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ISOLATION OF MICROORGANISMS TO ENHANCE THE DIGESTIBILITY OF ORGANIC SUBSTRATE FOR BIOGAS PRODUCTION

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Abstract

Agro-industrial waste is an abundant and renewable resource being an alternative for fossil fuels by production of biogas. This substrate has a high content of organic polymers and other high-mass substances, such as: starch, lignocellulose, proteins, lipids, and other compounds. For the degradation of these compounds, several microorganisms (bacteria and fungi) were isolated from the soil and characterized in terms of enzyme production: amylases, cellulases, laccases, proteases, and lipases. The microorganisms that had the highest enzyme indices were multiplied by cultivation in liquid media in order to highlight the degree of decomposition of the organic matter in the substrate. The aim of the article is to obtain at least 5 microbial strains with high degradative potential that can increase the degree of the substrate degradation through specific hydrolysis reactions of organic compounds.

Key words*: microorganisms, organic substrate, enzyme production.*

INTRODUCTION

Climate change is among the main global concerns, which are directly related to the burning of fossil fuels that generate greenhouse gas emissions. In this context, biomass plays an important role in mitigating the problems associated with the increased burning of fossil fuels (Yu et al., 2021). Thus, in recent decades there has been a growing interest in energy recovery from organic sources, such as animal waste, food waste, lignocellulosic biomass, and sewage sludge (Karrabi et al., 2023).

Biomass includes all organic matter that derives from different categories of feedstock, namely biomass from agro-zootechnical sector, forestry, food industry waste, algae and organic fraction of municipal solid waste (Janiszewska & Ossowska, 2022; Perea-Moreno et al., 2019). The biomasses generated by various agro-industries sectors have great potential as renewable energy sources since they can be used to produce biofuels that can be used for transportation, heating, and power generation (Nazar et al., 2022). In addition, biomass is one of the renewable resources with almost zero carbon dioxide (CO_2) emissions, because when it is formed it absorbs CO_2 from the atmosphere, so when it is burned, it does not contribute to global CO_2 emissions (Tursi 2019).

Anaerobic digestion process presents a promising solution to generate clean and sustainable energy through biogas production. This source of renewable energy offers multiple environmental benefits that are reflected in the reduction of greenhouse gas emissions, in the agronomic quality of the fertilizer obtained, and in the recovery of organic waste from landfills (Chaib et al., 2024; Scano et al., 2014).

Lignocellulosic biomass is an abundant raw material for conversion into biogas (Chaib et al., 2024). Agricultural by-products, mainly com-posed of straw, are common sources of lignocellulosic biomass. Such by-products are potential feedstocks for biofuel production due to their high availability and low cost (Nazar et al., 2022). Biogas production from lignocellulosic biomass has significant benefits, not only from the point of view of producing renewable energy, but also from the perspective of waste management (Hosseini Koupaie et al., 2019). The main problem of using lignocellulosic biomass for biogas production is its complex nature, which represents the resistance of the biomass to chemical and biological degradation (Ferdes et al., 2020). The main components of lignocellulosic

biomass are cellulose (crystalline and linear polymer with a rigid structure, difficult to decompose), hemicellulose (an amorphous heteropolysaccharide) and lignin (a heterogeneous phenylpropanoid macromolecule which is bound to both hemicellulose and cellulose, forming a tight physical structure that acts as an impenetrable barrier in the plant cell wall, giving it resistance against microbial attack), their proportions depending on the substrate used in the fermentation process (Amin et al., 2017; Chaib et al., 2024; Andlar et al., 2018).

Thus, the pretreatment of lignocellulosic biomass before the anaerobic digestion process is considered an important step for improving its biodegradability as well as biogas production.

When properly chosen, pretreatment methods can improve methane concentration and/or anaerobic digestion rate, thereby improving digester performance (Carrere et al., 2016). Various pretreatment techniques have been recommended to improve the digestibility of lignocellulosic biomass in order to enhance anaerobic digestion efficiency. The pretreatment methods are classified into physical, mechanical, chemical, and biological methods, these can be applied individually or in combination (Banu et al., 2021; Ferdes et al., 2020).

It is well known that microorganisms could produce complex enzymes that are crucial to the conversion of lignocellulose. Thus, biological pretreatment represents an attractive pretreatment method, with significant advantages, such as: it is conducted in mild environmental conditions, with low energy consumption, minimal or no inhibitor formation and no requirement to remove solvents after pretreatment (Gao et al., 2022). Fungal and bacterial pretreatment techniques are widely utilized for lignocellulosic biomass pretreatment. Cellulase or laccase enzymes are applied as biological pretreatment in order to improve the biogas yield (Rahmani et al., 2022).

Therefore, the main aim of this work is to obtain at least 5 microbial strains with high degradative potential that can increase the degree of substrate degradation through specific hydrolysis reactions of organic compounds. The efficiency of these strains was determined through enzyme activities: amylolytic, cellulolytic, proteolytic, lipolytic and laccases enzymes.

MATERIALS AND METHODS

Culture media. For isolation and storage of microbial culture nutritive agar medium (NA) was used.

To select the most productive microbial strains from the total of 16 isolated from the soil, the following culture media were used:

(1) for highlighting the amylolytic enzymes produced by the microbial colonies a solidified culture medium containing starch was used: yeast extract 4 g/L; soluble starch 10 g/L; K_2HPO_4 1 g/L; Mg $SO_4.7H_2O$ 0.5 g/L; agar – agar 20 g/L. Adding Lugol solution, the zone of hydrolysis remained uncoloured, whereas the non-hydrolysed starch was coloured dark blue.

(2) for cellulolytic enzymes the culture medium was prepared from: ammonium sulfate 1.4 g/L; peptone 0.5 g/L; K_2HPO_4 2 g/L; $CaCl_2$ 0.03 g/L; $MgSO_4$ 0.03 g/L; yeast-extract 0.75 g/L; CMC 10 g/L; agar 17.5 g/L. The medium is then colored with a Congo red solution, and the producing colonies become surrounded by a colorless area. If the tested microorganism produces extracellular cellulolytic enzymes, they diffuse into the culture medium and produce a clarification of the agar around the active colonies.

(3) for highlighting the proteolytic enzymes, a nutrient medium with casein was used: casein 2.5 g/L; Ca $(OH)_2$ 0.15 g/L; $CaCL_2$ 0.05 g/L; agar-agar 15 g/L. The colonies producing proteolytic enzymes determine around them a clear area of casein lysis in the culture medium.

(4) nutritive agar medium (NA) was used for highlighting the lipolytic enzymes. The lipolytic enzymes produced an opaque zone due to the formation of calcium salts in culture medium (NA) supplemented with Tween 80 and $CaCl_2$.

(5) the biosynthesis of laccases was assessed on Potato Dextrose Agar (PDA) supplemented with 0.04% guaiacol. The reddish-brown zones around fungal colonies are due to the oxidation of guaiacol to a coloured product (Ferdes et al. 2018; Ferdes and Ungureanu, 2009).

All the culture media were sterilized by autoclaving 15 minutes at 121°C then 15-20 mL are distributed in Petri dishes and allowed to solidify.

Methods

Isolation procedure. Soil collected from orchard, solar garden and fertilizer sample were used for the isolation of the potential enzyme producing microorganisms. All samples were collected from the Faculty of Biotechnical Systems Engineering, National University of Science and Technology Politehnica Bucharest. The isolations were carried out in Petri dishes, using the streak technique.

In this study, were initially isolated 16 strains from the three types of soil previously presented. After assessing the extracellular enzyme activity, were selected in the end five the most productive microbial strains.

Enzyme activity testing by the screening method

The culture media specific to the method were poured into Petri dishes and allowed to solidify. The inoculation of the media was done with the hook by central inoculation with a fragment of the inoculum from the stock cultures in test tubes. The Petri dishes were thermostated for 3-4 days until the complete development of the colonies.

Then, the diameters of the degradation zones of the substrate and the diameters of the colonies were measured, and their ratio was made (enzyme indices).

RESULTS AND DISCUSSIONS

After the first stage, that of isolating the microorganisms from the soil, several isolated microbial colonies were obtained along the striations from which a fragment of biomass was collected.

This was used for inoculating the culture medium in test tubes (Figure 2).

In Figure 1 it can be seen the isolation of microbial colonies from the solar garden, orchard and fertilizer that developed on the nutrient agar.

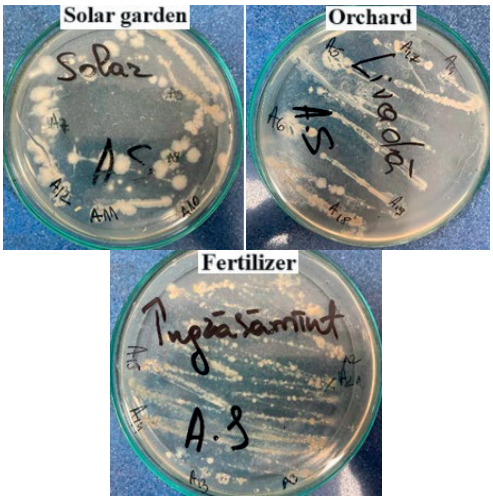

Figure 1. Isolation of the potential enzyme producing microorganisms

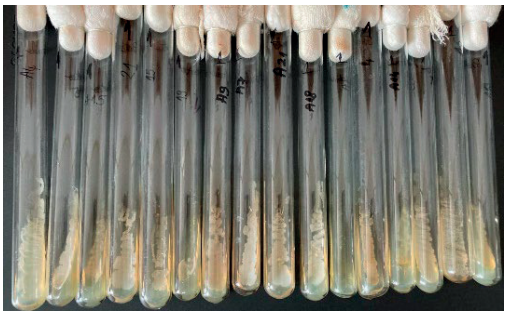

Figure 2. The isolated 16 strains from the three types of soil (in tubes)

Selection and identification of enzyme producing microorganisms.

In the preliminary stage, all 16 microbial strains were tested from the point of view of enzyme production, namely: amylolytic, cellulolytic, proteolytic, lipolytic and laccases. From these, depending on the enzyme indices, only 6 were selected, the best producers. Next, the 6 isolated strains were retested with the results shown in Table 1.

According to the result of extracellular enzyme activity assays, the most productive strains were selected (Figure 3).

Figure 3. The most productive microbial strains selected

The formation of a clear visible zone around the colony on the solid media supplemented with the suitable specific indicators demonstrated that isolated strains have enzymatic activity.

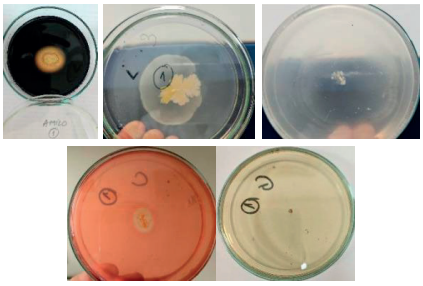

Figure 4. Appearance of colonies isolated from microbial strain no. 1 producing enzymes: amylase, lipase, protease, cellulase and laccase

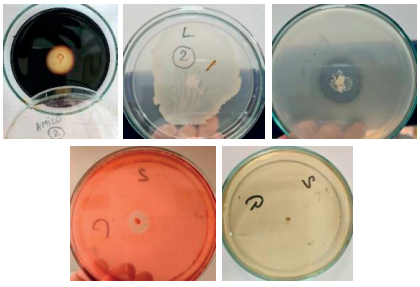

Figure 5. Appearance of colonies isolated from microbial strain no. 2 producing enzymes: amylase, lipase, protease, cellulase and laccase

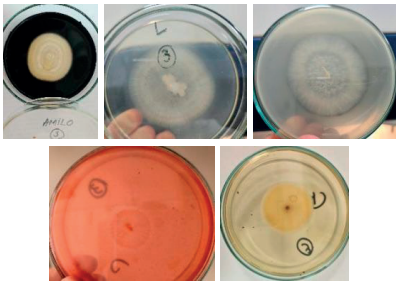

Figure 6. Appearance of colonies isolated from microbial strain no. 3 producing enzymes: amylase, lipase, protease, cellulase and laccase

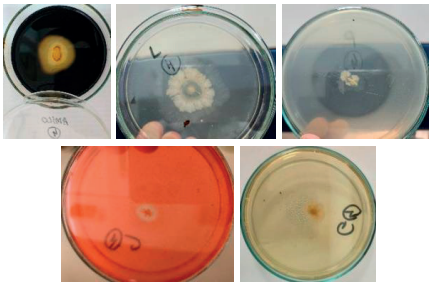

Figure 7. Appearance of colonies isolated from microbial strain no. 4 producing enzymes: amylase, lipase, protease, cellulase and laccase

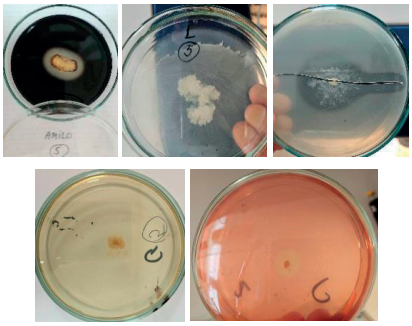

Figure 8. Appearance of colonies isolated from microbial strain no. 5 producing enzymes: amylase, lipase, protease, cellulase and laccase

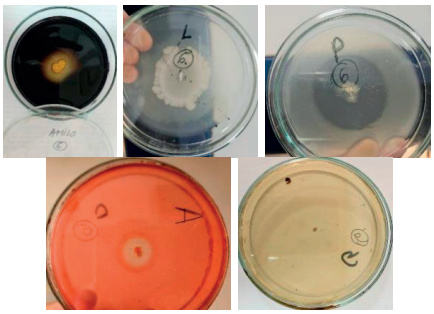

Figure 9. Appearance of colonies isolated from microbial strain no. 6 producing enzymes: amylase, lipase, protease, cellulase and laccase

For producing – laccase colonies, a slightly reddish colour is observed on the reverse side of the colony.

The selected bacterial strains exhibited different levels of enzyme activity. The enzymatic indices defined as the ratio of the diameter of hydrolysis zone and the diameter of colony has been calculated (Table 1).

Table 1. Enzymatic indices of selected strains (diameter of highlighted zone/ diameter of colony), mm mm^{-1}

Microbial strain no	Amylase index	Protease index	Lipase index	Cellulase index	Laccase production
1	2.5/1.2	0	0	1.2/0.8	-
2	2.7/1.6	2.4/1.3	0	1.3/0.4	+
3	3.3/2	3.6/3.5	6.8/5.6	2.1/2.1	+
4	4.5/1.5	4.5/0.9	0	1.1/0.5	+
5	3/1.2	3.2/2.4	0	1.2/0.6	+
6	3.5/1.2	4.4/1.2	8.2/5.2	1.4/0.5	+

After determining the enzyme indices, it was found that all the tested microbial strains showed amylase and cellulase enzyme activity. Proteolytic enzymes were absent in microbial strain no. 1. Only strains 3 and 6 synthesized lipolytic enzymes. Laccase biosynthesis was relatively low, but almost all microorganisms (except for microbial strain no. 1) had laccase activity (reverse colored reddish-brown). As shown in Table 1, bacterial strain no 6 showed the best enzymatic activity. Similar studies were carried out by Zhang et al. 2021, Wang et al., 2020 and Hossain et al. 2021.

Life cycle assessment of products and services is an environmental management technique to assess potential environmental impact associated with all life stages of a product, from the extraction of raw materials through material processing, production, distribution and use to waste management (Finnveden & Potting, 2014).

A lot of studies regarding the energy used and the environmental impact of bioenergy production were carried out in the literature (Osman et al. 2023; Yang et al., 2023; Ugwu et al. 2022; Ramirez-Arpide et al. 2018; Hijazi et al. 2016; Pacetti et al., 2015).

In the case of our study, the use of microorganisms increases the yield of the process because it hydrolyzes the substrate to a higher amount of nutrients which, probably, favors better growth of the microorganisms in the following stages.

Therefore, the final yield should be higher in the presence of hydrolytic and oxidation-reduction enzymes. We also appreciate that the CO_2 footprint should decrease when using these enzymes, thereby reducing the impact on the environment.

In addition, from an economic point of view, the isolation of these microorganisms from natural soils is reflected in the reduction of costs related to the pretreatment of the substrates and implicitly in the shortening the required time for the first stage of anaerobic digestion, that of hydrolysis.

CONCLUSIONS

In the present study, 16 bacterial strains were isolated from natural soils (orchard, solar garden and soil fertilizer) and the production level of amylolytic, cellulolytic, proteolytic, lipolytic and laccases enzymes was compared.

The selection of enzyme-producing microorganisms was carried out in several stages, namely: isolation from the soil, preliminary testing and then the selection of the best 6 strains that will be used in the following studies.

The best producer was microbial strain no. 6, which had an enzymatic index of 2.9 for amylolytic enzymes, 3.6 for proteolytic enzymes and 2.8 for cellulolytic enzymes.

The isolated enzymes producing microorganisms may be used for the effective pretreatment of lignocellulosic agricultural wastes for the production of biogas by anaerobic fermentation process.

The identification of such bacterial strains in natural soils is a process of actuality and is

necessary for the development of a sustainable and economically technology.

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