolnych do efektywnej degradacji celulozy różnopostaciowej, w warunkach beztlenowych i temperaturze 37°C.
- Ocena właściwości celulolitycznych wyizolowanych mikroorganizmów.
- Ocena możliwości przechowywania i ponownego wykorzystania wyizolowanych mikroorganizmów.
- Ocena wpływu inokulacji osadów ściekowych za pomocą autochtonicznych mikroorganizmów celulolitycznych i/lub produkowanych przez nie enzymów na produkcję metanu.
- Ocena wpływu obróbki osadów ściekowych wybranej metody biologicznej na parametry fizykochemiczne i stężenie EPS w badanym osadzie: porównanie krótko- i długotrwałego kontaktu.
- Określenie wpływu obróbki osadów ściekowych za pomocą wybranej metody biologicznej na zbiorowisko mikroorganizmów, obecne w badanym osadzie.

5. Metodyka badań

Sposób realizacji celów cząstkowych oraz kolejność prowadzonych badań przedstawiono na rysunku 2.



Etap 3



Wprowadzenie enzymów immobilizowanych i nieimmobilizowanych do osadu w warunkach laboratoryjnych.



Testowanie wpływu działania enzymów na intensyfikację potencjału metanowego osadu i degradacji celulozy.

Rysunek 2 Schemat przedstawiający podejmowane kroki, umożliwiające realizację celów cząstkowych, założonych w ramach rozprawy doktorskiej.

5.1.Publikacja 1: Sewage sludge pretreatment: current status and future prospects.

Przed rozpoczęciem badań opracowano przegląd literatury, dotyczący metod obróbki wstępnej osadów ściekowych, ze szczególnym uwzględnieniem ich wpływu na hydrolizę związków wielkocząsteczkowych oraz na produkcję metanu. W przeglądzie, przedstawiona została również efektywność poszczególnych metod, z uwzględnieniem stężenia lotnych kwasów tłuszczowych (LKT), chemicznego zapotrzebowania na tlen (ChZT)

oraz potencjału metanowego. Na podstawie przeglądu literatury, podjęta została decyzja o prowadzeniu badań w kierunku rozwoju biologicznych metod obróbki osadów ściekowych, z wykorzystaniem autochtonicznych mikroorganizmów celulolitycznych jako potencjalnej bioszczepionki do intensyfikacji hydrolizy i produkcji metanu z osadów ściekowych.

5.2.Publikacja 2: Cellulolytic Fungi from Sewage Sludge and Their Potential to Improve Methane Fermentation

Badania opisane w tej publikacji dotyczą izolacji, identyfikacji i charakterystyki autochtonicznych celulolitycznych grzybów mikroskopowych. Izolacja prowadzona była w temperaturze 37°C, w warunkach beztlenowych, z wykorzystaniem podłoży PDA (Potato Dextrose Agar, BTL Polska) i MEA (Malt Extract Agar, BTL Polska) z dodatkiem chloramfenikolu (30 mg/L). Izolacje grzybów prowadzono w warunkach

beztlenowych w komorze do posiewów beztlenowych Bactronez-2 (Sheldon Manufacturing, USA). Czyste kultury przeniesione zostały na podłoże CMC z dodatkiem czerwieni Kongo jako wskaźnika degradacji celulozy ((CMC 5 g/L, NaNO₃ 1 g/L, K₂HPO₄ 1 g/L, KCl 1 g/L, MgSO₄ 0.5 g/L, ekstrakt drożdżowy 0.5 mg/L, glukoza 1 g/L, agar 17 g/L, czerwień Kongo 1g/L). Wyizolowane grzyby celulolityczne testowane były następnie pod kątem zdolności degradacji celulozy w warunkach tlenowych i beztlenowych. Zdolność degradacji celulozy w różnych warunkach, nadawałaby wyizolowanym mikroorganizmom bardziej uniwersalny charakter. Obróbka wstępna osadów ściekowych mogłaby być wtedy prowadzona zarówno przed, jak i w trakcie fermentacji metanowej. Z grupy 12 wyizolowanych szczepów celulolitycznych 7 zdolnych było do wzrostu i degradacji celulozy zarówno w warunkach tlenowych, jak i beztlenowych w hodowli na podłożu stałym, jak i w hodowli płynnej. Ponadto, 5 z 7 szczepów udało się zidentyfikować za pomocą sekwencjonowania Sangera. Izolację DNA prowadzono z wykorzystaniem zestawu Genomic Mini Yeast Kit (A&A Biotechnology, Polska). Do amplifikacji regionu ITS (ang. Internal Transcribed Spacer) w reakcji PCR (ang. Polymerase Chain Reaction) wykorzystano startery ITS1F- TCCGTAGGTGAACCTGCGG i ITS4R- TCCTCCGCTTATTGATATGC dla fragmentów o długości ok. 550-700 par zasad, w zależności od gatunku grzyba (Zarrin i wsp., 2016). Reakcja PCR prowadzona była w 35 cyklach w termocyklerze BioRad C-1000 (BioRad, USA), w warunkach dobranych na podstawie literatury:

- wstępna denaturacja 95°C, 8 minut,
- denaturacja 95°C, 30 sekund,
- annealing 60°C 40 sekund,
- wydłużanie 72°C 40 sekund,
- końcowe wydłużanie 72°C, 5 minut.

Do testu degradacji czystej celulozy, wyizolowane grzyby hodowane były w płynnym podłożu z dodatkiem karboksymetylocelulozy przez 7 dni w temperaturze 37°C. Następnie, hodowlę odwirowano w 4400 rpm, 40 min, odpłukano i uzupełniono mineralnym podłożem Kojima (Na₂HPO₄ * 12 H₂O 3,78 g/L, KH₂PO₄ 0,5 g/L, NH₄Cl 5,0 g/L, MgSO₄ * 7 H₂O 0,2 g/L, ekstrakt drożdżowy 0.01 g/L) i inkubowano z 0,5 g filtra celulozowego stanowiącego źródło węgla (Whatman, Sigma Aldrich, USA). Warunki beztlenowe zostały zapewnione poprzez wypieranie tlenu azotem przez minutę.

Stężenie celulozy zostało oznaczone przy wykorzystaniu zmodyfikowanej metody PN-92/P-50092 i obliczone zgodnie ze wzorem 1:

$$C = \frac{m_d}{m_0 * \text{suchość}} \quad (1)$$

Gdzie, m_d - masa próbki po teście, m_0 - masa próbki przed testem, suchość= 99%.

Dla wyizolowanych autochtonicznych grzybów celulolitycznych, oznaczona została również aktywność enzymatyczna, z uwzględnieniem całkowitej aktywności celulolitycznej (ang. Filter-Paperase activity) i aktywności endoglukanaz (ang. CMCase activity), z wykorzystaniem metod rekomendowanych przez IUPAC (ang. International Union of Pure and Applied Chemistry). Do obliczenia całkowitej aktywności celulolitycznej wykorzystano wzór 2:

$$FPA \frac{FPU}{mL} = \frac{0.37}{E} \quad (2)$$

Gdzie, E- ilość enzymu potrzebnego do uwolnienia z 0.5 mg glukozy z bibuły filtracyjnej, w ciągu 1 godziny.

Do obliczenia aktywności enzymatycznej endoglukanaz, wykorzystano wzór 3:

$$CMC \frac{U}{mL} = \frac{0.185}{Ec} \quad (3)$$

Gdzie E_c - ilość enzymu potrzebnego do uwolnienia 0,5 mg glukozy.

Na podstawie oceny efektywności degradacji celulozy, szybkości wzrostu i aktywności enzymatycznej, do określenia potencjału metanowego, wybrany został jeden grzyb (*Aspergillus terreus* S011 (G3)). Test na potencjał metanowy przeprowadzony został z wykorzystaniem systemu Automatic Methane Potential Test (BPC Instruments AB, Szwecja). Podczas testu określony został również stopień degradacji celulozy z wykorzystaniem normy PN-92/P-50092, obliczony według wzoru 1.

5.3. Publikacja 3: Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia

Oprócz grzybów, do weryfikacji wpływu działania biologicznej obróbki osadów ściekowych, do intensyfikacji fermentacji metanowej wykorzystane zostały enzymy produkowane przez autochtoniczne bakterie potencjalnie celulolityczne. Opisane w **Publikacji 3** badania, obejmowały izolację bakterii celulolitycznych,

hodowanych w hodowli płynnej oraz analizę enzymów przez nie produkowanych, wykorzystywanych jako bioszczepionka wspomagająca hydrolizę osadów ściekowych. Dodatkowo, w publikacji wykazano wpływ działania tych enzymów w formie zimmobilizowanej i niezimmobilizowanej na zmiany stężenia celulozy i ilości produkowanego metanu.

Bakterie izolowane były z osadu ściekowego pobranego z WKF (wydzielona komora fermentacyjna), pracującej w Centralnej Oczyszczalni Ścieków w Gliwicach (Polska). Na osad w WKF składa się osad wstępny zagęszczony grawitacyjnie i nadmierny zagęszczony, podawany do reaktora w proporcji 35:65. Izolacja prowadzona była za pomocą pasażu w płynnym podłożu (karboksymetyloceluloza 5 g/L, NaNO_3 1 g/L, K_2HPO_4 1 g/L, KCl 1 g/L, MgSO_4 0.5 g/L, ekstrakt drożdżowy 0.5 mg/L, glukoza 1 mg/L). Na początku, do 40 mL podłoża dodano 10 mL osadu ściekowego i inkubowano przez 7 dni w temperaturze 37°C, z wytrząsaniem 100 rpm. Hodowlę prowadzono w warunkach beztlenowych, poprzez wyparcie tlenu azotem gazowym. Podłoże z karboksymetylocelulozą wymieniane było co 7 dni. Hodowlę płukano poprzez wirowanie przez minutę z prędkością 3000 rpm, następnie zawieszano w 0.9% NaCl. Zabieg powtórzono trzykrotnie. Następnie 20 mL hodowli mikroorganizmów ponownie mieszano z 20 mL płynnego podłoża z CMC. Po miesiącu prowadzenia hodowli pobrano próbki do izolacji DNA i sekwencjonowania następnej generacji (NGS). DNA zostało wyizolowane przy użyciu GeneMATRIX Soil DNA Purification Kit, zgodnie z instrukcją producenta (EurX, Polska). NGS przeprowadzone zostało przez firmę Genomed (Polska). Analiza metataksonomiczna została wykonana dla hiperzmiennego regionu V3-V4 genu kodującego 16SrRNA. Do amplifikacji użyte zostały startery 341F i 785R (Thijs i wsp., 2017).

Dla wyizolowanej hodowli określono również aktywność enzymatyczną za pomocą metod rekomendowanych przez IUPAC (aktywność celulolityczną i endoglukanaz), analogicznie jak opisano w metodyce w **Publikacji 2**. Efektywność działania enzymów izolowanych z mieszanej hodowli bakterii celulolitycznych, została porównana z działaniem mieszanki enzymów celulolitycznych dostępnych komercyjnie (Cellic CTec2, Novozymes, Dania).

Przeprowadzono test tworzenia biofilmu na naczyniu mikromiareczkowym. Do tego celu wykorzystany został fiolet krystaliczny i metanol czysty (99%). Wykorzystano 5 mL płynnego podłoża CMC i 100 μL mieszanej kultury bakterii. Mikroorganizmy inkubowane były przez 3 dni, w temperaturze 37°C z wytrząsaniem 100 rpm, w warunkach beztlenowych, zapewnionych poprzez wyparcie tlenu azotem

gazowym. Po 24. i 48. godzinnej inkubacji, medium na płytkach było wymieniane. Do określenia tworzenia biofilmu przy każdej wymianie medium z dołków po inkubacji 24, 48 i 72. godzinnej dodawano 5 mL metanolu i inkubowano przez 15 minut. Po tym czasie usuwano metanol, a dołki suszono w temperaturze pokojowej. Po suszeniu, dodawano 5 mL fioletu krystalicznego na 5 minut. Fiolet krystaliczny usuwano, płukano dołki dwukrotnie wodą destylowaną, a następnie dodawano 5 mL 33% kwasu octowego, stanowiło to roztwór do pomiaru absorbancji przy długości fali $\lambda = 570$ nm. Próbkę ślepą stanowił 33% kwas octowy.

Brak możliwości przechowywania i ponownego wykorzystania wyizolowanych bakterii, bez utraty ich właściwości, zdeterminował konieczność izolacji enzymów produkowanych przez te mikroorganizmy. Ekstrakcję enzymów z mieszanej hodowli bakterii, zdolnych do wzrostu i aktywności w płynnym podłożu CMC przeprowadzono z wykorzystaniem metody opisanej przez Hossain i wsp. (2021). Wyizolowane enzymy stabilizowane były za pomocą 0,05 M buforu cytrynianowego (Pol-Aura, Polska) w stosunku 1:2, 1:3, 1:4 i 1:5, gdzie część „1” stanowił bufor. Stężenie białka w wyizolowanej mieszaninie enzymów wyznaczono za pomocą Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA), z wykorzystaniem metody probówkowej, zgodnie z instrukcją producenta.

Celem zabezpieczenia do ponownego wykorzystania, enzymy zimmobilizowane zostały na nieorganicznym nośniku (węglu aktywnym). Immobilizowane i nieimmobilizowane enzymy izolowane z mieszanej hodowli mikroorganizmów celulolitycznych oraz enzymy komercyjne, testowane były pod kątem intensyfikacji degradacji celulozy w osadzie ściekowym, oraz wpływu ich dodatku na potencjał metanowy badanych osadów. Immobilizację przeprowadzono poprzez wytrząsanie wyizolowanych enzymów z węglem aktywnym (Sigma Aldrich, USA) przez 3 godziny, w temperaturze 20°C. Enzymy zimmobilizowane na nośniku zostały poddane charakterystyce termograwimetrycznej za pomocą termograwimetru TGA/SDTA 851 Mettler-Toledo (USA), w celu określenia efektywności ich związania.

Działanie enzymów w formie wolnej i zimmobilizowanej zostało zweryfikowane w teście długotrwałego kontaktu z osadem ściekowym. Do testu wykorzystane został osad zmieszany (wstępny zagęszczony grawitacyjnie i nadmierny zagęszczony w proporcji 35:65, stosunek dobrany na podstawie procesu prowadzonego w oczyszczalni ścieków) oraz osad z komory fermentacyjnej w proporcji 3:1 (na podstawie lotnych substancji). Test prowadzony był w butelkach 500 mL, całkowita objętość osadów wynosiła

400 mL. Test prowadzono w temperaturze 37°C, w warunkach beztlenowych. Warunki beztlenowe zostały zapewnione poprzez wyparcie tlenu azotem gazowym. W teście wykorzystano odpowiednio: immobilizowane enzymy izolowane z mieszanej hodowli autochtonicznych bakterii celulolitycznych, immobilizowane enzymy komercyjne, nieimmobilizowane enzymy izolowane z mieszanej hodowli autochtonicznych bakterii celulolitycznych, nieimmobilizowane enzymy komercyjne, węgiel aktywny. Enzymy dodawano w objętości obliczonej w odniesieniu do suchej masy osadu (1% suchej masy). Na początku i na końcu 15-dniowego testu, weryfikowane było stężenie celulozy oraz potencjał metanowy. Stężenie celulozy wyznaczone zostało za pomocą normy PN-92/P-50092, obliczonej według wzoru 1. Potencjał metanowy oznaczony został za pomocą Automatic Methane Potential Test (BPC Instruments AB, Szwecja). Potencjał metanowy osadów ściekowych obliczony został zgodnie ze wzorem 4:

$$\text{Potencjał metanowy} \left(\frac{NmLCH_4}{gVS} \right) = \frac{M_2 - M_1}{S_{VS}} \quad (4)$$

Gdzie, M_1 - średnia dziennej produkcji metanu w próbce uznanej za kontrolę; M_2 - dzienna produkcja metanu w próbce poddanej obróbce, S_{VS} - stosunek ilości substancji organicznej i chemicznego zapotrzebowania na tlen (ChZT) (g).

Na początku i na końcu testu weryfikowane było również stężenie EPS, które ekstrahowane były zgodnie z metodą termiczną opisaną przez Karapangiotis i wsp. (1989). Stężenie EPS oznaczono jako sumę węglowodanów (PS) i białek (PN). Stężenie węglowodanów wyznaczono zgodnie z metodyką opisaną przez Dubois i wsp. (1956), natomiast stężenie białek wyznaczono przy użyciu gotowego zestawu Pierce™ BCA Protein Assay Kit i procedury próbówkowej, zgodnie z instrukcją producenta (Thermo Fisher Scientific, USA).

5.4. Publikacja 4: Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych

W **Publikacji 4** opisany został wpływ krótkotrwałego kontaktu enzymów izolowanych z mieszanej kultury bakterii celulolitycznych i mieszaniny celulolitycznych enzymów komercyjnych (Cellic CTec2). Enzymy wykorzystywane do hydrolizy osadów ściekowych, testowane były w formie wolnej i immobilizowanej. Immobilizacja przeprowadzona została

na nośniku nieorganicznym, jakim był węgiel aktywny. Immobilizacja została opisana w **Publikacji 3**.

Test krótkotrwałego kontaktu enzymów z osadem ściekowym, prowadzony był w małej objętości (20 mL) w 3 powtórzeniach dla każdego wariantu. Test trwał 4 godziny, a próbki pobierane były w czasie 0, 60, 180 i 240 minut. Podczas testu, weryfikowane było stężenie lotnych kwasów tłuszczowych (LKT), chemicznego zapotrzebowania na tlen (ChZT) oraz polimerów zewnątrzkomórkowych (EPS). Stężenie LKT i ChZT oznaczano metodą spektrofotometryczną z wykorzystaniem spektrofotometru MERCK Spectroquant® NOVA60 i zestawów do szybkich testów (Merck Millipore, Niemcy). Stężenie polimerów zewnątrzkomórkowych oznaczono jako sumę węglowodanów (PS) i białek (PN). Stężenie węglowodanów wyznaczono zgodnie z metodyką opisaną przez Dubois i wsp. (1956), natomiast stężenie białek wyznaczono przy użyciu gotowego zestawu Pierce™ BCA Protein Assay Kit i metody probówkowej, zgodnie z instrukcją producenta (Thermo Fisher Scientific).

5.5.Publikacja 5: Comparative Enzymatic Pretreatment of Sewage Sludge:

Physicochemical Parameters and Microbial Community Effects

W badaniach opisanych w **Publikacji 5** wykorzystywane były enzymy izolowane z mieszanej hodowli bakterii celulolitycznych oraz komercyjnej mieszaniny enzymów celulolitycznych (Cellic CTec2, Novozymes, Dania). Izolacja enzymów izolowanych opisana została w **Publikacji 4**. Wpływ działania enzymów izolowanych i komercyjnych, był prowadzony dla enzymów immobilizowanych i nieimmobilizowanych na nośniku nieorganicznym (węglu aktywnym), jak opisano w **Publikacji 4**.

Do badań wykorzystany został osad ściekowy pobrany z Centralnej Oczyszczalni Ścieków w Gliwicach (Polska). Jako źródło substratu do testu wykorzystany został osad zmieszany (wstępny zagęszczony grawitacyjnie i nadmierny zagęszczony w proporcji 35:65).

Jako inokulum służył osad z komory fermentacyjnej. Substrat i inokulum zmieszane zostało w proporcji 1:3.

Wpływ działania enzymów izolowanych i komercyjnych weryfikowany był w 15-dniowym teście. Enzymy dodano do osadów w pierwszym dniu testu, w przeliczeniu na 1% suchej masy osadu (substratu).

W pierwszym i ostatnim dniu testu, określano zawartość suchej masy i suchej masy organicznej, zgodnie z normą PN-EN 12880:2004. Weryfikowane było również stężenie

azotu całkowitego (TN), azotu amonowego ($\text{NH}_4\text{-N}$), chemicznego zapotrzebowania na tlen (ChZT), lotnych kwasów tłuszczowych (LKT). Parametry fizykochemiczne oznaczano metodą spektrofotometryczną z wykorzystaniem spektrofotometru MERCK Spectroquant® NOVA60 i zestawów do szybkich testów (Merck Millipore, Niemcy). Ponadto, weryfikowany był również wpływ działania enzymów oraz ich immobilizacji na zmiany w zbiorowisku bakterii stanowiących inokulum i osadach wykorzystanych jako substrat i inokulum (1:3). W tym celu z próbek wyizolowane zostało DNA, za pomocą zestawu do izolacji DNA GeneMATRIX Soil DNA Purification Kit, zgodnie z instrukcją producenta (EurX, Polska). Wyizolowane DNA zostało przesłane do firmy Genomed, celem przeprowadzenia analizy metataksonomicznej zbiorowiska w oparciu o sekwencjonowanie nowej generacji. Podobnie jak opisano w **Publikacji 3**, analiza metataksonomiczna została wykonana w oparciu o hiperzmienny region V3-V4 genu kodującego 16SrRNA. Do amplifikacji fragmentu genu markerowego użyte zostały startery 341F i 785R (Thijs i wsp., 2017).

6. Podsumowanie wyników badań przedstawionych w cyklu publikacji

Postawiona hipoteza badawcza zakładała, że możliwa jest izolacja i efektywne wykorzystanie autochtonicznych mikroorganizmów celulolitycznych do poprawy hydrolizy osadów ściekowych, stabilizowanych w procesie fermentacji metanowej, poprzez intensyfikację degradacji celulozy obecnej w tych osadach.

W **Publikacji 1** opisane zostały metody obróbki wstępnej, stosowanej przed fermentacją metanową, z wyszczególnieniem wpływu ich stosowania na takie parametry osadu jak stężenie lotnych kwasów tłuszczowych, chemicznego zapotrzebowania na tlen (ChZT) i potencjału metanowego. Analiza wykorzystania metod biologicznych wykazała brak weryfikacji zależności pomiędzy wpływem stosowanej metody a funkcjonowaniem społeczności mikroorganizmów obecnych w osadzie i aktywnie prowadzących proces fermentacji. Ponadto zaobserwowano bardzo małą liczbę badań opisujących wpływ działania biologicznych metod na intensyfikację hydrolizy oraz na zwiększenie potencjału metanowego osadów ściekowych. Wśród zanalizowanych artykułów badawczych nie uwzględniano również degradacji celulozy. Przegląd literatury wykazał, że każda ze stosowanych metod posiada swoje wady i zalety, a wykorzystanie poszczególnych metod jest uwarunkowane możliwościami technologicznymi i potrzebami danego obiektu. Ze względu na trudność z implementacją w przemyśle, metody biologiczne wymagają odpowiedniego podejścia. Ich potencjał i przyjazny środowisku charakter stanowią bazę do rozszerzenia badań nad możliwością ich wykorzystania i próby opracowania efektywnej obróbki wstępnej osadów ściekowych w skali przemysłowej.

Spśród wszystkich mikroorganizmów, grzyby uznawane są za jedne z najbardziej efektywnie degradujących celulozę (Hermosilla i wsp., 2018). Wśród grzybów mikroskopijnych, za głównych przedstawicieli uznaje się grzyby z rodzaju *Trichoderma* i *Aspergillus*. Wyizolowane w badaniach opisywanych w **Publikacji 2** mikroorganizmy, to grzyby mikroskopowe (Kumar i Parikh, 2015), należące do rodzaju *Aspergillus*. Są to grzyby tlenowe, przy czym ich zarodniki mogą przetrwać w warunkach ograniczonego dostępu do tlenu. Ponadto, *Aspergillus fumigatus* wykazuje zdolność do adaptacji do hipoksji. W **Publikacji 2** opisywane grzyby przetestowane zostały pod kątem zdolności do degradacji celulozy w warunkach tlenowych i beztlenowych. Zaobserwowano, że inkubacja grzybów w warunkach beztlenowych

w medium hodowlanym, w którym jedynym źródłem węgla była karboksymetyloceluloza zapewnia efektywną degradację celulozy w postaci sączka celulozowego (o 8.57 %) (**Publikacja 2, Rysunek 2**). Ponadto, inokulacja osadu grzybem *Aspergillus terreus* S011 bezpośrednio przed testem na potencjał metanowy, bez dodatkowego czasu na obróbkę wstępną, wpłynęła na intensyfikację produkcji metanu o 11% (**Publikacja 2, Rysunek 3**). Inokulacja osadu z wykorzystaniem grzyba *Aspergillus terreus* S011 wpłynęła na intensyfikację degradacji celulozy o 6% w odniesieniu do osadu nieinokulowanego (**Publikacja 2, Rysunek 4**).

Grzyby z rodzaju *Aspergillus* są grzybami tlenowymi, jednak w niektórych przypadkach zdolne są do adaptacji i aktywnego funkcjonowania w warunkach ograniczonego dostępu do tlenu (Willger i wsp., 2009). Jednocześnie, literatura sugeruje, że wydłużenie czasu przebywania w komorze beztlenowej, prowadzi do redukcji liczby tych grzybów, a często również do redukcji liczby spor (Schnürer i Schnürer, 2006). Grzyby z rodzaju *Aspergillus* są grzybami patogenicznymi, jednak mimo tego, *A. terreus* wykorzystywany jest w przemyśle do produkcji np. kwasu itakonowego czy lowastatyny (Bizukojć i Ledakowicz, 2009). To wskazuje na możliwość efektywnego wykorzystania *A. terreus* w procesach intensyfikacji produkcji metanu w warunkach beztlenowych. Uwzględniając jednak na patogeniczny charakter grzybów z rodzaju *Aspergillus*, zanim podejmie się dalsze kroki, takie jak zwiększenie skali hodowli lub wykorzystanie tego grzyba w procesach biotechnologicznych, musi on zostać scharakteryzowany metodą PCR w celu wykrycia genów odpowiedzialnych za jego patogenność, tj. genów oporności na amfoterycynę B czy klastrów metabolitów wtórnych (BGC).

W badaniach wykorzystane zostały mikroorganizmy i ich strzępki, a nie same produkowane enzymy. Wynika to z łatwości inokulacji osadu w ten sposób, jednak biorąc pod uwagę możliwości implementacji i bezpieczeństwo potencjalnych pracowników, którzy odpowiedzialni byłiby za inokulację osadu grzybami, rozwinięcie tego rodzaju obróbki, wymaga wykorzystania samych enzymów produkowanych przez te grzyby. Dodatkowym czynnikiem jest fakt, że ze względu na preferencje *A. terreus* i *A. fumigatus* do wzrostu i aktywności w warunkach tlenowych, możliwe byłoby uzyskanie celulaz o większej aktywności enzymatycznej, niż oznaczonej w **Publikacji 2**. Inną możliwością jest zatrzymywanie i inokulacja osadu w warunkach tlenowych, przed wprowadzeniem go do wydzielonych komór fermentacyjnych (WKF), co jednak wiąże się z wydłużeniem czasu i koniecznością dodania osobnego reaktora. Takie rozwiązanie z punktu widzenia oczyszczalni ścieków, może być nieopłacalne.

Izolacja wyspecjalizowanych mikroorganizmów ze środowiska jest czasochłonna, wymaga precyzji i dokładnego planowania prowadzonych badań. Zgodnie z założeniami, wyizolowane bakterie powinny być zdolne do degradacji celulozy oraz wzrostu i aktywności w warunkach beztlenowych w temperaturze $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Podczas badań wstępnych udało się wyizolować i scharakteryzować dwie czyste kultury bakterii celulolitycznych. Bakterie zostały zidentyfikowane jako *Staphylococcus warneri* i *Staphylococcus epidermidis*. Następne badania nad wyizolowanymi szczepami dowiodły, że mrożenie i liofilizacja wpływają na ich aktywność celulolityczną. Mikroorganizmy po reaktywacji traciły właściwości lub degradacja celulozy trwała znacznie dłużej, niż w przypadku wykorzystania tych samych szczepów hodowanych w sposób ciągły. W związku z tym, przeprowadzona została ponowna izolacja celulolitycznych bakterii z osadu ściekowego. Wielokrotny pasaż umożliwił pozyskanie mieszanej kultury bakterii, zdolnej do wzrostu i aktywności w medium, w którym jedynym źródłem węgla była celuloza w formie karboksymetylocelulozy. Badanie metataksonomiczne wykazało, że w tym konsorcjum bakteryjnym występują przedstawiciele różnych gatunków, niekoniecznie zdolnych do degradacji celulozy, jednak najpewniej wspomagających i współpracujących z bakteriami celulolitycznymi podczas prowadzonych procesów. Skład mieszanej hodowli bakterii przedstawiony został na **Rysunku 2**, w **Publikacji 3**. Enzymy wyizolowane z mieszanej hodowli bakterii potencjalnie celulolitycznych wykazywały aktywność enzymatyczną na poziomie 1.69 FPU/mL w przypadku całkowitej aktywności enzymatycznej i 11.27 U/mL w odniesieniu do produkowanych endoglukanaz. Według informacji producenta, aktywność enzymatyczna komercyjnej mieszaniny enzymów celulolitycznych wynosi minimum 1000 U/g.

W badaniach opisanych w **Publikacji 4** weryfikowany był wpływ enzymów w formie immobilizowanej i nieimmobilizowanej, na osad ściekowy w pierwszych czterech godzinach od inokulacji. Na podstawie uzyskanych wyników możliwe było potwierdzenie oddziaływania enzymów w obu formach na parametry fizykochemiczne i odpowiedź mikroorganizmów osadu na zmianę warunków. W badaniach zaobserwowano stabilizujący wpływ działania węgla aktywnego na enzymy komercyjne, których działanie w wersji nieimmobilizowanej powodowało zaburzenie pracy osadu, nadmierną intensyfikację produkcji LKT i zakwaszanie osadu (wzrost stężenia o 1856% w 4. godzinie testu) i powstawania EPS (wzrost o ponad 200% w 4. godzinie testu) (**Publikacja 4, Rysunek 3**). W przypadku enzymów izolowanych, immobilizacja również

miała pozytywny wpływ na działanie enzymów, poprzez intensyfikację ich działania (wzrost stężenia LKT po 3 godzinach testu o 340%) (**Publikacja 4, Rysunek 7**).

Wpływ działania enzymów izolowanych i komercyjnych w formie immobilizowanej i nieimmobilizowanej zweryfikowany został również w teście długim (15 dni). W teście 15-dniowym weryfikowane było stężenie LKT, ChZT i EPS, a dodatkowo stężenie celulozy, potencjał metanowy, azot całkowity (TN) i azot amonowy (N-NH_4). Dodatkowo, analizowany był wpływ działania enzymatycznej obróbki osadów ściekowych i dodatku węgla aktywnego na społeczność mikroorganizmów osadu ściekowego. Przeprowadzone badania potwierdziły pozytywny wpływ działania enzymów zarówno izolowanych jak i komercyjnych na intensyfikację produkcji metanu. Wyniki opisane w **Publikacji 3** wskazują, że biologiczna obróbka osadów z wykorzystaniem enzymów skraca czas potrzebny do uzyskania takich samych wyników produkcji biogazu, jak w przypadku osadów niepoddawanych żadnej obróbce. Wykorzystanie immobilizowanych enzymów izolowanych wpłynęło na intensyfikację produkcji metanu o 18,9%, a w przypadku immobilizowanych enzymów komercyjnych o 27% (**Publikacja 3, Rysunek 3**). Dodatkowo, po 7 dniach testu, produkcja metanu w próbkach inokulowanych enzymami izolowanymi i komercyjnymi była na podobnym poziomie. W przypadku enzymów nieimmobilizowanych, znacznie intensywniejszy, dłuższy i bardziej stabilny wzrost produkcji metanu zaobserwowany był przy zastosowaniu enzymów komercyjnych. Ostatecznie odnotowano wzrost produkcji metanu o 43% w stosunku do osadu niepoddawanego obróbce. W przypadku nieimmobilizowanych enzymów izolowanych, wzrost produkcji wynosił 21,7%. Najintensywniejszy wzrost zaobserwowany był w piątym dniu testu, wtedy też produkcja metanu dla osadów poddawanych obróbce z wykorzystaniem enzymów izolowanych i komercyjnych była na podobnym poziomie. Stężenie EPS we wszystkich badanych próbkach, niezależnie od zastosowanej obróbki było niższe niż przed rozpoczęciem testu, przy czym najwyższe oznaczono w inokulum, niepoddawanym obróbce enzymatycznej (**Publikacja 3, Rysunek 4**). Stężenie celulozy oznaczone podczas testu w większości przypadków nie zmieniło się w znaczący sposób, poza stężeniem oznaczonym w próbkach inokulum zmieszanego z substratem (w stosunku 3:1), poddawanego obróbce z wykorzystaniem nieimmobilizowanych izolowanych enzymów (degradacja celulozy o $26,02\% \pm 0,75$), oraz w przypadku inokulum poddawanego obróbce z wykorzystaniem immobilizowanych komercyjnych enzymów (degradacja celulozy o $64,07\% \pm 0,37$).

Wykorzystanie immobilizowanych i nieimmobilizowanych enzymów w 15-dniowym teście wykazało brak znaczących różnic w wyznaczonych parametrach fizykochemicznych w próbkach kontrolnych i tych poddawanych obróbce enzymatycznej. Test długotrwałego kontaktu enzymów z osadem prowadzony był przez 15 dni. Po tym czasie ze względu na brak obserwowanej produkcji biogazu w próbkach z dodatkiem enzymów immobilizowanych i nieimmobilizowanych, test został zatrzymany. Na podstawie rezultatów ukazanych w **Publikacji 4** i **Publikacji 5** określono, że wykorzystana metoda obróbki osadów ściekowych wpłynęła na skrócenie czasu potrzebnego na wykorzystanie maksymalnego potencjału osadów do produkcji metanu. Zgodnie z założeniami, stężenie azotu całkowitego pozostawało na względnie stałym poziomie (**Publikacja 5, Rysunek 2**), z uwzględnieniem odchylenia pomiędzy 5-20%, który zaobserwowano w literaturze i uzasadniano migracją NH_3 ze strumieniem biogazu, sedymentacją części materii czy formowaniem struwitu (Chavez-Rico i wsp., 2022; Schievano i wsp., 2011). W przypadku $\text{NH}_4\text{-N}$ zmiany w stężeniu zaobserwowano jedynie w próbkach osadu zmieszanego i inokulum, poddawanego obróbce z wykorzystaniem immobilizowanych izolowanych enzymów, węgla aktywnego (**Publikacja 5, Rysunek 3**), oraz nieimmobilizowanych enzymów komercyjnych (**Publikacja 5, Rysunek 3**). Zastosowanie enzymów immobilizowanych i węgla aktywnego, spowodowało wzrost stężenia azotu amonowego ($\text{NH}_4\text{-N}$). Stężenie $\text{NH}_4\text{-N}$ w inokulum, czyli osadzie pobranym z komory fermentacyjnej, wzrosło po obróbce z wykorzystaniem immobilizowanych enzymów komercyjnych. Na tej podstawie, zakłada się, że intensyfikacja rozkładu białek na skutek obróbki enzymatycznej, nastąpiła tylko w próbkach, w osadach opisanych powyżej. Możliwe jest, że obróbka z wykorzystaniem nieimmobilizowanych enzymów wpłynęła również na intensyfikację degradacji substancji organicznej w pozostałych próbkach, jednak nie w tak znaczący sposób, aby mogło to być zaobserwowane po 15 dniach testu. W tym przypadku wraz z wydłużaniem czasu testu w osadach kontrolnych, stężenie po 15 dniach oznaczone zostało na podobnym poziomie co w osadach poddanych działaniu enzymatycznej obróbki z wykorzystaniem immobilizowanych enzymów komercyjnych oraz nieimmobilizowanych enzymów izolowanych. W przypadku stężenia azotu amonowego ($\text{NH}_4\text{-N}$) w próbkach substratu i inokulum, poddawanego działaniu nieimmobilizowanych enzymów komercyjnych, zaobserwowano spadek stężenia azotu amonowego ($\text{NH}_4\text{-N}$), co najprawdopodobniej wynika ze zbyt intensywnego działania

enzymu, wpływającego na wzrost produkcji substancji humusowych, będących inhibitorem fermentacji metanowej (Fernandes i wsp., 2014; Azman i wsp., 2015).

Zmiany w stężeniu lotnych kwasów tłuszczowych zaobserwowano jedynie w przypadku nieimmobilizowanych enzymów komercyjnych. To potwierdza wpływ na intensyfikację degradacji wielkocząsteczkowych związków organicznych, na skutek obróbki enzymatycznej (**Publikacja 5, Rysunek 4**).

Wzrost stężenia chemicznego zapotrzebowania na tlen (ChZT) (**Publikacja 5, Rysunek 5**) został zaobserwowany w próbkach inokulum, poddawanego enzymatycznej obróbce z wykorzystaniem enzymów immobilizowanych. W przypadku samego węgla aktywnego nie zaobserwowano różnicy w stężeniu w porównaniu z pozostałymi próbkami osadów, co świadczy o działaniu enzymów, nie węgla aktywnego. W przypadku nieimmobilizowanych enzymów, nie zaobserwowano żadnych różnic pomiędzy próbkami poddawanymi obróbce i niepoddawanymi obróbce.

Oznaczone parametry fizykochemiczne ściśle związane były ze zmianami w obfitości występowania mikroorganizmów osadu ściekowego. Wskaźniki różnorodności mikrobiologicznej Shannona i Simpsona (**Publikacja 5, Tabela 1 i 2**) na początku i na końcu testu wskazywały brak znaczących różnic wobec różnorodności występowania gatunków w zbiorowisku. Mimo to w próbkach poddanych działaniu immobilizowanych enzymów izolowanych zaobserwowano spadek bioróżnorodności i średniej obserwowanej liczby OTU (**Publikacja 5, Tabela 1**). Zwykle oznaczane wartości indeksu Shannona mieszczą się w przedziale 1,5-3. W badaniach prowadzonych w ramach rozprawy doktorskiej oznaczony wskaźnik Shannon przekraczał 4 niezależnie od formy użytego enzymu (immobilizowane/ nieimmobilizowane) występowania enzymów czy ich rodzaju (izolowane/ komercyjne), co wskazuje na dużą różnorodność w próbkach badanego osadu. W **Publikacji 5, Rysunek 6** przedstawiony został skład zbiorowiska osadu ściekowego przed testem w osadzie pracującym w WKF, osadzie przed fermentacją metanową (zmieszany) oraz po 15-dniowym teście produkcji metanu osadów inokulowanych i nieinokulowanych enzymami. Na podstawie **Rysunku 6** można zaobserwować, że enzymatyczna obróbka osadów ściekowych nie wpłynęła na znaczące ograniczenie liczebności żadnego z gatunków, występujących w próbkach, których obfitość byłaby większa niż 1%. Wskaźnik Simpsona (**Publikacja 5, Tabela 2**) oznacza się w skali 0-1. Indeks zbliżony do 1 wskazuje na brak dominującego gatunku w zbiorowisku. Indeks Simpsona mówi o tym, że w przypadku wylosowania dwóch osobników, będą oni należeć do jednego gatunku. W osadach poddawanych obróbce z wykorzystaniem

immobilizowanych i nieimmobilizowanych izolowanych i komercyjnych enzymów, a także osadach niepoddawanych obróbce indeks Simpsona wynosił 0,98, wskazując na dużą różnorodność w badanym zbiorowisku.

Weryfikacja zależności stężenia parametrów oznaczonych w osadach poddawanych działaniu enzymów i obfitości występowania bakterii w osadzie ściekowym przeprowadzona została z wykorzystaniem korelacji Pearsona. Najbardziej obficie występującą gromadą były *Bacteroidota* i *Proteobacteria*. W tabeli 1 przedstawiono korelacje pomiędzy stężeniem celulozy i objętością wyprodukowanego metanu, a obfitością występowania poszczególnych bakterii (klasyfikacja na poziomie gromady) w próbkach osadu poddawanego działaniu enzymów izolowanych. Kolorem niebieskim oznaczone zostały pozytywne korelacje, a czerwonym negatywne. Korelacja pomiędzy stężeniem celulozy w próbkach traktowanych izolowanymi immobilizowanymi enzymami wskazuje na negatywną korelację z gatunkami z typów *Acetothermia*, *Caldatribacteriota* i *Desulfobacterota*, a więc spadkiem jednego parametru przy wzroście drugiego. Na podstawie Tabeli 2 (**Publikacja 5**) przedstawiającego zmiany w stężeniu celulozy w próbce inokulum i substratu poddanych działaniu izolowanych enzymów celulolitycznych, obserwowany był spadek stężenia celulozy na poziomie 10%. Równocześnie, obserwowany był wzrost obfitości wymienionych wyżej grup bakterii (**Publikacja 5, Rysunek 6**). W przypadku stężenia celulozy w próbkach traktowanych izolowanymi enzymami nieimmobilizowanymi, obserwowany był znaczny spadek stężenia i w odróżnieniu od próbek traktowanych immobilizowanymi enzymami izolowanymi, korelacja z obfitością występowania typów bakterii jest w większości przypadków negatywna. Pozytywna korelacja obrazuje spadek stężenia celulozy i spadek obfitości występowania *Chloroflexi* i *Proteobacteria*. W przypadku negatywnej korelacji, obserwuje się wzrost obfitości występowania poszczególnych typów bakterii przy spadku stężenia celulozy. Większość ze zidentyfikowanych grup bakterii w osadzie ściekowym pozytywnie korelowała z wydajnością metanu, zarówno w próbkach traktowanych immobilizowanymi, jak i nieimmobilizowanymi enzymami izolowanymi.

Tabela 1 Wyznaczone korelacje pomiędzy obfitością występowania poszczególnych bakterii (klasyfikacja na poziomie typu) w osadzie ściekowym a stężeniem celulozy i objętością wyprodukowanego metanu w próbkach osadu ściekowego poddawanego działaniu enzymów izolowanych. Kolor czerwony wskazuje na negatywną korelację, kolor niebieski na pozytywną.

typ	Celuloza-immob.	Celuloza-nieimmob.	Wydajność metanu-immob.	Wydajność metanu-nieimmob.
<i>Acetothermia</i>	-0.31	-0.94	-0.91	0.98
<i>Acidobacteriota</i>	1.00	0.22	0.62	-0.06
<i>Actinobacteriota</i>	1.00	-0.67	0.73	0.78
<i>Armatimonadota</i>	0.94	-0.41	0.37	0.56
<i>Bacteroidota</i>	1.00	-0.29	0.63	0.44
<i>Caldatribacteriota</i>	-0.23	-0.61	-0.87	0.47
<i>Caldisericota</i>	0.82	-0.18	0.12	0.34
<i>Chloroflexi</i>	0.71	0.90	-0.04	-0.96
<i>Cloacimonadota</i>	0.93	-0.80	0.89	0.89
<i>Desulfobacterota</i>	-0.10	-0.72	-0.80	0.82
<i>Firmicutes</i>	0.94	-0.08	0.39	0.24
<i>Patescibacteria</i>	0.94	-0.06	0.89	0.23
<i>Planctomycetota</i>	0.96	-0.32	0.85	0.47
<i>Proteobacteria</i>	0.94	0.92	0.88	-0.84
<i>Spirochaetota</i>	1.00	-0.83	0.73	0.91
<i>Thermotogota</i>	0.99	-0.48	0.79	0.62
<i>Verrucomicrobiota</i>	0.99	-0.91	0.55	0.97

W przypadku enzymów komercyjnych, obserwuje się znaczące różnice w porównaniu z enzymami izolowanymi. W obfitości występowania poszczególnych typów dominuje negatywna korelacja ze stężeniem celulozy w próbkach immobilizowanych i nieimmobilizowanych enzymów. Przeprowadzony test na stężenie celulozy wykazał znaczącą statystycznie degradację na poziomie 10% i wzrost obfitości występowania typów z wyznaczoną negatywną korelacją. W próbkach poddanych działaniu nieimmobilizowanych komercyjnych enzymów zależność pomiędzy obfitością występowania poszczególnych typów a wydajnością metanu jest nieznaczna, z wyjątkiem *Caldatribacteriota*, *Chloroflexi* i *Proteobacteria*, wykazujących silną korelację negatywną. Obserwowany był znaczący statystycznie spadek obfitości występowania tych

grup we wskazanych próbkach. W przypadku immobilizowanych enzymów komercyjnych, dominowały silne pozytywne korelacje.

Tabela 2 Wyznaczone korelacje pomiędzy obfitością występowania poszczególnych typów bakterii w osadzie ściekowym a stężeniem celulozy i objętością wyprodukowanego metanu w próbkach osadu ściekowego poddawanego działaniu enzymów komercyjnych. Kolor czerwony wskazuje na negatywną korelację, kolor niebieski na pozytywną.

typ	Celuloza-immob.	Celuloza-nieimmob.	Wydajność metanu-immob.	Wydajność metanu-nieimmob.
<i>Acetothermia</i>	-0.89	-0.61	-0.96	-0.18
<i>Acidobacteriota</i>	-0.64	-0.45	0.08	-0.36
<i>Actinobacteriota</i>	0.15	-0.62	0.80	-0.16
<i>Armatimonadota</i>	0.97	-0.63	0.52	-0.15
<i>Bacteroidota</i>	-0.65	-0.95	0.07	0.40
<i>Caldatibacteriota</i>	0.05	0.80	-0.66	-0.98
<i>Caldisericota</i>	-0.94	-0.81	-0.44	0.12
<i>Chloroflexi</i>	0.56	0.84	-0.17	-0.97
<i>Cloacimonadota</i>	0.06	-0.94	0.74	0.38
<i>Desulfobacterota</i>	-1.00	-0.85	-0.71	0.19
<i>Firmicutes</i>	-0.79	-0.92	-0.13	0.33
<i>Patescibacteria</i>	0.38	-0.99	0.92	0.54
<i>Planctomycetota</i>	0.26	-0.92	0.86	0.33
<i>Proteobacteria</i>	0.45	0.36	0.95	-0.93
<i>Spirochaetota</i>	0.08	-0.73	0.75	-0.01
<i>Thermotogota</i>	-0.18	-0.91	0.56	0.31

Wpływ na rodzaj korelacji pomiędzy obfitością występujących bakterii i parametrami fizykochemicznymi w osadzie miała forma enzymu, a więc immobilizacja lub obecność węgla aktywnego. Kiedy osady poddawano obróbce z wykorzystaniem enzymów immobilizowanych, korelacja była pozytywna, w przypadku enzymów nieimmobilizowanych można było spodziewać się korelacji negatywnej. Zależność taką obserwowano niezależnie od tego czy wykorzystywane były enzymy izolowane, czy komercyjne.

Podsumowując, badania przeprowadzone w ramach rozprawy doktorskiej pozwoliły na opracowanie metody intensyfikacji fermentacji metanowej z uwzględnieniem degradacji

trudno rozkładalnego polimeru jakim jest celuloza, która może stanowić uniwersalną metodę stosowaną w oczyszczalniach ścieków. Zastosowanie autochtonicznych mikroorganizmów i enzymów przez nie produkowanych, z założenia dopasowanych do indywidualnych potrzeb oczyszczalni ścieków, może zapobiegać niepożądanemu zaburzeniu pracy osadu podczas fermentacji metanowej.

7. Wnioski

Wszystkie z założonych w ramach rozprawy doktorskiej celów zostały zrealizowane. W trakcie badań potwierdzono, że:

- możliwe jest wyizolowanie pojedynczych szczepów bakterii celulolitycznych z osadu ściekowego, jednak ich przechowywanie, poprzez mrożenie, liofilizację i pasaż prowadzi do utraty właściwości celulolitycznych lub wydłużenia czasu wzrostu.

- Niektóre szczepy bakterii celulolitycznych tracą również właściwości po odseparowaniu ich od innych bakterii, z którymi wzrastały na podłożu stałym. Izolacja autochtonicznych bakterii w podłożu płynnym z dodatkiem karboksymetylocelulozy wykazała obecność celulolitycznych bakterii, jak również innych szczepów, które swoją obecnością w mieszanej hodowli, najpewniej wspierały wzajemnie swoje działania.

- Enzymatyczna obróbka osadów ściekowych, z wykorzystaniem mieszaniny enzymów izolowanych z mieszanej hodowli bakterii zdolnych do degradacji celulozy wpływa na intensyfikację produkcji metanu, degradacji celulozy i poprawę hydrolizy osadów ściekowych, prowadząc do możliwości skrócenia czasu potrzebnego na fermentację.

- Zaszczepienie osadu ściekowego autochtonicznym celulolitycznym grzybem *Aspergillus terreus* pozwoliło na intensyfikację produkcji metanu w badanym osadzie o 11%.

- Zastosowanie immobilizowanych enzymów izolowanych z osadu ściekowego spowodowało wzrost potencjału metanowego badanego osadu ściekowego o 18,9%.

- Wykorzystanie grzybów mikroskopowych o właściwościach celulolitycznych w postaci strzępek, jako biologicznej metody obróbki osadów ściekowych wpływa na intensyfikację degradacji celulozy i potencjału metanowego tych osadów.

- Immobilizacja enzymów na węglu aktywnym stabilizuje ich działanie poprzez wydłużenie działania enzymów izolowanych i zmniejszenie intensywności działania w przypadku enzymów komercyjnych, zabezpieczając proces przed nadmiernym zakwaszaniem,

na skutek zwiększonej produkcji LKT.

- Zastosowanie enzymatycznej obróbki osadów ściekowych z wykorzystaniem enzymów izolowanych i komercyjnych, w formie immobilizowanej i nieimmobilizowanej w objętości 1% suchej masy substratu, nie wpływa w znaczącym stopniu na skład zbiorowiska bakterii zasiedlających badany osad ściekowy.

8. Kontynuacja badań

Rezultaty przeprowadzonych badań jednoznacznie wskazują na konieczność ich kontynuacji oraz rozszerzenia o szczegółową analizę losów mikroorganizmów wprowadzanych do osadu poddawanego fermentacji metanowej, a także o pogłębioną charakterystykę autochtonicznych zbiorowisk mikrobiologicznych obecnych w tym środowisku.

Dotychczasowe eksperymenty realizowano w skali laboratoryjnej, co uzasadnia potrzebę przejścia do etapu badań w skali półtechnicznej, umożliwiającego ponowną i bardziej miarodajną ocenę efektywności proponowanej metody w warunkach zbliżonych do rzeczywistych.

W odniesieniu do mikroorganizmów tworzących mieszaną hodowlę bakterii zdolnych do wzrostu w podłożu, w którym karboksymetyloceluloza stanowi źródło węgla, niezbędne jest przeprowadzenie pogłębionej analizy struktury i funkcjonowania zbiorowiska, ze szczególnym uwzględnieniem interakcji oraz zależności pomiędzy mikroorganizmami. Analogiczne badania powinny zostać rozszerzone na układy osadu ściekowego poddawanego biologicznej obróbce z zastosowaniem zarówno enzymów immobilizowanych, jak i nieimmobilizowanych.

Istotnym kierunkiem dalszych prac jest również opracowanie wydajnej i powtarzalnej metody wprowadzania oraz odzysku enzymów immobilizowanych po zakończeniu procesu fermentacji, co może przyczynić się do istotnego ograniczenia kosztów operacyjnych.

Ponadto, kluczowym elementem dalszych badań pozostaje weryfikacja skuteczności proponowanej strategii dozowania enzymów na poziomie 1% w odniesieniu do suchej masy osadów, z uwzględnieniem zróżnicowanego pochodzenia osadów z różnych oczyszczalni ścieków.

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Rysunek 1 Odwracalne i nieodwracalne metody immobilizacji enzymów. Na podstawie Maghraby i wsp., 2023. (Ćwierniewicz-Wojciechowska i wsp., 2024).....	18
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10. Inne osiągnięcia naukowe

10.1. Publikacje naukowe

Banach Wiśniewska A., Ćwiertniewicz-Wojciechowska M., Ziemińska-Buczyńska A.: Effect of temperature shifts and anammox biomass immobilization on sequencing batch reactor performance and bacterial genes abundance, International Journal of Environmental Science and Technology, 18, 1719-1730, 2021. <http://dx.doi.org/10.1007/s13762-020-02957-w>. IF₂₀₂₄= 3,4; MNiSW₂₀₂₅= 70

Gamoń F., Ćwiertniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.: Ocena zdolności wybranych nanomateriałów węglowych do usuwania antybiotyków ze środowiska wodnego, Współczesne problemy ochrony środowiska i energetyki 2020, Publikacja zbiorowa 2021, 395-40, Gliwice, Politechnika Śląska, ISBN 978-83-950087-8-8. MNiSW₂₀₂₅= 20.

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Ćwiertniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.: Hydroliza osadów ściekowych z wykorzystaniem enzymów immobilizowanych na węglu aktywnym, Współczesne problemy ochrony środowiska i energetyki 2022, 2023, Gliwice, Politechnika Śląska, 25-35, ISBN 978-83-964116-3-1. MNiSW₂₀₂₅= 20.

Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.: Wspomaganie hydrolizy celulozy obecnej w osadach ściekowych jako metoda intensyfikacji fermentacji metanowej, Balon B. (Ed.) Interdyscyplinarne badania młodych naukowców, Gliwice, Politechnika Śląska, 2024, 87-97. ISBN 978-83-7880-983-8. MNiSW₂₀₂₅= 20.

Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.: Wpływ enzymów celulolitycznych na przebieg fermentacji metanowej osadów ściekowych, Innowacyjna Zielona Gospodarka. Cz. 6, Klimat i bioróżnorodność, Katowice, Główny Instytut Górnictwa, 2025, 56-66. ISBN 978-83-65503-67-1. MNiSW₂₀₂₅= 20.

Gamoń F., Ćwierniewicz-Wojciechowska M., Muszyński-Huhajło M., Miodoński S., Ziemińska-Buczyńska A., Cema G., Tomaszewski M.: Low-Temperature Anammox Supported by Zero-Valent Iron (ZVI): Microbial and Physicochemical Changes during Treatment of Synthetic and Municipal Wastewater, Microbial Ecology, 88(108), 2025. <https://doi.org/10.1007/s00248-025-02615-z>. IF= 4.0, MNiSW₂₀₂₅= 100.

10.2. Projekty

- Subwencja na utrzymanie i rozwój potencjału badawczego Młodzi Naukowiec 2020/2021 „Ocena skuteczności wpływu zastosowanych metod obróbki biomasy na hydrolizę osadów ściekowych.” 08/070/BKM21/0007
- Grant projakościowy, finansowany w ramach programu Inicjatywa Doskonałości – Uczelnia Badawcza na dofinansowanie rozpoczęcia działalności naukowej w nowej tematyce badawczej „Izolacja i charakterystyka autochtonicznych grzybów mikroskopowych z osadów ściekowych do rozkładu celulozy.” 32/014/SDU/10-22-09
- Subwencja na utrzymanie i rozwój potencjału badawczego Młodzi Naukowiec 2023/2024 „Wpływ enzymów celulolitycznych na efektywność fermentacji metanowej i społeczność mikroorganizmów obecnych w osadzie ściekowym.” 08/070/BKM24/0033

10.3. Staże naukowe i szkoły letnie

- 30.08-30.09.2021: zagraniczny staż naukowy na Uniwersytecie w Tartu, Estonia (2021)
- 23-27.05.2022: uczestnictwo w Szkole Letniej: Resource Recovery and Biotechnology, realizowanej na Uniwersytecie w Borås, Szwecja (2022)
- 22-26.04.2025: krajowy staż naukowy na Politechnice Gdańskiej, Polska (2025)

- 08-10.07.2025: uczestnictwo w Szkole Letniej, Politechnika Śląska, Gliwice (2025)

10.4. Prezentacje na konferencjach naukowych, krajowych i międzynarodowych

- XXIIInd Gliwice Scientific Meetings 2018, Gliwice 16-17.11.2018, pH and temperature effect on the absorption capacity of the sodium alginate-polyvinyl alcohol carriers (poster)
- IX Environmental Protection & Energy Conference, Gliwice 10.12.2021, Polska; Wpływ obecności grzybów mikroskopowych w osadach ściekowych na rozkład celulozy (wystąpienie ustne)
- XI Ogólnopolska Konferencja Hydromikrobiologiczna: HYDROMICRO 2021. Mikroorganizmy wodne - zagrożenia i nadzieje, Sopot 9-11.06.2021, Polska; Izolacja i charakterystyka szczepów celulolitycznych pochodzących z osadów ściekowych do biotechnologicznych zastosowań (intensyfikacja hydrolizy) (poster)
- The 5th Symposium on Biotransformations for Pharmaceutical and Cosmetic Industry. Kraków-Częstochowa, 13-15.06.2022, Polska; The cellulolytic potential of fungi isolated from sewage sludges (poster)
- X Environmental Protection & Energy Conference, Gliwice 09.12.2022, Polska; Hydroliza osadów ściekowych z wykorzystaniem enzymów immobilizowanych na węglu aktywnym (wystąpienie ustne)
- IX International Interdisciplinary Conference of PhD students of Technical Universities – InterTechDoc'2024, Ustroń, 20-21.05.2024, Polska; Wspomaganie hydrolizy celulozy obecnej w osadach ściekowych jako metoda intensyfikacji fermentacji metanowej (wystąpienie ustne)
- XII International Environmental Protection & Energy Conference, Gliwice 06.12.2024, Polska; Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych (wystąpienie ustne)
- IV Konferencja pt.: Klimat i Bioróżnorodność z cyklu Innowacyjna Zielona Gospodarka. Główny Instytut Górnictwa - Państwowy Instytut Badawczy, Katowice 25.10.2024, Polska; Wpływ enzymów celulolitycznych na przebieg fermentacji metanowej osadów ściekowych (poster)
- XIII Ogólnopolska Konferencja Hydromikrobiologiczna HYDROMICRO 2025, Bydgoszcz 25-27.06.2025, Polska; Autochtoniczne grzyby celulolityczne i ich potencjał intensyfikacji produkcji metanu (wystąpienie ustne)

10.5. *Nagrody i wyróżnienia*

- X Environmental Protection & Energy Conference, 2022- Nagroda II stopnia w kategorii: najlepszy temat.
- XIII Ogólnopolska Konferencja Hydromikrobiologiczna HYDROMICRO 2025- Wyróżnienie za wystąpienie ustne.
- Three Minute Thesis- Polish your English, 2025- Nagroda publiczności i nagroda inżynierska.

11. Wkład autorski

Publikacja 1 (70%):

- Konceptualizacja badań,
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- Przygotowanie manuskryptu roboczego,
- Przygotowanie wszystkich grafik i tabel,
- Edycja manuskryptu, przygotowanie odpowiedzi dla recenzentów.

Publikacja 2 (80%):

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- Przeprowadzenie analiz laboratoryjnych i analiza wyników badań,
- Przygotowanie manuskryptu roboczego,
- Przygotowanie wszystkich grafik i tabel,
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- Przeprowadzenie przeglądu literatury,
- Przeprowadzenie analiz laboratoryjnych i analiza wyników badań,
- Przygotowanie manuskryptu roboczego,
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- Przygotowanie wszystkich grafik i tabel,
- Edycja manuskryptu, przygotowanie odpowiedzi dla recenzentów.

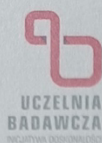
12. Wkład pozostałych autorów

W tabeli 3. przedstawiony został pełny udział procentowy dla poszczególnych publikacji.

Tabela 3. Lista współautorów i ich procentowy wkład w publikacje wchodzące w skład pracy doktorskiej.

Tytuł publikacji	Magdalena Ćwiertniewicz- Wojciechowska	Aleksandra Ziemińska- Buczyńska	Grzegorz Cema	Anna Wolny	Sebastian Jurczyk
Publikacja 1	70%	15%	15%	-	-
Publikacja 2	80%	10%	10%	-	-
Publikacja 3	70%	10%	10%	5%	5%
Publikacja 4	80%	10%	10%	-	-
Publikacja 5	75%	10%	10%	5%	-

Deklaracje i wkład wszystkich pozostałych współautorów publikacji wchodzących w zbiór opublikowanych i powiązanych tematycznie artykułów niniejszej pracy doktorskiej, zostały zamieszczone poniżej.



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Prof. dr hab.
Aleksandra Ziemińska-Buczyńska

Gliwice, 03.03.2026

Deklaracja indywidualnego i merytorycznego wkładu współautora

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Publikacji 1 Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.,
Sewage sludge pretreatment: current status and future prospects: Environmental Science
and Pollution Research, 2023, 30, 88313-88330. IF2022= 5.8; MNiSW2022= 100
wynosił 15% i obejmował:

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- edycję i przygotowanie ostatecznej wersji manuskryptu.

Publikacji 2 Ćwierniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.,
Cellulolytic Fungi from Sewage Sludge and Their Potential to Improve Methane
Fermentation, Biomass Conversion and Biorefinery, 10.1007/s13399-026-07082-1.
IF2026= 4,1; MNiSW2026= 70 wynosił 10% i obejmował:

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Publikacji 3 Ćwierniewicz-Wojciechowska M., Wolny A., Jurczyk S., Cema G.,
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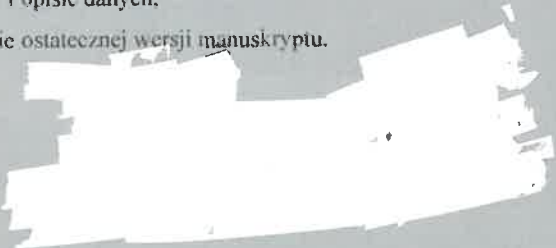
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Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych: Współczesne
problemy ochrony środowiska i energetyki, 2024, 101-110, Gliwice, Politechnika Śląska.
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Publikacji 5 Ćwiertniewicz-Wojciechowska M., Wolny A., ~~Chlebek A.~~, Cema G.,
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Gliwice, 03.03.2026

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Publikacji 1 Ćwiertniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A., Sewage sludge pretreatment: current status and future prospects: Environmental Science and Pollution Research, 2023, 30, 88313-88330. IF₂₀₂₂= 5.8; MNiSW₂₀₂₂= 100 wynosił 15% i obejmował:

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Publikacji 2 Ćwiertniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A., Cellulolytic Fungi from Sewage Sludge and Their Potential to Improve Methane Fermentation, Biomass Conversion and Biorefinery, 10.1007/s13399-026-07082-1. IF₂₀₂₆= 4,1; MNiSW₂₀₂₆= 70 wynosił 10% i obejmował:

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Publikacji 3 Ćwiertniewicz-Wojciechowska M., Wolny A., Juszczak S., Cema G., Ziemińska-Buczyńska A., Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia (w trakcie recenzji) wynosił 10% i obejmował:


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Publikacji 4 Ćwiertniewicz-Wojciechowska M., Cema G., Ziemińska-Buczyńska A.,
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problemy ochrony środowiska i energetyki, 2024, 101-110, Gliwice, Politechnika Śląska.
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Buczyńska A., Comparative Enzymatic Pretreatment of Sewage Sludge:
Physicochemical Parameters and Microbial Community Effects (w trakcie recenzji) 10%
i obejmował:

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- Pomoc w interpretacji i opisie danych literaturowych,
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- Immobilizację enzymów.

Publikacji 5 Ćwiertniewicz-Wojciechowska M., Wolny A., Cema G., Ziemińska-Buczyńska A., Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and Microbial Community Effects, (w trakcie recenzji) wynosił 5% i obejmował:

- Immobilizację enzymów.

Dr

Sebastian Jurczyk

Gliwice, 15.03.2026

Deklaracja indywidualnego wkładu współautora

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Publikacji 3 **Ćwiertniewicz-Wojciechowska M.**, Wolny A., Jurczyk S., Cema G., Ziembińska-Buczyńska A., Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia, (w trakcie recenzji) wynosił 5% i obejmował:

- Przeprowadzenie analiz TGA enzymów immobilizowanych na nośniku.

13. Publikacje

W dalszej części pracy zamieszczono artykuły naukowe, wraz z materiałami uzupełniającymi. Stanowią one cykl publikacji monotematycznych niniejszej rozprawy doktorskiej, w podanej kolejności:

PUBLIKACJA 1: **Ćwierniewicz-Wojciechowska M.**, Cema G., Ziemińska-Buczyńska A., Sewage sludge pretreatment: current status and future prospects: Environmental Science and Pollution Research, 2023, 30, 88313-88330. IF₂₀₂₂= 5.8; MNiSW₂₀₂₂= 100

PUBLIKACJA 2: **Ćwierniewicz-Wojciechowska M.**, Cema G., Ziemińska-Buczyńska A., Cellulolytic Fungi from Sewage Sludge and Their Potential to Improve Methane Fermentation, Biomass Conversion and Biorefinery, 10.1007/s13399-026-07082-1. IF₂₀₂₆= 4,1; MNiSW₂₀₂₆= 70

PUBLIKACJA 3: **Ćwierniewicz-Wojciechowska M.**, Wolny A., Juszczak S., Cema G., Ziemińska-Buczyńska A., Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia, w trakcie recenzji.

PUBLIKACJA 4: **Ćwierniewicz-Wojciechowska M.**, Cema G., Ziemińska-Buczyńska A., Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych: Współczesne problemy ochrony środowiska i energetyki, 2024, 101-110, Gliwice, Politechnika Śląska. ISBN 978-964116-6-2. MNiSW₂₀₂₄= 20

PUBLIKACJA 5: **Ćwierniewicz-Wojciechowska M.**, Wolny A., Cema G., Ziemińska-Buczyńska A., Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and Microbial Community Effects, w trakcie recenzji.



Sewage sludge pretreatment: current status and future prospects

Magdalena Ćwiertniewicz-Wojciechowska¹ · Grzegorz Cema¹ · Aleksandra Ziemińska-Buczyńska¹

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Abstract

Sewage sludge is regarded by wastewater treatment plants as problematic, from a financial and managerial point of view. Thus, a variety of disposal routes are used, but the most popular is methane fermentation. The proportion of macromolecular compounds in sewage sludges varies, and substrates treated in methane fermentation provide different amounts of biogas with various quality and quantity. Depending on the equipment and financial capabilities for methane fermentation, different methods of sewage sludge pretreatment are available. This review presents the challenges associated with the recalcitrant structure of sewage sludge and the presence of process inhibitors. We also examined the diverse methods of sewage sludge pretreatment that increase methane yield. Moreover, in the field of biological sewage sludge treatment, three future study propositions are proposed: improved pretreatment of sewage sludge using biological methods, assess the changes in microbial consortia caused with pretreatment methods, and verification of microbial impact on biomass degradation.

Keywords Anaerobic digestion · Methane fermentation · Hydrolysis · Biogas production · Wastewater · sCOD · Volatile fatty acids · Methane yield

Introduction

Continuously expanded exploitation of fossil fuels is leading to their depletion; hence, exploration into novel sources and methods for the provision of infinite electrical energy and heat inflow, while maintaining comparative usage to conventional sources, is of vital importance. Moreover, the exploitation is not only leading to depletion but also impacts the environment during the sourcing and use. Non-renewable fossil fuels affect climate changes by releasing large amounts of carbon dioxide into the air when are burned. That also forces us to minimize its usage and search for alternative solutions.

In general, human activity creates a vast amount of wastes, which gives rise to potential opportunities in the circular economy. The wastewaters are produced daily in domestic households, industries, and entertainment sectors, to name a few, which are treated in a wastewater treatment

plant. However, a common side product of such treatment is the generation of sewage sludge that has the potential to be useful in biogas production. In recent years, biogas production by anaerobic digestion and its further usage is ever expanding and delving deeper in to unique and interesting areas of science. Figure 1 shows the number of papers published using the following keywords: sewage sludges, pretreatment, and anaerobic digestion.

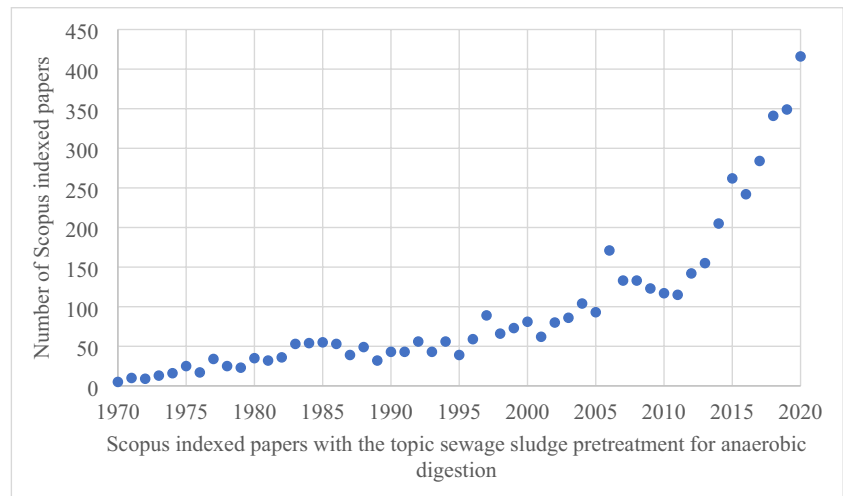
Methane formation is an anaerobic microbial process that decreases the amount of dry and organic matter of substrates, causing stabilization of the properties of the sewage sludge. During this process, biogas is released which contains mainly methane and carbon dioxide at levels of 50–70% and 30–50% (v/v), respectively; however, residuals of hydrogen, hydrogen sulfide, and volatile organics also reside (Shrestha et al. 2020; Czatzkowska et al. 2020). The amount and quality of biogas are determined by the substrate characteristics and the ability of the fermenting microorganisms to conduct the following four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. According to reports, methane production during anaerobic digestion is mostly dependent on the composition of the sewage sludge used in the fermentation process and methods used to treat the biomass (Li et al. 2019; Park et al. 2021; Liu et al. 2021b; Akbay et al.

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Fig. 1 Numbers of research papers dealing with sewage sludge pretreatment for anaerobic digestion published between 1970 and 2022, according to Scopus



2021). A high amount of organic matter influences the efficacy and rate of the anaerobic digestion, thus sludge treatment. Moreover, usually, the sludges produced by the wastewater treatment plants contain considerable quantities of heavy metals, pathogens, and bacteria (Liew et al. 2022). Generally, recalcitrant and low degradable biomass limits the hydrolysis stage, which impacts anaerobic digestion. During this stage, cell walls are destroyed and extracellular polymeric substances (EPSs) are released and degraded by acidogenic microorganisms, which employ EPSs as organic material. This is an important mechanism due to compounds in sewage sludge being considered relatively unfavorable substrates for microbial degradation. This stems from their structure, which protects the cell from osmotic lysis (Appels et al. 2010). EPSs are known as the dewaterability disruptor of sludge that bind with bacterial cell walls or remain in suspension. They prevent desiccation of the bacterial cell and promote water binding, making sludges difficult to dewater. In the case of thermal methods, bonded and intracellular water is more easily released compared to other methods, i.e., chemical method (Pilli et al. 2014). Additionally, depending on the sewage sludge composition and limitations of microorganism consortia, it is difficult to convert all raw materials during hydrolysis and avoid organic matter in post-fermented sludges. Research has shown that hydrolysis is a slow and limiting phase of anaerobic digestion (Li et al. 2019; Junior et al. 2021); therefore, it is important to study over improvement of pretreatment methods or hydrolysis before anaerobic digestion. The utilization of biological, chemical, thermal, or mechanical methods for the disintegration of sludge flocs and the release of simpler compounds increase biogas production (Lindmark et al. 2012; Tsapekos et al. 2015; Shrestha et al. 2017; Liang et al. 2021). Nevertheless, anaerobic digestion is a

popular treatment process due to proven efficiency, reduction of pollutants, stabilization of sludges, and reduction of sludges demanding utilization (Pilli et al. 2014; Patinvo et al. 2016). Thus, it is important to develop biomass pretreatment that will allow more effective usage of biomass and increase biogas production using an eco-friendly method and low financial outlay. The first phase of fermentation determines the effectiveness of the whole process, where the decomposition of macromolecular compounds into monomers and smaller molecules used by microorganisms in the following stages (Ziemiński and Frac, 2021; Neumann et al. 2016). In 1978, Haug et al. conducted the first pretreatment of biomass via thermal methodology for sludge dewaterability and biodegradability under anaerobic conditions. Since this development, numerous publications on this subject have been published, which propose a plethora of combined methods and the utilization of biomass sources. However, even though pretreatment research focusing on hydrolysis improvement has been conducted for the last 40 years, comprehensive verification and testing has yet to be achieved. Nowadays, the majority of studies examine various biomass and comparison of methods in order to enhance methane production, as well as degradation or transformation of hard to decompose compounds. Moreover, pretreatment methods must consider ecological and economic aspects, including the removal of heavy metals and pharmaceuticals. In addition, there are increasing opportunities to recover various useful substances such as volatile fatty acids (VFAs), nutrients (nitrogen, phosphorus, potassium) and enzymes (Ye et al. 2020; Sichler et al. 2021). This review examines sewage sludge pretreatment methods for methane fermentation with view on substances that present limitations on the process, i.e., cellulose, hemicellulose, proteins, and lipids. Furthermore, the advantages and disadvantages of

sludge pretreatment are reviewed and their influence on the methane production yield.

Methane fermentation of sewage sludge

The instantly increasing human population has caused an increase in wastewater and sewage sludge volumes generated post treatment (Duan et al. 2011). In 2010, the world's population was 6.956.823.603, and in 2020, it increased to 7.794.798.739. On November 2022, the population reached 8 billion. According to Population Division of the Department of Economic and Social Affairs United Nations, the number of people will increase over the next few years, which will influence heavily the production of wastes and sewage sludge. According to World Population Prospects (2022), the growth to 8.5 billion in 2030 and 9.7 billion is expected by 2050.

In the last decade, the volumes of sewage sludge production generated in wastewater treatment plants (WWTPs) were increasing continuously, which is caused by the increasing population. Table 1 shows sludge production in chosen European countries between 2010 and 2019 (Eurostat, the statistical office of the European Union). That is why biogas production through anaerobic digestion becomes more interesting.

Factors impacting on the sewage sludge management

In biogas production, various sources of substrates are used depending on availability, geography, optional price and demand, type of active industry in the area, and possibilities for usage. These factors impact methane yield in relation

to microorganisms' potential such as the ability to grow in extreme conditions, incubation temperature, and environmental preferences (Passos et al. 2015). In methane fermentation, easily fermented substrates include livestock manure, food-processing wastes, and sewage sludge (Patinvoh et al. 2016; Córdoba et al. 2018).

Sewage sludges are composed of organic macromolecule compounds, varying in concentration and proportion. Wastewater flowing into WWTP is not only from private households but also from breweries, dairy factories, and paper industries, to name a few (Aski et al. 2020), which dictate the high concentration of lipids, proteins, cellulose and hemicellulose, and other polysaccharides in wastewater and then in sewage sludge. All sewage sludges are produced during wastewater treatment; hence, the composition of the sewage sludge is directly related to the composition of the wastewater that goes directly into the wastewater treatment plant. Thus, it can contain a qualitative and quantitative variety of lipids, pathogens, pharmaceuticals and their derivatives, or heavy metals. Methane fermentation of sewage sludge, other than its positive aspect for sludge stabilization and biogas production, creates wastes. These wastes depend on the degree of fermentation, calorific value, and element content such as phosphorus, potassium, calcium, and heavy metals, which can be managed, burned, or used as fertilizer. Moreover, wastewater treatment plants must contend with the presence of challenging compounds, such as insoluble proteins (about 30%, w/w TS in municipal primary sludge) (e.g., keratin) and polysaccharides (about 26%, w/w TS in municipal primary sludge) (e.g., cellulose) (Glińska et al. 2020). Recalcitrant form and difficulties in degradation of these compounds hinder the large amounts of dry matter and organic matter present in the post-fermented sewage sludge. As with almost every resource, sewage sludges must

Table 1 Total sludge production in Europe (thousand tones), years 2010–2019 (Eurostat, the statistical office of the European Union, 2022)

Country	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Bulgaria	49.80	51.40	59.30	60.30	54.90	57.40	65.80	68.60	53.10	n.a.
Czechia	196.30	217.90	263.30	260.10	238.59	210.24	206.71	223.27	228.22	221.09
Ireland	89.99	85.65	72.43	64.55	53.54	58.39	56.02	58.77	55.23	58.63
Germany	1 893.64	1 946.29	1 848.85	1 808.72	1 830.82	1 820.57	1 794.36	1 785.55	1 761.62	1 749.86
Estonia	18.80	18.30	21.70	17.00	19.91	19.11	18.65	20.94	25.54	24.94
Spain	1 355.10	1 331.60	1 233.40	1 122.60	1 131.60	1 152.60	1 174.40	1 192.00	1 210.40	n.a.
Lithuania	21.39	19.76	20.11	22.82	22.08	21.92	25.92	24.94	24.59	24.18
Hungary	170.34	168.33	160.60	170.47	163.12	177.70	217.96	266.84	233.66	227.89
Austria	262.80	n.a.	266.30	n.a.	239.04	n.a.	237.94	n.a.	234.48	233.56
Poland	526.70	519.20	533.30	540.30	556.00	568.00	568.33	584.45	583.07	574.64
Rumania	82.10	114.10	85.40	172.80	192.33	210.45	240.41	283.34	247.76	230.59
Slovakia	54.76	58.72	58.71	57.43	56.88	56.24	53.05	54.52	55.93	54.83
Finland	142.70	140.90	141.20	95.20	115.70	146.00	146.99	161.19	146.62	n.a.

n.a. data not available

be pretreated to become efficient and safe biomass during and after the process of fermentation. These treatments must be cost-efficient and effective for various types of sewage sludge. Three groups of methods of sludge pretreatment are used, i.e., chemical, physical (including thermal and mechanical), and biological methods. Each provides different advantages and disadvantages in sewage sludge treatment, presented in Table 2. The applied impact is related to the amount of biogas produced and the level of stability of the sewage sludge. According to the literature, the most effective are thermal methods, which provide improvement in methane yield production up to 1000% (Appels et al. 2008; Nguyen et al. 2021), although the costs of energy needed to heat the sludges need to be considered. Mechanical methods provide usually not less than 100% improvement in methane production, and their use in the industry is most common (Yue et al. 2021). Usage of the chemical methods provides around 50% of the better methane production, but they are regarded to be harmful for the environment and sewage sludge itself (Zhang et al. 2023). Biological methods are not as effective as other methods mentioned above; however, they are regarded to be the safest and can be improved with other methods at a relatively low cost from other methods (Wandera et al. 2019; Liang et al. 2021). As mentioned previously, wastewater treatment plants choose pretreatment methods depending on their financial and technological possibilities, but also according to the sludge composition, mainly the presence of hard-to-decompose substances. The main purpose of the anaerobic digestion is to decrease the amount of organic substances present, i.e., in the sewage sludges, so their products could be used further in the future. Without the proper treatment, the large amount of organic matter residues remains, and eventual deposition in the environment may be dangerous for the soil and water. Sewage sludges that are not treated properly can cause odors, environmental pollution due to the recalcitrant leakage, and finally potentially hazardous influence on the human and animal health (Nguyen et al. 2021). Proper pretreatment provides not only the possibility for decreasing sludges volume directed from WWTPs but also their further usage due to stabilization of the sewage sludge properties during the anaerobic digestion, and the production of the biogas is its beneficial feature. The usage of the sewage sludges as a source for the methane production and providing its better composition contributes in a circular economy strategy.

Lignocellulose complex

Cellulose is a troublesome compound, which is a part of a lignocellulose complex that also contains hemicellulose and lignin. The combinative effect of these compounds negatively influences the effectiveness of lignocellulose decomposition. Lignin is regarded as the most recalcitrant

compound to undergo biochemical decomposition (Wagland et al. 2011; Li et al. 2021). Lignocellulose contains ca. 40–60% cellulose and 20–40% hemicellulose; hence, it is a potential carbon source for biogas production (Kang et al. 2014). According to Zhen et al. (2017), the utilization of lignocellulose as a substrate promotes the production of biogas containing approx. 50–70% CH₄ and 25–50% CO₂. Lignocellulose degradation process is mainly associated with bacteria and fungi performance, and isolation of enzymes produced by microorganisms (Zhang et al. 2018). Although lignocellulose is a limiting compound, thus for proper usage, it is necessary to convert it into simpler and more soluble compounds, which requires lignin removal methodology (Shah et al. 2018). Wagland et al. (2011) showed that high temperature is the most efficient way for the decomposition of such biomass. In the case of other approaches, including chemical methods, pretreatment with high energy may assist the degradation process. An alternative for high-temperature pretreatment or assisted temperature pretreatment is the chemical method, where incorporation of acids and bases removes or degrades various compounds. For lignin removal or pretreatment from sewage sludges, the alkaline pretreatment approach is preferred, which mainly utilizes KOH, Ca(OH)₂, and NaOH (Dai et al. 2018). Ethanol, benzene, and ethylene glycol have also been investigated for lignin removal (Taherzadeh and Karimi, 2008). The possibility of biomass fermentation increases in the presence of bases due to lignin degradation (Patinvoh et al. 2016). However, alkaline treatment decreases the degree of polymerization of lignocellulosic biomass compounds, promoting lignin removal, and increases organic matter solubility in sludge and easier cellulose degradation by enzymatic and microbial impact (Liu et al. 2021a). Thus, alkaline treatment method is a promising and efficient way to lignocellulosic biomass decomposition. In the reference to the chemical treatment, the utilization of acids in pretreatment and assisted pretreatment methodology increases the fragility of the biomass in fermentation and promotes intramolecular bond cleavage between lignin, hemicellulose, and cellulose in the cell wall (Taherzadeh and Jeihanipour 2012). The disturbance over chemical construction enables the more efficient operation of cellulolytic enzymes. It is known that the presence of acids contributes to the hydrolysis of celluloses and hemicelluloses but does not dissolve lignin, only contributing to its partial destruction (Solarte-Toro et al. 2019). Fernandes et al. (2012) reported that biomass adapted to 4.9 g NH₄-N*/L and a lack of inhibition of the hydrolysis of cellulose after the addition of ammonia concentration 2.4–7.8 g NH₄-N*/L. Free ammonia addition is known as an effective method to improve anaerobic methane production. Using it as a pretreatment method can enhance the fermentation process but also adapt biomass to handle any ammonia concentration (Wei et al. 2017a).

Table 2 Comparison of biomass pretreatment methods described in the “Methods of sewage sludge treatment” section

Method	Used tools	Advantages	Disadvantages/challenges	Effectiveness	References
Chemical methods	HCl, H ₂ SO ₄ , H ₃ PO ₄ , HNO ₃ , HNO ₂	-Hemicellulose degradation, improvement of proteinaceous biomass treatment	-The expensive process of acids recovery, destroying of devices (corrosion), occurrence of inhibitors	~50%	Dai et al. (2018), Taherzadeh and Jeihanipour (2012), Solarte-Toro et al. (2019), Zhang et al. (2023)
	NaOH, KOH, Ca(OH) ₂ , Mg(OH) ₂ , CaO, and ammonia	-Degradation of the lignin structure, internal surface area increase	-High costs of bases, inhibition of the methanogenesis using inappropriate concentration, occurrence of inhibitors	>100%	Zawieja et al. (2019); Özön and Erdinçler (2019); Liu et al. (2020); Yue et al. (2021); Kazimierowicz et al. (2022)
Physical methods	Mechanical: ultrasonic, microwaves, milling, beating	-Increase of surface area, decrease of biomass size, decrease of biomass recalcitrance, possibility of using heat and electrical energy from biogas production, disruption of chemical bonds in the cell wall and membrane, increase of solubilization of sludge, increase of VS degradation, increase of methane production, reduction of retention time	-High investment and operating costs, consumption of enormous electric energy		
Biological methods	Thermal hydrolysis: steam explosion, 60–270°C, pressure 6–25 bar	-Partial solubilization and disintegration of biological cells, partial removal of micropollutants, high breakdown of the cell structure of sludge, release of intracellular bound water	-Odor generation, high operating costs, occurrence of inhibitors (i.e., ammonia)	Up to 1000%	Myszograj and Pluciennik-Koropeczuk (2023); Kakar et al. (2022); Gahlot et al. (2022); Taboada-Santos et al. (2019); Nguyen et al. (2021)
	Bacteria, fungi, enzymes	-Degradation of lignocellulosic biomass (hemicellulose, cellulose, and lignin), reduction of inhibitors	-A long time of culturing and high cultivating demands, long treatment time, difficulties with process modelling, high costs of enzymes, low solubilization yield	~10%	Agabo-García et al. (2019); dos Santos Ferreira et al. (2020); Kumar and Gopal (2015); Jiang et al. (2022)

Proteins and lipids

Usually, the main part of the sewage sludge organic matter consists of carbohydrates; hence, numerous studies focus on the hydrolysis and decomposition of such compounds. However, in the case of proteinaceous sewage sludge, the amount of protein content exceeds that of carbohydrates. Proteinaceous sewage sludge originates from the dairy industry, and it is characterized as a valuable source for methane production, due to its high VFA content. In some industries, protein content in dairy wastewater is over 40% of total oxygen demand, but the addition of carbohydrate matter can increase protein conversion in the sewage sludge. Moreover, pH influences methane production through the composition of VFA (Liu et al. 2021a). The use of sludges rich in protein sources, such as keratin wastes (e.g., feathers), contains over 90% of crude protein instead, or mixed with sewage sludge may provide higher biogas production. Due to the insolubility of the crude protein, it is necessary to provide access for microorganisms, which allow effective decomposition and conversion into soluble oligomers (Patinvoh et al. 2016). According to Ma et al. (2019), alkaline fermentation leads to efficient VFA enhancement production providing a carbon source for biological nutrient removal and increased degradation of difficult to biodegrade organic matter. Reports have shown that increased VFA concentration contributes to intensification of biogas production (Worwag and Kwarciak-Kozłowska 2019).

The prevalence of proteins in sewage sludge can also inhibit the process. Inhibition of bacterial activity can occur when substrates and products are in significant concentrations. Thus, the properly chosen pretreatment method may be beneficial for the process, from the point of troubleshooting and economy of the treatment. Under such conditions, more VFA is produced, which may lead to inhibition of the process (Shah et al. 2018). When VFA concentration is too high (>3.6 g*/L (Zhang et al. 2001)), inhibition of methanogenesis and hydrolysis/acidogenesis occurs due to organic overloading (Fotidis et al. 2012). Methanogens are not able to remove hydrogen and volatile organic acids faster than they are produced, resulting in this compound accumulation that causes changes in pH and disturbance of the hydrolysis and acidogenesis stages (Ponsá et al. 2008). High concentrations of ammonia can also inhibit methanogenesis, which is commonly present in complex wastes, mainly in animal manures, black water, or waste oil from gastronomy (Belmonte et al. 2011). Shi et al. (2017) observed that the presence of free ammonia (0.045 g*/L) caused an inhibitory effect of methanogenesis and accumulation of VFAs. Belmonte et al. (2011) showed that the concentration of free ammonia above 0.04 g $\text{NH}_3\text{-N}^*/\text{L}$ caused IC_{50} (concentration at which a substance exerts half of its maximal inhibitory effect) of 56–84% for

methanogenic bacteria present in raw swine wastewater and 84–94% for treated swine wastewater.

Methods of sewage sludge treatment

The pretreatment methods of sewage sludges are used to support the process of methane fermentation (Patinvoh et al. 2016; Wluka et al. 2021; Nguyen et al. 2021; Machnicka and Grübel 2023). Methods used for the pretreatment of biomass for biogas production on a large scale are chemical, or physical, including thermal and mechanical. Recently, more attention gains also biological methods, based on microorganisms possessing particular features and enzymes produced by them (Kumar and Gopal 2015; Junior et al. 2021; Nguyen et al. 2021). For now, biological methods find their appliance only in the lab scale. The effectiveness of these methods can be compared only with the impact on the precise substrate (Lee et al. 2010). The potential advantages and disadvantages of the proposed methods are compared in Table 2.

All described methods demand high investment or operating costs, but in the long-term appliance, advantages may be overall the negative aspects. There is no universal pretreatment method for all biomass types; thus, the methods described in Table 2 and the potential of their usage are usually different depending on the composition of biomass sources. Nevertheless, each of the presented provides an increase in methane production and improved sewage sludge stabilizations, which decrease WWTP overall costs.

Chemical methods

Pretreatment using chemical methods commonly employs acids and bases, which decrease recalcitration, and improves decomposition or degradation of biomass. Depending on the composition and origin of the biomass, various solutions of different concentrations are used to increase decomposed solid compounds and methane yield. Unoptimized and unbalanced pretreatment methods can lead to the production of inhibitors, such as furfurals during high thermal acidic pretreatment of the lignocellulosic substrate in order to degrade cellulose and hemicellulose (Chen et al. 2007), or VFA during alkali pretreatment of proteinaceous sludge. From a financial point of view, chemical methods are highly desirable. They are easily modified and result in high methane yield. In most reported cases, chemical pretreatment avoids subjection to thermal conditions. The most commonly used chemicals are HCl, H_2SO_4 , H_3PO_4 , HNO_3 , and HNO_2 for acid pretreatment and NaOH, KOH, $\text{Ca}(\text{OH})_2$, $\text{Mg}(\text{OH})_2$, CaO, and ammonia for alkali pretreatment. Lü et al. (2007) examined alkali pretreatment and determined that using ammonia in protein hydrolysis caused its biodegradation. In the case of lipids, different concentrations of

ammonia displayed varying degrees of influence on easy and hard biodegradable lipids. Wei et al. (2017a) verified the influence of free ammonia on methane production, where the addition of 250 mg $\text{NH}_3\text{-N/L}$ of the free ammonia was effective in enhancing methane fermentation. The greatest improvement of biochemical methane potential and hydrolysis rate was determined for 420–680 mg $\text{NH}_3\text{-N/L}$.

Shah et al. (2018) studied alkali reagents of various concentrations in conjunction with different heating processes, including water bath, autoclaving and short time microwaving. The results showed that the highest delignification, compared to the other methods, was obtained using 2% NaOH and short time microwave heating process, providing biogas yield of 560 mL/g VS.

Wei et al. (2017b) showed that during acid pretreatment, addition of N/L free nitrous acid increased methane production by $16\pm 1\%$ and 16–17% reduction of dewatered sludge for final disposal. According to Zahedi et al. (2016), addition of free nitrous acid (2.49 mg $\text{N-HNO}_2\text{/L}$) and incubation for 5 h increased the solubility of organic matter and methane production up to 25%.

Liu et al. (2021b) reported that pH regulation and chemical pretreatment were achieved using NaCl and NaOH. They determined that at pH = 11.0 the concentration of soluble proteins and VFA was higher than that at the pH = 3.0. Hence, pH modification impacted not only on VFA production but also the percentage of individual VFA. All acids and bases described above are used for sewage sludge treatment in research and industry. Dai et al. (2018) researched chemical and biological pretreatment of rice straw. Pretreatment using cellulase with NaOH, HCl, and $\text{CO(NH}_2)_2$ increased biogas production under anaerobic conditions. HCl dissolved hemicellulose by approx. 12.5–7.1%. After cellulase pretreatment, cellulose degradation was 38.3–10.9%. Pretreatment using 6% NaOH resulted in TS and VS removal of 53.8% and 36.8%, respectively. Song et al. (2014) compared seven chemicals alkali and acid pretreatment methods of various concentrations: H_2SO_4 , HCl, H_2O_2 , and CH_3COOH at concentrations of 1%, 2%, 3%, and 4% (w/w) for acid pretreatment and NaOH, Ca(OH)_2 , and $\text{NH}_3\cdot\text{H}_2\text{O}$ at concentrations of 4%, 6%, 8%, and 10% (w/w) for alkaline pretreatment. The results showed that H_2O_2 and Ca(OH)_2 were the most efficient, giving 216.7 and 206.6 mL of $\text{CH}_4\text{/g VS}$.

Physical methods

The complex structure of sewage sludge compounds prevents effective processing, forcing mechanical, ultrasound, microwave, or explosion treatment before anaerobic digestion. The main goal for pretreatment of sewage sludge is to increase the accessibility of the surface area and pore size, and cell walls break down, allowing microorganisms access to nutrients, thus improving biomass decomposition (Zhen

et al. 2017). Physical pretreatment methods are preferable to treat difficult biomass, such as hard food wastes; agricultural residues from straw, crops, and wood; and forest residues (Rodriguez et al. 2017). Mechanical methods of disintegration reduce particle size due to external stress and pressure, converting compact masses of biomass into more soluble material (Shrestha et al. 2020). The size of particles impacts biogas production, where larger particles result in lower chemical oxygen demand (Nguyen et al. 2021). Ultrasound is considered an effective mechanical method. During ultrasonication, microbubbles that form collapse after reaching the critical size and cavitation occur. This process increased with increasing temperature and pressure, initiating hydro-mechanical shear forces and the presence of reactive radicals ($\text{H}\cdot$ and $\cdot\text{OH}$). The decomposition of sludge flocs and release of intercellular material by physical methods have the hydro-mechanical shear forces and oxidizing influence of radicals, but the greatest impact has hydro-thermal shear forces (Pilli et al. 2011).

Another commonly used method of pretreatment involves microwaves, where the generated heat causes disintegration of compounds via hydrogen bond cleavage due to changes in dipole orientation at the polarized side chains in the cell membranes of macromolecules (Park et al. 2004, Serrano et al. 2016). Microwave is considered a promising pretreatment method and is normally used in crop straw pretreatment (Liu et al. 2021a). It is a rapid heating source, promoting thermal and nonthermal effects, resulting in enhanced biogas production, and it is regarded a supporting method for chemical treatment (Xu, 2015; Zaidi et al. 2019). Elalami et al. (2020) determined that using a single microwave or ultrasound pretreatment was less effective than the combined methods with alkali pretreatment. Moreover, mild microwave or ultrasonic pretreatment did not affect lignin degradation; however, the utilization of NaOH showed a substantial effect. Furthermore, alkali pretreatment reduces lipid content by saponification.

For mechanical pretreatment, various beaters or mills can be employed. Rodriguez et al. (2018) used the Hollander beater for anaerobic digestion of algae *Pelvetia canaliculata*. This resulted in maximum methane production of 283 mL/g VS and increased methane yield up to 43% in reference to non-pretreated algae. The Hollander beater was used also by Tedesco et al. (2014), where after pretreatment at 50°C for 10 min, biogas production increases by 52% and methane yield by 53% compared to the control sample. Rice straw biogas production using biological pretreatment supported by milling was performed by Mustafa et al. (2017). First biomass was treated with fungal strains and then prior into a mill. This combination increased methane production by 165%. It is known that particle size matters. Izumi et al. (2010) verified its effect on anaerobic digestion of food wastes. Bead milling was used and enhanced

methane production by 28%, compared to the non-pretreated sample. Depending on the used biomass, physical method may give different results, so also comparison inside the group of methods is studied. Suresh et al. (2013) showed higher methane production using green algae *Ettlia* sp. as a substrate with the pretreatment under autoclave conditions compared to using sonicated residue and 250 W microwave. Montingelli et al. (2015) verified the influence of microwaves, ball milling, and beating on micro-algae biomass and determined that beating increased methane yield up to 37%, but ball milling and microwave method gave lower methane yield compared to biomass without pretreatment.

Among all pretreatment methods, thermal hydrolytic pretreatment reduces the VS and enhances biogas production (Chen et al. 2018). Thermal hydrolysis is a commonly used pretreatment method in industry for effective sludge treatment (Liang et al. 2021). Such pretreatment methodologies improve dewaterability, solubilization, and liquefaction of organic matter (Pilli et al. 2014). Additionally, thermal hydrolysis decreases particle size and viscosity, which increases access of the microbes and enzymes to the substrate (Chen et al. 2019). Reports have shown that thermal hydrolysis used on a full scale at WWTP increases the net electrical production of biogas by 20% (Tyagi and Lo 2013; Liang et al. 2021). The temperature range of thermal pretreatment is between 60 and 270°C, which categorizes pretreatment into low- (below 100°C) and high-temperature thermal pretreatment (above 100°C). High-temperature thermal pretreatment destroys sewage sludge particles and releases intracellular water, which is also achieved by particle collisions that causes gel structure destruction (Chu et al. 1999). Furthermore, an increase in temperature increases the chemical oxygen demand (Perez-Elvira et al. 2010). An increase in carbohydrate concentration was observed up to 130 °C and then decreases above 165°C. The results were obtained using spectrophotometric techniques by quantifying carbonyl groups (Pilli et al. 2014). The best results in sludge biodegradability, dewaterability, and biogas production (an increase of 40–80%) were obtained using pretreatment in 160 for 30 min and 180°C for 60 min. Low- and high-temperature pretreatment differs in the treatment time which is needed to obtain the same biogas generation. In the case of low-temperature pretreatment, the time period use can be hours or even days, with an increase of 20–50% in biogas production in comparison to samples never treated. The anaerobic pretreatment may be conducted in mesophilic and thermophilic conditions, or of their combination as two-stage anaerobic digestion. Under these conditions, hyperthermophilic chamber is used, followed by a mesophilic digester, causing an increase of solid-state hydrolysis, acidogenesis in thermophilic conditions, and then methanogenesis in a mesophilic environment (Handous et al. 2019). Ruffino et al. (2020) compared temperature-phased

anaerobic digestion (TPAD) regarding conventional anaerobic digestion and showed higher efficiency in volatile solid (VS) reduction and methane generation. Amodeo et al. (2021) studied two different organic fractions of municipal solid wastes in co-digestion with digested sludge and determined an increase of methane production for both substrates at 55°C of solid retention time. Xiao et al. (2018) compared TPAD and food wastes with single-stage digestion in mesophilic and thermophilic anaerobic digestion and determined that lower methane yields were obtained when temperature-phased pretreatment was used. All thermal pretreatment methods influence methane production, enhance VFA generation, solubilization, and soluble chemical oxygen demand (sCOD) (Pilli et al. 2014; Junior et al. 2021). Low-temperature pretreatment increases sCOD, proteins, and sludge solubilization, but at a very low level, compared to high-temperature treatment (Chen et al. 2018). Thermal pretreatment consists of heat energy provided by heat exchangers or steam explosion, generating high pressure and heat. Such pretreatment methods enhance solubilization. The highest methane yield was observed after pretreatment for 15 min at 10 bar and 10 min at 15 bar. Similarly, studies conducted by Lizasoain et al. (2016) confirmed a positive influence of steam explosion regarding reed biomass, in which increased methane yield up to 89% was obtained after 15 min pretreatment at 200°C. A comparative study of steam explosion at 170°C and regular thermal sludge treatment in the autoclave (70, 100, and 125°C) was conducted by Liu et al. (2019). It was found that steam explosion had the potential to pretreat granular aerobic biomass with low methane yield caused by high mineral content. According to reports, the method that considers mineral content may be useful for resource recovery.

Biological methods

Over the past few years, biological method using microorganisms and enzymes under aerobic and anaerobic conditions is a promising biomass pretreatment method. During the process, extensive degradation of lignin conducted by white-rot fungi and their enzymes responsible for the decay was observed (Adney et al. 2009). Fungi are considered to be the most effective microorganisms in biodegradation of lignocellulosic biomass, but other microorganisms have also their part. The influence of microorganisms is highly effective and improves both the pretreatment and fermentation process. Under optimal conditions, microorganisms stop the activity of inhibitors if they are biodegradable, or accumulated it while the concentration of non-biodegradable inhibitors is low. A high concentration of inhibitors leads to microbe death (Chen et al. 2007). Microorganisms can originate from various environments such as soil, manure, compost, food wastes, and sewage sludges (Kumar and

Gopal 2015; Pessuto et al. 2016; Rodriguez et al. 2017; Baba et al. 2017; Zhang et al. 2018). Since microorganisms are already present in the environment, their addition cannot lead to microbial pollution, but the improper number, causing disturbance in the community, may decrease process efficiency. Similarly, as with chemical and physical pretreatment, biological methods may provide positive or negative biogas production effects. Microbial consortia during biogas production undergo various processes, which depend on the accessibility of nutrients and proper process conditions. Any disturbance in the microbial balance or presence of inhibitors may stop the biogas production process, or change its composition. As mentioned, microorganisms can take part in substrate treatment under aerobic or anaerobic conditions. Aerobic pretreatment consists of oxygenation, through the injection of oxygen into the pre-fermentation reactor, which improves the hydrolysis of macromolecular organic compounds by increasing the autochthonous microorganisms. Oxygenation, also called micro-aeration pretreatment, not only impacts microbial activity and diversity, causing hydrolysis increase, but also methane production (Nguyen and Khanal 2018). Micro-aeration stabilizes the liberation of exoenzymes, which biodegrade compounds that remain recalcitrant under anaerobic conditions (Ahn et al. 2014). Supporting impact on organic compound treatment in the aerobic pretreatment has a high temperature, which stimulates microbial consortia to produce enzymes (i.e., proteases) responsible for improving biomass solubility and organic compound degradation (Neumann et al. 2016). Nguyen et al. (2019) suggested that such pretreatment method could be used as an effective control strategy when the organic loading rate was high, thus, confirming rapid VFA conversion, followed by methane production, conducted by facultative anaerobic microorganisms and hydrogenotrophic methanogens. Using limited micro-aeration in to corn straw anaerobic digestion produces the following maximum results: 216.8 mL/g VS of methane yield and 54.3% removal of VS (Fu et al. 2016). Fu et al. (2016) showed changes in the microbial community structure and improvement of specific methanogenic activity after micro-aeration. Under micro-aerobic conditions, increased numbers of phylum *Firmicutes*, class *Clostridia*, and order *Clostridiales* were observed (Mustapha et al. 2018). As mentioned, micro-aeration at high temperature generates positive results and high potential, but research (Table 3; Ding et al. 2017; Liang et al. 2021) confirms high methane yield when using only high temperature and microorganisms. Biological pretreatment can proceed by the aforementioned methods described, using inoculation of the biomass with microorganisms, their activity products (isolated enzymes), or commercially available enzymes. According to reports, the basic assumption is that microorganisms present in the substrate use available compounds as food, allowing the use of the biodegradable

products by other microorganisms, which undergo the following stages. The addition of extra microorganisms can disturb the balance in the microbial community of sewage sludge but also improve the process. The influence of microorganism inoculation is noticeable when using autochthonous microorganisms according to Pessuto et al. (2016) for anaerobic digestion of swine manure, where an increase in biogas and methane production was determined.

Supporting the pretreatment with enzymes has gained lot of interest. The addition of hydrolytic enzymes provides enhanced hydrolysis and increases biogas production, sludge solubilization, and degradation of the extracellular polymeric substances (EPSs) (Yin et al. 2016). Enzymes may be an alternative for thermochemical methods of hydrolysis, diminishing overall costs, as the enzymes may be produced by a wide range of bacteria and fungi (Miao et al. 2013; Mahdy et al. 2014).

There are 4 ways of enzyme addition methods according to Brémond et al. (2018), which rely on (1) addition to a dedicated pretreatment vessel, (2) direct addition to the hydrolysis and acidification vessel over a two-stage process, (3) direct addition to the digester in a single-stage process, and (4) addition to the recirculated anaerobic digestion leachate. Depending on the substrate, the composition and parameters of enzymes used should be optimized, such as isoelectric point, specificity, activity, and temperature (Divya et al. 2015). The research conducted by Zhao et al. (2018) compared chemical and biological pretreatment using enzymes, fungi, and alkali (NaOH) and combined NaOH with both fungi and enzymes. The combination of the two enzymes gave methane yield of approx. 276.16 and 273.75 mL/g TS, respectively. The presence of NaOH gave the same result as the first obtained during enzyme pretreatment, confirming that chemical pretreatment could be replaced by a safer method. NaOH combined with one enzyme pretreatment method increased methane production to over 20.24% compared to the control, but fungal treatment decreased biogas production and inhibited the process. Enzymes were used also by Moon and Song (2011), where hydrolysis reaction was conducted for 10 h. A mixture of enzymes (carbohydrase, protease, and lipase in ratio 1:2:1) was examined, generating 0.35 L CH₄/gCOD. Biological methods have already been studied, but compared to chemical or physical pretreatment, they seem to be equally popular.

Biological methods using enzymes are a promising approach, but their price is high, and the time of producing enzymes by microorganisms is long. Hence, the production and subsequent recovery of enzymes are reasonable from the economical point of view (Marín et al. 2018). Cellulases were extracted from the substrate, which was further tested for methane production. The authors obtained 552±66, 543±47, and 663±40 mL biogas/g VS for apple pomace, orange peel, and rice fiber after 25 days, respectively, confirming the positive

Table 3 Effect of soluble oxygen demand and VFA concentration and methane yield depending on the pretreatment method

Methods type	Biomass	Methods of treatment	Time of treatment	Temperature	sCOD before pretreatment	sCOD after pretreatment	VFA before pretreatment	VFA after pretreatment	Biogas yield, % (calculated)	References
Chemical methods	Sewage sludge	0.2 g β -cyclodextrin/g VS	8 days	35 $\pm$ 2 °C	53.7 $\pm$ 6 mg/L	-	220 mg COD/L	4200 g/g VS/g β -cyclodextrin	β -cyclodextrin inhibited methanogens activity (methane production decreased by 27.9%)	Yang et al. (2012)
	Waste-activated sludge	pH= 10	10 min	60 °C	2071 mg/L	~14019 mg/L	-	-	Methane production increased by 43.61%	Tulun and Bilgin, (2019)
		pH= 5	15 min	40 °C	-	9403 mg/L	-	-	Methane production increased by 12.34%	
	Sewage sludge + seeding sludge	pH= 10	7 days	27–34 °C	940 mg/L	13390 mg/L	60.2 mg COD/L	2145 mg COD/L	Methane production increased by 51.94%	Presti et al. (2021)
Physical methods/mechanical methods		pH= 10	8 days	37 °C	124.7 $\pm$ 14.34 mg/L; 398 $\pm$ 31.25 mg/L	6448 $\pm$ 72.37 mg/L	722.3 $\pm$ 34.21 mg COD/L	3762 $\pm$ 204.86 mg COD/L	Lack of production	Ma et al. (2019)
	Waste-activated sludge	Ultrasonic density (1.0 kW/L) + pH= 10	72 h	20 $\pm$ 1 °C	-	-	1275 mg COD/L	3109.8 mg COD/L	-	Yan et al. (2010)
		Thermal pretreatment	1 h	170 °C	-	-	-	8340 mg COD/L	Biogas production increased by 67.8%	Qiao et al. (2011)
	Sewage sludge (1 g VS substrate/g VS inoculum)	Thermal pretreatment	15 min	120 °C	17020 $\pm$ 180 mg/L	19680 + 540 mg/L	1800–2200 mg COD/L	2200–2600 mg COD/L	-	Iglesias-Iglesias et al. (2019)
	Primary sludge	Steam explosion (10 bar)	15 min	280 °C	Increase of sCOD by 53%	-	-	-	Methane production increased by 224% (380 mL CH ₄ /g VS)	Aski et al. (2020)
		Steam explosion (15 bar)	10min	-	Increase of sCOD by 57%	-	-	-	Methane production increased by 205% (358 mL CH ₄ /g VS)	
	Waste-activated sludge	Steam explosion (10 bar)	15 min	-	Increase of sCOD by 52%	-	-	-	Methane production increased by 169% (315 mL CH ₄ /g VS)	
		Steam explosion (15 bar)	10min	-	Increase of sCOD 54%	-	-	-	Methane production increased by 185% (334 mL CH ₄ /g VS)	
	Sewage sludge	Ultrasounds (27 kHz, 200 W/L)	2.5 min	20 °C	620 mg/L	2100 mg/L	-	-	Methane production increased by 151% (8.09 mL CH ₄ /g sCOD)	Grönroos et al. (2005)
			10 min	60 °C	-	4200 mg/L	-	-	-	

Table 3 (continued)

Methods type	Biomass	Methods of treatment	Time of treatment	Temperature	sCOD before pretreatment	sCOD after pretreatment	VFA before pretreatment	VFA after pretreatment	Biogas yield, % (calculated)	References
Physical method/thermal method	Thickened sludge	Low-temperature thermal treatment	15 min	70 °C	400 mg/L	650 mg/L	75 mg COD/L	111 mg COD/L	Lack of significant increase of methane production	Appels et al. (2010)
			30			800 mg/L		618 mg COD/L		
			60			1150 mg/L		1071 mg COD/L		
			15	80 °C		1350 mg/L		988 mg COD/L		
			30			2050 mg/L		1177 mg COD/L	Methane production increased by 37% (48.02 mL/g ODS)	
			60			8200 mg/L		1277 mg COD/L	Methane production increased by 117% (75.64 mL/g ODS)	
			15	90 °C		1600 mg/L		1733 mg COD/L	Methane production increased by 120% (76.69 mL/g ODS)	
			30			7200 mg/L		1963 mg COD/L	Methane production increased by 307% (141.94 mL/g ODS)	
			60			10250 mg/L		2744 mg COD/L	Methane production increased by 984% (377.56 mL/g ODS)	
	Waste-activated sludge	Low-temperature thermal treatment + alkali pretreatment (NaOH/Ca(OH) ₂ =1:4)	6	80 °C	765 mg/L	-	225.4 mg COD/L	~700 mg COD/L	Methane production increased by 129.1%	Zou et al. (2020)
								~700 mg COD/L	Methane production increased by 146.1%	
								~700 mg COD/L	Methane production increased by 154.6%	
		Low-temperature thermal treatment + alkali pretreatment (NaOH/Ca(OH) ₂ =1:1)						~730 mg COD/L	Methane production increased by 163.8%	Zheng et al. (2021)
								~670 mg COD/L	Methane production increased by 171.7%	
								-	Methane production increased by 31% (117.5 mL/g VS)	
								-	Methane production increased by 75% (156.4 mL/g VS)	

Table 3 (continued)

Methods type	Biomass	Methods of treatment	Time of treatment	Temperature	sCOD before pretreatment	sCOD after pretreatment	VFA before pretreatment	VFA after pretreatment	Biogas yield, % (calculated)	References
Biological methods	Raw sewage sludge	Microbial electrochemical system	6 days	35 ± 2 °C	Increase of sCOD by 31%	-	-	-	Methane production increased by 47 and 56%	Vu and Min, (2019)
	Secondary sludge	Biological hydrolysis	6 days	35 °C	-	-	24.5 mg/L	666.5 mg/L	~95 mL/h	Chen and Chang, (2018)
				42 °C	-	-	-	918.4 mg/L	Biogas production increased by 5% with increase of the temp. (~100 mL/h)	
				55 °C	-	-	-	1398.5 mg/L	Biogas production increased by 10% with increase of the temp. (~ 105 mL/h)	
	Thickened sludge	Hydrolytic microorganisms + thermal treatment	6 days	35 °C	175.2 ± 38.2 mg/L	3314.5 ± 683.4 mg/L	100 mg/L	1021 ± 155.2 mg/L	Very low methane yield, decreasing with increase of temperature	Ding et al. (2017)
42 °C				-	2857 ± 193.5 mg/L	-	1362.5 ± 165.2 mg/L	-		
55 °C				-	4518.5 ± 190.6 mg/L	-	1423 ± 117.4 mg/L	-		
Thickened sludge	Hyperthermophilic hydrolysis + acetogenic microorganisms	5 days	70 °C -> 35 °C	7100 ± 1400 mg/L	43600 ± 17500 mg/L	1920 ± 400 mg COD/L	57000 ± 9000 mg COD/L	Methane production increased by 462% (152 mL CH4/g VS)	Mahdy et al. (2019)	
			50±1 °C	244.21 ± 12.65 mg/L	7850.96 mg/L	190.85 ± 1.93 mg COD/L	5699.44 mg COD/L	-	Xiong et al. (2012)	
			70 °C -> 37 °C	8600 ± 2000 mg/L	12000 ± 2000 mg/L	-	2567 ± 440 mg COD/L	Methane production increased by 375% (176 mL/g VS)	Wandera et al. (2019)	
Waste-activated sludge	Thermal hydrolysis, fungal fermentation (<i>Aspergillus niger</i>)	3 h (1 h each temp.)	140 °C	-	-	-	-	Methane production increased by 7% (302.9 ± 19.9 mL biogas/g COD)	Liang et al. (2021)	
			160 °C	-	-	-	-	Methane production increased by 13% (312.3 ± 21.2 mL biogas/g COD)		
			180 °C	-	-	-	-	Methane production increased by 5% (298.1 ± 11.0 mL biogas/g COD)		

sCOD soluble chemical oxygen demand, VFA volatile fatty acids

influence of enzymes. The authors tested recovered enzymes, which determined their potential reusability. Table 3 shows examples of pretreatment methods, the concentration of VFAs, sCOD, and methane yield, depending on the used biomass and total and volatile solids.

Pretreatment methods are useful not only for increasing biomass fragility but also for heavy metals or pharmaceutical removal from the sludges to the liquid phase; such methods include steam explosion pretreatment. This approach employs high pressure and heat causing break down of sludge structures and contained compounds. The steam explosion eliminates the need for chemical usage; hence, it can be considered as an eco-friendly pretreatment method (Aski et al. 2020). Aski et al. (2020) confirmed that this method allowed for pharmaceutical and heavy metal removal, such as ibuprofen (in 65 and 69%), acetaminophen (in 66 and 70%), amoxicillin (in 66 and 70%), lead (in 78 and 70%), and cadmium (in 79%) removal from primary sludge and waste-activated sludge by facilitating the leaching them out into the liquid phase. Physical pretreatment methods can be applied to sewage sludge pretreatment, as well as reduction of dangerous impact of pharmaceuticals, antibiotics, and heavy metals. Zhang et al. (2019) described that an influencing factor that reduces antibiotic resistance genes (ARGs) was present in chicken manure, when supported with activated carbon. The authors noted that ARG removal rate was obtained 87–95% in a digester with microwave pretreatment supported with activated carbon and 34–58% in the digester with substrate only microwaved. In other studies, Bao et al. (2020) showed an increase of VFA and sCOD concentration after ultrasound-alkali pretreatment of waste-activated sludge and methane yield of approx. $97 \pm 1.85\%$ in reference to the control reactor. The research was conducted using a combined process of microbial electrolysis cell and anaerobic digestion. The obtained results suggest that such pretreatment not only supports the degradation of organic compounds and increases biogas production but also supports VFA recovery that becomes precursors for the production of higher-value biofuel and biochemical generation.

Currently, the methane produced in anaerobic digestion is used by wastewater treatment plants for its purposes, to provide heat and energy for WWTPs' infrastructure, or it is simply burned. Future perspective of using biogas as green energy source for larger group of consumers should be considered. Improved production of biogas may fulfil the energy demand with environmentally friendly replacement for fossil fuels.

Conclusion

Sludges for fermentation may contain inhibitors, indigestible, and hard to degrade compounds, but their presence should not determine the low effectiveness of the process.

An efficient process may be possible with proper supporting methods, providing effective treatment of substrates, with high energy benefits. One of the critical aspects is high methane potential of the substrate, which is determined only after all possible pretreatment methods are tested, because certain methods may not impact, increase, or decrease biogas and methane production. All described methods improve hydrolysis and methane yield, but the best effects are obtained using mechanical methods. Such methods are expensive and require high energy contribution, which, in the circular economy, can be returned in biogas production. Chemical methods may be less effective when used separately, can cause the production of inhibitors, and destroy equipment and large investment and exploitation costs. Biological methods produce comparative methane yields to other pretreatment methods but may not be yet considered as an alternative method for chemical or physical pretreatment on a large scale. Moreover, they are costly and cannot be easily transformed from a lab scale into the industry. All methods provide higher efficiency when they are used in combination with the other techniques, from a different category. As methane fermentation is a biological process, any change may cause microbial consortia balance disturbance; thus, it should be obligatory to verify the condition of the biomass activity before and after pretreatment, revealing the pretreatment effect. Both, the influence of pretreatment on microorganisms consortium present in the sewage sludges, and the influence of the microorganisms on the biodegradability of sludge and composition changes. Additionally, the analysis for determining hydrolysis effectiveness should be systematized, which will allow for proper comparison of possible methods. The greatest challenge for sustainable and efficient anaerobic digestion is possessing energy and economic balance, when considering ecological aspects. Moreover, very often efficient pretreatment methods are expensive when tested and used already in lab scale; therefore, they may not be attractive for industries. Potential influence on the pretreated substrate environment should be considered and the possibility of recovery compounds, substrates, or as circular economy attitude. For future studies, more attention should be given to the improvement of biological pretreatment methods without using chemical supported methods, as well as conducting additional recovery of used materials for a more eco-friendly approach.

Figures and tables of this word can be found in online version of the paper

Availability of data and materials Not applicable

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Declarations

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Cellulolytic fungi from sewage sludge and their potential to improve methane fermentation

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Abstract

Fungi possess strong cellulolytic abilities, producing enzymes that can biodegrade complex compounds like cellulose, lignin, and hemicellulose. These abilities are valuable in industries, particularly those generating large amounts of waste, such as wastewater treatment plants, where hard-to-degrade compounds hinder efficient waste processing. Inoculation of the sewage sludge with autochthonous cellulolytic fungi may improve the hydrolysis and consequently methane yield, produced during anaerobic digestion. The usage of autochthonous fungi is dictated by its easier implementation not only at lab, but also at larger, industrial scale as a natural element of the substrate used. Moreover, such microorganisms usage will be not only beneficial for the process efficacy, but also safer for total community integrity regardless to scale. For the inoculation of sludge, twelve fungi strains with cellulolytic abilities were isolated, with seven capable of degrading cellulose under both aerobic and anaerobic conditions. Five strains, which showed the highest and similar to each other biodegradation effectivity in the pure cellulose degradation test were identified as *Aspergillus terreus* (3 strains) and *Aspergillus fumigatus* (2 strains). All isolated anaerobic fungi have been confirmed to produce Carboxymethyl Cellulase Assay for endo- β -1,4-glucanase (CMCase) and Filter Paper Assay for saccharifying cellulase (FPU Assay/FPase). The highest FPase (0.37 FPU/mL) was provided by G11 (*A. fumigatus*), and CMCase (1.49 U/mL) by G9 (*A. terreus*). The most stable degradation of the pure cellulose in anaerobic conditions and temperature of 37 °C in a 30-day test was provided by *Aspergillus terreus* (G3) (8.57%). Utilizing *Aspergillus terreus* in the Automatic Methane Potential Test System (AMPTS) resulted in an 11% increase in methane yield compared to untreated sludge, indicating enhanced degradation of organic matter during the anaerobic digestion. Methane yield in inoculated sludge was 164.2 N mL CH₄/g VS, whereas control samples showed 146.63 N mL CH₄/g VS. These findings highlight the potential of cellulolytic fungi to improve methane production from waste substrates.

Keywords Cellulose · Anaerobic digestion · Biomethane production · Cellulases · *Aspergillus terreus* · *Aspergillus fumigatus*

1 Introduction

Sewage sludge is a type of biomass with high potential as a substrate for energy production in biorefinery systems of wastewater treatment plants [1]. It shows great prospects for biogas production in the methane fermentation process, which is a four-stage process: hydrolysis, acidogenesis,

acetate phase and methanogenesis. All four phases are linked together, as products of one stage are used during the following stage [2]. Thus, the effectiveness of methane production mainly depends on hydrolysis, where the most difficult compounds are decomposed, and the effectiveness of this process influences substrate availability for further reactions [3]. The high level of hard-to-decompose substances in wastewater and sewage sludges makes this biomass relatively difficult to use for biogas production. Moreover, hard-to-decompose sludges cannot be used further, for composting or fertilizing, causing the necessity for its burning. Thus, a large number of complex organic compounds generates high costs and technical difficulties during wastewater and sewage sludge treatment, challenging

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researchers and technologists in the way of improving its treatment and methane production [4].

The most difficult to degrade in sewage sludges are lignocellulose complexes and their individual components i.e., cellulose, hemicellulose and lignin. According to the literature, cellulose concentration is about 200 mg/L in municipal wastewater, based on the amount of wastewater flowing into the wastewater treatment plant (WWTP) [5, 6]. In consequence, sewage sludges contain about 10–30% cellulose based on suspended solids [7]. On the other hand, renewable lignocellulose complex is considered as a great source for biogas production [8]. It is economical and carbon-neutral, and its composition provide valuable products for biofuels production and bio-sourced compounds [9, 10]. Thus, searching for methods to improved lignocellulosic biomass degradation, gives a possibility for its usage not only for decrease of organic matter in digested sewage sludges, but also for improved biofuels production.

There are different methods to manage the cross-linked structure of the lignocellulose complex, i.e. chemical, physical or biological techniques of pretreatment of the sludges, which provide degradation of the sludge components and improvement of the quality of the sludge [11–14]. All of them have their advantages and disadvantages, thus depending on the cost and equipment possibilities WWTPs decide on different (single or combined) solutions [12]. Chemical methods using acids (i.e. HCl, H₃PO₄, HNO₃, HNO₂), bases (NaOH, KOH, Ca(OH)₂, Mg(OH)₂, CaO, NH₃) or ionic liquids gets most research interest due to their effectiveness in biodegradation of complex matter [15, 16]. For the lignocellulosic complex, acids provide degradation of cellulose and hemicellulose, without any particular effect on lignin. Thus, degradation only of freely present environment cellulose can be received. Alkalis have a decomposition effect on lignin and in smaller part on hemicellulose, thus facilitating the release of cellulose from the lignocellulosic complex. For the complex degradation, usually combined methods are used with for example thermal-chemical treatment [17]. Although, despite high biomass degradational level, they are dangerous to the environment and equipment, and their presence can inhibit methanogenesis [18]. More secure arrangements of chemical methods may be ionic liquids, depicted as green chemistry During its generation, there are little or no substances, that are harmful to humans and the environment. Using ionic fluids for hydrolysis gives non-covalent interactions between the lignocellulosic components without its corruption. Expanded availability to the cellulose, lignin and hemicellulose can be at that point influenced by the cellulolytic enzymes. Essentially to the acids or bases, ionic fluids must be recuperated after the hydrolysis handle, since indeed little sums of them may become a chance within the improved utilize of hydrolyzed biomass

i.e. through inhibition of methanogens [19]. The mechanical pretreatment using physical and thermal pretreatment methods (i.e. microwaves, milling, griding, steam explosion) is considered as the best choice for the industrial appliance, providing high degradation level of organic matter, including lignocellulosic and cellulosic biomass [20]. Moreover, the mechanical pretreatment directly impact on the increase of methane production improvement, as implementation of physical methods provides disintegration of the lignocellulosic complex as a result of increased accessibility to the surface area. Breaking the bonds of the complex allows the microorganisms and the enzymes they produce to more easily access the individual components [21]. Yet those methods usually generates high investment and operating costs. Latest trends occur to use environmental friendly options, which can be biological methods using microorganisms and enzymes which they produce have become more popular [22, 23]. Their potential relates to the non-harmful impact of this method on the equipment and environment. Moreover, biological methods are considered to be equally efficient as chemical or physical methods. In some cases, biological pretreatment may outperform physical and chemical pretreatment [14]. In the case of lignocellulosic biomass, which is considered a limiting factor during anaerobic digestion, biological methods provide effective de-lignification of the lignocellulosic materials. Pretreatment allows to expose carbohydrates for anaerobic digestion and increases the access to cellulose [8]. The greatest disadvantage of biological pretreatment is a longer period of sludge treatment before anaerobic digestion, which makes this type of method uneconomic for the industry [24]. Pretreatment time significantly increases the time of the entire fermentation, making it an inefficient and unprofitable form for the industry. Moreover, conducting additional hydrolysis in a separate tank may be conceptually convenient, but it should be remembered, that the anaerobic digestion is a simultaneous process and de-lignification products are converted by other microorganisms and/or enzymes [25]. Pretreating the sludge before anaerobic digestion may also lead to the loss of fermentable carbohydrates as a result of its digestion by microorganisms [26]. More efficient could be enriching the community working in the inoculum with specialized microorganisms and remodeling the current community by creating an environment with microorganisms effectively degrading lignin in the lignocellulose complex.

The biological methods for lignocellulosic biomass pretreatment use bacteria, fungi or enzymes that they produces [27, 28]. Between bacteria and fungi, the latter mentioned have great capability in the hydrolysis of sewage sludges, due to their ability to produce a variety of enzymes and thus degrade complex and problematic compounds, i.e., cellulose, hemicellulose and lignin [29, 30]. Among all

microorganisms, fungi are the major cellulose or lignin degrading microorganisms, converting cellulose and lignocellulose biomass [31]. Its addition to the sewage sludge can significantly improve cellulose degradation, as according to the literature, there are not many cellulolytic microorganisms in sewage sludge, because that carbohydrate is not the main component of the mixture [32]. Lignocellulose complex degradation by fungi is conducted using different mechanisms, depending if those organisms are brown, white or soft rot fungi [33]. The recalcitrant structure resistant to degradation (e.g. lignocellulosic materials) or complex macromolecules hard to degrade, limits the methane production during an anaerobic digestion, when conducting the process without any pretreatment. Hard for depolymerization structure of lignocellulosic biomass is caused with presence of hemicellulose and lignin protective barrier around the cellulose, but also with the crystalline structure of cellulose. The lignocellulose complex can be degraded by enzymes produced by many aerobic and anaerobic microbes to monosaccharides, first by the removal of lignin to provide an access to hemicellulose and cellulose and their further degradation [30]. An effective break down of lignin in agricultural waste are considered white and brown rot fungi of class *Basidiomycetes*, and other fungi i.e. *Aspergillus terreus*, *Trichoderma reesei*, *Phanerochaete chrysosporium*, *Arillaria* sp. or *Polyporus* sp [34–36]. Fungi are known for breaking down organic matters during an anaerobic digestion, providing metabolic precursors (such as H_2 and CO_2) and formate for methanogenesis and increasing efficiency of the system [37]. Different species of *Aspergillus* are able to produce various types of hydrolases that improves hydrolysis of lignocellulosic substrates during the anaerobic digestion [38]. Moreover, fungi are more resistant for the temperature changes and ammonium concentration in comparison to the bacterial community, what can protect the process from limitation of hydrolysis or inhibition of methanogenesis resulted with accumulation of VFA [37]. It is predicted, that *Penicillium* and *Aspergillus* can use polysaccharides as substrates to produce VFAs, and further degrade fatty acids and lipids during natural food fermentation, resulting in improved fermentation in case of controlled fatty acids accumulation [26, 39, 40]. VFAs are intermediate metabolites produced during acidogenesis and acetogenesis, as a result of microbial conversion of organic matter. Those acetic, propionic and butyric acids can be potentially used by fungi as a carbon source for their cultivation [41]. The improved degradation of organic matter directly impact on the methane production. Other biological methods uses microbial consortia, enzymes and co-digestion [42]. An enzymatic hydrolysis significantly increases a methane production (15–35% increase) and degradation of resistant organic matter [43]. Biological methods gain more attention

also due to the fact that they can be specifically oriented for the enhancing methane production and degradation of particular matter, i.e. due to precisely chosen enzymes [44]. Bacterial degradation of lignocellulose complex in environment is usually simultaneous with fungi. Bacteria can degrade lignocellulose on their own but the process is very slow. Microbial consortia and mixed cultures among cellulolytic organisms can significantly improve conducted processes. Bacterial cooperation with fungi, provides essential vitamins and growth-promoters to plant-decaying fungi, or enhance of mycelial growth as a result of atmospheric nitrogen fixing by some plant-inhabiting bacteria [33]. An addition of external precisely prepared organisms for biological treatment can be replaced with inoculation of the biomass with wastes during co-fermentation, providing high degradation of organic compounds and an increase in methane yield [45]. The disadvantage of the using biological method despite its great potential in the methane production and degradation of organic matter is upscaling. The isolation and incubation of the bacteria or fungi is a long term process which should be conducted in specially prepared conditions, with the provision of a suitable culture medium, what generate costs among the culturing. Moreover, addition of the organisms into the sludge demand proper preparation i.e. removing the metabolites from the medium suspending the microorganisms in the liquid for the inoculation. Biological treatment using enzymes is a extremely expensive and also demand long time for the production of demanded amount of them. An alternative is simultaneous production of the enzymes, what requires additional equipment and costs for the investment and operating costs [44]. It is even considered as not suitable for upscaling [46], yet combining multiple fungal species or co-cultivation with other microorganisms can help in scaling up [47].

Cellulose is a polymer resistant to degradation and its decomposition needs to be performed in several steps. Total enzymatic degradation of cellulose is possible with the presence of the three enzymes: 1,4-D-glucan-4-glucanohydrolase or endoglucanase, 1,4-D-glucan-glucohydrolase or exoglucanase and D-glucoside-glucohydrolase or β -glucosidase [48]. Fungi, which can be isolated as pure cultures, produces those enzymes effectively and are present in sewage sludges, belong usually to *Penicillium*, *Trichoderma* and *Aspergillus* genera [49]. The presented study aimed to isolate anaerobic cellulose-degrading fungi and verify their ability to improve hydrolysis and methane production from sewage sludges. As cellulose causes such difficulties in management, its degradation has remained a subject of intense research and implementation of applications, which will help to decrease the volume of problematic sludges [50]. Cellulose paper materials (as filter paper) are not as difficult to degrade as lignocellulosic plant-based materials present

in sewage sludges, because there are no lignin bonds which protect the complex [24]. However, the free form of cellulose is also present in sewage sludges, and it is reasonable to use the fungus with the highest efficiency in pure cellulose degradation, for verification of its influence on methane production. Moreover, methane fermentation of sewage sludges is a microbiological process during which different bacteria and fungi work together to degrade various compounds, thus the addition of the fungus specialized in cellulose degradation may not only improve the hydrolysis of cellulose but support the work of other microorganisms present in the tested sludge. On the other hand, indigenous microorganisms live with other bacteria and fungi in equilibrium. Any disturbance in the harmony may limit or stop the process as the composition of the consortium changes after inoculation [22]. Isolated fungi are *A. terreus* and *A. fumigatus*. Both are aerobic organisms but were isolated from the sludge already working in an anaerobic chamber. Further verification of their abilities provided effective degradation of cellulose in anaerobic conditions. Due to that, we decided that they can be able to degrade multimolecular compounds like cellulose for a short time after inoculation. The anaerobic environment during methane fermentation may be helpful. Both used fungi are potentially hazardous to human health, yet their presence in the beginning can improve anaerobic digestion. Due to anaerobic conditions, later those fungi and their spores cannot effectively work and exist in such conditions, yet it is not certain that they won't be present in the soil after redistribution of the sludges to the environment [51]. The proposed pretreatment of the sewage sludge during anaerobic digestion may help increase the hydrolysis of the sewage sludge, methane production and degradation level of cellulose, using potentially dangerous fungi, which may be neutralized in the process itself.

2 Materials and methods

2.1 Isolation and culturing of anaerobic cellulolytic fungi

The fungi were isolated from sewage sludge (WWTP Gliwice) on Potato Dextrose Agar (PDA P-0134) and Malt Extract Agar (MEA P-0415) (BTL, Poland) medium with 30 mg/L chloramphenicol without dilution and 10^{-1} – 10^{-6} . All plates were duplicated and incubated both in anaerobic and aerobic conditions at 37 °C for 7 days. After that time, isolation of pure cultures using PDA and MEA medium was performed. The transferring onto new medium was performed every 3 days. Pure cultures were transferred and incubated in 37 °C for 7 days onto carboxymethylcellulose (CMC) medium with Congo Red (carboxymethylcellulose

sodium salt 5 g/L, NaNO₃ 1 g/L, K₂HPO₄ 1 g/L, KCl 1 g/L, MgSO₄ 0.5 g/L, yeast extract 0.5 mg/L, glucose 1 mg/L, agar 17 g/L, Congo Red 1 g/L) to determine cellulolytic potential. To provide anaerobic conditions, fungi were isolated and cultured using a Bactronez-2 Anaerobic chamber (Sheldon Manufacturing, USA). The composition of Anaerobic Mixed Gas for Bactronez-2 was 5% H₂, 5% CO₂, and 90% N₂, according to the manufacturer's recommendations. The humidity during the incubation was 40–50%. Cellulolytic fungi able to degrade cellulose in aerobic and anaerobic conditions were transferred into liquid CMC medium (carboxymethylcellulose sodium salt 5 g/L, NaNO₃ 1 g/L, K₂HPO₄ 1 g/L, KCl 1 g/L, MgSO₄ 0.5 g/L, yeast extract 0.5 mg/L, glucose 1 mg/L) and incubated in 37 °C. The mycelial culture floating on the surface of the liquid cultures was separated from the medium with centrifugation (4000 rpm for 10 min). The sediment as mycelium sediment was weighed and used for the determination of methane production. The weight of each mycelium sediment replicate was 0.2 ± 0.01 g. The OD₆₀₀ was measured using a UV-VIS UV5100 spectrophotometer (BioSens, Poland) in each liquid flask before centrifugation was 1 ± 0.02 .

2.2 DNA isolation from fungal strains

For DNA isolation, fungi were transferred in liquid CMC medium (carboxymethylcellulose sodium salt 5 g/L, NaNO₃ 1 g/L, K₂HPO₄ 1 g/L, KCl 1 g/L, MgSO₄ 0.5 g/L, yeast extract 0.5 mg/L, glucose 1 mg/L) in anaerobic conditions in Bactronez-2 and protected with parafilm. Then, fungi were incubated for 7 days at 37 °C. After incubation, fungal DNA was isolated using an A&A Genomic Mini AX Yeast (A&A Biotechnology, Poland) according to the manufacturer's protocol. The concentration of the DNA was quantified using a Qubit® Fluorometer (Invitrogen, USA), and stored at –20 °C. Isolated DNA was used for PCR amplification and Sanger sequencing.

2.3 DNA sequencing-based identification of fungi

For molecular identification and phylogenetic studies, the preferred barcode in fungi classification is the Internal Transcribed Spacer (ITS) region, as it has high interspecies and low intraspecies variability. These fragments are used successfully as molecular markers in genus and species identification [44]. By PCR, the ITS1/ITS4 region was amplified with primers ITS1F - TCCGTAGGTGAACCTGCGG and ITS4R - TCCTCCGCTTATTGATATGC [52]. The PCR reaction was performed in a reaction volume of 30 µL, containing 18.2 µL water, 6 µL reaction buffer, 1.3 µL of each dNTP, 1.2 µL MgCl₂, 1 µL of each primer, 0.3 µL of GoTaq® DNA Polymerase (Promega, Poland), and a

Table 1 Characteristics and identification of the isolated anaerobic fungi. Size of the clearing zone as a result of growing and metabolite production by fungi strains on the CMC medium with congo Red, ∞- a full cover of media plate by a clear zone

Marking used in the text	Fungus name	Strain designation	DNA sequence identities, (%)	Size of the clearing zone, (mm)	NCBI GenBank accession number
G2	n.i.	n.i.	n.i.	1 ± 0	-
G3	<i>Aspergillus terreus</i>	S011	100 to the <i>Aspergillus terreus</i> MT077141.1	5 ± 1	PQ066109
G5	<i>Aspergillus terreus</i>	AKF2	100 to the <i>Aspergillus terreus</i> KJ685810.1	∞	PQ066110
G6	n.i.	n.i.	n.i.	∞	-
G9	<i>Aspergillus terreus</i>	Iz11	100 to the <i>Aspergillus terreus</i> KT803070.1	4 ± 1	PQ066111
G10	<i>Aspergillus fumigatus</i>	C1946	100 to the <i>Aspergillus fumigatus</i> MK623263.1	4 ± 1	PQ066112
G11	<i>Aspergillus fumigatus</i>	V-2	99.67 to the <i>Aspergillus terreus</i> MK490926.1	5 ± 2	PQ066113

n.i.- non identified



Fig. 1 Phylogenetic dendrogram of isolated fungi. The dendrogram was made with the NJ method [53]. Scale: 0.08. The Bootstrap set-up for the NJ method was 100

1 µL of DNA. PCR was run for 35 cycles in the DNA thermal cycler - BioRad C1000 Touch thermal cycler (Bio-Rad, USA). Conditions during denaturation: 95 °C for 8 min, amplification: 95 °C for 30 s., 60 °C for 40 s., 72 °C for 40 s., and denaturation 72 °C for 5 min. Products of the PCR were visualized using agarose gel electrophoresis (1%, w/v) under UV light. PCR products for sequencing were dispatched to Genomed company (Poland). Species identification was performed by Sanger sequencing and analysis of the bacterial genome based on the ITS1 sequence fragment. PCR products were subjected to matrix purification, sequencing of both DNA strands of the ITS1 fragment, and sequence analysis (assembled in consensus and compared with the GeneBank database, Standard Nucleotide BLAST).

Sequence analysis was performed using the ABI Prism “BigDye” Terminator Cycle Sequencing kit (Applied Biosystems™, ThermoFisher Scientific, USA). Sequences were

compared to the NCBI GenBank deposited sequences using the BLASTn algorithm. Moreover, obtained sequences were deposited in the NCBI GenBank. The accession numbers are collected in Table 1.

Based on the DNA sequences the dendrogram was prepared (Fig. 1). The method used for dendrogram construction was the Neighbor-Joining (NJ) method, as it is the most widely used distance-based algorithm for cases where the rate of sequence similarity is not extremely high or low. The Neighbor-Joining method allows us to outline the phylogenetic relationship between the sequences [53].

2.4 Pure cellulose degradation

Growth of the fungi was performed in the liquid CMC medium and incubated in 50 mL Falcon tubes for 7 days at 37 °C. After incubation samples were centrifuged for

40 min at 4400 rpm, and the supernatant was poured off. Samples with fungi were placed into mineral medium with mineral Kojim medium ($\text{Na}_2\text{HPO}_4 \times 12 \text{ H}_2\text{O}$ 3.78 g/L, KH_2PO_4 0.5 g/L, NH_4Cl 5.0 g/L, $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$ 0.2 g/L, yeast extract 0.01 g/L) and Whatman® filter paper (Sigma-Aldrich, USA) as a carbon source to determine the cellulolytic potential. The test was conducted for 30 days at 37 °C with 110 rpm shaking in anaerobic conditions in triplicates. Anaerobic conditions were created by displacing oxygen with nitrogen gas. Determination of the cellulolytic potential of microorganisms was performed according to PN-92/P-50,092. The procedure assumes the use of 1 ± 0.001 g sample weight. The samples were placed in flasks and each sample was flooded with a mixture consisting of 6 mL acetylacetone, 2 mL dioxane and 1.5 mL hydrochloric acid. The flasks were placed in a boiling water bath for 30 min, ensuring that the bottom of the flask was submerged in water. After incubation, 30–40 mL of methyl alcohol was added, and the whole sample was transferred to a funnel, connected to a vacuum pump. Then, the sample was washed with 40 mL of dioxane, 40 mL of hot distilled water, again with 40 mL of dioxane and 40 mL of ethanol. After each addition of the rinsing agent, the liquid drained freely, and the residue was extracted using a vacuum. The last wash was performed with 40 mL of ethyl ether and again left to drain freely; the excess was extracted using a vacuum pump. The sample crucibles were transferred to a drying oven and dried at 105 °C for about 3 h. Concentration was calculated with the Eq. 1:

$$C = \frac{m_d}{m_0 * \text{dryness}} \quad (1)$$

Where: m_d is the mass of the sample after the analysis, and m_0 is the mass of the sample before analysis. Dryness is 99%.

2.5 Enzyme assay procedure

Fungi are known as effective enzyme producers. In the case of cellulolytic abilities, necessary is determination of the amount of cellulose-degrading enzymes. The enzyme assays for two cellulolytic enzymes isolated from obtained pure cultures of fungi in this experiment were performed. The enzymes: filter paper cellulase activity (FPase), and carboxymethylcellulase (endoglucanase), were examined by methods, recommended by the International Union of Pure and Applied Chemistry (IUPAC) [54]. After 7 days of incubation at 37 °C, fungi with the Whatman® filter paper for FPase and 2% sodium carboxymethylcellulose (Sigma-Aldrich, USA) in Kojim medium (20 mL) were centrifuged

at 4400 rpm for 10 min and the supernatant was transferred into the new tubes. The centrifuged fungi and filter paper were flooded with 20 mL of citrate buffer, to dilute enzyme solutions and homogenize cells in case any enzymes were cell bounded (pH = 4.8, Chempur, Poland) and incubated for 2 h, and centrifuged as previously. The mixed supernatant was used as an enzyme mixture.

2.5.1 Filter paper cellulase (FPase) activity

The hydrolytic ability of mixed enzymes, used for the hydrolysis of cellulolytic biomass is determined using Ghose's [54] procedure. The total activity of cellulase was assayed by measuring the filter paper assay (FPase) using Whatman® filtration paper (Sigma-Aldrich, USA) liberated in the reaction mixture. For the determination of cellulases assay of the enzyme preparations 1 mL of 0.05 M Na-citrate buffer (Chempur, Poland), pH 4.8 and Whatman filtration paper no. 1 (50 g) were added to a test tube. Then, 0.5 mL of diluted enzyme was added to the tube. Two dilutions were made of each enzyme sample. Concentrations of the glucose to determine the standard curve were 2.0, 3.3, 5.0, 6.7 mg/mL. The tubes were incubated at 50 °C for 1 h. At the end of the incubation, the cellulase reaction was stopped by the addition of 3.0 mL of 3, 5- dinitrosalicylic acid (DNS) (Pol-Aura, Poland) with glucose as a standard. All tubes were boiled at 100 °C for exactly 5.0 min in a vigorously boiling water bath. Finally, after the colored solution was diluted with 20 mL of H_2O , the absorbance at 540 nm was measured. One unit of FPase is defined as an amount of enzyme which releases 1 μmol of glucose per minute.

FPA was calculated using Eq. 2:

$$FPA \frac{FPU}{mL} = \frac{0.37}{E} \quad (2)$$

Where: E is the concentration of the enzyme released from 0.5 mg of glucose from filter paper in 1 h.

2.5.2 Carboxymethylcellulase (CMCase) activity

For determining the CMCase activity (endoglucanase activity) was used Ghose's [54] procedure. A volume of 0.5 mL of the enzyme was diluted in 0.5 mL citrate buffer and incubated for 30 min at 50 °C. Then, 3 mL of 3, 5-dinitrosalicylic acid (DNS) was added and well mixed with the samples. The prepared mixture was boiled at 100 °C for 5 min and transferred to cold water. After cooling, 20 mL of deionized water was added, and well mixed. The absorbance was measured at 540 nm wavelength. The absorbance was determined in relation to glucose production during the reaction, using a glucose standard curve. The concentration of the

enzyme is estimated by plotting glucose liberated against enzyme concentration. One unit of CMCase is defined as an amount of enzyme which releases 1 μmol of glucose per minute.

CMC concentrations were calculated using the following Eq. 3:

$$CMC \left(\frac{U}{mL} \right) = \frac{0.185}{Ec} \quad (3)$$

Where: Ec - enzyme concentration to release 0.5 mg of glucose.

2.6 Methane production potential test

Fungi that occurred with the most stable growth were used in the Automatic Methane Potential Test System (AMPTS) (BPC Instruments, Sweden), to determine its impact on the sewage sludges on biomethane production. The test was performed in 3 replicates with 0.2 g fungi G3 (*Aspergillus terreus*) and without bioaugmentation. The volume of one replicate was 400 mL in a 0.5 L bottle. The test lasts for 24 days at 37 °C. As a substrate for the AMPTS was the sludge taken from the wastewater treatment plant (Gliwice, Poland), and directed to the methane digester: gravity-thickened preliminary sludge mixed with thickened surplus sludge. The sludge proportions were 65% of surplus sludge and 35% of preliminary sludge. Municipal sludge from the digester was used as the inoculum for the test. The test was performed with (1) inoculum + mixed sludge + fungus G3, (2) inoculum + fungus G3, (3) inoculum + mixed sludge and (4) inoculum. The ratio of mixed sludge and inoculum for the methane production was 1:3. In the Fig. 4 we present the comparison between inoculum + mixed sludge inoculated and non-inoculated (control) with fungi G3.

The amount of the sludges used for the AMPTs was determined based on the total solids (TS) of the sludges. The total solids (TS) value was determined by overnight oven drying at 105 °C. After drying, the porcelain crucible with sludge was placed in a desiccator to cool and weighted. The volatile solids were determined by placing the porcelain crucible into a muffle furnace and drying it at 550 °C for 2 h. After drying, samples were left in a desiccator to cool and weighted. The VS and TS were calculated using Eqs. 4 and 5:

$$TS (\%) = \frac{\text{weigh dried at } 105^\circ C}{\text{wet weigh}} * 100 \quad (4)$$

$$VS (\%TS) = \frac{\text{dried weigh at } 105^\circ C - \text{dried weigh at } 550^\circ C}{\text{dried weigh at } 105^\circ C} * 100 \quad (5)$$

The TS and VS were determined to calculate the amount of substrate and inoculum for the test. TS and VS parameters are presented in Table S1 (see supplementary materials).

Methane production was conducted for substrate/inoculum = 1:3 ratio.

The initial TS of inoculum without the addition of mixed sludge or fungus G3 was used to calculate the real amount of produced biomethane. The real amount of methane yield was calculated using Eq. 6:

$$\text{Methane yield} (N \text{ mL } CH_4/g \text{ VS}) = \frac{M_2 - M_1}{S_{VS}} \quad (6)$$

Where: M_1 is a mean of daily methane production of inoculum, concerned as a control sample.

M_2 is the daily methane production from a treated sample, and S_{VS} is a substrate VS/COD amount [g].

2.7 Cellulose concentration in sewage sludge

Cellulose concentration and loss were determined using the modified procedure according PN-92/P-50,092 as described in Sect. 2.4. For cellulose concentration in sewages, samples of sludge with and without inoculation with cellulolytic fungi were used, to determine its impact on methane production and cellulose concentration. Determination of the cellulose concentration and degradation was verified at the beginning and end of the AMPTS (24 days, 37 °C). Concentration was calculated with the Eq. 1. The control sample in the test was mixed (65% of surplus sludge and 35% of preliminary sludge) sludge. The test was performed in triplicates for each tested variant.

2.8 Statistical analysis

All results are presented as the mean $\pm$ standard deviation (SD) from the experiments performed in triplicates. The standard deviations of mean values were calculated using the Microsoft Office Excel 365 function. STATISTICA was used for statistical significance analysis (StatSoft, Inc. STATISTICA (data analysis software system), version 13. www.statsoft.com). ANOVA to determine the significance of the difference between the influence of the *A. terreus* (G3) in the degradation of pure cellulose and cellulose contained in sewage sludge, and the influence on methane production. The significant level of p -value was <0.05 .

3 Results and discussion

3.1 Isolation of the fungi

During this experiment, 12 fungal strains were isolated from sewage sludge and cultured in the laboratory in aerobic and anaerobic conditions using MEA and PDA medium. Cellulolytic abilities were tested using media with carboxymethylcellulose medium (CMC) as a source of carbon. Among all isolated fungi, 7 were able to grow and degrade cellulose in aerobic and anaerobic conditions in the Bactronez-2 Anaerobic chamber. Five of them were identified as *Aspergillus* sp. and two were non-identified in Sanger sequencing.

3.2 Identification and characteristics of the isolated fungi

Determination of the cellulolytic fungi was performed using Kongo Red dye added to a solid carboxymethylcellulose (CMC) medium. Kongo Red dye added to a CMC medium allows to determine the ability of microorganisms to degrade cellulose by visible decolored zones around the colonies producing cellulolytic enzymes (Figure S2 and S3, see supplementary materials). The clearing zone seen on plates allowed to confirm the cellulolytic ability of isolated microorganisms. Bright spots on plates were measured after 7 days of incubation and presented in Table 1. Figures S2 and S3 (supplementary materials) show the plates after incubation prolonged up to 14 days.

DNA of all 7 anaerobic fungi were isolated for their identification in Sanger sequencing. Results showed that 3 isolated fungi were *Aspergillus terreus* (100%) and two *Aspergillus fumigatus* (100 and 99.67%). The dendrogram based on the sequence was constructed by the NJ (Neighbor-Joining) method (Fig. 1) and shows that G3, G5, G9, G10 and G11 are located in the node of *Aspergillus*.

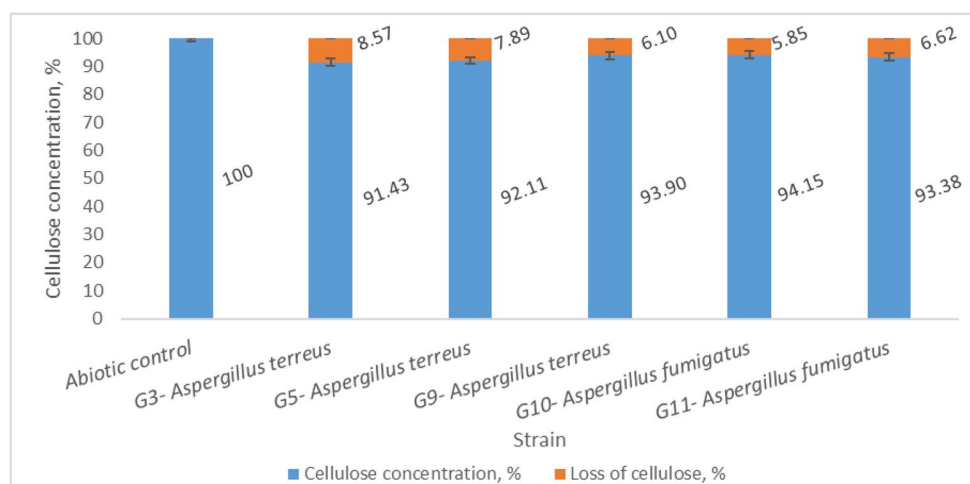
Fungi G2 and G6 were not identified, due to heterogeneous sequence readings. Based on their growth on the CMC medium, appearance under the microscope after lactophenol blue staining, and effectiveness in cellulose degradation, similar to identified fungi, they could belong to *Aspergillus* sp. Strains G3, G5 and G9 were growing similarly on the CMC medium with and without Congo Red. On the medium, colonies grew from beige to cinnamon buff, with the release of yellow pigments (Figure S1, see supplementary materials), which is characteristic of *A. terreus* [55]. Strains G10 and G11 also showed similar growth as black colonies on the plates with CMC and Congo Red medium (Figure S2, see supplementary materials) with characteristics for *A. fumigatus* greenish conidia.

According to the literature, fungi from the genus *Aspergillus* are commonly dominant in difficult environments i.e.,

sewage sludges, contaminated soils, or industrial wastes [56]. *Aspergillus* sp. representatives are aerobic organisms, yet they can grow in the environment with limited access to oxygen, showing even better growth of *Aspergillus* hyphae with 6 O₂–10% CO₂ than with the access to the room air [57]. Literature suggest that fungi can adapt to prevailing O₂ conditions [58], and show enzymatic activity e.g., β-glucosidase production) in the context of anaerobic silage fermentation [59]. Moreover, Stief et al. [60] determined, that *Aspergillus terreus* (isolate An-4) is capable of dissimilatory nitrate reduction to ammonium under anoxic conditions. Both *A. terreus* and *A. fumigatus* are acute threats to human health. *Aspergillus fumigatus* has a high potential for tissue invasion, causing diseases due to mycotoxin production [55]. The most common places to isolate *A. fumigatus* are hospitals, garden decaying materials, and soil of vastly different environments, from the northern tundra to the tropics [61–63]. Worldwide, the highest number of these fungi was detected in Europe [64]. *Aspergillus terreus* can cause human diseases, usually resistant to the antifungal drug amphotericin B [65]. It can be found in soil, plants, or animal tissues, as an effect of infecting and colonizing plants' roots by producing a secondary metabolite called terrain [66].

Aspergillus representatives are aerobic organisms, thus surviving during an AD (organisms and spores) is challenging for them, yet we found them in the digester sludge as they can tolerate lower oxygen concentrations. Presence of the fungi and other organisms is required for an efficient process. Although, when the organisms are pathogenic, it would be convenient if they would not survive an anaerobic digestion. Some fungi are able to persist through the whole AD process, but even then, the abundance of the community change, depending on the nutrients availability and fungal ability to adapt to the bioreactor environment [37]. Moreover, despite they can survive, it is not clear whether they are active. Obi et al. [67] observed that an anaerobic digestion does not impact the abundance of obligate aerobic fungi, and *Aspergillus* was observed among all anaerobic digestion process, but after an AD they noticed drastic reduction in the diversity of *Aspergillus*. From the other point of view in research conducted by Guo et al. [68] the addition of *Aspergillus sydowi* 8 L-9-F02 had an beneficial effect in increasing *Methanosaeta* and *Metanobacterium* abundance leading methanogenesis, improvement of extracellular polymeric substances and coenzyme F420 concentration. On the other hand, lovastatin secreted by *Aspergillus terreus* and other *Aspergillus* strains can inhibit growth of *Methanobrevibacter smithii* and the CH₄ production [69]. There are not much studies that describe fungal presence in anaerobic digestion and its impact on the microbial community structure [37, 51, 70, 71], thus it is necessary to

Fig. 2 Cellulose concentration change after 30-day treatment with 7 cellulolytic strains in anaerobic conditions: G3, G5 and G9 identified as *A. terreus*, and G10, G11- *A. fumigatus* respectively. Error bar represent standard deviation



verify the path of the fungi among the phases of methane fermentation closely. With this in mind, we would like to draw special attention to the necessity of sewage sludge hygienisation, in order to inactivate potentially pathogenic microorganisms found in sewage sludge in large quantities. They are a potential threat to the environment when used for agricultural purposes. Considering used biomass, the users should be always aware of the risk and focused on minimizing the danger.

3.3 Cellulolytic abilities of isolated fungi

Fungi are well-known as microorganisms that degrade organic matter, showing the ability to synthesise cellulases during growth on cellulosic materials [72]. The ability of isolated fungi to degrade cellulose is demonstrated on plates with CMC medium and Congo red, as referenced in Figure S3 (see supplementary materials). Among the twelve isolated fungi strains, seven demonstrated cellulolytic abilities under both anaerobic and aerobic conditions, as detailed in Table 1. Five strains have shown similar biodegradation levels in the 30-day test with the cellulose test (presented in Fig. 2).

The research of cellulose degradation was determined in a liquid test, with filter paper as a substrate. The obtained results are presented in Fig. 2.

Two strains (G3 and G5) identified as *A. terreus* provided the highest degradation of pure cellulose among all tested strains. Post hoc Turkey HSD test revealed nonsignificant difference between all cellulose degradation changes, using isolated fungi. The highest loss of cellulose was noticed for the strain named G3 (*A. terreus*) at 8.57%. A lower ability was observed in the test with strain G5 (*A. terreus*) 7.89%. Yet, the degradation percent of those two strains, with standard deviation on level 5% suggests very similar result in cellulose degradation. *A. terreus* G9 and *A. fumigatus* G11 provided cellulose degradation by 6.1% and 6.62%,

Table 2 FPase and CMC activity determined for *A. terreus* and *A. fumigatus*

Marking used in the text	Fungus name	FPase activity (FPU/mL)	CMC activity (U/mL)
G3	<i>A. terreus</i>	0.34	1.1
G5	<i>A. terreus</i>	0.32	1.16
G9	<i>A. terreus</i>	0.25	1.49
G10	<i>A. fumigatus</i>	0.36	1.02
G11	<i>A. fumigatus</i>	0.37	0.99

respectively. The lower ability for pure cellulose degradation was observed in the test with *A. fumigatus* G10- 5.85%. That suggest that all *Aspergillus* strains degradation ability in these conditions is on the similar level, slightly better for *Aspergillus terreus*. The differences in ability of the cellulose degradation are not significant between the tested strains but are not the result of measurement errors. Isolated cellulolytic fungi were able to grow on the plate together, as a mix of microorganisms and to grow and effectively degrade cellulose after separating pure culture. Thus, confirmed was their ability to work with other microorganisms.

3.4 Enzyme assays

Aspergillus spp. are considered to play an important role in lignocellulose degradation as producers of enzymes and organic acids for industrial purposes [73]. *Aspergillus terreus* was determined as able to biodegrade lignocellulose wastes and cellulose residues. FPase and CMC (endoglucanase) activity are presented in Table 2.

Results presented in this paper show CMCase production at level 1 U/mL. The CMC and FPU results for G3 and G5 are very close, what as in the test with cellulose degradation, indicates no significant differences in their abilities. Other authors confirm higher CMCase production by the same fungi but using different types of sludges and biomass. That is caused by the amount of amorphous and crystalline

cellulose. The endoglucanases prefer to degrade amorphous parts of the cellulose [74]. Shahriarinnour et al. [75] verified the potential of cellulase production by *A. terreus*. They cultured the fungus in different medium compositions with delignified oil palm empty fruit bunch fiber as a substrate. Results showed FPase activity of 0.76 U/mL and CMCase activity of 8.64 U/mL with yeast extract (6 g/L) and delignified oil palm empty fruit bunch fiber (10 g/L). Moreover, they noticed that the addition of Tween 80 (2 mL/L) increased the production of cellulase by two times, compared to the culturing without Tween 80 [75]. Tween 80, as it is a non-ionic surfactant, can help in enzyme recycling during enzymatic hydrolysis of lignocellulose and decrease of amount of enzyme that needs to be used for hydrolysis of cellulose [6]. Sohail et al. [48] verified the potential of *A. terreus* to produce cellulases with different sources of cellulose, among others CMC, and filter paper. They noticed an increase in endoglucanase and FPase production during culturing with CMC (as it is a water-soluble form of cellulose) (0.38 U/mL) and an increased level of β -glucosidase (0.56 U/mL) in presence of salicin, due to presence of an extra complex source of carbon [48].

Other research determined the potential for degradation of the lignocellulolytic biomass by *Aspergillus fumigatus* isolated from the compost [76]. The authors used lignocellulolytic materials as a substrate in solid-state fermentation, determining CMCase and FPase activity. The highest concentrations of enzymes were 526.3 and 144.6 U/g dry matter for CMCase and FPase at 50 °C, 80% initial moisture and pH 4.0, confirming good cellulase activity in temperature in the range of 40–55 °C and pH between 4.5 and 5.5. Results obtained by those researchers show more effective enzyme production than any other reports provided for this genus. Such great results were obtained on corn straw, while far lower on other substrates. The reason for that is that corn stover is highly contained with hemicellulose and other compounds, such as nitrogen, phosphorus, potassium or magnesium. Moreover, organic sources of nutrients are more suitable to produce lignocellulolytic enzymes than their inorganic representatives [76]. This gives a great opportunity for our isolated fungi, as sewage sludges contain large amounts of organic substances. Based on the obtained results, the amount of the produced cellulolytic enzymes depends strictly on the biomass used as a source of carbon. Moreover, the most effective production of the enzymes by fungi will be fulfilled in conditions similar to the environment from which it comes.

Degradation of cellulose and lignocellulosic biomass using fungi remain as sustainable alternative to other used methods, yet low microbial enzyme production rate limits its use in industries and agriculture waste management and biofuel production. The low enzyme activity can be

increased using various methods, i.e. random mutagenesis via exposing fungi to physical mutagens like radiation or chemical mutagens [36] or using gene-editing methods such as CRISPR-Cas9 [77].

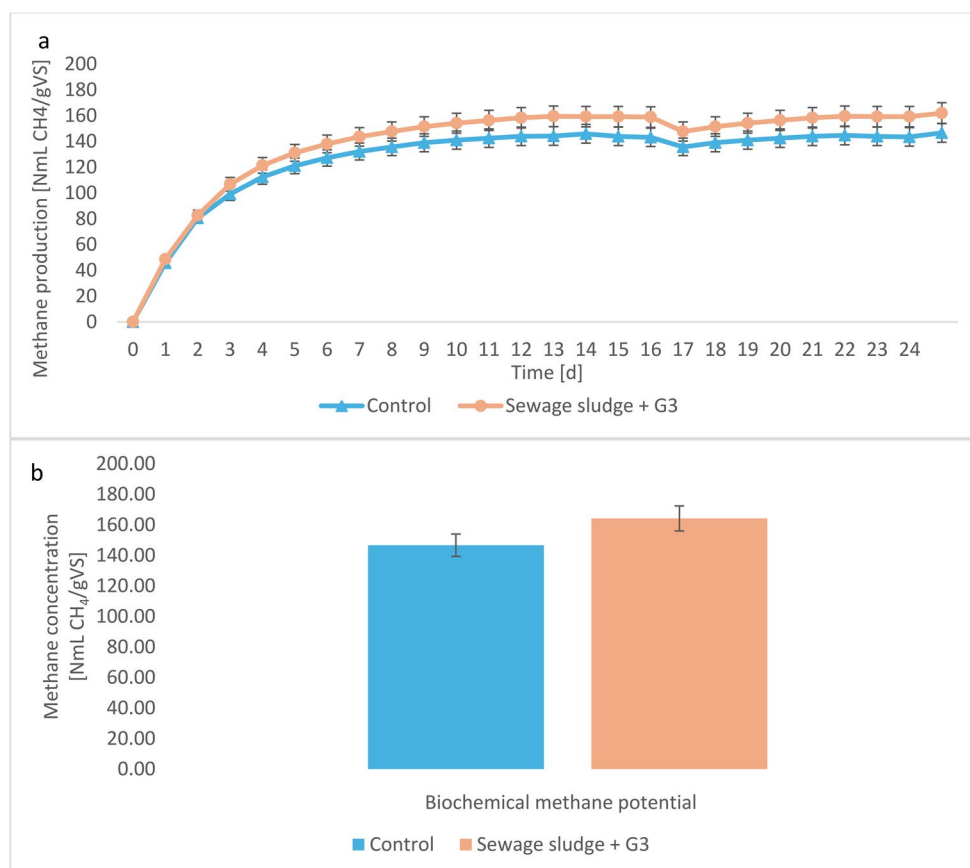
3.5 Methane production using automatic methane potential tests system

Methane production efficacy was investigated using the AMPTs test. The methane production was recorded for 24 days. Production was stable and undisturbed for the whole process, except day 17 when an insignificant drop in methane yield was noticed. The decline was due to a short-term equipment problem. Presented in Fig. 3 the methane production and increased methane yield after inoculation with fungi G3 (*Aspergillus terreus*) confirm its positive appliance in the process. In the current study, the biochemical methane potential (BMP) for the inoculated samples is higher by 17.57 N mL CH₄/g VS (about 11%) for the whole process, showing 164.2 ± 7.07 N mL CH₄/g VS ($p < 0.05$). The BMP without the inoculation was 146.63 ± 16.12 N mL CH₄/g VS. Obtained increase in methane production is comparable to increase in methane yield obtained by Noonari et al. [78]. The authors tested supernatant from the culturing 3 different *Aspergillus* fungi as pretreatment of rice straw, incubated together for 7, 15 and 30 days, and obtained increase in methane yield between 10 and 13% using 7 days pre-pretreatment. We did not conducted incubation of fungi together with the sludge before anaerobic digestion, thus comparison of the results suggests that our method using mycelia added directly to the methane production process, without the pre-pretreatment can provide the same results as using pre-pretreatment, that allow to shorten the time. Moreover, the authors noticed that using *Aspergillus terreus* provided the highest methane production, comparing to *A. niger* and *A. sojae*.

Based on the results for cellulose degradation and enzyme assay, we can suspect that results for methane production using other *A. terreus* (G5 or G9) strain would give as close effect. Considering the results for *A. fumigatus* as well, we could assume that under these conditions similar methane yield increase would be obtained for any strain of *Aspergillus*.

This research verifies whether the inoculation of sewage sludge with a single fungal strain influences the improvement of the overall fermentation process. Lima et al. [79] provided in their research, that enzymatic hydrolysis with *A. terreus* assures higher methane yield production than raw sewage by 47%. Although, Basak et al. [26] verified the influence of the *Aspergillus fumigatus* pretreatment of polysaccharidic wastes in a dual-stage process during the production of the hydrogen (5–20%), methane (80–95%)

Fig. 3 Amount of daily methane production from sewage sludge with and without the addition of fungi G3 (a), and biochemical methane potential of control and sludge inoculated with cellulolytic fungi G3 (b). Error bar represent standard deviation

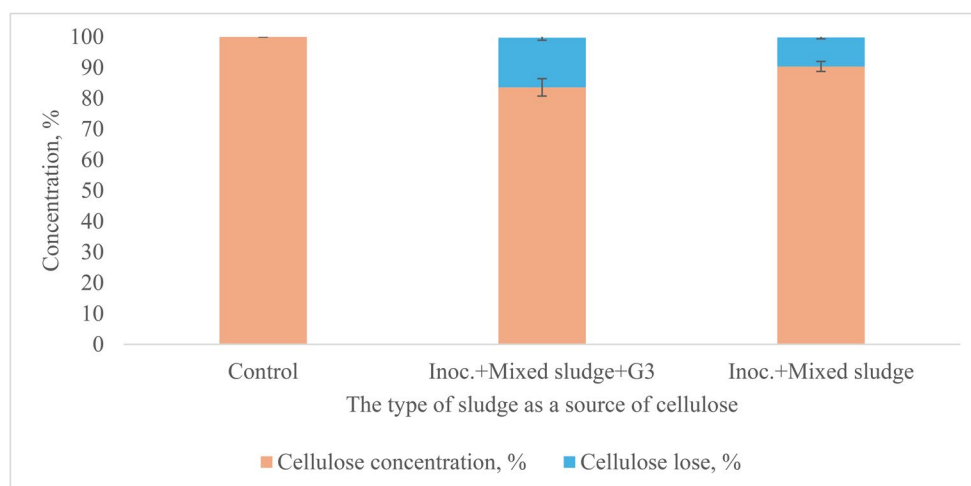


and mixture (biohythane), and observed 214.13 mL/g VS of the biogas yield. Their results confirm that fungi from the genus *Aspergillus* are effective and have great potential to enhance different gas production in anaerobic digestion. Ali and Sun [80] determined that pretreatment of the cellulosic biomass provided increased methane production from 102.6 to 125.9 mL/gVS, providing about 22% of the enhanced production. The authors used mixed pretreatment of chemical (2.5% NaOH and 2.5% NH₄OH), mechanical (milling) and biological (combination of the *A. terreus* and *Trichoderma viride*) methods. Pérez-Rodríguez et al. [81] also verified the influence of the mixed pretreatment (chemical and biological) in the hydrolysis of the agricultural biomass (raw corn cob). They determined that alkali (0.4% sodium hydroxide) and fast extrusion followed by enzymatic hydrolysis (enzymatic cocktail produced by *Aspergillus*) treatment provided the highest increase of the methane yield by 22.3% in comparison to the methane yield obtained in anaerobic digestion of the raw corn cob. Noonari et al. [78] determined, that pretreatment of the buffalo dung and rice straw in co-digestion with the addition of *Aspergillus niger*, *Aspergillus sojae* and *Aspergillus terreus* provides an increase in the methane yield. The heaviest increase in methane production was observed in pretreatment with *A. terreus* (about 15% after 7 days, 63% after 15 days and 43% after 30

days). The increase in methane yield obtained for treatment using fungal mycelia is comparable to other pretreatment methods. Pagés-Díaz et al. [82] received increase in methane yield up to 11–13% for the slaughterhouse wastewater treated with granular biochar. In other research, addition of biochar provided increase in biogas yield over 30%, when the used biomass was dairy manure [83]. Anaerobic digestion of thermally pretreated sewage sludge or sludge filtrate, co-digested with food wastes, provided an increase in methane yield between 9.7 and 69.7% and 11–101.1.1% respectively [84]. In reference to the lignocellulosic biomass, the increase in yield production is determined between 10 and 60% using mechanical pretreatment, 30–85% using chemical pretreatment, 5–65% using thermal pretreatment and between 15 and 100% using biological pretreatment [44].

Comparing results obtained in the test and presented in the literature on the treatment of wastes with *Aspergillus terreus* and *Aspergillus* sp. it is confirmed that using this strain as a biological method provides similar results to combined pretreatment (using chemical or mechanical methods). The biological pretreatment methods give promising results and additionally are well received due to their non-toxicological and environmentally friendly character, and low energy requirements. Although the long pretreatment time caused by the rate of biological hydrolysis is a great disadvantage

Fig. 4 Cellulose degradation during AMPTS with the addition of fungi G3. The results are the average of three samples. Error bar represent standard deviation



of biological methods, mixed pretreatment may be a reasonable solution from the economic point of view [85]. Thanks to that, it could be possible to decrease the amount of energy requirements and the amount of harmful chemicals.

Based on the literature, it can be observed that the overall result of methane production depends on various impacts, i.e. availability of the substrates, composition of the inoculum or activity of the microorganisms. The differences in methane yield increases are noticeable, although not repetitive for every kind of biomass. Sun et al. [86] noticed in their research, that the degradation rate of cellulose and straw differ depending on the used inoculum. That indicated that the microbial community in inoculum differs between the wastewater treatment plants significantly. Due to that, using fungi at different WWTPs will have different impact on the community structure and hydrolysis efficiency. They observed methane production between 307 ± 54 to 376 ± 8 N mL CH₄/g VS for cellulose and from 233 ± 38 to 316 ± 37 N mL CH₄/g VS for straw. Thus, using the same microorganisms in pretreatment may not provide a similar result for every kind of waste or sewage.

3.6 Degradation of cellulose in sewage sludge

The degradation of cellulose contained in sewage is more difficult than the degradation of pure cellulose, as it occurs in the environment mainly in the form of a lignocellulosic complex [7]. Moreover, in the environment present there are other compounds that could be more attractive as a source of carbon to the G3 fungus, than the cellulose. Degradation of cellulose present in sewage sludge by fungi was carried out in sewage sludge taken from the same wastewater treatment plant from which the fungi were isolated. Thanks to that, we provided them potentially familiar environment. The mixed sludge used for the test was preliminary and thickened sludge, as previously. Additionally, we used sludge from the digester as an inoculum, which provided

continuously working microorganisms for methane fermentation. As presented in Fig. 4 the highest cellulose degradation was observed in samples with the addition of fungus G3 (16.13%). The degradation was higher by over 7% than in samples with mixed sludge and inoculum. The verification of cellulose degradation using fungi G3 revealed increased removal of cellulose contained in lignocellulosic complex. In reference to that, we can assume, that addition of this fungus, under used conditions improves cellulose through: (1) direct consumption of cellulose contained in the sludge, released from the lignocellulosic complex as a result for mixing the sludge during the treatment or (2) release of the cellulose from the complex. *Aspergillus terreus* produces effective cellulases, hemicellulases or ligninases, thus both solutions are possible [87, 88]. Nevertheless, the degradation of cellulose by microorganisms present in the inoculum was at level 9%. Erdal and Hakkarainen [89] noticed that during the 28-day test in closed bottles, microorganisms present in the raw sewage were able to degrade 28% of cellulose, and 58% after prolonging the test up to 110 days. When the test was led as semicontinuous flow-activated sludge, the degradation of the carboxymethyl cellulose was over 90%. The obtained results show that in samples with lower concentrations of cellulose, there is observed higher methane yield. Thus, the obtained results suggest that the addition of the fungus G3 provided more effective degradation of organic substances by microorganisms present in the inoculum and did not disturb the anaerobic digestion process. It could be suspected that fungus G3 was able to adapt to the already balanced consortium, enhancing its effectiveness. This part of the experiment requires further investigation.

The total degradation of cellulose during methane fermentation lasts several weeks, thus during the conventional anaerobic digestion at the wastewater treatment plant, less than 60% of the cellulose can be decomposed at a solids retention time of 4–5 weeks [90, 91]. Obtained results suggest that even short-time biological treatment of the sewage

sludge during anaerobic digestion increases the amount of degraded cellulose and improves the production of the biomethane. The issue of improper composition of fermented sludge is a common problem, which causes difficulties with the management of sewage sludges. The use of microorganisms and enzymes that they produce could improve the anaerobic digestion effectiveness. However, in the case of biological pretreatment, it is hard to scale the process due to the size of the digesters, and microbial growth time. The solution for that could be the usage of a well-defined amount of mycelium, taken from the surface of the liquid fungal culture. The mycelium formed by fungi targets and degrades organic pollutants, conducting transport of nutrients and enzymes. Moreover, mycelium releases extracellular enzymes and acids able to break down the cellulose and lignin [52, 92]. For effective and efficient use of the proposed solution, the application would demand continuous fungal cultivation with an additional tank at the WWTP, what would provide undisturbed growth of fungi, producing concentrated amount of active enzymes and metabolites released by mycelium.

3.7 Statistical analysis

All experiments were conducted in triplicates. The ANOVA one-way was conducted for the tests of cellulose degradation and methane production. Table S2-S4 (see supplementary materials) presents ANOVA used to examine the significance of the differences between cellulose concentration and methane production in the sludge treated with the fungus G3.

The difference among strains regarding pure cellulose degradation was insignificant. Statistical analysis confirms the significance of the differences between the influence of the fungus G3 on pure cellulose degradation and cellulose contained in sewage sludge, which proves that, despite isolated *A. terreus* is able to degrade different forms of cellulose, the effectiveness of the degradation differs. Moreover, findings revealed that cellulose degradation and methane production was significantly different considering the variants with addition of G3 fungus into the sludge with indigenous microorganisms and without. The experimental design focused primarily on screening for cellulolytic potential under anaerobic conditions, and the methane potential was evaluated only for the most promising isolate (G3). Due to the limited number of replicates and the qualitative nature of the screening stage, a statistically sound comparison between strains was not feasible within the scope of this study.

4 Conclusion

Fungi isolated from the sewage sludge can degrade pure cellulose and cellulose present in sewage sludge at various efficacy. Inoculation of the sewage sludges with the mycelium of indigenous fungi gives the potential for enhanced methane production during the methane fermentation process in wastewater treatment plants. The addition of *Aspergillus terreus* (G3) for improvement of the methane fermentation process provides an increase of the methane yield by about 11% in the tested sludge, while simultaneously enhancing cellulose degradation in the sewage sludge by over 6%. The usage of this method can provide shortening the time needed for biological methods consisting of long-term pretreatment. Its application can be combined with mechanical methods, to enhance the methane yield, or other microorganisms. Moreover, inoculation of sewage sludge with fungi, not the cellulolytic enzymes that they produce, gives more opportunities, as those fungi may improve the degradation of other organic compounds. Despite that *Aspergillus terreus* is characterized as aerobic fungi, in this study it was able to degrade cellulose in aerobic and anaerobic conditions. Thus, its abilities should be also verified in short pretreatment (i.e. HRT= 1 d) before anaerobic digestion without any other supporting pretreatment method. The determined enzymatic activity of isolated fungi was not spectacular, yet its addition provided an increase in cellulose degradation and methane production. Thus, further research should be expanded with treatment of the sludge using other isolated fungal species, or combining them into a mixed culture as one of the solution for increasing its effective methane production increase. Moreover, it should be also verified if inoculation of the sludge with fungal in different form, for example using immobilized strains, will provide improved methane production. Further research in the field of the potential pathogenicity, the fate of the inoculated strain in the total community, and the community structure in the fermentation chamber are required. We are planning to improve conducted research with verification of the abundance of the *Aspergillus* during anaerobic digestion, considering the organisms and spores. Moreover, it is needed to verify the possibility of scaling up the process considering all obstacles.

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Author Contribution Magdalena Ćwiertniewicz-Wojciechowska: conceptualization, data curation, formal analysis, methodology, visualization, validation and writing. Grzegorz Cema: formal analysis, supervision, validation and review and editing. Aleksandra Ziemińska-Buczyńska: formal analysis, supervision, validation, review and editing.

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Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia

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Enhancing methane production during anaerobic digestion of sewage sludge using enzymes produced by autochthonous microbial consortia

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Abstract

Sewage sludge is a key biomass in wastewater treatment plants, where anaerobic digestion enables energy recovery in the form of methane. However, the initial phase of digestion is often limited by the slow hydrolysis of complex organic matter. This study investigates the potential of enzymes produced by autochthonous microbial consortia, naturally occurring in digested sewage sludge, to enhance methane production during anaerobic digestion. The effects of non-immobilized and immobilized autochthonous enzymes were evaluated in batch methane potential tests and compared with commercial cellulolytic enzymes.

The application of non-immobilized autochthonous enzymes increased methane yield by up to 21.7% compared to untreated sludge, while immobilization on activated carbon provided a more stable but less pronounced enhancement. The methane production achieved with autochthonous enzymes was comparable to that obtained with commercial enzyme preparations. Although no significant differences in cellulose degradation were observed, enzymatic addition accelerated the initial phase of methane production, leading to shorter effective digestion times. These results indicate that enzymes derived from autochthonous sludge microorganisms can serve as a low-input strategy to improve anaerobic digestion performance without the need for external enzyme supplementation.

Keywords: activated carbon; anaerobic digestion; autochthonous enzymes; enzyme immobilization; methane production; sewage sludge

1. Introduction

Current global challenges related to the increasing demand for heat and electricity combined with fossil fuel depletion, have intensified the search for renewable and environmentally friendly energy sources. At the same time, enormous amounts of sewage sludge, which are considered an efficient biomass source, are produced at the wastewater treatment plants and need to be properly managed. Across the EU, around 2–3 million tons of sewage sludge are produced annually, which represents a major management challenge and a potential substrate for biomethane production [13]. This biogas may be used as a source of energy by WWTPs or sold to be used as a Renewable Natural Gas (RNG) for transportation. An anaerobic digestion of sewage sludge provides effective biogas production and stabilizes sludge properties for further use. However, due to the high content of sewage sludges, its effective treatment is often limited without additional pretreatment step (Wan et al., 2022). Among different ways for sewage sludge pretreatment, including mechanical, chemical, and biological approaches, enzymes treatment has been reported to enhance the hydrolysis of complex organic compounds, improve biogas production, while remaining non-detrimental to equipment and the environment [52]. Among the organic components present in sewage sludge, cellulose constitutes a significant fraction of the total dry organic matter. Utilization of cellulose requires its conversion into glucose. Moreover, cellulose in sewage sludge is typically embedded within a lignocellulosic matrix, which limits its accessibility due to its crystalline and amorphous

structure [5]. Thus, the complex structure of lignocellulosic matter limits its biodegradability and poses challenges for efficient conversion during anaerobic digestion. Current studies confirm significantly improved disintegration of hard-to-degrade compounds using a various enzymatic approach, including enzyme mixtures or single hydrolytic enzymes [37, 48, 53]. Enzymatic pretreatment can increase soluble chemical oxygen demand and improve volatile fatty acids yield in fermentation [2]. An important aspect of enzymatic pretreatment is also the stability of the enzymes. The sludge environment and processes that are led during anaerobic digestion can cause the denaturation of enzymes used for enzymatic pretreatment; thus, their properties can be stabilized using immobilization. The appropriate selection of a carrier can further enhance anaerobic digestion performance, with activated carbon being one of the inorganic materials commonly applied for this purpose. Due to its inorganic character, it does not increase the organic load of the sludge, and its properties can promote acidogenesis and methanogenesis by stabilizing the microbial consortium, resulting in increased methane yield [20, 56, 63]. During anaerobic digestion, the microbial community in sewage sludge cooperate in the bioconversion of complex organic matter into methane and carbon dioxide. From the other point of view, methane fermentation is a biological process in which microorganisms cooperate in equilibrium. That equilibrium can be disturbed by treatment, i.e., due to a high concentration of ammonia, causing inhibition of methanogenesis or even stopping methane production [7]. WWTPs search for solutions that will increase the potential of the used sewage sludge and limit the costs that they need to spend on its management. This study focuses on enzymatic treatment during methane fermentation of a mixture of thickened primary and waste activated sludge (WAS), with the following objectives: 1) assessment of the impact of enzymatic treatment on the anaerobic digestion process, 2) comparison of enzymatic treatment using non-immobilized and immobilized enzymes with an activated carbon as a carrier, 3) comparison of enzymes derived from autochthonous microorganisms isolated from digested sludge with commercial enzyme preparations. Moreover, during the test, selected indicators related to organic matter transformation and changes in extracellular polymeric substances (EPS) were analyzed.

2. Materials and methods

Figure 1 presents a schematic overview of the study design. The whole experiment consists of three phases. First, digested sludge was collected from the WWTP and used as a source material for the enrichment of the autochthonous cellulolytic bacteria. In the second stage, the microbial community was characterized using NGS and determined their cellulolytic enzymatic activity was assessed using Ghose's saccharification procedure [15]. At the end, we isolated enzymes produced by a mixed bacterial culture. In a third stage, both the autochthonous enzyme preparation and a commercial cellulolytic enzyme preparation were immobilized on activated carbon. The impact of the enzymes in non-immobilized and immobilized forms was tested for 15 days using Automatic Methane Potential Test System II (AMPTS II). Samples were collected at the beginning and the end of the test, to analyze changes in EPS and cellulose concentration, and methane yield.

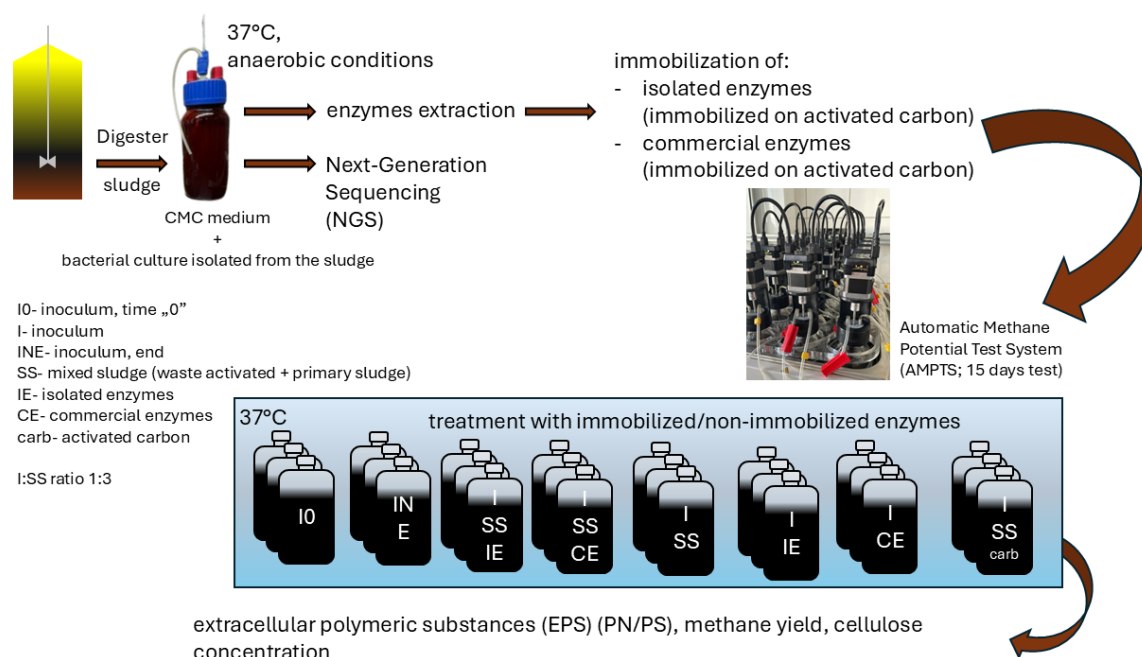


Figure 1. Schematic diagram of the steps taken to achieve the implementation of the planned experiment.

2.1. Enrichment and cultivation of the autochthonous cellulolytic microorganisms

Cellulolytic autochthonous microorganisms were isolated from the mixed sewage sludge (SS) collected from the communal WWTP (Gliwice, Poland). Mixed culture of autochthonous microorganisms was isolated by several series of passaging on bacteriological media (CMC medium - contained carboxymethylcellulose sodium salt 5 g/L, NaNO₃ 1 g/L, K₂HPO₄ 1 g/L, KCl 1 g/L, MgSO₄ 0.5 g/L, yeast extract 0.5 mg/L, glucose 1 mg/L). Mixed sludge consisted of thickened primary and waste activated sludge (35%:65%). 10 mL of SS and 40 mL of liquid medium (CMC) were incubated for 7 days at 37°C and 100 rpm in a 50 mL tube. To remove oxygen, samples were flushed with N₂. A new medium was provided every 7 days. The culture was centrifuged for 1 minute at 3000 rpm, suspended in sterile 0.9% NaCl in triplicate to remove metabolites. Then, microbial culture and sterile 0.9% NaCl were mixed together in a ratio of 1:1 and incubated as described above. Samples were collected for DNA isolation into sterile Eppendorf tubes and protected with RNeasy lysis solution (Invitrogen™, ThermoFisher Scientific, USA) in a ratio of 2:1, where 1 part was a sludge sample. After 21 days of the experiment, the inoculation on solid medium (CMC medium solidified with bacteriological agar 17.0 mg/L) was performed to verify the possibility of isolating a pure culture and incubation on plates. The remaining culture was fed with the new portion of liquid CMC medium and incubated at 37°C and 100 rpm.

The cellulose-degrading ability of the microorganisms was confirmed by inoculating 0.1 mL of the liquid mixed-culture on Congo-Red agar media containing CMC medium solidified with agar (CMC medium solidified with agar 17 g/L and Congo Red 1g/L), pH 6.8-7.2.

After isolation, samples were stored at -20°C until later use.

2.2. Metataxonomic analysis of enrichment bacterial culture

Metataxonomic analysis was performed on total bacterial DNA isolated from the autochthonous mixed culture. DNA was isolated using the GeneMATRIX Soil DNA Purification Kit, according to the manufacturer's protocol (EurX, Poland). Metataxonomic sequencing was performed under contract by the Genomed company (Poland). The metagenomic analysis of bacterial and archaeal communities was performed based on the hypervariable V3-V4 region of the 16S rRNA gene. The primer pair for amplification was 341F and 785R [51]. The PCR reaction was performed using Q5 Hot Start High-Fidelity 2X Master Mix (conditions according to the manufacturer's instructions) (New England Biolabs). Sequencing was performed on a MiSeq, paired-end (PE), 2x300nt, using Illumina's v3 kit, according to the manufacturer's protocol. Automatic ascending data analysis was performed on a MiSeq sequencer using MiSeq Reporter (MSR) v2.6 software. Bioinformatic analysis to ensure the classification

of reads to the species level was carried out with QIIME 2, based on sequences referential base Silva 138. A programming package was also used to distinguish sequences of biological origin from those newly created during the sequencing process. This package was also used to derive unique sequences of biological origin, or so-called amplicon sequence variants (ASV).

2.3. Extraction of the enzymes

Enzyme extraction was performed by using 50 mL of the mixed microbial culture isolated for this experiment ($OD_{600} > 0.9$) and 150 mL of the CMC medium, incubated at 37°C with shaking: 160 rpm for 2 days, and 120 rpm for the following 5 days. The mixed culture was deprived of oxygen by displacing it with nitrogen (for 3 minutes). Further steps of the extraction were performed based on Kokila et al. [30]. Extraction was performed using heat at 80°C and centrifugation [25]. Enzymes were isolated with Na-citrate buffer (Pol-Aura, Poland; 0.05 M, pH 6) at a ratio of 1:2, 1:3, 1:4, and 1:5, where 1 part was a buffer. Reaction was performed for 30 minutes at 37°C and 120 rpm. The samples were then centrifuged at 10000 rpm for 15 minutes, and the supernatant was used as a source of enzymes [27, 46].

The protein content was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA), according to the manufacturer's protocol for the test-tube procedure.

2.3.1. The activity of isolated enzymes based on filter paper assay (FPase) and endoglucanase (CMC)

The enzyme activity of filter paper assay (FPase) and endoglucanase (CMC) was determined using Ghose's procedure [15]. The total activity of cellulase was assayed by measuring the filter paper assay using Whatman® filtration paper (Sigma-Aldrich, USA) liberated in the reaction mixture. Cellulase assay was determined with the preparation of 1 mL of 0.05 M Na-citrate buffer (Pol-Aura, Poland), pH 6, and 2-3 cm Whatman filter paper no. 1 (Sigma-Aldrich, USA), added to a test tube. We prepared two dilutions (1:2 and 1:5) of each enzyme sample with glucose as a standard. Concentrations of glucose for the standard curve were 0.2, 1, 2.5, and 5 mg/mL. The tubes were incubated at 50°C for 1 hour, and then the cellulase reaction was terminated by the addition of 3.0 mL of 3,5-dinitrosalicylic acid (DNS; Pol-Aura, Poland). All tubes were boiled at 100°C for 5.0 min in a vigorously boiling water bath. Finally, the obtained solution was diluted with 20 mL of H₂O, and the absorbance was measured at 540 nm. One unit of FPase is defined as an amount of enzyme that releases 1 μmol of glucose per minute.

FPA was calculated using Equation 1:

$$FPA \frac{FPU}{mL} = \frac{0.37}{E} \quad (1)$$

Where: E is the concentration of the enzyme released from 0.5 mg of glucose from filter paper in 1h. The calculations were performed according to the Ghose procedure [15].

For endoglucanase (CMC) activity, enzymes were incubated for 3 minutes in 0.5 mL Na-citrate buffer at room temperature. Then, the 0.5 mL of 2% CMC with 0.05 M sodium citrate buffer was added to the enzyme, mixed, and incubated for 30 minutes at 50°C. After the incubation, 3 mL of 3.5-DNS was added and mixed with the sample to determine reducing sugars [40]. The prepared mixture was boiled at 100°C for 5 minutes. After cooling down, 20 mL of deionized water was added and mixed with the sample. The absorbance was measured at 540 nm wavelength in reference to glucose production during the reaction, using a glucose standard curve. The concentration of the enzyme is estimated by plotting glucose liberated against enzyme concentration. One unit of CMCase is defined as an amount of 1 μmol of glucose liberated per ml of enzyme per minute.

CMC concentrations were calculated using Equation 2:

$$CMC \frac{U}{mL} = \frac{0.185}{Ec} \quad (2)$$

Where: Ec - enzyme concentration to release 0.5 mg glucose.

2.4. Enzymes immobilization

Immobilization of the mix of isolated enzymes and commercial enzymes (Celllic CTec2, Novozymes) was performed on activated carbon (Sigma-Aldrich, USA). Enzymes were prepared for immobilization in 5 different concentrations: initial, diluted in proportions 1:2, 1:3, 1:4, and 1:5 with Na-citric buffer (Pol-Aura, Poland; 0.05 M, pH 6).

2.4.1. Physical immobilization of enzymes on activated carbon

Activated carbon (0.4 g), commercial enzymes or isolated enzymes diluted in citric buffer (1:2, 1:3, 1:4, 1:5; v/v) (0.5 mL) were stirred in a 25 mL round-bottom flask in a thermostatic shaker (250 rpm) at 20°C for 3 h. Then, biomaterials were filtered off, washed with citric buffer (3×10 mL), and dried under vacuum using a Schlenk line (25°C, 24 h).

2.4.2. Thermogravimetric analysis (TGA)

Thermal characteristics of the enzymes immobilized on the activated carbon were determined using the TGA/SDTA 851 Mettler-Toledo thermogravimeter. Tests were performed from 25°C to 800°C at a heating rate of 10°C/min in a stream of nitrogen (60 mL/min). Samples of approximately 10 mg were introduced into 70 µL aluminium oxide crucibles. The obtained TGA data were analyzed using the Mettler-Toledo STARe System SW 15.00.

2.5. Verification of methane production using the Automatic Methane Potential Test System (AMPTS)

The sewage sludge for the test was taken from the communal wastewater treatment plant (Gliwice, Poland). Sewage sludge for the Methane Potential Test was mixed sludge: thickened primary sludge mixed with waste activated sludge. The sludge proportions were 65% of WAS sludge and 35% of primary sludge. Digested sludge from the anaerobic digester was used as the inoculum for the test. The test was conducted in the Automatic Methane Potential Test System (AMPTS) (BPC Instruments, Sweden) According to the procedure, the test was carried out until the daily gas production dropped below 5 NmL/d [33]. Each replicate for the methane production test was carried out in a 500 mL bottle with a working volume of 400 mL. The test was performed with enzymes isolated from the cellulolytic autochthonous bacteria and the cellulase enzyme blend (Cellic CTec2) with 200 FPA U/g (SAE0020, Sigma-Aldrich, USA), both immobilized and non-immobilized [22]. The concentration of the commercial enzyme was a minimum of 1200 U/mL for the 50mL package size, according to the manufacturer's information. Based on the manufacturer recommendations, for usage of the commercial enzyme regarding cellulose concentration, and further preliminary test for three different Cellic CTec2 concentration (0.2, 0.5 and 1%) (data not shown) we estimated the amount of immobilized and non-immobilized enzymes. The enzymes were dosed at a level of 1% of the TS. All samples were flushed with nitrogen gas for one minute to ensure anaerobic conditions and then incubated at a mesophilic temperature of 37°C. AMPTS settings included a mixer speed of 60%, with 60 seconds on and 180 seconds off intervals. At the beginning and the end of the test, all samples were centrifuged for 20 minutes at 3300 rpm and filtered through a 0.45 µm filter membrane (ChemLand, Poland). Control samples containing only inoculum (without substrate or enzymes) were used to determine net biomethane production, with Equation 3.

$$\text{Methane yield } \left(\frac{\text{NmLCH}_4}{\text{gVS}} \right) = \frac{M_2 - M_1}{S_{VS}} \quad (3)$$

Where M_1 is the mean of the daily methane production of the inoculum, considered as a control sample, M_2 is the daily methane production from a treated sample, and S_{VS} is a substrate volatile solids/chemical oxygen demand (VS/COD) amount [g].

2.6. Extracellular polymeric substances (EPS)

Extraction of tightly bound extracellular polymeric substances was performed by the heating method according to Raszka et al. [44] after Karapanagiotis et al. [25]. Samples of sewage sludge were centrifuged at low speed (20 minutes, 3000 rpm). The supernatant was poured off, and the sludge pellets were resuspended in distilled water to the initial volume. Well-mixed samples were placed into a water bath (80°C) for 1 hour. After the incubation, the samples were centrifuged first at 3000 rpm, and then at 3300 rpm for 20 minutes each time. The remaining extract was analyzed for proteins and saccharides. The protein substance concentration (PN) was determined using the BCA Protein Assay Kit (ThermoFisher Scientific, USA). Determination of polysaccharides (PS) was performed using the modified Dubois et al. (1956) [11] method, where instead of 80% phenol, we used 5% phenol as described by Raunkjær et al. [45] as a safer but still effective compound. The results were compared for immobilized and non-immobilized enzymes, in reference to Cellic CTec2. Absorbance was measured with wavelength $\lambda=490$ nm.

2.7. Cellulose concentration in sewage sludge

Cellulose concentrations were determined using the PN-92/P-50092 for cellulose contained in lignocellulosic complexes, by dissolving the lignin. The procedure uses $\text{CH}_3\text{COCH}_2\text{COCH}_3$, $\text{C}_4\text{H}_8\text{O}_2$, HCl , CH_3OH , $\text{C}_2\text{H}_5\text{O}_2\text{H}_5$. 1 ± 0.001 g of each sample was placed in flasks and flooded with a mixture consisting of 6 mL acetylacetone, 2 mL dioxane, and 1.5 mL hydrochloric acid. The flasks were placed in a boiling water bath for 30 minutes, provided that the bottom of the flask was submerged in water. After incubation, 30-40 mL of methyl alcohol was added, and the sample was transferred to a funnel, connected to a vacuum pump. Samples were washed with 40 mL of dioxane, 40 mL of hot distilled water, again 40 mL of dioxane, and 40 mL of ethanol. After each washing, the liquid drained freely, and the residue was extracted using a vacuum. The last wash was performed with 40 mL of ethyl ether and again left to drain freely. The excess was extracted using a vacuum pump. The samples in crucibles were transferred to a drying oven and dried at 105°C for about 3 h. Concentration was calculated using Equation 4:

$$C = \frac{m_d}{m_0 * \text{dryness}} \quad (4)$$

Where: m_d is the mass of the sample after the analysis, and m_0 is the mass of the sample before analysis. Dryness is 99%.

2.8. Statistical analysis

The experimental results were expressed as the mean $\pm$ standard deviation for each triplicate experiment. The data processing was conducted by the Microsoft Office Excel 365 function. R software (<https://www.r-project.org>). Two-way ANOVA was used to test the significance of results for immobilized and non-immobilized enzymes, and also for the immobilized enzymes and activated carbon, regarding physicochemical parameters. For methane concentration, the Kruskal-Wallis test was used. The significance level of the p -value was <0.05 .

3. Results and discussion

3.1. Isolation, identification, and characteristics of the bacterial mixed culture

Biochemical cycles performed by microorganisms in nature are based on microbial cooperation; thus, it is widely known that in the environment, microbes cooperate while living in communities. Their proper growth and development depend not only on the substrate availability but also on the ecophysiological relationships among various groups of microbes cooccurring in their biotope. These relationships are not possible to be mimic in laboratory conditions. That is why most environmental bacteria, among which a large part could be biotechnologically useful, cannot be obtained as pure cultures in lab conditions. Moreover, even if the obtained pure culture is able to grow and degrade a particular substrate (i.e., cellulose) in laboratory conditions, their development and enzymatic activity are relatively low and ineffective in comparison to their performance in the community. Such a situation probably occurred in this experiment. What is more, after re-inoculation of the obtained bacteria onto fresh medium, these microbes lost their abilities for effective cellulose degradation and/or could not degrade cellulose, which could be explained by the lack of microbial cooperation mentioned above. Metataxonomic sequencing of the sludge samples allowed to indicate the most abundant group of microorganisms present in the samples. In this group at the genus level: *Bacteroides* sp., *Dysgonomonas* sp., *Petrimonas* sp., *Proteiniphilum* sp., *Desulfovibrio* sp., *Pleomorphomonas* sp., *Paracoccus* sp., *Bordetella* sp., *Castellaniella* sp., *Limnobacter* sp., *Alicyclophilus* sp., *Klebsiella* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Sphaerochaeta* sp. were indicated.

The composition of a community created as a mixed culture of isolated bacteria (by genus), producing cellulolytic enzymes, used in this study, is presented in Figure 2.

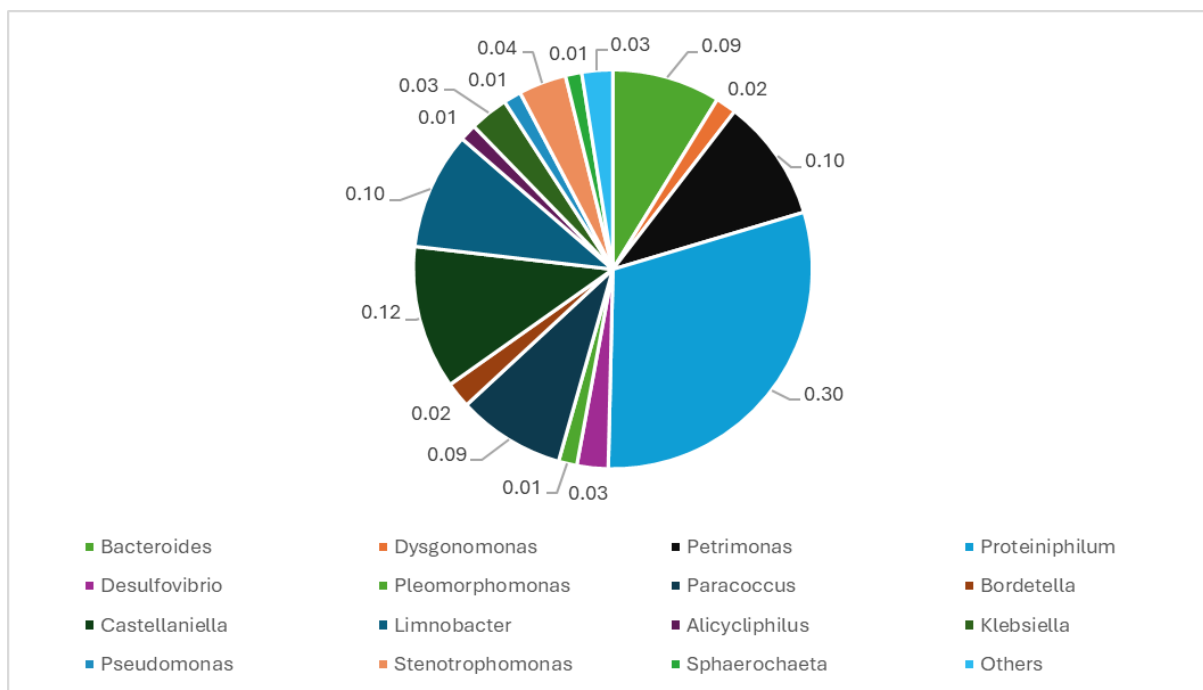


Figure 2. The relative abundance of bacteria at the genus level in the cellulolytic community isolated from the sewage sludge from the digester. Sequences that accounted for less than 1% of the community were grouped into the category “Others”.

The community was able to be cultured in the medium with the sodium carboxymethylcellulose as a source of carbon, consisting of various bacteria, with abilities useful in the hydrolysis of macromolecular organic compounds. The dominant bacterial genus in the sludge samples is *Proteiniphilum*, representing the *Porphyromonadaceae* family. *Porphyromonadaceae* are known to be able to degrade complex carbohydrates, proteins, and peptides. Moreover, they participate in the production of volatile fatty acids during acidogenesis [18]. Granada et al. [17] noticed that representatives of *Porphyromonadaceae* were linked with the high methane production in their research and characterized those microorganisms as bacteria that drive biogas production from slaughterhouse wastewater anaerobic digestion, containing blood, manure, hair, fat, feathers, and bones.

The second most abundant genus was *Alcaligenes*, which is a heterotrophic microorganism able to oxidize and remove ammonia from wastewater [14, 57]. Although most of the *Alcaligenes* representatives are aerobic bacteria, some strains are capable of anaerobic respiration in the presence of nitrite and nitrate [3]. Zheng et al. [64] noticed in their research that *Alcaligenes* sp., *Pseudomonas* sp., and *Clostridium* sp. were the key microorganisms that took part in sludge hydrolysis improvement and enhanced short-chain fatty acids production, as they are able to excrete extracellular proteases.

Bacteroides sp. abundance was 8.78% in total. *Bacteroides graminisolvens* is a strictly anaerobic bacteria able to utilize saccharides [42]. The strains produce acetate and propionate [19, 31].

Limnobacter spp. strains are considered to contribute to the anaerobic oxidation of the methane community, providing sulfate from sulfur oxidation [6].

The bacteria from the genus *Paracoccus*, also abundant in the sludge samples, have a high potential for volatile fatty acids production from the waste-activated sludge [67].

Some strains from the genus *Stenotrophomonas* have high nitrogenase activity and are able to grow on a nitrogen-free culture medium [24, 43]. Various research confirms positive results for nitrate reduction, citrate utilization, or hydrolysis of various carbons [16].

Klebsiella sp. produces large amounts of extracellular polymeric substances, which can help protect the microbial community from stressful conditions and can be helpful in the degradation of organic matter. Bacteria from this genus can degrade lipids and increase methane production [35, 48].

Some bacteria from the genus *Desulfovibrio* sp. can remove COD by breaking down the organic compounds, sulfate, and/or using nitrate as a terminal electron acceptor [32].

Bordetella sp. in a mixed culture takes part in the degradation of various cellulosic materials and increases methane production [62].

Dysgonomonas degrade saccharides and produce short-chain fatty acids [29, 59]. They are able to degrade various substrates, i.e., lignin, alginate, or naphtha [21, 28].

Pleomorphomonas sp. are nitrogen-fixing bacteria [60]. Damo et al. verified them as phosphate-solubilizing bacteria [8]. It plays a role in lignocellulose degradation [9]. *Pleomorphomonas oryzae* is able to consume glucose anaerobically [12].

Pseudomonas sp. is considered to play a key role during anaerobic digestion for all main methanogenic pathways, mainly due to carbohydrate hydrolysis [4]. Moreover, they can produce and secrete alkaline proteases, an important enzyme for the hydrolysis of sludge proteins [26].

Alicyclophilus representatives can use low-molecular-weight organic acids and amino acids as carbon and nitrogen sources [38, 39]

Also, representatives of *Sphaerochaeta* are able to ferment mono- and polysaccharides [47].

Bacteria present in the mixed culture show some similarities, i.e., utilizing a broad range of saccharides, or like *Alcaligenes* sp., *Pseudomonas* sp., and *Dysgonomonas* sp., showing enhanced short-fatty acids production. Despite the bacteria with lower abundance having similar abilities to the bacteria with higher abundance, their presence in a bacterial mixture suggests that it is still possible for them to grow and use some nutrients, and their presence is supportive. Moreover, most of the identified microorganisms are not strictly cellulolytic, but anaerobic and able to degrade various organic compounds, which helps in using products from cellulose degradation. Thus, their presence in the mixed culture and the enzymes that they produce may be supportive for the hydrolysis process due to the degradation of various organic compounds.

3.2. Characteristics of cellulolytic enzymes and the effect of immobilization

The TGA analysis allowed us to confirm the presence of enzymes on the surface of the activated carbon carrier. After the first immobilization, performed for cellulases, the enzyme content on the carrier was 6.06% for enzymes isolated from autochthonous bacteria and 22.28% for the commercial enzyme (Cellic CTec2). Dilution of the enzymes using citric buffer did not affect immobilization efficiency. The amount of enzymes immobilized with the activated carbon for the conducted tests was balanced. The literature confirms positive application of the activated carbon to absorb various components and compounds, i.e., heavy metals, ammonia, or sulfates [41], but also proteins, VFAs, or polysaccharides [23]. That, as a result, enhances methane production [1, 58]. The enzyme content detected on the carrier indicates free spaces, which can be filled with compounds present in the tested environment, which can enhance the process [63].

The determined concentration of the protein assay was 3.1 mg/mL. Isolated mixed autochthonous bacteria produced enzymes, whose activity of FPA is 1.69 FPU/mL and CMC 11.27 U/mL, which, according to the literature, effectively increases cellulose enzymatic degradation [68].

3.3. Verification of methane production in the sludges, using the Automatic Methane Potential Test System

In Figure 3, we present the methane production obtained with immobilized and non-immobilized enzymes. During tests with immobilized enzymes, methane yield increased in all treated samples compared to the untreated control. The methane yield of the control sample was 137.09 ± 49.02 NmL CH₄/gVS. Samples treated with immobilized commercial enzymes showed a methane yield of 174.02 ± 7.28 NmL CH₄/gVS, while those treated with immobilized isolated enzymes reached 163.1 ± 1.36 NmL CH₄/gVS. This corresponds to a 27% increase for commercial enzymes and an 18.9% increase for isolated enzymes relative to the control. Samples treated with activated carbon produced 6.75% more methane than the control. In contrast, samples treated with non-immobilized enzymes showed substantially higher methane yields: 274.1 ± 7.14 NmL CH₄/gVS for commercial enzymes and 233.99 ± 17.5 NmL CH₄/gVS for isolated enzymes.

The methane yield in untreated samples was 192.32 ± 11.24 NmL CH₄/gVS. For non-immobilized enzymes, the increase in methane yield was higher than that observed for immobilized enzymes. Relative to the control, methane yield increased by 21.7% for isolated enzymes and by 43% for commercial enzymes. The differences in methane yield observed between control samples (untreated samples in the immobilized and non-immobilized enzyme experiments) are due to sewage sludge being collected at two different times. Because of equipment limitations, it was necessary to divide the experiment into two sets: one for immobilized enzymes and one for non-immobilized enzymes, to allow for replication.

Although the two-way ANOVA test indicates that there is a non-significant difference in the methane potential regarding the used enzymes, significance lies in whether we use immobilized or non-immobilized ($p < 0.05$). That indicates that enzymes produced by microorganisms present in the sludge can enhance methane production on a similar level as commercial enzymes and provide cheaper solutions for sludge treatment.

In samples with immobilized enzymes, we can observe that after 7 days of AMPTS, the isolated enzymes obtained the same level of methane production as commercial enzymes, and then the production of biomethane in those samples remained at the same level until the end of the test. That suggests that the addition of those immobilized isolated enzymes in the used dose can enhance the methane production for 7 days, and after this time, further improvement needs another inoculation. For non-immobilized enzymes, the concentration of produced methane is the same for isolated and commercial enzymes for the first 3 days, and then again, the peak time of the daily biogas production is visible on day 5. After that time, we can see stabilization or even a slight decrease in methane production, which can be related to consumption of VFA [34, 36, 65].

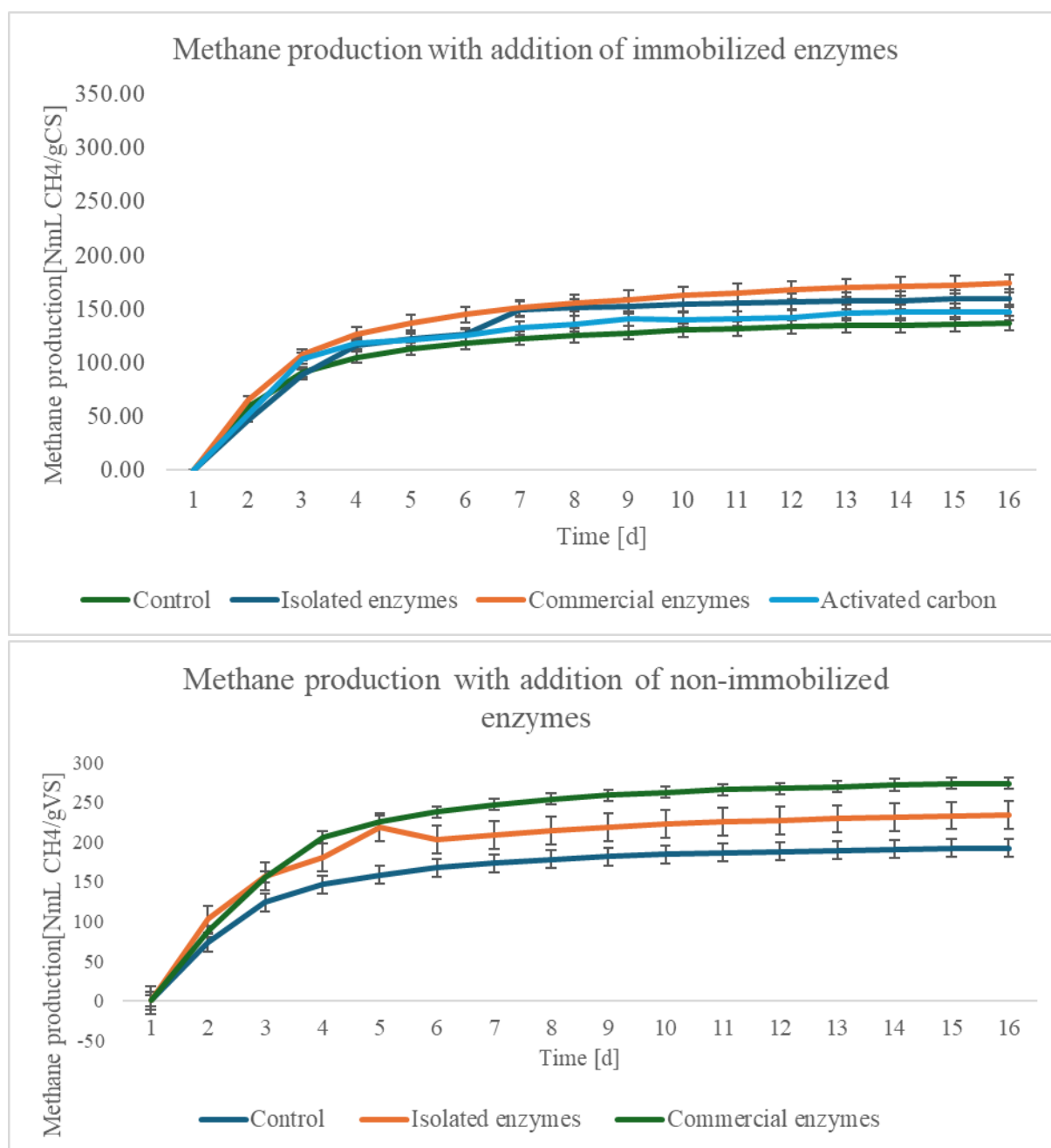


Figure 3. Methane production over time, measured in the sewage sludge treated with immobilized and non-immobilized enzymes in the Automatic Methane Potential Test (AMPTS).

Based on the methane production day by day, we can assume that using isolated enzymes caused increased degradation around the 4th day using immobilized and non-immobilized enzymes, and additionally, the 7th day for immobilized enzymes, causing increased sudden acidification as a result of VFA production, which inhibited the effective process [10]. That may also suggest that the immobilization delayed or stabilized the effect of the enzymatic impact. From around the 8th to 9th day, methane production was stable in all samples.

3.4. *Tightly bound EPS*

Tightly bound extracellular polymeric substances (Figure 4) concentration was determined for sludges treated with immobilized and non-immobilized enzymes (Figure 3). The PN was the dominant component of EPS, and the PS concentration was relatively low. The protein concentration decreased during anaerobic digestion from an initial concentration of 2183.6 mg/g VSS to 583.9 mg/g VSS for untreated samples and 547.52 mg/g VSS treated with isolated enzymes and commercial enzymes (404.22 mg/g VSS). For saccharides, the concentration decreased from 87.6 mg/g VSS to 61.6 mg/g VSS for untreated samples, 52.1 mg/g VSS for samples treated with immobilized isolated enzymes, and 39.1 mg/g VSS for samples treated with immobilized commercial enzymes. The decrease in the EPS concentration can be caused by the adsorption of the extracellular polymeric substances on activated carbon, which was also observed by Sun et al. [49]. They also noticed an enhanced methane production (by 10%) connected with the EPS removal within the first 6 h of the reaction. In our preliminary research, we noticed a decrease in EPS concentration in samples with activated carbon during the 4 h test. That is similar to Sun et al. [40] observation, although they used GAC (granulated activated carbon), and we used powdered activated carbon. Jiang et al. [23] noticed that higher molecular weight substances, i.e., proteins and saccharides, are better adsorbed on granular activated carbon than small molecules such as VFA or glucose. Even though we are using powdered activated carbon, maybe the lack of changes in VFA concentration in samples with immobilized enzymes and activated carbon, could also be caused with the absorption of the multimolecular substances on the carrier. Although, that hypothesis should be verified in a test with measuring VFA concentration in a time.

A decrease in the EPS concentration (Figure 4) in samples with enzymes can be caused with adsorption on the free spaces of the carrier. The decrease in EPS concentration is also observed in samples with non-immobilized enzymes. From the initial 1193.22 mg/g VSS for proteins, the concentration decreased to 806.4 mg/g VSS for untreated samples, 768.12 mg/g VSS for isolated enzymes, and 774.8 mg/g VSS for commercial enzymes. The concentration of the EPS saccharides (PN) remained at a similar level in all samples, as the initial concentration was 87.6 mg/g VSS, and determined in untreated samples with enzymes was 86.21 mg/g VSS, 87.3 mg/g VSS for isolated enzymes, and 85.7 mg/g VSS for commercial enzymes. EPS concentrations were determined only at the beginning and at the end of the test; therefore, the observed changes are interpreted in reference to literature data indicating limited variation of tightly bound EPS during anaerobic digestion [61]. On the other hand, the decrease in PN/PS ratio is also noticed in samples with untreated inoculum, but that can be a result of degradation of the EPS caused by a lack of additional substrate before anaerobic digestion.

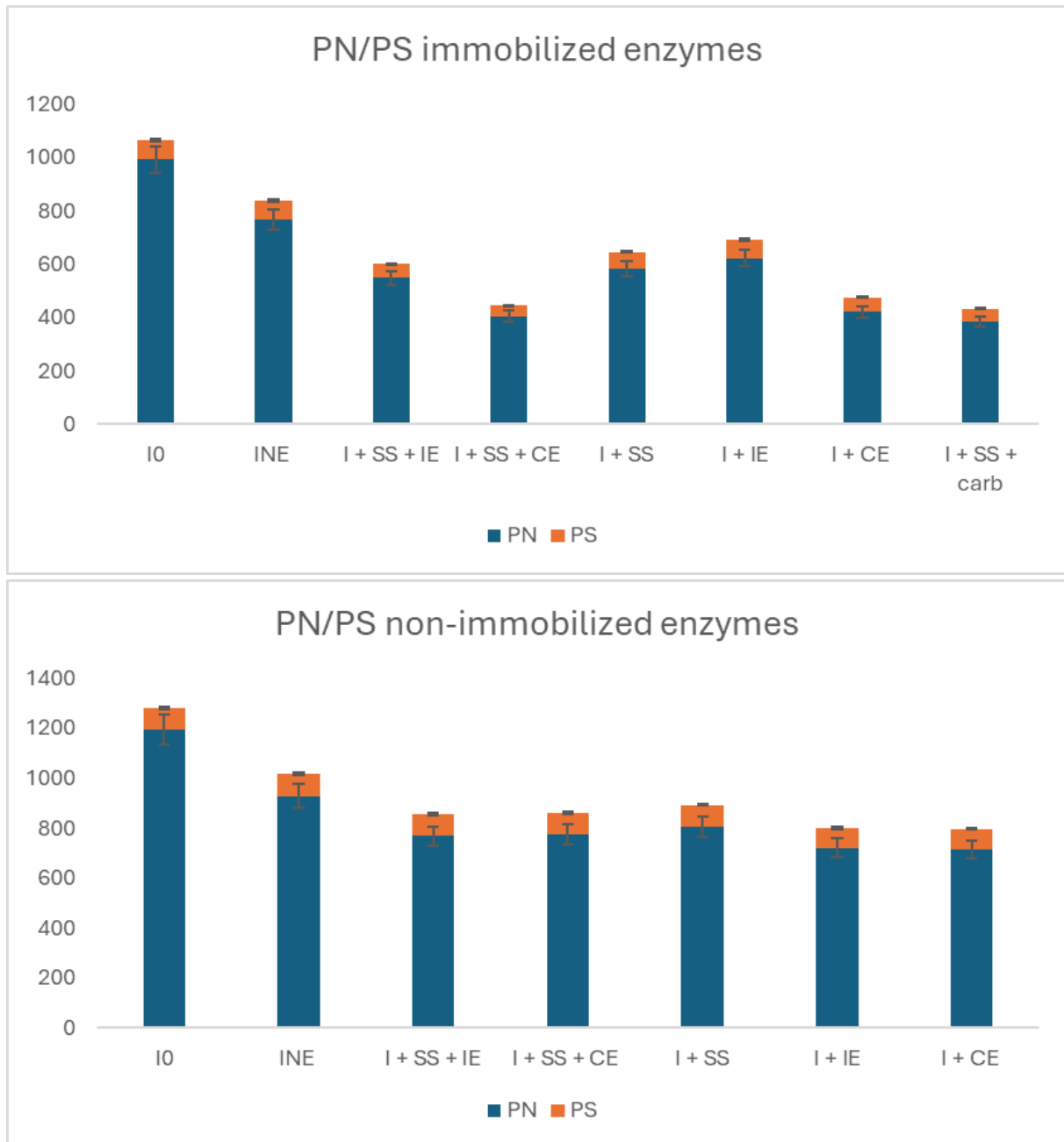


Figure 4. Proteins and saccharides ratio, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production. I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon.

The highest EPS decrease among the samples is noticed in sludge with immobilized isolated enzymes with mixed sludge as a substrate and immobilized enzymes with inoculum, without the addition of a substrate. EPS concentration can be the factor that indicates the influence of the enzymes on the methane production in this research, as the removal of EPS enhances the hydrolysis and further the fermentation of the sludge [54].

Table 1 presents the PN/PS ratio of sludges treated with enzymes. The PN/PS ratio decreases for all samples, regardless of the treatment. The greatest decrease is observed for the sludge treated with activated carbon and is similar to the samples with commercial immobilized enzymes unfed with mixed sludge. PN/PS ratio for untreated

with enzymes mixed sludge (9.48 and 9.35) was lower than in samples of inoculum (11.13 and 10.24), suggesting that the addition of the new portion of substrate for the inoculum caused stable degradation of EPS, whereas in samples without addition of the mixed sludge, previously stored EPS was degraded after depletion of multimolecular organic compounds.

Table 1. PN/PS ratio of sludges before and after an Automatic Methane Potential Test (AMPTS), treated with immobilized and non-immobilized isolated and commercial enzymes. I- Inoculum, I0- at time “0”, INE- after AMPTS, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme, carb- activated carbon.

	I0	INE	I+SS	I+SS+IE	I+SS+CE	I+IE	I+CE	I+SS+carb
Immobilized	13.6	11.13	9.48	10.51	10.35	8.68	7.78	7.88
Non-immobilized	13.6	10.24	9.35	8.79	9.04	9.03	8.41	-

The impact of the enzymes, depending on their form (immobilized or non-immobilized) and type (commercial or isolated), is statistically significant with a p -value<0.05.

3.5. *Effect of enzyme pretreatment on the cellulose concentration in the sewage sludges*

The PN-92/P-50092 norm for the cellulose concentration allows for the determination of the concentration of cellulose released from the lignocellulosic complex by dissolving the lignin. Due to that, the results must be considered in reference to the initial concentration of cellulose in the inoculum and to the I + SS. The concentration of free cellulose decreased after AMPTS in all samples, regardless of enzyme or activated carbon addition. The extent of cellulose reduction was similar across all treatments (approximately 90%, considering standard deviation), indicating that neither enzymes nor activated carbon significantly affected cellulose content within the lignocellulosic complex. The observed decrease relative to the initial inoculum is most likely due to microbial activity in the sludge.

A more pronounced decrease in cellulose concentration was observed following the addition of immobilized commercial enzymes to the inoculum, suggesting that enzyme application after digestion or at a different stage than fresh sludge addition may enhance the degradation of complex organic compounds. This effect may result from prior microbial disruption of the lignocellulosic structure, increasing cellulose accessibility. Mixing during the AMPTS test may have further contributed by reducing structural recalcitrance and facilitating microbial degradation.

Overall, cellulose degradation appears to be driven primarily by microbial processes rather than by the used treatments. PS analysis indicated no increased saccharide production, suggesting that the applied doses of cellulolytic enzymes and activated carbon did not significantly influence digestion. Although both immobilized and non-immobilized enzymes enhanced methane production, this effect is likely due to microbial stabilization or support rather than increased conversion of cellulosic compounds.

Table 2 shows the cellulose concentrations of sludges used in the AMPTS tests. In samples treated with non-immobilized commercial enzymes, approximately 10% cellulose degradation was observed in all cases except I+SS+IE. This suggests that activated carbon may protect immobilized enzymes from the degradative conditions of digested sludge. In samples treated with non-immobilized isolated enzymes, the observed changes may result from the utilization of other organic compounds rather than direct cellulose degradation. Because lignin is removed during cellulose analysis, the measured cellulose content represents total cellulose in the sludge. Therefore, the absence of differences between initial and final cellulose concentrations may be due to the release of cellulose from the lignocellulosic complex rather than its degradation during the methane potential test.

Table 2. Cellulose concentrations determined before and after an Automatic Methane Potential Test (AMPTS) for the inoculum (digested sludge) and a mix of the thickened primary and waste activated sludge, treated with immobilized and non-immobilized enzymes: isolated and commercial. I- Inoculum, I0- at time “0”, INE- after AMPTS, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme, carb- activated carbon.

Type of sludge and/or mix of sludge with enzymes	Cellulose concentration (% TS)	
	Immobilized enzymes	Non-immobilized enzymes
I0	31.20±3.63	30.52±0.81
INE	28.28±1.84	28.61±0.33
I+SS	29.93±0.69	27.53±1.94
I+SS+IE	28.09±1.92	22.58±0.75
I+SS+CE	28.87±0.96	27.98±0.79
I+carb	27.88±1.0	-
I+IE	28.17±3.38	29.64±2.63
I+CE	11.21±0.37	28.56±0.88

The statistical analysis indicates that the differences between immobilized and non-immobilized isolated enzymes are significant (p -value = 0.01117). As the concentration of PS (Figure 4) was almost unchanged in the samples before and after AMPTS, we can assume that there was a degradation of organic compounds with the production of saccharides, which were consumed at the same time and remained at the initial level.

Based on the results of the produced biogas and degraded cellulose, it is not possible that the enzymes and activated carbon did not influence the anaerobic digestion at any level. The lack of differences can be caused by the amount of used enzymes, which was sufficient to enhance the degradation at the beginning of the process, but with time, the microorganisms already present in the sewage sludge were able to degrade similar level to the organic matter, but from easily accessible matter. It is possible that in treated and untreated samples, different compounds were degraded. This is confirmed by the literature, which argues that the addition of activated carbon enhances the hydrolysis and methane production [55, 56]. Additionally, the lack of differences could be caused by the nature of the enzymes. In reference to the non-immobilized enzymes, introduced enzymes could be degraded by proteases produced by the microorganisms of the sewage sludge [50, 66].

4. Conclusion

In this study, we analyzed the influence of the enzymes isolated from the cellulolytic autochthonous bacteria, commercial enzymes, non-immobilized and immobilized on an activated carbon as a carrier and activated carbon itself on methane production. The enzymatic treatment during anaerobic digestion resulted in increased methane production using immobilized and non-immobilized enzymes, with the used dose of enzymes equal to 1% per TS. Although no significant differences were observed between commercial and isolated enzymes, the form of enzyme application (immobilized vs non-immobilized) significantly affected methane production. Using isolated enzymes provided an increase of 18.9% (using immobilized enzymes) and 21.7% (using non-immobilized enzymes). The treatment used did not influence the cellulose concentration in the lignocellulose complex. Further research needs to investigate combining enzymatic pretreatment with different mechanical methods for increased decomposition of lignocellulosic biomass.

Statements and Declarations

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Dataset generated in Next-Generation Sequencing is available in the GenBank, under the link: <https://www.ncbi.nlm.nih.gov/biosample/52862516>.

Other datasets used and/or analysed during the current study are available from the corresponding author on a request.

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Wpływ enzymów celulolitycznych na hydrolizę osadów ściekowych

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Streszczenie

Wykorzystanie różnego typu enzymów hydrolitycznych stanowi obiecującą alternatywę lub formę wspomagania obecnie stosowanych metod obróbki osadów ściekowych. Zastosowanie biologicznych metod może w znaczący sposób wspomóc degradację wielkocząsteczkowych związków organicznych obecnych w osadach ściekowych i tym samym zwiększyć efektywność procesu fermentacji oraz ilość produkowanego biogazu. Celem prowadzonych badań była weryfikacja wpływu biologicznej obróbki osadu za pomocą enzymów celulolitycznych na efektywność hydrolizy surowca do fermentacji. Porównane zostało działanie immobilizowanych i nieimmobilizowanych enzymów: produkowanych przez bakterie osadu ściekowego, oraz mieszaniny komercyjnych enzymów celulolitycznych. Ocenę efektywności wpływu badanych enzymów na hydrolizę osadów ściekowych dokonano na podstawie stężenia polimerów zewnątrzkomórkowych (EPS), lotnych kwasów tłuszczowych (LKT) i chemicznego zapotrzebowania na tlen (ChZT). Wyniki uzyskane podczas testu dostarczają informacji na temat efektywności degradacji substancji organicznych w trakcie hydrolizy. Otrzymane wyniki wskazują na intensyfikację hydrolizy po zastosowaniu enzymów celulolitycznych produkowanych przez bakterie osadu ściekowego oraz mieszaninę enzymów komercyjnych. Immobilizacja enzymów pozwoliła na zwiększenie stabilności enzymów, zwiększając jednocześnie intensywność działania enzymów komercyjnych w badanym osadzie.

Słowa kluczowe: fermentacja metanowa, hydroliza, osady ściekowe, enzymy celulolityczne

1. Wprowadzenie

W trakcie procesu oczyszczania ścieków powstają osady ściekowe, których skład zależy ściśle od rodzaju ścieków dopływających do oczyszczalni. Odpowiednie zagospodarowanie osadów stanowi dla zakładów oczyszczania ścieków duży problem, generując koszty sięgające około 50% ogólnych wydatków. Jedną z najpowszechniej stosowanych w Polsce metod, która pozwala na późniejsze wykorzystanie osadów ściekowych jest fermentacja beztlenowa [5], czyli proces mikrobiologiczny, polegającym na rozkładzie substancji organicznych przez mikroorganizmy w warunkach beztlenowych, z wydzielaniem metanu [18]. Na proces składają się 4 etapy, z których pierwszy - hydroliza jest najdłuższy i to on ma wpływ na przebieg całej fermentacji. W trakcie hydrolizy rozkładane są nierozpuszczalne związki organiczne do prostych związków rozpuszczalnych w wodzie, przy wykorzystaniu enzymów wytwarzanych przez mikroorganizmy hydrolityczne [4; 11]. Prawidłowy i jak najbardziej efektywny przebieg hydrolizy warunkuje ilość rozłożonych związków organicznych, ponieważ to produkty hydrolizy właśnie wykorzystywane są w kolejnych etapach fermentacji [18]. Fermentacja metanowa ma na celu zmniejszenie objętości osadów i ich stabilizację. Niestety często zdarza się tak, że parametry osadów po procesie fermentacji określone w uwarunkowaniach prawnych, nie pozwalają na wykorzystanie ich do wybranych celów. Najczęściej związane jest to z dużą zawartością suchej masy organicznej w przefermentowanych osadach. Jedną z substancji wpływającą negatywnie na ten parametr jest celuloza, czyli trudno rozkładalny polimer najpowszechniej występujący w środowisku [19]. Niemożliwość wykorzystania przefermentowanych osadów, determinuje konieczność stosowania dodatkowych metod obróbki osadów ściekowych przed fermentacją, do których zaliczyć można chemiczne, termo-chemiczne, mechaniczne i biologiczne metody [2]. Te ostatnie, zyskują w ostatnim czasie coraz większą uwagę, ze względu na ich przyjazny środowisku charakter. Osady ściekowe są zbiorem mikroorganizmów, produkujących różnego rodzaju enzymy,

które efektywnie rozkładają wiele substancji. Dlatego w prowadzonych badaniach podjęto decyzję o weryfikacji wpływu celulolitycznych enzymów na hydrolizę osadów ściekowych. Ze względu jednak na to, że enzymy są dosyć wrażliwe i mogą łatwo ulegać degradacji, zweryfikowane zostało ich działanie w formie nieimmobilizowanej i immobilizowanej na nieorganicznym nośniku.

2. Materiały i metody

2.1. Ekstrakcja i immobilizacja enzymów

Do ekstrakcji enzymów wykorzystano 50 ml mieszanej hodowli bakterii celulolitycznych i 150 ml płynnego podłoża, w którym jedynym źródłem węgla była karboksymetyloceluloza w postaci soli sodowej (5 g/L) (Sigma-Aldrich). W tabeli 1 przedstawiono pełny skład wykorzystanego podłoża. Hodowlę prowadzono w warunkach beztlenowych, w 37°C przez 7 dni z wytrząsaniem 100 rpm. Ekstrakcję przeprowadzono z wykorzystaniem wirowania. Próbkę osadu wirowano przez 20 minut przy 2000 rpm w 4°C. Pozostała ciecz nadosadowa stanowiła źródło enzymów. Oznaczono stężenie białka, które wynosiło 3089,3 mg/L.

Celem porównania efektywności wyizolowanych enzymów w badaniach wykorzystano mieszaninę komercyjnie dostępnych celulolitycznych enzymów zawierających celulazy, β -glukozydazy i hemicelulazy (Sigma Aldrich).

Tab. 1. Skład podłoża do hodowli bakterii celulolitycznych i produkcji enzymów w hodowli płynnej.

Składnik	Stężenie, g/L
Karboksymetyloceluloza (CMC)	5
NaNO ₃	1
K ₂ HPO ₄	1
KCl	1
MgSO ₄	0,5
Ekstrakt drożdżowy	0,5
Glukoza	1

Wyizolowane enzymy poddano immobilizacji z wykorzystaniem nieorganicznego nośnika, jakim był węgiel aktywny.

2.2 Testy porcjowe krótkotrwałego wpływu enzymów na badane parametry osadu ściekowego

Immobilizowane i nieimmobilizowane enzymy dodano do 15 ml zmieszanego osadu ściekowego (osad nadmierny zagęszczony: osad wstępny zagęszczony grawitacyjnie w stosunku 65%:35%) w temperaturze 37°C 120 rpm przez 60, 180 i 240 min. Do 15 ml osadu dodano odpowiednio 0,001g immobilizowanych enzymów izolowanych lub komercyjnych i 500 μ l nieimmobilizowanych enzymów izolowanych lub komercyjnych.

2.2. Ekstrakcja polimerów zewnątrzkomórkowych (EPS)

Izolację EPS prowadzono z wykorzystaniem ekstrakcji cieplnej przy 80°C i wirowania ([10]). Próbkę osadu wirowano przez 20 minut przy 2000 rpm. Zlano ciecz nadosadową, uzupełniono wodą destylowaną do objętości początkowej próbki, a następnie poddano ogrzewaniu przez godzinę w 80°C. Po zakończonej inkubacji ponownie uzupełniono odparowaną ciecz do objętości początkowej, próbkę wirowano przez 20

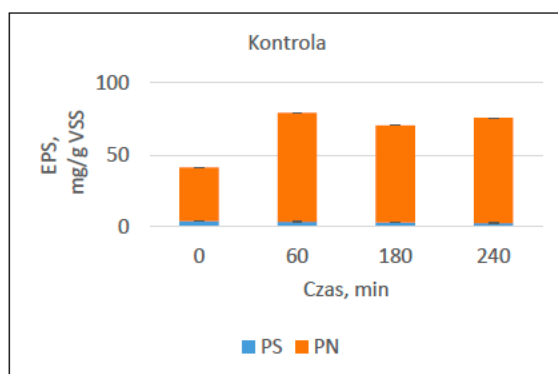
min przy 2000 rpm oraz 20 min przy 3000 rpm. Supernatant uzyskany po wirowaniu stanowił ekstrakt polimerów zewnątrzkomórkowych.

2.3. Oznaczenie polimerów zewnątrzkomórkowych, lotnych kwasów tłuszczowych i chemicznego zapotrzebowania na tlen

W próbkach oznaczano EPS związane, lotne kwasy tłuszczowe i chemiczne zapotrzebowanie na tlen. EPS oznaczano jako sumę sacharydów (PS) i białek (PN). Oznaczenie zawartości cukrów wykonano według Dubois i wsp., (1956). Zamiast 80% roztworu fenolu, wykorzystano fenol o stężeniu 5% [14]. Do oznaczenia białek wykorzystano gotowy zestaw Pierce™ BCA Protein Assay Kit i procedurę próbówkową zgodnie z instrukcją producenta (Thermo Fisher Scientific). Oznaczenie LKT i ChZT wykonano z wykorzystaniem gotowych zestawów zgodnie z instrukcją producenta (Merck).

3. Wyniki i dyskusja

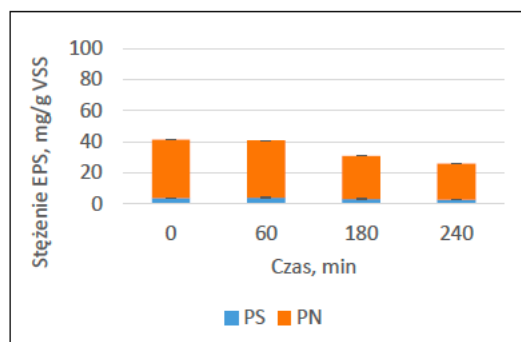
Przeprowadzone badania pozwoliły porównać wpływ enzymów celulolitycznych izolowanych z mieszanej hodowli bakterii celulolitycznych bytujących w osadzie ściekowym, oraz celulolitycznych enzymów komercyjnych. Na rysunku 1 przedstawiono zmiany stężenia polimerów zewnątrzkomórkowych w próbkach niepoddawanych działaniu żadnych czynników (enzymów czy nośnika). Próbki te stanowiły kontrolę.



Rys. 1. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w kontrolnej próbce osadu ściekowego, nienarażonego na działanie enzymów.

Na wykresie zaobserwowano wzrost stężenia EPS po 60 minutach inkubacji i nieznaczny spadek w kolejnym wyznaczonym czasie pomiaru. Jedynym czynnikiem wpływającym na potencjalne zmiany w stężeniach EPS w próbce kontrolnej mogło być wytrząsanie, które wymusiło na mikroorganizmach konieczność wzmożonej produkcji EPS celem ochrony przed zmianami w środowisku. Ze względu jednak na to, że oddziaływanie nie było dla mikroorganizmów nadmiernie stresujące, w kolejnych godzinach nie zaobserwowano wzrostu stężenia sacharydów i białek, a sumaryczny poziom EPS zaczął się stabilizować.

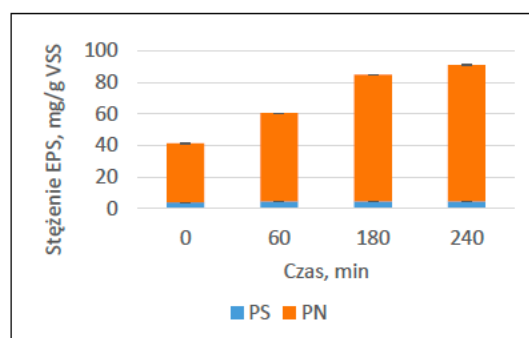
Na rysunku 2 przedstawiono zmiany stężenia EPS w próbkach wystawionych na działanie nieimmobilizowanych enzymów izolowanych z mieszanej hodowli bakterii celulolitycznych.



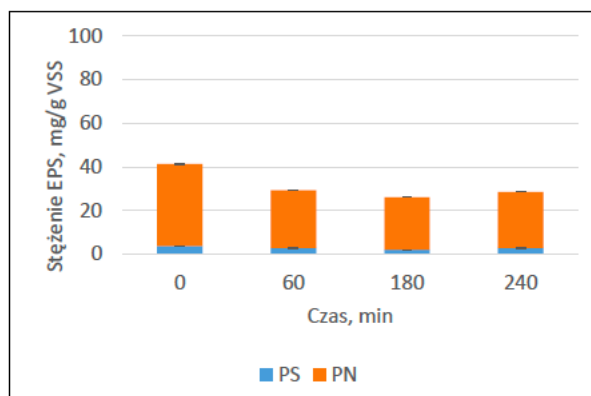
Rys. 2. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w próbce osadu ściekowego poddawanej na działanie nieimmobilizowanych enzymów izolowanych z celulolitycznych mikroorganizmów osadu czynnego.

Po 180 minutach kontaktu osadu z enzymami obserwowany jest spadek stężenia EPS, co może świadczyć o degradacyjnym wpływie enzymów na badane polimery zewnątrzkomórkowe. Konsekwencją degradacji EPS w osadzie ściekowym może być zakwaszenie osadu, co wspomaga hydrolizę, a w konsekwencji proces fermentacji metanowej [16]. Po 240 minutach stężenie EPS było niższe o 38%, w porównaniu ze stężeniem zmierzonym na początku eksperymentu.

W przypadku nieimmobilizowanych enzymów komercyjnych obserwowany jest wzrost stężenia EPS (rys. 3.). Najprawdopodobniej jest to odpowiedź mikroorganizmów na stresujące warunki, wywołane wprowadzeniem do próbki enzymów celulolitycznych lub zwiększonej ilości łatwo przyswajalnych substancji, powstałych jako produkty rozkładu złożonych związków przez enzymy [12]. W skład komercyjnej mieszaniny enzymów celulolitycznych wchodzi celulaza, β -glukozydazy i hemicelulazy, które efektywnie rozkładają celulozę, hemicelulozę, ale także inne cukry [6]. Na rysunku 4 przedstawiony został wpływ węgla aktywnego na stężenie EPS.



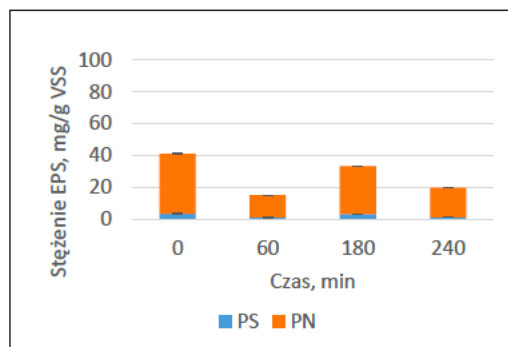
Rys. 3. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w próbce osadu ściekowego poddawanej na działanie nieimmobilizowanych celulolitycznych enzymów komercyjnych.



Rys. 4. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w próbce osadu ściekowego poddawanego na działanie węgla aktywnego.

W przypadku węgla aktywnego obserwowany jest spadek stężenia EPS, co może być związane z właściwościami węgla aktywnego, czyli zdolnością do sorbowania różnych substancji i związków na swojej powierzchni. Jiang i wsp. [2021] zaobserwowali, że lepiej adsorbują się na granulowanym węglu aktywnym substancje o większej masie cząsteczkowej, tj. białka, sacharydy, niż małe cząsteczki, takie jak LKT czy glukoza. Podobnie rezultaty obserwowane są w opisywanych przez nas badaniach, w których wykorzystywany był sproszkowany węgiel aktywny. Węgiel aktywny sprzyja poprawie różnorodności mikroorganizmów bytujących w osadach ściekowych, poprawiając stabilność struktury społeczności najprawdopodobniej na skutek adsorpcji mikroorganizmów na powierzchni węgla aktywnego [8; 15]. Dzięki temu mikroorganizmy obecne w osadzie są w stanie efektywniej hydrolizować dostępne związki organiczne, a przez to intensyfikować proces fermentacji metanowej [17].

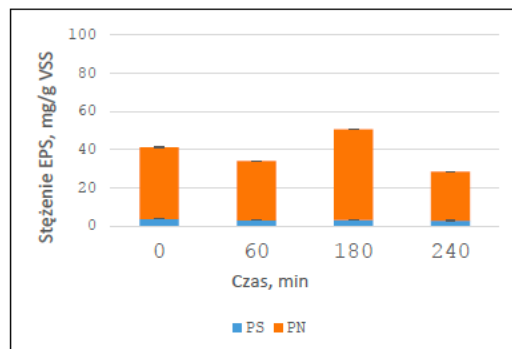
Wpływ immobilizowanych enzymów celulolitycznych przedstawiono na rysunku 5. Uzyskane stężenia również potwierdzają degradacyjny wpływ enzymów na obecne w osadzie polimery zewnątrzkomórkowe. W porównaniu z nieimmobilizowanymi enzymami, spadek stężenia EPS obserwuje się już w 60 minucie testu. Spadek zaobserwowano również przy wykorzystaniu samego węgla aktywnego, co może świadczyć o wsparciu enzymatycznej obróbki przez nośnik sam w sobie. Powierzchnia wykorzystywanego nośnika nie została w pełni pokryta przez enzymy, pozostawiając na nim wolne przestrzenie, przez co w próbkach z immobilizowanymi enzymami obserwowany był wpływ zarówno enzymów jak i samego nośnika. Stężenie EPS w próbkach osadu ściekowego z immobilizowanymi enzymami izolowanymi spadło o 62% po 60 minutach kontaktu. W przypadku wykorzystania samego węgla aktywnego, maksymalny procent degradacji zaobserwowano po 180 minutach kontaktu (o 36%).



Rys. 5. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w próbce osadu ściekowego poddawanej na działanie immobilizowanych enzymów izolowanych z celulolitycznych mikroorganizmów osadu czynnego.

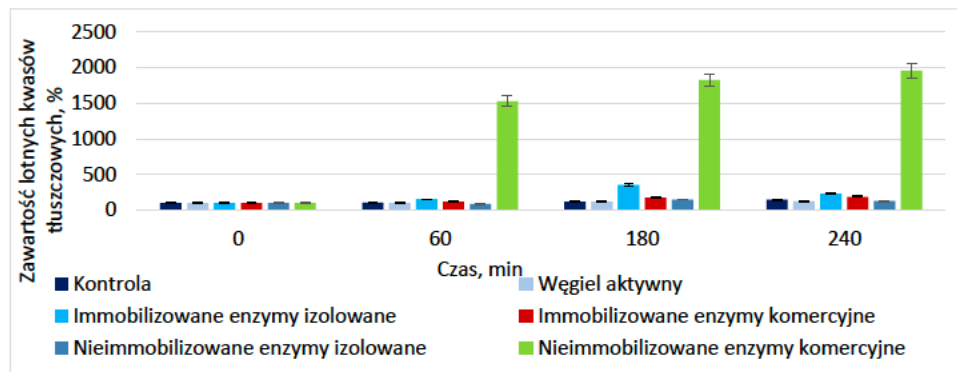
Immobilizowane enzymy komercyjne pozwoliły na degradację EPS w 17% w ciągu pierwszych 60 minut. W trakcie kolejnego pomiaru po 180 minutach, kontakt komercyjnych nieimmobilizowanych enzymów celulolitycznych doprowadził do wzrostu produkcji EPS w badanym osadzie o 25% w stosunku do początkowej zawartości EPS w osadzie i o ponad 50% w stosunku do stężenia obserwowanego w czasie 60 min od rozpoczęcia testu. Wzrost stężenia EPS może świadczyć być spowodowany zmianą warunków panujących w środowisku na skutek zwiększonej degradacji związków organicznych obserwowanych na podstawie wyznaczonego stężenia ChZT (rys. 7), co przyczyniło się do zwiększonej produkcji EPS. Obniżenie stężenia EPS po 240 minutach kontaktu z enzymami może być konsekwencją kontynuacji degradacyjnego wpływu enzymów na EPS, których wartości ukształtowały się ponownie na poziomie stężenia obserwowanego po 60 minutach inkubacji. W przypadku immobilizowanych enzymów izolowanych i komercyjnych widoczne są spadki stężenia EPS po 60 minutach i ponowny wzrost po 180 minutach. W przypadku enzymów izolowanych spadek jest większy, niż w przypadku enzymów nieimmobilizowanych co sugeruje wzmacniający i stabilizujący efekt działania węgla aktywnego. W przypadku enzymów komercyjnych, immobilizacja enzymów na nośniku mogła wpłynąć na ograniczenie intensywnego wpływu na rozkład substancji organicznej, przez co mikroorganizmy nie zostały wystawione na działanie stresujących dla nich warunków. Wydłużanie czasu kontaktu enzymów z osadem bezpośrednio wpływający na wydłużanie czasu inkubacji w fazie stacjonarnej może prowadzić do obniżenia stężenia EPS [13]. Ponowny spadek po 240 minutach testu mógł być spowodowany wyczerpaniem wielkocząsteczkowych związków organicznych obecnych w osadzie ściekowym, przez co enzymy zaczęły degradować polimery zewnątrzkomórkowe.

Podobne wahania stężenia EPS, jak w przypadku immobilizowanych enzymów izolowanych, zaobserwowano w próbkach z immobilizowanymi enzymami komercyjnymi, przedstawionymi na rys. 6.



Rys. 6. Zmiany stężenia EPS w czasie 0, 60, 180 i 240 min jako suma sacharydów (PS) i białek (PN) w próbce osadu ściekowego poddawanego na działanie immobilizowanych celulolitycznych enzymów komercyjnych.

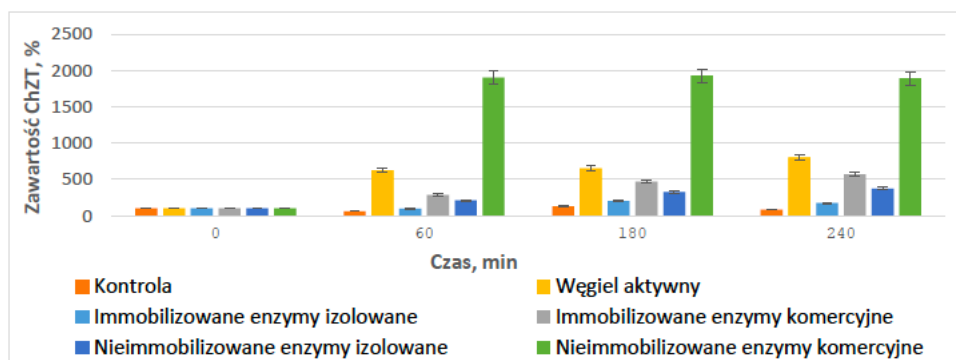
Na wykresie przedstawiającym stężenie EPS w osadzie poddanym działaniu immobilizowanych enzymów komercyjnych (rys. 6) widoczny jest spadek stężenia w 60 minucie i ponowny wzrost po 180 minutach prowadzenia testu. Po 240 minutach kontaktu osadu z immobilizowanymi enzymami komercyjnymi obserwuje się spadek. Przebieg procesu dla immobilizowanych enzymów izolowanych i komercyjnych jest podobny, z tą różnicą, że w przypadku enzymów komercyjnych zmiany stężenia są większe. Po 240 minutach kontaktu enzymów komercyjnych z osadem, stężenie EPS spadło do poziomu obserwowanego w próbkach z węglem aktywnym po tym samym czasie, co może sugerować wyczerpanie się enzymu na nośniku lub jego denaturację na skutek zakwaszenia środowiska i wspomaganie procesu jedynie na skutek działania samego nośnika. To sugerowałoby, że immobilizacja enzymu komercyjnego nie zwiększa jego stabilności. Spadek stężenia EPS w próbkach z dodatkiem immobilizowanych enzymów izolowanych obserwuje o 38% pomiędzy czasem 180 i 240 min. W przypadku enzymów komercyjnych spadek stężenia wynosi 45%. Wyniki stężenia EPS łączą się również z poziomem stężeń lotnych kwasów tłuszczowych oznaczonych w próbkach. Procentową zawartość lotnych kwasów tłuszczowych przedstawiono na rys. 7.



Rys. 7. Procentowa zawartość LKT w badanym osadzie ściekowym, poddanym działaniu węgla aktywnego i enzymów nieimmobilizowanych i immobilizowanych.

Wzrost stężenia LKT prowadzi do zakwaszania osadu i świadczy o efektywnej hydrolizie tłuszczu. Na rysunku 8 widoczny jest znaczny wzrost stężenia LKT w przypadku nieimmobilizowanych enzymów komercyjnych. Obserwowany jest już po 60 minutach, co potwierdza przypuszczenia o zakwaszaniu osadu. Dodatek nieimmobilizowanych enzymów komercyjnych doprowadził do wzrostu stężenia EPS, który scharakteryzowany był wcześniej jako odpowiedź mikroorganizmów na stresujące warunki. Mogły one zostać właśnie wywołane nagłym pojawieniem się dużej ilości lotnych kwasów tłuszczowych, których stężenie w ciągu pierwszych 60 minut testu wzrosło o ponad 1500%, a w 240 minucie o około 1856% w porównaniu z próbą kontrolną. Wzrost stężenia LKT w przypadku immobilizowanych enzymów izolowanych był większy, niż w przypadku immobilizowanych enzymów komercyjnych o 50% po 180 minutach trwania testu. Zmiany stężenia LKT zmierzone w próbkach z immobilizowanymi enzymami izolowanymi 180 minucie odpowiadają wzrostowi stężenia EPS, co było najprawdopodobniej czynnikiem zmuszającym bakterie do produkcji EPS jako ochrony przed niepożądanym czynnikiem. W 240 minucie stężenie LKT obniżyło się, jak i stężenie EPS. Stężenie LKT w próbkach z węglem aktywnym wzrosło o 250% po 180 minutach trwania testu, co potwierdza doniesienia literaturowe o zakwaszającym działaniu węgla aktywnego na wczesnym etapie [17].

Parametrem pozwalającym określić efektywność rozkładu substancji organicznej w osadzie było stężenie chemicznego zapotrzebowania na tlen (rys. 8.).



Rys. 8. Procentowa zawartość ChZT w badanym osadzie ściekowym, poddanym działaniu węgla aktywnego i enzymów nieimmobilizowanych i immobilizowanych.

Wszystkie badane czynniki wpłynęły na wzrost stężenia ChZT, co świadczy o wzroście biodegradowalności osadu [1]. Ponownie największe stężenie obserwowane było przy kontakcie osadu z nieimmobilizowanymi enzymami komercyjnymi. Wskazuje to na intensywny wzrost substancji organicznej, co łączy się ze wzrostem EPS. Wysoki poziom stężenia ChZT obserwowany był również w próbkach z samym węglem aktywnym i immobilizowanymi enzymami komercyjnymi. Dodatek węgla aktywnego sprawia, że struktura społeczności mikroorganizmów osadu czynnego jest bardziej stabilna, przez co efektywniej wykorzystuje materię organiczną [7]. W próbkach z izolowanymi enzymami immobilizowanymi obserwuje się mniejsze stężenie ChZT, niż w próbkach z samym węglem aktywnym, co wskazuje na wspomaganie degradującego działania enzymów izolowanych.

4. Podsumowanie

Enzymy celulolityczne produkowane przez autochtoniczne bakterie osadu ściekowego i celulolityczne enzymy komercyjne wspomagają hydrolizę i poprawiają stopień biodegradowalności osadu ściekowego.

Na podstawie przeprowadzonych badań stwierdzono efektywną degradację EPS w osadzie ściekowym, poddanym działaniu nieimmobilizowanych enzymów celulolitycznych produkowanych przez bakterie osadu ściekowego. Zaobserwowano również, że nawet krótkotrwały kontakt osadu z enzymami wpływa pozytywnie na degradację substancji organicznych i poprawę hydrolizy badanych osadów. Jednocześnie, sam węgiel aktywny efektywnie wspomaga hydrolizę osadów, przez co wykorzystanie go jako nośnika dla enzymów celulolitycznych przynosi dodatkową korzyść. Wykorzystanie węgla aktywnego jako nośnika dodatkowo stabilizuje działanie enzymów produkowanych przez bakterie osadu ściekowego i enzymy komercyjne. Uzyskane wyniki zachęcają do rozszerzenia badań o kolejne parametry charakteryzujące efektywność tego rodzaju obróbki osadu i weryfikację możliwości przeskalowania eksperymentu.

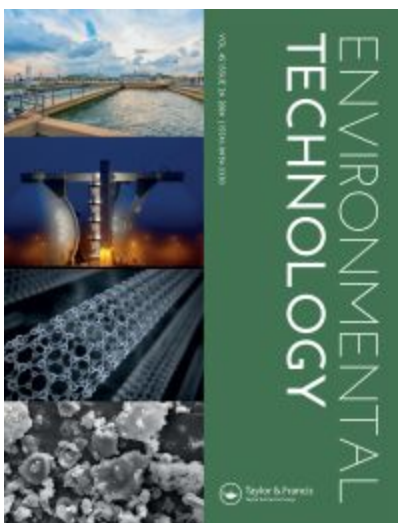
Dodatkowe informacje

Badania wspierane przez Politechnikę Śląską, Wydział Inżynierii Środowiska SUT 08/070/BKM24/0033 (BKM-715/RIE7/2024) w ramach subwencji na utrzymanie i rozwój potencjału badawczego Młodzi Naukowcy. A. Ziemińska-Buczyńska jest finansowana z projektu Politechniki Śląskiej nr: 08/070/BK_24/0031 (BK-217/RIE7/2024).

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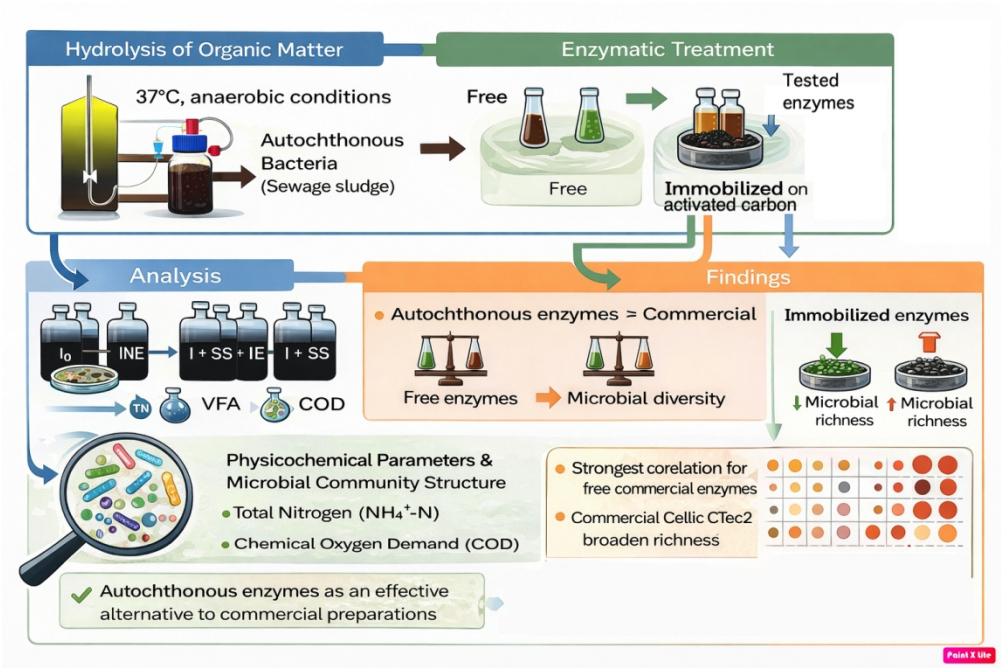
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Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and Microbial Community Effects

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Keywords:	sewage sludge, autochthonous cellulolytic enzymes, commercial cellulases, biological treatment, microbial community





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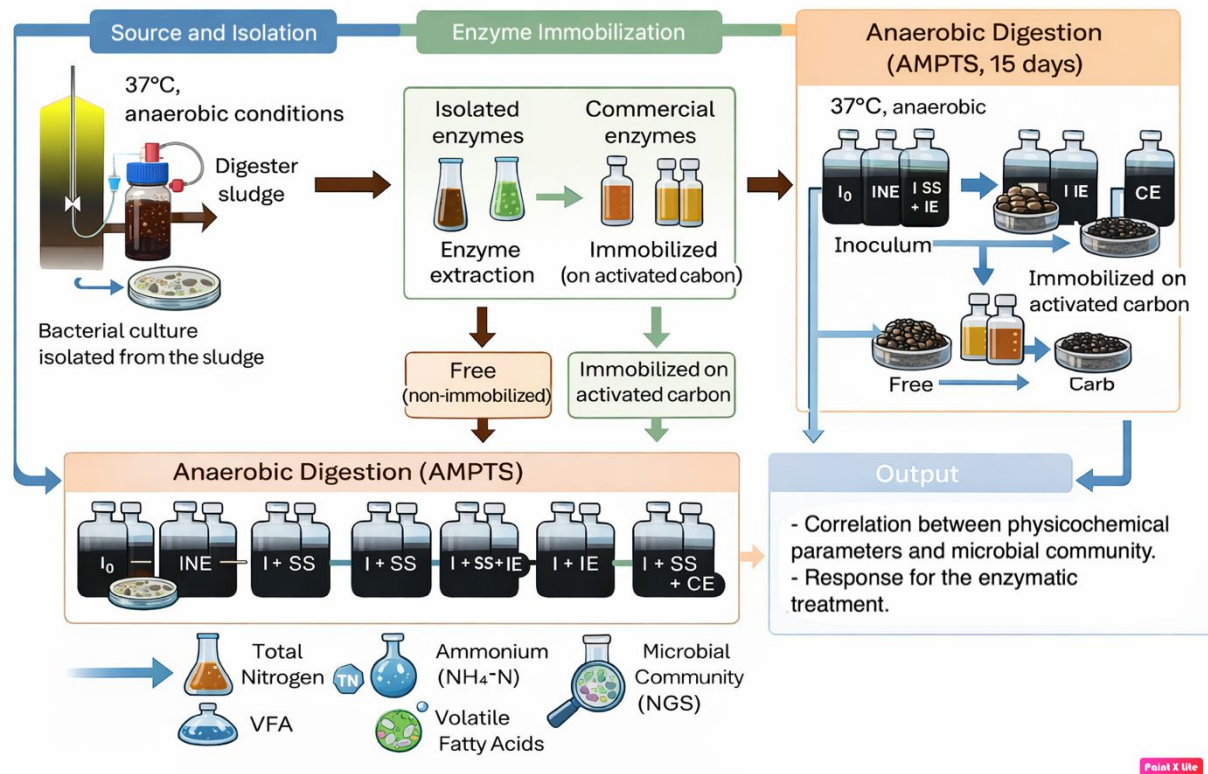


Figure 1. Schematic diagram of the steps taken to achieve the implementation of the planned experiment.

The abbreviations used for the experimental variants are explained in Table 1.

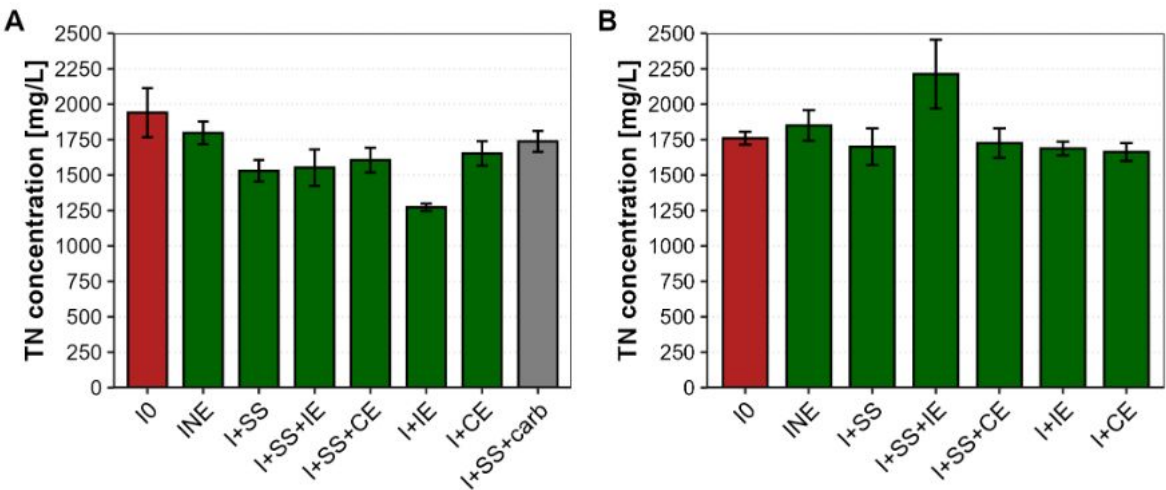


Figure 2. Total nitrogen concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations (n = 4). Error bars represent the standard errors.

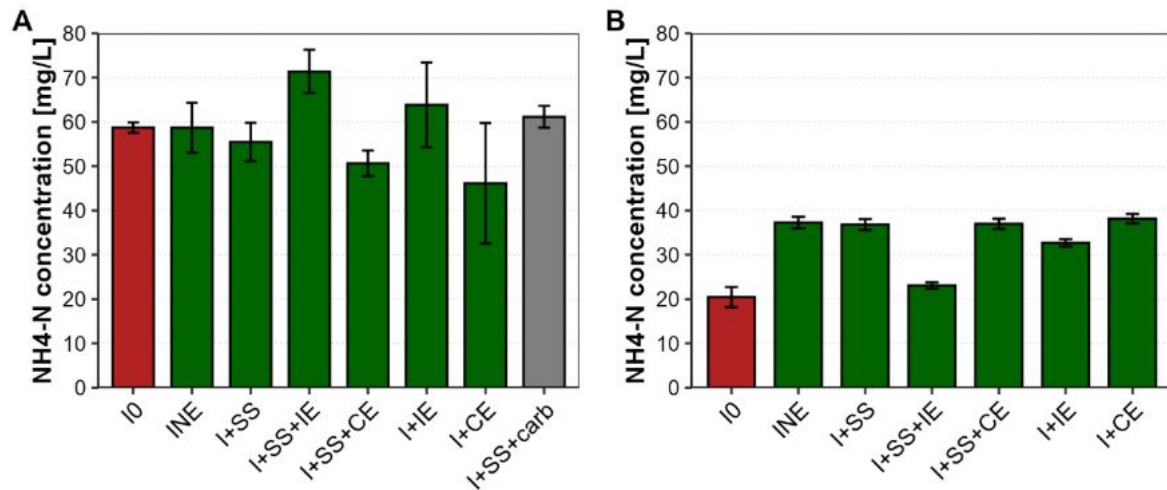


Figure 3. Ammonium concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production, measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations (n = 4). Error bars represent the standard errors.

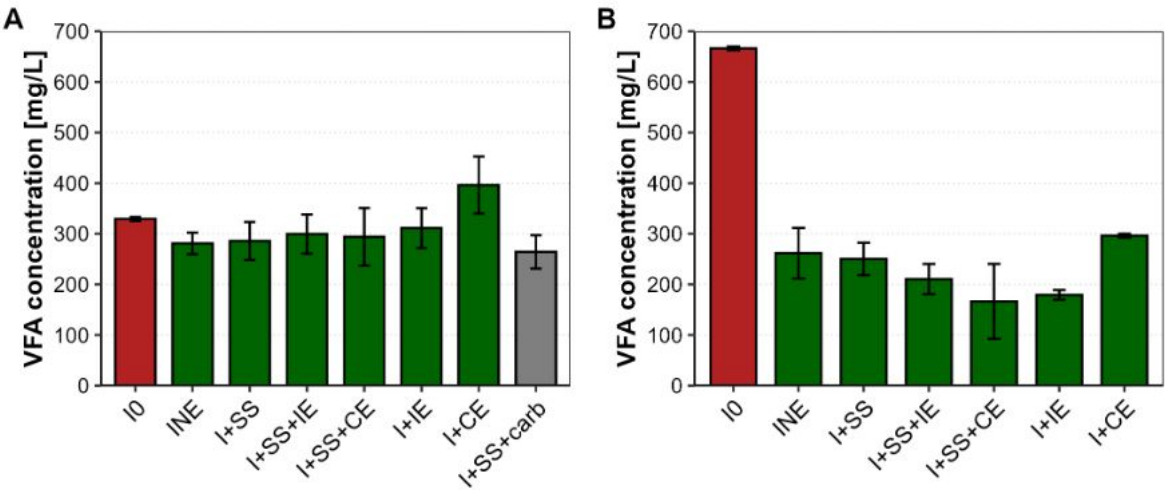


Figure 4. Volatile fatty acids concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations (n = 4). Error bars represent the standard errors.

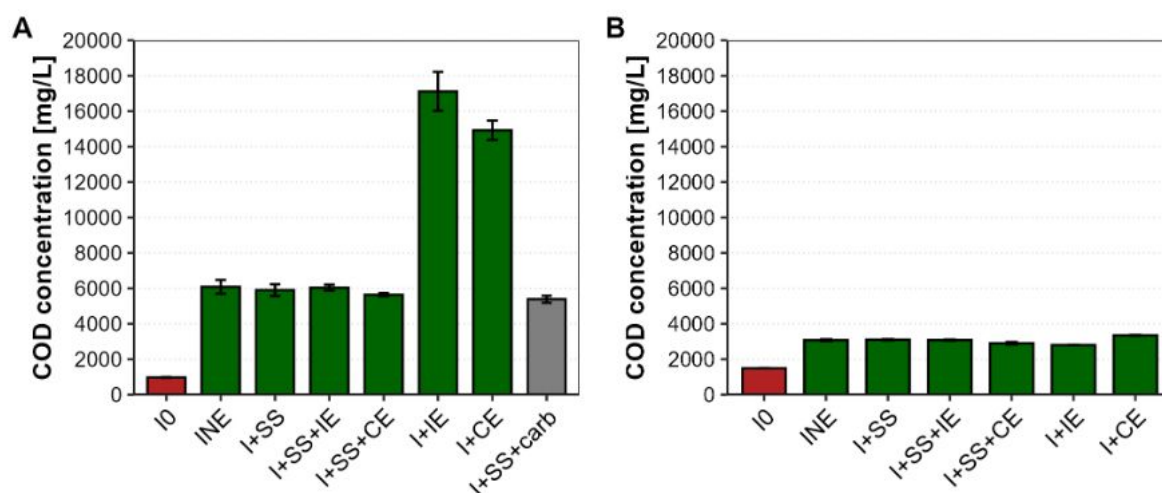


Figure 5. Chemical oxygen demand concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations ($n = 4$). Error bars represent the standard errors.

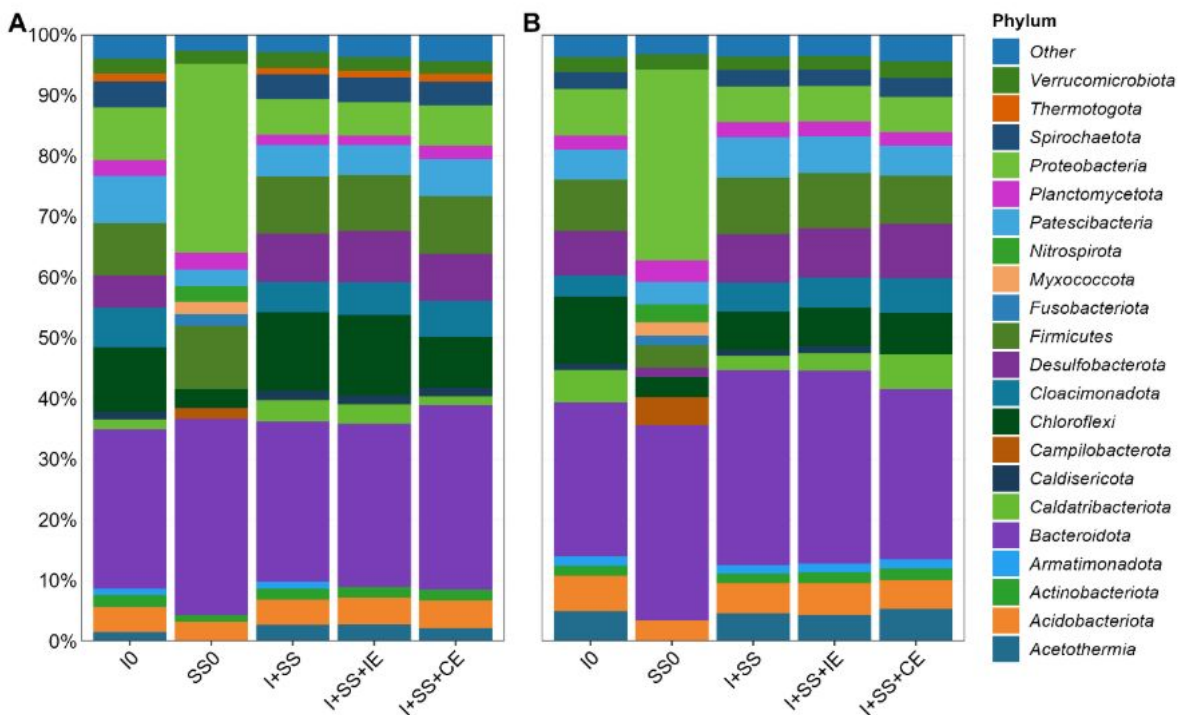


Figure 6. Microbial community structure at the phylum, regarding the 22 most abundant groups of bacteria in the sludges. Sequences that accounted for less than 1% of the population were grouped into the category “Others”.

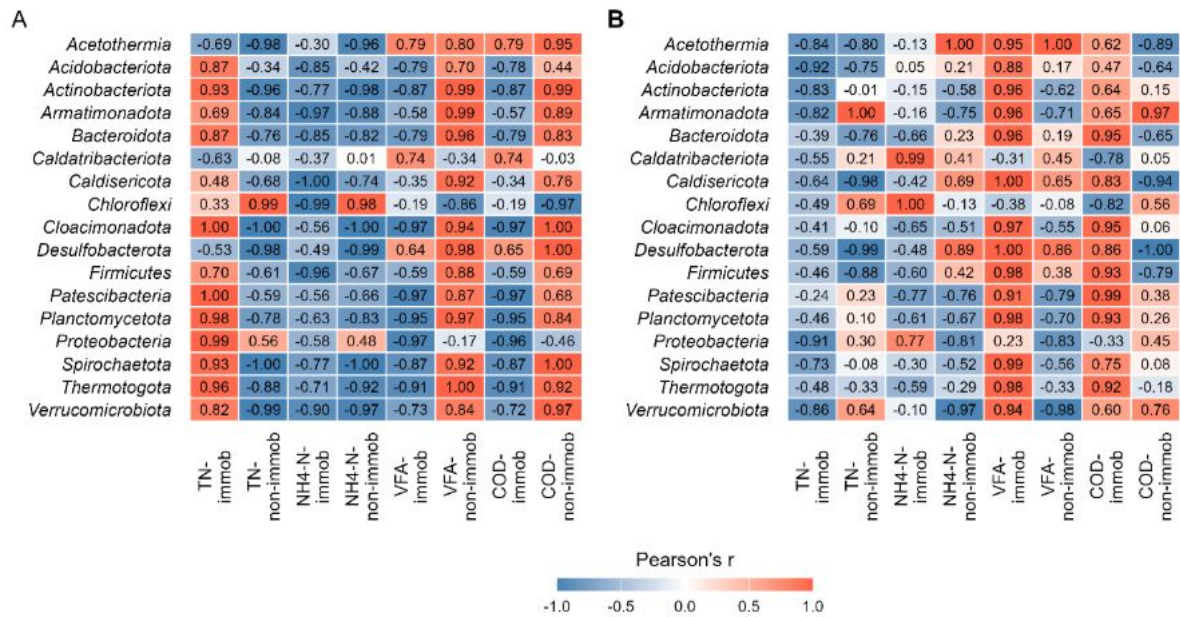


Figure 7. Pearson correlation coefficient between physicochemical parameters and microbial abundance ratio in the consortia at the phylum level using isolated (A) and commercial (B) enzymes. The red color indicates a decrease in one parameter with an increase in the second parameter. The blue color indicates a relation between the increase or decrease of two parameters simultaneously, microbial abundance and physicochemical parameters.

Supplementary Materials
For
Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and Microbial Community Effects

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Table S1. Abundance of the bacteria present in the sewage sludge, treated with immobilized, isolated, and commercial enzymes, among the genus. I0- inoculum before the AMPTS, SS0- mixed sludge (thickened primary + waste activated sludge before the AMPTS), I + SS- sample of inoculum and mixed sludge after AMPTS, during the test with addition of immobilized enzymes for remaining samples, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme).

I0		SS0		I + SS		I + SS + IE		I + SS + CE	
<i>Acetothermiia</i>	1.509%	<i>Bacteroidales</i>	1.009%	<i>Acetothermiia</i>	2.151%	<i>Acetothermiia</i>	2.668%	<i>Acetothermiia</i>	2.754%
<i>Aminicenantales</i>	1.227%	<i>Bacteroides</i>	2.016%	<i>Aminicenantales</i>	1.888%	<i>Aminicenantales</i>	1.791%	<i>Aminicenantales</i>	1.669%
<i>Thermoanaerobaculum</i>	1.387%	<i>Prevotella</i>	8.991%	<i>Thermoanaerobaculum</i>	1.373%	<i>Thermoanaerobaculum</i>	1.195%	<i>Thermoanaerobaculum</i>	1.437%
<i>Bacteroidetes</i> vadinHA17	4.757%	<i>Terrimonas</i>	1.043%	<i>Bacteroidetes</i> vadinHA17	7.94%	<i>Bacteroidetes</i> vadinHA17	6.548%	<i>Bacteroidetes</i> vadinHA17	6.61%

<i>Prolixibacteraceae</i> ; g__uncultured	3.524%	f__ <i>Saprospiraceae</i> ; g__uncultured	6.671%	<i>Prolixibacteraceae</i> ; g__uncultured	6.803%	<i>Prolixibacteraceae</i> ; g__uncultured	5.992%	<i>Prolixibacteraceae</i> ; g__uncultured	6.309%
DMER64	2.021%	SJA-28	1.628%	DMER64	2.641%	DMER64	2.209%	DMER64	2.518%
dBlvii28_wastewater- sludge_group	4.321%	<i>Arcobacter</i>	1.124%	Blvii28_wastewater- sludge_group	6.066%	Blvii28_wastewater- sludge_group	5.756%	Blvii28_wastewater- sludge_group	5.663%
f__ <i>Saprospiraceae</i> ; g__uncultured	2.575%	<i>Butyrivibrio</i>	2.305%	<i>Saprospiraceae</i> ; g__uncultured	1.011%	<i>Lentimicrobium</i>	1.892%	<i>Lentimicrobium</i>	1.876%
<i>Lentimicrobium</i>	1.726%	<i>Ruminococcus</i>	1.012%	<i>Lentimicrobium</i>	1.963%	JS1	3.501%	JS1	3.149%
SJA-28	1.231%	<i>Acidaminococcaceae</i> ; g__uncultured	1.261%	SJA-28	1.167%	<i>Leptolinea</i>	2.95%	<i>Leptolinea</i>	2.869%
JS1	1.643%	<i>Leptotrichia</i>	1.771%	JS1	1.318%	<i>Anaerolineaceae</i> ; g__uncultured	6.943%	<i>Anaerolineaceae</i> ; g__uncultured	7.076%
<i>Leptolinea</i>	1.776%	<i>Nitrospira</i>	2.648%	<i>Leptolinea</i>	1.997%	Candidatus <i>Cloacimonas</i>	2.573%	Candidatus <i>Cloacimonas</i>	2.774%
f__ <i>Anaerolineaceae</i> ; g__uncultured	5.393%	<i>Novosphingobium</i>	1.044%	<i>Anaerolineaceae</i> ; g__uncultured	4.065%	W5	1.355%	W5	1.419%
Candidatus <i>Cloacimonas</i>	3.457%	<i>Sphingomonadaceae</i> ; g__uncultured	1.114%	Candidatus <i>Cloacimonas</i>	2.751%	<i>Smithella</i>	2.874%	<i>Smithella</i>	3.131%
W5	1.685%	f__ <i>Comamonadaceae</i>	1.418%	W5	1.839%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.223%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.306%
<i>Smithella</i>	1.856%	<i>Rhodoferrax</i>	1.641%	<i>Smithella</i>	2.606%	<i>Syntrophorhabdus</i>	2.106%	<i>Syntrophorhabdus</i>	2.388%
o__ <i>Syntrophales</i> ; f__uncultured; g__uncultured	1.153%	<i>Dechloromonas</i>	1.051%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.308%	<i>Gracilibacteraceae</i> ; g__uncultured	2.489%	<i>Gracilibacteraceae</i> ; g__uncultured	2.334%

<i>Syntrophorhabdus</i>	1.777%	SC-I-84	1.08%	<i>Syntrophorhabdus</i>	2.086%	<i>Sedimentibacter</i>	1.277%	<i>Sedimentibacter</i>	1.679%
f__ <i>Gracilibacteraceae</i> ; g__uncultured	2.832%	<i>Acinetobacter</i>	2.801%	<i>Gracilibacteraceae</i> ; g__uncultured	2.642%	DTU014	2.162%	DTU014	2.185%
DTU014	1.412%	<i>Dokdonella</i>	2.263%	<i>Sedimentibacter</i>	1.18%	<i>Dojkabacteria</i>	3.372%	<i>Dojkabacteria</i>	3.126%
<i>Dojkabacteria</i>	4.456%			DTU014	2.185%	<i>Spirochaetaceae</i> ; g__uncultured	3.097%	<i>Spirochaetaceae</i> ; g__uncultured	3.153%
<i>Spirochaetaceae</i> ; g__uncultured	3.119%			<i>Dojkabacteria</i>	3.931%	ADurb.Bin063-1	1.398%	ADurb.Bin063-1	1.261%
				<i>Spirochaetaceae</i> ; g__uncultured	2.877%				
				ADurb.Bin063-1	1.327%				

Table S2. Abundance of the bacteria present in the sewage sludge, treated with non-immobilized isolated and commercial enzymes, among the genus. I0- inoculum before the AMPTS, SS0- mixed sludge (thickened primary + waste activated sludge before the AMPTS), I + SS- sample of inoculum and mixed sludge after AMPTS, during the test with addition of immobilized enzymes for remaining samples, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme).

I0		SS0		I + SS		I + SS + IE		I + SS + CE	
<i>Acetothermiia</i>	4.943%	<i>Bacteroides</i>	4.048%	<i>Acetothermiia</i>	4.549%	<i>Acetothermiia</i>	4.298%	<i>Acetothermiia</i>	5.25%
<i>Aminicenantales</i>	1.817%	<i>Macellibacteroides</i>	1.821%	<i>Aminicenantales</i>	2.178%	<i>Aminicenantales</i>	2.268%	<i>Aminicenantales</i>	2.094%
<i>Thermoanaerobaculum</i>	2.744%	37-13	1.743%	<i>Thermoanaerobaculum</i>	1.931%	<i>Thermoanaerobaculum</i>	2.05%	<i>Thermoanaerobaculum</i>	1.92%
<i>Bacteroidetes</i> vadinHA17	4.836%	<i>Saprospiraceae</i> ; g__uncultured	10.54%	009E01-B-SD-P15	1.19%	009E01-B-SD-P15	1.141%	009E01-B-SD-P15	1.192%

<i>Prolixibacteraceae</i> ; g__uncultured	4.07%	<i>Chitinophagales</i> ; f__uncultured; g__uncultured	1.01%	<i>Bacteroidetes</i> vadinHA17	6.797%	<i>Bacteroidetes</i> vadinHA17	6.794%	<i>Bacteroidetes</i> vadinHA17	6.3%
DMER64	1.616%	NS9 marine group	1.265%	<i>Prolixibacteraceae</i> ; g__uncultured	7.63%	<i>Prolixibacteraceae</i> ; g__uncultured	8.194%	<i>Prolixibacteraceae</i> ; g__uncultured	6.452%
Blvii28_wastewater- sludge_group	4.897%	<i>Cloacibacterium</i>	1.737%	DMER64	2.009%	DMER64	2.162%	DMER64	1.886%
<i>Saprospiraceae</i> ; g__uncultured	1.716%	SJA-28	1.807%	Blvii28_wastewater sludge_group	5.763%	Blvii28_wastewater- sludge_group	5.07%	Blvii28_wastewater- sludge_group	4.974%
<i>Lentimicrobium</i>	2.098%	<i>Arcobacteraceae</i>	1.214%	<i>Lentimicrobium</i>	2.866%	<i>Lentimicrobium</i>	2.759%	<i>Lentimicrobium</i>	2.928%
JS1	5.36%	<i>Arcobacter</i>	2.883%	SJA-28	1.71%	SJA-28	1.633%	SJA-28	1.081%
<i>Leptolinea</i>	1.987%	<i>Leptotrichia</i>	1.128%	JS1	2.242%	JS1	2.861%	JS1	5.781%
<i>Anaerolineaceae</i> ; g__uncultured	5.575%	<i>Nitrospira</i>	2.934%	<i>Leptolinea</i>	1.345%	<i>Leptolinea</i>	1.576%	<i>Leptolinea</i>	1.831%
Candidatus <i>Cloacimonas</i>	1.223%	<i>Aeromonas</i>	2.795%	<i>Anaerolineaceae</i> ; g__uncultured	3.183%	<i>Anaerolineaceae</i> ; g__uncultured	3.038%	<i>Anaerolineaceae</i> ; g__uncultured	2.907%
W5	1.168%	<i>Comamonas</i>	1.056%	Candidatus <i>Cloacimonas</i>	1.729%	Candidatus <i>Cloacimonas</i>	1.709%	Candidatus <i>Cloacimonas</i>	1.904%
<i>Smithella</i>	2.819%	<i>Dechloromonas</i>	1.025%	W5	1.654%	W5	1.711%	W5	2.249%
<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.063%	Candidatus <i>Competibacter</i>	1.122%	<i>Smithella</i>	2.531%	<i>Smithella</i>	2.58%	<i>Smithella</i>	2.785%
Syntrophorhabdus	1.749%	<i>Acinetobacter</i>	3.492%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.28%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.462%	<i>Syntrophales</i> ; f__uncultured; g__uncultured	2.905%

Gracilibacteraceae; g__uncultured	1.886%	Verrucomicrobiaceae; g__uncultured	1.408%	Syntrophorhabdus	2.415%	Syntrophorhabdus	2.489%	Syntrophorhabdus	2.6%
DTU014	1.616%			Gracilibacteraceae; g__uncultured	1.724%	Izemoplasmatales	1.006%	Izemoplasmatales	1.075%
Dojkabacteria	2.559%			Sedimentibacter	1.714%	Gracilibacteraceae; g__uncultured	1.979%	Gracilibacteraceae; g__uncultured	1.795%
Spirochaetaceae; g__uncultured	1.977%			DTU014	1.517%	Sedimentibacter	1.356%	DTU014	1.505%
LD1-PA32	1.141%			Dojkabacteria	3.964%	DTU014	1.485%	Dojkabacteria	2.498%
				Spirochaetaceae; g__uncultured	2.003%	Dojkabacteria	3.504%	Spirochaetaceae; g__uncultured	2.404%
				ADurb.Bin063-1	1.164%	Spirochaetaceae; g__uncultured	1.938%	ADurb.Bin063-1	1.488%
						ADurb.Bin063-1	1.09%		

Table 1. Abbreviations of experimental variants used in the study

<i>Abreviation</i>	<i>Description</i>
<i>SS0</i>	mixed sludge before AMPTS
<i>I0</i>	inoculum before AMPTS
<i>INE</i>	inoculum after AMPTS
<i>I+SS</i>	inoculum with mixed sludge
<i>I+SS+IE</i>	inoculum with mixed sludge and isolated enzyme
<i>I+SS+CE</i>	inoculum with mixed sludge and commercial enzyme
<i>I+IE</i>	inoculum with isolated enzyme
<i>I+CE</i>	inoculum with commercial enzyme
<i>I+SS+carb</i>	inoculum with mixed sludge and activated carbon

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Table 2. The microbial diversity indices, Shannon and Simpson, of bacteria present in the sludge during the methane fermentation measured in an automatic methane potential test system (AMPTS) with the addition of immobilized enzymes, with an average of observed OTUs. I- Inoculum, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme.

	Shannon (H')	Simpson (S)	Richness	Average of observed OTUs
I + SS	4.69	0.98	603	200.04
I + SS + IE	4.61	0.98	474	193.79
I + SS + CE	4.74	0.98	678	219.74

Table 3. Microbial diversity indices, Shannon and Simpson of bacteria present in the sludge during the methane fermentation measured in an automatic methane potential test system (AMPTS) with the addition of non-immobilized enzymes, with an average of observed OTUs. I- Inoculum, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme.

	Shannon (H')	Simpson (S)	Richness	Average of observed OTUs
I + SS	4.69	0.98	622	208.63
I + SS + IE	4.7	0.98	679	236.67
I + SS + CE	4.61	0.98	612	228.79

Comparative Enzymatic Pretreatment of Sewage Sludge: Physicochemical Parameters and
Microbial Community Effects

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Abstract

Effective biological treatment of sewage sludge depends on the use of methods that support the hydrolysis of organic matter. In this study, we evaluated the effects of enzymes isolated from autochthonous cellulolytic bacteria naturally present in sewage sludge and compared them with commercial cellulases (Cellic CTec2, Novozymes). We analyzed their influence on selected physicochemical parameters of sludge and on microbial community structure. Both free enzymes and enzymes immobilized on activated carbon were tested. The use of autochthonous cellulolytic enzymes did not cause significant changes in the final values of the analyzed physicochemical parameters and did not clearly disturb the microbial community structure. However, the type of enzyme and the way it was applied affected microbial richness and diversity. Immobilized isolated enzymes reduced community richness, while immobilized commercial enzymes increased it. For non-immobilized enzymes, regardless of their origin, an increase in microbial diversity was observed. Correlation analysis showed relationships between most of the analyzed physicochemical parameters, except NH₄-N, and the dominant bacterial groups. The strongest relationships were found in variants with non-immobilized commercial enzymes. These results suggest that autochthonous enzyme systems may be an alternative to commercial preparations in the biological treatment of sewage sludge.

Keywords: sewage sludge, autochthonous cellulolytic enzymes, commercial cellulases, biological treatment, microbial community

Key points:

- The effects of free and immobilized cellulolytic enzymes on sewage sludge were evaluated.

- Enzymatic treatment influenced microbial diversity and correlations with physicochemical parameters.
- Autochthonous enzyme systems may be an alternative to commercial preparations.

Introduction

The proper management of sewage sludge and digested sewage sludge is a common problem, mainly due to their large amounts (Salva et al., 2025). It causes financial, technical, and logistical problems for the wastewater treatment plant (WWTP). Due to that, there is a need to improve proper sewage sludge management, which will help to decrease the amount of digested sewage sludge, improve its quality, and decrease the costs that need to be covered for the previously described purposes. Management of the sewage sludges demands stabilizing their properties and their volume. For that, methane fermentation is usually used. Methane fermentation is a microorganism-driven biological process consisting of four main stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During hydrolysis, complex organic compounds such as proteins, lipids, and carbohydrates are broken down into simpler, water-soluble molecules, which are further converted into volatile fatty acids and alcohols. The efficiency of the process depends on the balanced activity of microbial communities, and any disturbance can inhibit methane production and overall process stability. (Li et al., 2023a, Liang et al., 2021). Pretreatment or treatment methods lead to an increase in the hydrolysis of the compounds contained in the sewage sludge by improving the degradation of macromolecular organic substances. As the complex compounds are degraded during the hydrolysis, it is highly important to conduct treatment in a way that won't disturb this phase. There is limited information in the literature about the impact of the biological pre-treatment methods on the shifts in microbial communities of the treated sewage sludge, as well as the correlations between sludge communities and physiochemical parameters of this sludge, especially using biological treatment with enzymatic mixtures.

Various chemical, mechanical, and biological methods are used to pretreat sewage sludge and improve methane fermentation efficiency by enhancing the degradation of complex organic compounds (Zhour et al., 2024). These methods differ in their impact on the process, equipment, and environment. Although biological methods are the most environmentally friendly, their high cost, time requirements, and technical limitations hinder their implementation in WWTPs (Khanh Nguyen et al., 2021). These challenges, including the need for additional equipment and limited sludge storage time, drive the search for more efficient and cost-effective solutions. One promising approach is applying biological treatment during

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methane fermentation, which can reduce operational costs and provide an alternative to conventional methods associated with high energy demand, harmful by-products, and equipment degradation (Ćwiertniewicz-Wojciechowska et al., 2023).

Sewage sludge contains diverse organic and inorganic components that influence treatment efficiency and the final dry organic matter content. Cellulose, a major recalcitrant polymer comprising up to 50% of insoluble wastewater fractions, significantly limits degradation and biogas production (Djordjevićová et al., 2023). Enhancing its hydrolysis is therefore crucial for improving anaerobic digestion. While most studies focus on long-term pretreatment, a more practical industrial approach is to support hydrolysis during anaerobic digestion through in situ treatment of incoming sludge (Zhou et al., 2024). Biological methods involve the use of bacteria, fungi, or their enzymes (Khanh Nguyen et al., 2021). Among these, enzymatic treatment is most suitable for industrial applications due to its simplicity and safety. Given the high cellulose content of sewage sludge, cellulolytic enzymes are particularly advantageous, as they enhance cellulose degradation and thereby improve the breakdown of other organic compounds (Wang et al., 2020).

However, due to enzyme instability, high cost, and the variable, often harsh conditions of sewage sludge, immobilization on inorganic carriers is a promising strategy. Immobilization enhances enzyme stability, reusability, and recyclability (Bijoy et al., 2022). Activated carbon is an effective carrier due to its large surface area, high pore volume, and low toxicity, as well as its ease of use and low cost (Torres et al., 2017). Its addition during methane fermentation has been shown to increase methane production, enhance volatile fatty acid utilization, and stimulate microbial activity (Ma et al., 2020; Aamir and Hassan, 2025). Furthermore, to reduce costs and support circular economy principles, biochar derived from biomass pyrolysis can be used as an alternative carrier (De Almeida et al., 2022).

Presented research verifies the impact of the enzymatic treatment, supported and stabilized with activated carbon as an immobilization carrier, on the physiochemical parameters of the sewage sludge before and after the 15-days test, and check the shifts in the microbial community structure caused with: 1) inoculation of the sewage sludge with enzymes, 2) inoculation of the sewage sludge with enzymes immobilized on the activated carbon as a carrier, 3) changes among the physiochemical parameters of sewage sludge. The paper reveals the impact of enzymatic treatment on the physicochemical parameters of the sludge during the 15-day test and presents an investigation of the potential changes in community structure caused by the enzymatic treatment, which can influence the methane production efficacy. A 15-day

test was conducted using the Automatic Methane Potential Test (AMPTS), as a system to ensure conditions of mimicking methane fermentation.

Materials and methods

Figure 1 shows the experimental scheme used in this study. In a first step, sludge collected from the digester of a municipal wastewater treatment plant was used as the source material for the isolation of autochthonous cellulolytic bacteria. The obtained mixed culture was then incubated under controlled conditions, and the enzymes produced by these microorganisms were extracted. In the next stage, the isolated enzymes were immobilized on activated carbon used as a carrier, and their effects were compared with those of commercial cellulolytic enzyme preparation. Free and immobilized enzymes were then applied in batch tests performed under anaerobic conditions using the Automatic Methane Potential Test System (AMPTS). Samples were collected before and after a 15-day test to determine: total nitrogen (TN), ammonium nitrogen ($\text{NH}_4\text{-N}$), chemical oxygen demand (COD), volatile fatty acids (VFA), and a bacteria community structure.

Figure 1. Schematic diagram of the steps taken to achieve the implementation of the planned experiment.

The abbreviations used for the experimental variants are explained in Table 1.

Table 1. Abbreviations of experimental variants used in the study

Isolation of an autochthonous mixed culture capable of degrading cellulose

To isolate the cellulolytic autochthonous microorganisms, sewage sludge (SS) from the communal WWTP (Gliwice, Poland) was used. Mixed culture of autochthonous microorganisms was isolated by several series of passaging on bacteriological media (CMC medium- contained carboxymethylcellulose sodium salt 5 g/L, NaNO_3 1 g/L, K_2HPO_4 1 g/L, KCl 1 g/L, MgSO_4 0.5 g/L, yeast extract 0.5 mg/L, glucose 1 mg/L). Mixed sludge consisted of thickened primary and waste activated sludge (35%:65%). SS and liquid medium (CMC) were mixed in a ratio of 1:4 and incubated for 7 days at 37°C and 100 rpm. To remove oxygen, samples were flushed with N_2 . A new medium was provided every 7 days. The culture was centrifuged for 1 minute at 3000 rpm, suspended in sterile 0.9% NaCl in triplicate to remove metabolites. Then, microbial culture and sterile 0.9% NaCl were mixed in a ratio of 1:1 and incubated as described above. Samples of sludge were collected for DNA isolation into sterile

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3 Eppendorf, and protected with RNAlater (Invitrogen™, ThermoFisher Scientific, USA).
4 Solutions were mixed in a ratio of 2:1, where 1 part was microbial culture. After 21 days of the
5 experiment, the inoculation on solid medium (CMC solidified with agar 17.0 g/L) was
6 performed to verify the possibility of isolating a pure culture and incubation on plates. The
7 remaining culture was fed with the new portion of liquid CMC medium and incubated at 37°C
8 and 100 rpm.
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10 The cellulose-degrading ability of the microorganisms was confirmed by inoculating 0.1 mL of
11 the liquid mixed-culture on Congo-Red agar media containing CMC medium solidified with
12 agar (CMC with agar 17 g/L and Congo Red 1g/L), pH 6.8-7.2.
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14 After isolation, samples were stored at -20°C until later use.
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17 *Extraction of the enzymes from the autochthonous mixed culture*

18 50 mL of the autochthonous mixed culture, able to degrade cellulose ($OD_{600} > 0.9$), and 150 mL
19 of the CMC medium were mixed and incubated at 37°C. For the first 2 days at 160 rpm, and
20 the next 5 days at 120 rpm. The mixed culture was deprived of oxygen by displacing it with
21 nitrogen (for 3 minutes). Extraction was performed using heat at 37°C and a centrifuge (Hossain
22 et al., 2021). Enzymes were isolated with Na-citrate buffer (Pol-Aura, Poland) (0.05 M, pH 6)
23 at a ratio of 1:2, 1:3, 1:4, and 1:5, where 1 part was a buffer. Reaction was performed for 30
24 minutes at 37°C and 120 rpm. The samples were then centrifuged at 10000 rpm for 15 minutes,
25 and the supernatant was used as a source of enzymes (Khan et al., 2017; Ravichandran and
26 Vimala, 2012).
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28 The protein concentration was determined using Pierce™ BCA Protein Assay Kit
29 (ThermoFisher Scientific, USA), according to the manufacturer's protocol for the test-tube
30 procedure.
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33 *Enzyme immobilization with activated carbon as a carrier*

34 For the immobilization, a mix of enzymes isolated from the sewage sludge microbial
35 community and the enzyme blend of cellulases (Cellic CTec2, Novozymes) was used. Enzymes
36 were prepared for immobilization in 5 different concentrations: initial, diluted in proportions
37 1:2, 1:3, 1:4, and 1:5 with citric buffer. 0.4 g of activated carbon as a carrier; commercial and
38 isolated enzymes were stirred in a 25 mL round-bottom flask in a thermostatic shaker at 250
39 rpm, 20°C for 3 hours. After this time, biomaterials were filtered off, washed with a citric buffer
40 (3×10 mL), and dried under vacuum using a Schlenk line (25°C, 24 h).
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Analytical methods for sewage sludge during the long-term test

For the batch experiment, mixed sludge composed of thickened primary sludge and waste activated sludge (35%:65%) was used as a substrate, while sludge from the digester was used as a substrate. The substrate and an inoculum were mixed in a ratio of 1:3 based on volatile suspended solids (VSS). The sludge was treated with free and immobilized isolated enzymes; free and immobilized commercial enzymes, activated carbon alone, and untreated samples were used as a control. The experiment was carried out for 15 days using the Automatic Methane Potential Test System (AMPTS, BPC Instruments AB, Sweden). Samples were collected at the beginning and the end of the test for determination of TN, NH₄-N, VFA, COD. For physicochemical analyses, the liquid fraction of the samples collected before and after the 15-day test. TN, NH₄-N, COD, and VFA were determined using photometric tests (MERCK Millipore, USA) and spectrophotometer (MERCK Spectroquant® NOVA60, USA) according to the manufacturer's protocol.

Metataxonomic analysis of environmental samples

For the metataxonomic analysis of the sludge, samples were collected at the beginning and the end of the 15-day test. Samples were stored using RNAlater in a ratio of 3:1, where one part was a sludge sample. DNA isolation was performed using GeneMATRIX Soil DNA Purification Kit, according to the manufacturer's protocol (EurX, Poland). The metagenomic analysis of total prokaryotic communities was performed based on the hypervariable V3-V4 region of the 16S rRNA gene. The primer pair for amplification was 341F and 785R (Thijs et al., 2017). The PCR reaction was performed using Q5 Hot Start High-Fidelity 2X Master Mix (conditions according to the manufacturer's instructions) (New England Biolabs). Sequencing was performed on a MiSeq, paired-end (PE), 2x300nt, using Illumina's v3 kit, according to the manufacturer's protocol. Automatic ascending data analysis was performed on a MiSeq sequencer using MiSeq Reporter (MSR) v2.6 software. Bioinformatic analysis to ensure the classification of reads to the species level was carried out with QIIME 2, based on sequences referential base Silva 138. A programming package was also used to distinguish sequences of biological origin from those newly created during the sequencing process. This package was also used to derive unique sequences of biological origin, or so-called amplicon sequence variants (ASV) sequences. As a quantitative measure of community richness, Shannon and Simpson's diversity indices were used.

All sequences are deposited in NCBI under the link <http://www.ncbi.nlm.nih.gov/bioproject/1347622>.

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Statistical analysis

The experimental results for physicochemical methods were expressed as the mean $\pm$ standard deviation for each triplicate experiment. The data processing was conducted by the Microsoft Office Excel 365 function. R software (<https://www.r-project.org>). Two-way ANOVA was used to test the significance of the results for immobilized and non-immobilized enzymes, and for the immobilized enzymes and activated carbon, regarding physicochemical parameters. The significance level of the p -value was <0.05 . For the Shannon index, we calculated the Hutcheson t-test, with the significance level of the p -value <0.05 . Pearson correlation analyses were carried out between the environmental factors using the 17 most abundant phyla.

Results

Effect of enzyme treatment on sewage sludge hydrolysis and physicochemical parameters

Total nitrogen concentration

The concentration of nitrogen determined in the samples of sludge before and after the anaerobic digestion indicates a stable process, because there are no noticeable changes in the concentration of total nitrogen. The concentration of TN in the inoculum, as a sludge from the digester, remained at 1873.3 mg/L. Figure 2 presents the changes in total nitrogen concentration, depending on the applied treatment.

Figure 2. Total nitrogen concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations (n = 4). Error bars represent the standard errors.

Statistical analysis confirmed there are no significant differences between immobilized and non-immobilized enzyme samples regarding total nitrogen concentration (p -value >0.05).

Although the p -value calculated using immobilized enzymes and activated carbon was 0.0511. $F = 4.21$ [0:4.2565]. The test was confirmed using Tukey-Kramer, indicating there is no significant difference between the means of any pair.

Ammonium nitrogen concentration

In an anaerobic digestion system, the total ammonia nitrogen (TAN) is mainly composed of free ammonia nitrogen (FAN) and ionized ammonium nitrogen (NH_4^+) (Dai et al., 2017). Due to its high ability to penetrate the cell membranes, free ammonia nitrogen is the dominant inhibitor of microorganisms (Yang et al., 2024). Microorganisms working in the anaerobic chamber need nitrogen in the form of ammonium to produce new cell mass, and its presence is also an important parameter for the buffer capacity in an anaerobic reactor (Chavez-Rico et al., 2022). In all samples, we did not notice FAN greater than 0.49 mg/L (in a sample with immobilized commercial enzymes, inoculum, and mixed sludge), and lower than 0.27 mg/L (in a sample with immobilized isolated enzyme and inoculum). The concentration of $\text{NH}_4\text{-N}$ is presented in Figure 3.

Figure 3. Ammonium concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production, measured in the automatic methane potential test system (AMPTS). I_0 - inoculum before the AMPTS, SS_0 - mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE - sample of inoculum after AMPTS, $I + SS + IE$ - inoculum + mixed sludge + isolated enzyme, $I + SS + CE$ - inoculum + mixed sludge + commercial enzyme, $I + IE$ - inoculum + isolated enzymes, $I + CE$ - inoculum + commercial enzymes, $I + SS + carb$ - inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations ($n = 4$). Error bars represent the standard errors.

Statistical analysis confirmed there are no significant differences between immobilized and non-immobilized enzyme samples regarding $\text{NH}_4\text{-N}$ concentration ($p\text{-value} > 0.05$). P -value for $\text{NH}_4\text{-N}$ concentration using immobilized enzymes and activated carbon was $p = 0.0002013$. According to Tukey-Kramer, the means are significantly different.

Volatile fatty acids concentration

Figure 4 presents the concentration of volatile fatty acids, which decreased during the 15-day test from 329.3 ± 3.9 mg/L up to ~ 300 mg/L in samples with immobilized enzymes.

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Figure 4. Volatile fatty acids concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I₀- inoculum before the AMPTS, SS₀- mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE- sample of inoculum after AMPTS, I + SS + IE- inoculum + mixed sludge + isolated enzyme, I + SS + CE- inoculum + mixed sludge + commercial enzyme, I + IE- inoculum + isolated enzymes, I+CE- inoculum + commercial enzymes, I + SS + carb- inoculum + mixed sludge + activated carbon. Note: Data are presented as means ± standard deviations (n = 4). Error bars represent the standard errors.

There are no statistically significant differences, both between immobilized and non-immobilized enzymes, but also between isolated enzymes, commercial enzymes, and activated carbon as a carrier (p -value>0.05)

Chemical Oxygen Demand

For the immobilized enzymes, the concentration of COD after AMPTS remains at a similar level, around 6000 mg/L (~270 mg/g VSS) in samples with activated carbon, commercial and isolated enzymes, as well as in the sample without treatment, with an initial 1468.3 mg/L (44 mg/g VSS). The greatest increase in the COD concentration presented in the Figure 5 is visible in samples with inoculum and enzymes (17116.6±1100 mg/L (778.03 mg/g VSS) for isolated enzymes, 14925±544 mg/L (678.41 mg/g VSS) for commercial enzymes), confirming the suggestion that the use of the immobilized enzymes would increase treatment of organic compounds remaining in digested sludge during post-treatment in anaerobic conditions. In preliminary tests, an increase in COD concentration was observed during the first 4 hours after inoculation with enzymes. In samples with immobilized isolated enzymes, the concentration of COD after 240 minutes was 73% greater than in the initial sample. In samples with commercial immobilized enzymes, we noticed an increase of 470%. The addition of activated carbon caused an increase in COD concentration by 704%.

During the 4 h test, we did not notice any significant changes in the control samples (Ćwiertniewicz-Wojciechowska et al., 2024). In the research described in this paper, at the end of the test, the concentration of both treated and untreated samples was at a similar level. Based on the preliminary test, we assume that the addition of the enzymes and activated carbon as a

carrier enhances the treatment of the sewage sludge in the beginning, but process elongation allows for obtaining similar results for untreated and treated sludges (Wu et al., 2022). A similar situation is observed with non-immobilized enzymes. The concentration of COD for untreated sludge with enzyme samples was 3097.5 ± 32.02 mg/L (169.26 mg/g VSS), treated with isolated enzymes 3077.5 ± 29.8 mg/L (168.17 mg/g VSS), and with commercial enzymes 2900 ± 73.94 mg/L (158.47 mg/g VSS). The results determined during preliminary tests with non-immobilized enzymes, in the case of isolated enzymes, we observed an increase by 272%, while for commercial enzymes, 1792% (Ćwiertniewicz-Wojciechowska et al., 2024). In reference to the obtained results, further research should focus on determining the detailed composition of the sludge during anaerobic digestion, which will help to better understand the rate of sludge degradation treated with those enzymes.

Figure 5. Chemical oxygen demand concentration, measured in the sewage sludge treated with immobilized and non-immobilized enzymes, before and after methane production measured in the automatic methane potential test system (AMPTS). I_0 - inoculum before the AMPTS, SS_0 - mixed sludge (thickened primary + waste activated sludge before the AMPTS), INE - sample of inoculum after AMPTS, $I + SS + IE$ - inoculum + mixed sludge + isolated enzyme, $I + SS + CE$ - inoculum + mixed sludge + commercial enzyme, $I + IE$ - inoculum + isolated enzymes, $I+CE$ - inoculum + commercial enzymes, $I + SS + carb$ - inoculum + mixed sludge + activated carbon. Note: Data are presented as means $\pm$ standard deviations ($n = 4$). Error bars represent the standard errors.

Results for COD concentration are statistically significant regarding the usage of immobilized enzymes and the activated carbon - p -value was 0.00242 ($p < 0.05$). Based on that, we can confirm the influence of enzymes immobilized with activated carbon in reference to the activated carbon, regarding the COD concentration. The impact of the immobilization on the concentration of the COD shows p -value=0.5851, $F=8.8919$ [0, 10.128].

Microbial diversity in the sludge community after inoculation with enzymes

In Tables 2 and 3, we present the Shannon (H') and Simpson (S) diversity indices calculated for the results of metataxonomic analysis of the sludge microbial communities used in the AMPTS, and the number of observed OTUs. The Shannon index informs about the richness of the community, and the Simpson index measures the probability that two individuals drawn from the sample will belong to the same species. OTU number gives information about the

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number of closely related individuals. We compared results for the Shannon index, using the Hutcheson t-test. Based on the determined *p*-values, there are no significant differences between samples I+SS, but the differences between samples treated with immobilized, non-immobilized, isolated, and commercial enzymes are statistically significant, *p*-value <0.05. Based on the H' and S indices, it could be suspected that the addition of the enzymes or the carrier didn't disturb the equilibrium of the microorganisms present in the sewage sludge, but in reference to the richness and average of observed OTUs we can see the increase in diversity and richness in the sludge treated with immobilized commercial enzymes, and non-immobilized isolated enzymes. Among all tested samples, H' and S' indicate high biodiversity among the tested samples, with no dominant individual. In Supplementary Material, we show an abundance of the bacteria present in the sewage sludge, treated with enzymes, among the genus (See Supplementary Materials, Table S1 and Table S2). Although inoculation of the sludge using immobilized isolated enzymes (I+SS+IE, Table 2) caused a decrease in diversity and richness, as well as treatment using a non-immobilized commercial enzyme (I+SS+CE, Table 3).

Table 2. The microbial diversity indices, Shannon and Simpson, of bacteria present in the sludge during the methane fermentation measured in an automatic methane potential test system (AMPTS) with the addition of immobilized enzymes, with an average of observed OTUs. I- Inoculum, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme.

Table 3. Microbial diversity indices, Shannon and Simpson of bacteria present in the sludge during the methane fermentation measured in an automatic methane potential test system (AMPTS) with the addition of non-immobilized enzymes, with an average of observed OTUs. I- Inoculum, SS- mixed sludge, IE- isolated enzyme, CE- commercial enzyme.

Figure 6 shows the microbial community structure at the phylum level regarding the 22 most abundant bacteria in the sludges. The most abundant among all samples are 2 phyla: *Bacteroidota* (*Bacteroidetes*), which enhances biogas production and degrades lignocellulosic biomass in various environments (Wright and Klieve, 2011; Tukanghan et al., 2021), and in the mixed sludge, also *Proteobacteria*, whose presence is crucial in anaerobic digestion due to the degradation of organic molecules, by consumption of glucose, propionate, butyrate, and acetate (Guo et al., 2015; Kara Murdoch et al., 2018). The Pearson correlation also determined that the results are statistically significant (*p* (2-tailed) < 0.05). Also, the obtained results indicate that

the addition of the activated carbon and enzymes in the used doses does not impact positively or negatively on the microbial community structure during the 15-day test.

Figure 6. Microbial community structure at the phylum, regarding the 22 most abundant groups of bacteria in the sludges. Sequences that accounted for less than 1% of the population were grouped into the category “Others”.

Pearson correlation (Table 3) determined that some changes in the concentration of single physicochemical parameters were significantly correlated to the changes in the microbial consortium, and changes in the microbial consortium have a strong correlation with physicochemical parameters. The red color indicates a decrease in one parameter with an increase in the second parameter. The blue color indicates a strong relation between the increase or decrease of two parameters simultaneously, microbial abundance and physicochemical parameters.

In reference to the isolated enzymes, the correlation results show that immobilization of enzymes changes the correlation between certain bacteria and determined physicochemical parameters. In samples treated with isolated immobilized enzymes, we can see that the concentration of $\text{NH}_4\text{-N}$, VFA, and COD depends on the microbial abundance, and the correlation is negative. It is similar in samples treated with non-immobilized enzymes, except for VFA and COD concentration, where we can see that an increase in the abundance causes an increase in the concentration of those parameters, or *vice versa*. That would suggest an increased activity of those bacteria in response to the addition of the isolated non-immobilized enzymes.

Figure 7A shows the Pearson correlation between samples treated using immobilized and non-immobilized isolated enzymes, and Figure 7B shows the Pearson correlation coefficient between physicochemical parameters and microbial abundance ratio in the consortia treated using commercial enzymes.

Figure 7. Pearson correlation coefficient between physicochemical parameters and microbial abundance ratio in the consortia at the phylum level using isolated (A) and commercial (B) enzymes. The red color indicates a decrease in one parameter with an increase in the second

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parameter. The blue color indicates a relation between the increase or decrease of two parameters simultaneously, microbial abundance and physicochemical parameters.

Discussion

Nitrogen in anaerobic digestion is an important factor for the formation of new microbial biomass, and in the form of ammonia for stabilization of the pH value in the reactor (Fricke et al., 2007). Moreover, it is a compound that is the most susceptible to transformations, affecting the risk of unproductive losses, with the production of metabolic products, ie. NH_3 , NH_4^+ , N_2O , NO_2^- or NO_3^- , which may cause problems (Möller, 2015; Fricke et al., 2007). The decomposition of organic matter results in nitrogen release, as proteins, amino acids, and urea are the main carriers of nitrogen (Suschka and Grübel, 2014).

The total nitrogen is mostly conserved during anaerobic digestion but can decrease up to 10% (Chavez-Rico et al., 2022). Schievano et al. (2011) showed a decrease of N concentration between 5-20%, suspecting the gaseous NH_3 migration with the biogas flux. Moreover, they suggest the possibility of losses caused by the partial organic/inorganic matter sedimentation, struvite formation (Siciliano et al., 2020), and precipitation and subsequent retention (Massé et al., 2007). In the test with immobilized enzymes, we observed a decrease in total nitrogen concentration in samples with the mixed sludge, but also in samples with inoculum and enzymes (Figure 2). In a sample with the immobilized enzymes, a stronger decrease (more than 10%) can be caused by the adsorption of the nitrogen on the surface of the activated carbon (Zhu et al., 2012; Kabuba et al., 2023). In the sample with non-immobilized commercial enzymes, there is an increase in total nitrogen concentration, which suggests that the enzymes enhanced the amount of nitrogen after its addition into the sewage sludge, as a result of the decomposition of the matter present in the mixed sewage sludge.

As a result of protein degradation during anaerobic digestion, the nitrogen present in the sewage sludge is converted into ammonium nitrogen ($\text{NH}_4^+\text{-N}$). Due to that, the concentration of ammonium nitrogen increases, and its typical content is about 60-80% of the total nitrogen in the digestate (Morozova et al., 2020; Manau et al., 2021). Low concentrations of NH_4^+ favor anaerobic digestion, and high concentrations can lead to digestive system failure and lessen the effectiveness of the entire fermentation process (Wang et al., 2023). An increase in the $\text{NH}_4\text{-N}$ (Figure 3) we observe only in samples with immobilized isolated enzymes with mixed sewage sludge, in inoculum with immobilized isolated enzymes, and samples with activated carbon, which suggests improvement of protein degradation during the anaerobic digestion in 15 days of the process. For the remaining samples with immobilized enzymes, the concentration of $\text{NH}_4\text{-}$

N is at a similar level to the concentration in the inoculum in the beginning. In a sample with non-immobilized enzymes, $\text{NH}_4\text{-N}$ concentration remains unchanged in samples with commercial enzymes and mixed sludge after the AMPTS. The initial concentration of $\text{NH}_4\text{-N}$ in mixed sludge was 19.9 mg/L.

The ammonium nitrogen is a sensitive form of nitrogen, and temperature, pH, and hydration state of the sludge affect the $\text{NH}_4\text{-N}$ concentration. The addition of the commercial cellulases could increase solubilization of the sewage sludge, resulting in increased degradation of organic compounds and the appearance of humic acids, which are considered inhibitors of the methane fermentation process (Fernandes et al., 2014; Azman et al., 2015). Moreover, too low or too high concentration of the enzymes used for the sludge treatment also inhibits anaerobic digestion (Bhatnagar et al., 2019).

An increase in the VFA leads to acidification of the sludge because of effective hydrolysis of the lipids, but can stop the methanogenesis (Ma et al., 2020). The concentration of the VFA (Figure 4) in mixed sludge with immobilized enzymes remains at a similar level for all samples after AD. In the preliminary test (Ćwiertniewicz-Wojciechowska et al., 2024), where we verified the short-term impact of the enzymatic treatment on the sewage sludge, similar to the one described in this paper, we confirmed that the addition of the enzymes impact on the increased production of the VFA. We observed an increase in VFA concentration during the 4 h test in all samples, except the samples untreated with enzymes. The concentration of VFA in samples treated with immobilized isolated enzymes increased by 128%, and with immobilized commercial enzymes by 85% (Ćwiertniewicz-Wojciechowska et al., 2024). That suggests that enzymatic treatment enhances the degradation of organic compounds. The VFA concentration in control samples was at a similar level as in treated samples after an AD, which suggests that treatment of the sludge with an enzyme can decrease the time needed to obtain the same results. An increase in VFA concentration is the same for inoculum and in samples with mixed sludge as a substrate, indicating that the substrate was not used efficiently by microorganisms. The increase of VFA concentration (396.25 ± 56.4 mg/L (18.01 mg/g VSS)) is noticed in the sample with inoculum and immobilized commercial enzymes. That can suggest that the addition of this commercial mix of cellulolytic enzymes can enhance the production of VFA when the enzymes are inoculated into a digester at a different time than the addition of the fresh sludge for the digestion, or can enhance post-treatment of the digested sludges, i.e., during composting. Literature confirms the involvement of activated carbon in anaerobic digestion, i.e., the acidifying effect of activated carbon at an early stage (Wu et al., 2022), while at the same time shows alleviating excessive acidification by accelerating VFA consumption

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(Ma et al., 2020). In preliminary tests, we observed an increase in VFA production by 24% using only activated carbon as an immobilization carrier. That result is similar to the obtained increase of 25% when using treatment with non-immobilized isolated enzymes. Although treatment with non-immobilized commercial enzymes provided an increase in VFA concentration by 1856% during the 4 h test (Ćwiertniewicz-Wojciechowska et al., 2024). Comparing the results for VFA concentration of samples with immobilized and non-immobilized enzymes, the concentration in those first mentioned was similar among all samples and differed between samples with non-immobilized enzymes. The presence of activated carbon as a carrier may have been dominant in the process, so the effect of enzymes on the VFA consumption was not observed. On the other hand, the addition of activated carbon and immobilized enzymes could impact the activity of microorganisms present in the sewage sludge, inhibiting the biodegradation of multimolecular organic compounds (Jiang et al., 2021). The concentration of VFA after AMPTS in samples treated with non-immobilized enzymes remained at a level detected in the initial inoculum. The concentration of VFA in samples with non-immobilized isolated and commercial enzymes was lower by 61% than in samples with immobilized enzymes. Thus, the lower concentration of the VFA in samples with non-immobilized enzymes could be related to the increased consumption of acetic acid to produce methane, undisturbed with the presence of activated carbon (Dong et al., 2023). The addition of enzymes, in the beginning, could have helped to decompose organic matter, allowing microorganisms to effectively use created products as a substrate. Moreover, different ratios of VFA production between samples with immobilized and non-immobilized enzymes can be caused by different abundance of microorganisms in the sludge, which is presented in Figure 4. In section 2.2, we present the correlation between microorganisms and the determined factors.

The decrease in the VFA concentration in the sludge with non-immobilized enzymes is noticed in all samples. The obtained result indicates effective anaerobic digestion. The addition of the non-immobilized enzymes may have an impact on the increase in degradation of the organic matter, resulting in the depletion of the matter to degrade, accelerating the fatty acids consumption and the fast end of acidogenesis (Ma et al., 2020). Then, the increase of fatty acids and the acidic environment led to degradation of enzymes and/or inhibition of the bacteria, which can degrade complex macromolecules into small organic molecules.

According to the literature, activated carbon promotes the improvement of the diversity of microorganisms residing in sewage sludge, improving the stability of the community structure, most likely due to the adsorption of microorganisms on the surface of activated

carbon. As a result, the microorganisms present in the sludge are able to hydrolyze the available organic compounds more efficiently, thus intensifying the methane fermentation process (He et al., 2019; Wang et al., 2019; Wu et al., 2022). Yet in our results, greater community richness is presented in the sludges treated with non-immobilized enzymes (Table 3) than immobilized enzymes (Table 2). We cannot confirm increased diversity caused by the addition of activated carbon in our research, as there was no observed increase in diversity in samples with immobilized enzymes during the 15-day test. In samples with immobilized isolated enzymes, we can see a decrease in richness and average number of OTUs, with a slight decrease in H' index, in reference to the sludge untreated with enzymes or treated with commercial enzymes. Nevertheless, the potential shifts in microbial communities should be verified in a longer test. We have observed that on the abundance of the dominant phylum, *Bacteroidota*, a greater impact had a type of enzyme, not immobilization. In samples treated with commercial immobilized and non-immobilized enzymes, the abundance of *Bacteroidota* had increased, whereas in samples treated with isolated non-immobilized enzymes the abundance decreased. The greatest difference between commercial and isolated enzymes can be noticed in the concentration of TN and VFA in samples treated with immobilized enzymes. In the first case, we can see that in isolated enzymes, there is a strong, positive correlation between bacteria and the concentration of the total nitrogen. Among isolated immobilized enzymes, the correlation between bacteria abundance and TN is mostly positive. In samples with commercial enzymes, correlation is negative, which confirms a decrease in the abundance of some phyla, like *Caldatibacteriota* and *Chloroflexi* (Figure 6). In the second case, VFA concentration in samples treated with isolated enzymes is negative, and in samples treated with commercial enzymes, positive, suggesting that the concentration of the VFA rises with an increase in microbial abundance, all except *Caldisericota* and *Chloroflexi*. In samples treated with non-immobilized commercial enzymes, the correlation mostly depends on the individual bacteria, indicating an impact of the enzymatic treatment on them. Based on that, we assume that the presence of the activated carbon, solo and with an enzyme immobilized onto it, protected and supported the work of the whole community of the sewage sludge from the intensive impact of the enzymes. The impact of the enzymatic treatment on the individuals may disturb the methane fermentation process, as an enhancement or decrease of the abundance of one phylum may disturb the balance or degrade enzymes originally produced by the microbial consortia from the sewage sludge (Zhou et al., 2024).

Phylum *Acetothermia* presents a strong correlation with all physicochemical parameters except $\text{NH}_4\text{-N}$ and cellulose concentrations using immobilized isolated enzymes. Regarding commercial enzymes, *Acetothermia* shows a strong correlation with all parameters except $\text{NH}_4\text{-N}$ and methane yield in samples with non-immobilized enzymes. The correlation was confirmed by Hao et al. (2018), who verified that members of this phylum conduct fermentation of peptides, amino acids, and simple sugars to acetate, formate, and hydrogen, indicating that changes in the abundance of those bacteria are related to the changes in the concentration of determined parameters. *Acidobacteriota* shows a strong correlation with all parameters except TN, $\text{NH}_4\text{-N}$, and COD, in samples treated with non-immobilized isolated enzymes. *Acidobacteriota* correlates with TN and VFA-non-immobilized, and with $\text{NH}_4\text{-N}$ and immobilized commercial enzymes. Representatives of this phylum are not well studied in reference to their abundance in the wastewater treatment process; however, they were already described in anaerobic digestion chambers (Hao et al., 2018). Kristensen et al. (2021) determined that members of *Acidobacteriota* show potential involvement in nitrogen and phosphorus removal. Moreover, they are a group of microorganisms that show various metabolic capabilities to break down organic matter and support other microbial communities, which conduct methane production (Li et al., 2021). De sousa e Silva et al. (2025) noticed an increase in the abundance of *Acidobacteriota*, but *Chloroflexi* and *Bacteroidota* were also connected with the greater methane production. *Actinobacteriota* shows a strong correlation among all determined parameters in samples with isolated enzymes. Phylum *Actinobacteriota* shows a correlation with TN and VFA with non-immobilized commercial enzymes. *Armatimonadota* correlates with all parameters except $\text{NH}_4\text{-N}$ using non-immobilized commercial enzymes. *Armatimonadota* is described as capable of performing acetogenesis and participating in nitrogen and sulfur cycling, confirming a strong correlation between their abundance and determined physicochemical parameters (Carlton et al., 2023). In samples with commercial enzymes, *Bacteroidota* correlates strongly with VFA and COD concentration determined in samples with immobilized enzymes, and for $\text{NH}_4\text{-N}$ with non-immobilized enzymes. *Bacteroidota* were the most abundant phylum among all samples, indicating effective acidogenic fermentation (Peña-Picola et al., 2024). Bacteria from this phylum are one of the main cellulose degraders (Chen et al., 2017). The abundance of the *Bacteroidota* decreased in samples with immobilized enzymes when comparing the initial and final abundance. In samples with non-immobilized enzymes, the abundance of those bacteria increased. That may suggest that the presence of non-immobilized enzymes caused increased hydrolysis of complex organic matter (Jackson et al., 2020). *Caldatribacteriota* shows a strong correlation with VFA and COD

in samples treated with immobilized isolated enzymes. There is also an increased abundance of this phylum (Figure 6) in a sample of non-immobilized isolated enzymes. *Caldatribacterioitas*' abundance decreased in samples treated with immobilized and non-immobilized commercial enzymes, in reference to not enzymatically treated sludge. In samples with commercial enzymes, those bacteria correlate with $\text{NH}_4\text{-N}$ and COD concentration in samples with non-immobilized enzymes. Members of this phylum can utilize a broad spectrum of carbohydrates, including cellulose, and they participate in the degradation of the organic matter, which can be used by the methanogens (Zhang et al., 2023). *Caldisericota* is not well described in literature. According to Mori et al. (2009), it is an anaerobic, thermophilic, and thiosulfate-reducing bacterium. We observed its strong correlation with TN, VFA, and COD concentration in samples treated with immobilized isolated enzymes. Moreover, *Caldisericota* abundance correlates with TN, $\text{NH}_4\text{-N}$ with immobilized commercial enzymes, with VFA, and COD using non-immobilized enzymes. An abundance of *Chloroflexi* is strongly related to TN, $\text{NH}_4\text{-N}$, VFA, and COD, with the presence of non-immobilized isolated enzymes. Those bacteria are commonly found in full-scale anaerobic reactors and can ferment carbohydrates or amino acids (Bovio-Winkler et al., 2021). In samples with commercial enzymes, *Chloroflexi* correlates with $\text{NH}_4\text{-N}$ and COD concentration using non-immobilized enzymes. *Cloacimonadota* abundance is related to TN, VFA, and COD concentration treated with immobilized and non-immobilized enzymes, and with $\text{NH}_4\text{-N}$ only in samples treated with non-immobilized enzymes, when considering isolated enzymes. In samples with commercial enzymes, *Cloacimonadota* correlates with VFA and COD concentration when sludge was treated with non-immobilized enzymes, and methane yield in samples treated with immobilized enzymes. Similarly to *Chloroflexi*, they use amino acids as carbon and energy sources during anaerobic digestion (Johnson and Hug, 2021). *Desulfobacterota* shows a strong correlation with all parameters regarding treatment with isolated immobilized and non-immobilized enzymes. Calculated is also a strong correlation between *Desulfobacterota* and all determined parameters except TN and $\text{NH}_4\text{-N}$ in samples with commercial non-immobilized enzymes. Members of this phylum can transfer electrons to other microorganisms present in the community, which later use the electrons to reduce carbon dioxide to methane (Zheng et al., 2021). Phylum *Firmicutes* show a strong correlation with TN and $\text{NH}_4\text{-N}$ in samples treated with isolated immobilized enzymes, and with VFA with non-immobilized enzymes. When samples were treated with commercial enzymes, *Firmicutes* correlated strongly with $\text{NH}_4\text{-N}$ concentration with immobilized enzymes, and with VFA and COD concentration with non-immobilized enzymes. Chen et al. (2016) noticed that *Firmicutes* and *Bacteroidetes* abundance, despite VFA concentration, is also

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related to organic loading rate and methane production. Those microorganisms break down complex organic matter, using the produced cellulases, lipases, and proteases (Barros et al., 2017). *Planctomycetota* abundance correlates with the concentration of TN, VFA, and COD using isolated immobilized and non-immobilized enzymatic treatment, $\text{NH}_4\text{-N}$ with immobilized enzymes, and cellulose concentration using non-immobilized enzymes. The abundance of members of this phylum correlates strongly with VFA concentration in samples treated with commercial non-immobilized enzymes, and COD in samples treated with both forms of enzymes. They are relevant to nitrogen and carbon cycles and have genes for reactions involved in methanogenesis (Storesund et al., 2020). *Patescibacteria* correlates with the same parameters in samples with isolated enzymes as *Firmicutes*, and additionally, VFA in samples treated with immobilized enzymes. In samples with commercial enzymes, a strong correlation is visible with VFA and COD for immobilized and non-immobilized enzymes and TN concentration for immobilized enzymes. *Proteobacteria* correlation is strong for TN (positive), VFA, and COD concentration (negative) using isolated immobilized enzymes. An abundance of *Proteobacteria* correlates with the concentration of TN and $\text{NH}_4\text{-N}$ for non-immobilized enzymes. *Spirochaetota* abundance shows a strong correlation with all physicochemical parameters in samples with isolated enzymes. Those are microorganisms that have a strong relationship with fermentation parameters (Dhakal et al., 2024). *Spirochaetota*'s abundance correlates strongly with TN, VFA, and COD treated with non-immobilized enzymes. *Thermotogota* abundance correlates with all parameters determined in sludges treated with isolated immobilized or non-immobilized enzymes. In samples with commercial enzymes, *Thermotogota* correlates with VFA and COD, determined only in samples treated with non-immobilized enzymes. Members of the phylum *Thermotogota* are usually present in thermophilic fermentation (Li et al., 2023b), although the genus *Mesotoga*, which we found, is strictly mesophilic (Pollo et al., 2015). *Verrucomicrobiota* correlates strongly with all determined parameters and forms of isolated enzymes. *Verrucomicrobiota* abundance in samples with commercial enzymes correlates with TN and VFA concentration for immobilized and non-immobilized enzymes, and for COD concentration in samples treated with immobilized enzymes. Those are microorganisms that use methane as an energy source (Fenibo et al., 2023).

Summing up, bacteria presented in Figure 6 and their abundance changes, depending on the enzymatic treatment. The most abundant are *Bacteroidota* and *Proteobacteria*, which correlates strongly with all determined physicochemical parameters. The immobilization of enzymes strongly impacts the correlation between microorganisms and the determined physicochemical

parameters. The differences between isolated and commercial enzymes are noticed mainly among the immobilized forms of them, especially TN, VFA, and COD. Considering the above, a greater impact on the process and correlation between physicochemical parameters and bacterial abundance had an immobilization than the composition of the enzymatic mixture.

Conclusion

In this study, we evaluated the effects of enzymes isolated from cellulolytic autochthonous bacteria, commercial enzymes, and activated carbon on the physicochemical properties of sewage sludge during a 15-day anaerobic digestion process. The results demonstrate that autochthonous enzymes exert a stronger and more consistent influence on the relationships between bacterial abundance and key physicochemical parameters compared to commercial preparations, highlighting their potential as a site-specific and adaptive biotechnological tool.

A high correlation between process parameters was observed in sludge treated with both immobilized and non-immobilized isolated enzymes, indicating a more integrated and stable system response. In contrast, sludge treated with commercial enzymes showed correlations primarily at the level of individual taxa rather than the overall microbial community, emphasizing the importance of maintaining high microbial diversity for process resilience under variable operational conditions.

The findings further reveal that enzyme immobilization on activated carbon enhances methane fermentation efficiency by promoting the degradation of organic matter in sewage sludge. This combined approach represents a promising and sustainable strategy for improving anaerobic digestion performance.

Future research should focus on long-term experiments to better understand microbial community shifts induced by enzymatic treatment, as well as on integrating enzymatic and mechanical pretreatment methods to further enhance lignocellulosic biomass degradation. Additionally, detailed investigation of enzyme effects on volatile fatty acid (VFA) production is necessary, given their variable behavior and critical role in the methane fermentation process.

Declarations

Ethical Approval

Not applicable

Funding

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Availability of data and materials

Data supporting this study are included within the article and/or supporting materials. The sequence of raw data is available under the submission number PRJNA1347622 in NCBI, under the link <http://www.ncbi.nlm.nih.gov/bioproject/1347622>.

Conflict of interest statement

The authors declare no conflicts of interest regarding this manuscript.

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