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STUDY ON BIOTECHNOLOGICAL POTENTIAL OF THERMOPHILIC GRAM-POSITIVE BACTERIUM BREVIBACILLUS SP. BZ

STUDIUM BIOTECHNOLOGICKÉHO POTENCIÁLU TERMOFILNÍ GRAM-POSITIVNÍ BAKTERIE BREVIBACILLUS SP. BZ

MASTER'S THESIS

DIPLOMOVÁ PRÁCE

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ABSTRACT

The subject of the Master's theses is to study the biotechnological potential of the thermophilic Gram-positive bacterium *Brevibacillus borstelensis* BZ. The theoretical part contains a general characterization of thermophilic organisms and their derived thermozymes, and describes their adaptive molecular mechanisms in protein thermostability. The final part of the theoretical part focuses on biodegradation of waste substrates, biobased and fossil-based polymers. The first experimental part deals with the production of hydrolytic enzymes on various original sources and waste substrates. *Brevibacillus borstelensis* BZ is reputed to be a highly promising source of thermostable enzymes, namely xylanases and cellulases, due to its high enzymatic production on original sources. Secondly, the strain BZ was capable of producing a thermostable hydrolytic enzyme on waste substrates. On these substrates, a selected strain preferably produces xylanases. As a consequence, thermostable xylanase has gained a special attention, and, subsequently, it was assayed for the identification of its optimal pH and temperature. The final part of the experimental work discovers the biodegradation capacity of *Brevibacillus borstelensis* BZ toward selected biobased and fossil-based polymers. The strain BZ provided new insight into biodegradation of polyethylene terephthalate (PET), amorphous fraction of polylactic acid (PLA), semicrystalline PLA, and polyhydroxyalkanoates (PHA). Scanning electron microscope (SEM) observations confirmed an occurrence of roughness surface, the presence of grooves, and an utter penetration of the bacterium through the PET film. Regarding biobased polymers, PHA granules treated with the strain BZ were completely degraded. By studying the surface morphology of both types of PLA, the results indicated a clear deterioration of the structure and the presence of pits and cracks on the surfaces.

KEYWORDS

Brevibacillus borstelensis, biodegradation, hydrolytic enzymes, xylanase, polyethylene terephthalate, polylactic acid, polyhydroxyalkanoates, scanning electron microscope

ABSTRAKT

Předmětem předložené diplomové práce je studium biotechnologického potenciálu termofilní grampozitivní bakterie *Brevibacillus borstelensis* BZ. Teoretická část obsahuje obecnou charakterizaci termofilních organismů a jejich termozymů. Popisuje také adaptivní molekulární mechanismy, které zajišťují termostabilitu těchto proteinů. Závěr teoretické části je věnován biodegradaci odpadních substrátů a polymerů na bázi přírodních a fosilních zdrojů. První část experimentální práce se zabývá produkcí hydrolytických enzymů na různých původních zdrojích a odpadních substrátech. Díky intenzivní enzymatické produkci na původních zdrojích je *Brevibacillus borstelensis* BZ považován za velmi slibného producenta termostabilních enzymů, konkrétně xylanáz a celuláz. Testovaný bakteriální kmen BZ byl schopen produkce termostabilních enzymů i na odpadních substrátech. Na vybraných substrátech kmen BZ přednostně produkoval xylanázy. Díky tomu byla xylanázám věnována zvláštní pozornost, kdy bylo testováno teplotní a pH optimum. Závěrem experimentální práce byla testována schopnost bakterie *Brevibacillus borstelensis* BZ degradovat vybrané polymery na bázi přírodních a fosilních zdrojů. Kmen BZ poskytl nový pohled na biologický rozklad polyethylentereftalátu (PET), amorfní frakce kyseliny polymléčné (PLA), semikrystalické PLA a polyhydroxyalkanoátů (PHA). Pozorováním povrchu PET fólie skenovacím elektronovým mikroskopem (SEM) se potvrdilo zdrsnění materiálu, přítomnost rýh a naprosté pronikání bakterie skrz fólii. Pokud jde o polymery na bázi přírodních zdrojů, PHA granule byly zcela degradovány. Studiem morfologie povrchu obou zmíněných PLA bylo prokázáno jasné zhoršení jejich struktury přítomností jam a trhlin na povrchu polymerů.

KLÍČOVÁ SLOVA

Brevibacillus borstelensis, biodegradace, hydrolytické enzymy, xylanáza, polyethylentereftalát, kyselina polymléčná, polyhydroxyalkanoáty, skenovací elektronový mikroskop

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PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Diplomová práce z hledisku majetkem Fakulty chemické VUT v Brně a může být použita ke komerčním účelům jen se souhlasem vedoucího práce a děkana FCH VUT.

DECLARATION

I declare that the diploma's thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology, and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

…….....

Anastasiia Filimonova

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CONTENTS

1. INTRODUCTION

Extremophiles are a large group of organisms with the ability to thrive in various environmental conditions, including extremes of temperature, pressure, salinity, desiccation, and pH. The discovery of such organisms has gained the center of attention due to the remarkable benefits they may have for humanity. Regarding to the best accessed knowledge, merely the minority extremophile organisms on Earth have been discovered and, consequently, exploited [1; 2]. The majority of extremophiles that have been identified to date belong to the Archaea domain. The extremophiles' survival mechanisms are being investigated in order to meet the challenges presented in white, red, and grey biotechnologies. However, extremophilic microorganisms are reputed to be a good source of extremophile-derived enzymes, or extremozymes, and extremolytes. These metabolites have the potential to be a valuable resource for a novel improvement of the bio-based economy through their applications in the chemical, food, and pharmaceutical industries. Regarding extremozymes, they play a pivotal role in functional stability in economically preferable conditions for industrial applications [1; 2; 3]. For instance, extremozymes obtained from acidophilic and acidothermophilic microorganisms might remain stable at low pH and high temperatures. These extremozymes are capable of catalyzing chemical reactions under harsh industrial conditions, such as those that were previously not considered to be conductive for enzymatic activity. Therefore, due to the activity and stability of extremozymes under extreme conditions, they may offer new catalytic alternatives for current industrial applications. Furthermore, they are reputed to be the cornerstone for the development of environmentally friendly, efficient, and stable industrial technologies. Many advantages in industrial biocatalysis have been achieved in recent years and far more will be discovered in the nearest future [4; 5].

2. THEORETICAL PART

2.1. Overview of extremophiles

2.1.1. Extremophiles

Extremophiles are defined as organisms, mainly microorganisms, that live in challenging extreme environments. According to the developed abilities in adapting to extreme conditions, they were classified as psychrophiles, thermophiles, alkaliphiles, acidophiles, halophiles, barophiles, psychrophiles, etc. [1]. Table 1 presents the classification of extremophiles and their adaptational capabilities due to their selected environmental parameter.

Table 1: *Classification and examples of extremophiles [1; 3]*

Environmental parameter	Type	Definition	Examples
Temperature	Hyperthermophile	Growth > 80 °C	*Pyrolobus fumarii*, 113 °C
	Thermophile	Growth 45 – 80 °C	*Synechococcus lividis*
	Psychrophile	< 15 °C	*Psychrobacter*
Radiation	–	–	*Deinococcus radiodurans*
Desiccation	Xerophile	Anhydrobiotic	*Artemia salina*
Salinity	Halophile	Salt-loving (2 – 5 M NaCl)	*Dunaliella salina*
pH	Alkaliphile	pH > 9	*Natronobacterium* *Bacillus firmus* OF4 *Spirulina* spp. (all pH 10,5)
	Acidophile	low pH-loving	*Cyanidium caldarium* *Ferroplasma* sp. (both pH 0)
Oxygen tension	Anaerobe	Cannot tolerate O_2	*Methanococcus jannaschii*
	Microaerophile	Tolerates some O_2	*Clostridium*
Chemical extremes	Gases	–	*C. caldarium* (pure CO_2)
	Metals	Can tolerate high concentration of metal (errantotolerant)	*Ferroplasma acidarmanus* (Cu, As, Cd, Zn); *Ralstonia* sp. CH34 (Zn, Co, Cd, Hg, Pb)

Some extremophiles may introduce simultaneous adjustment to multiple stress factors. For instance, a thermoacidophile maintains its growth under both conditions: high temperature and low pH. In this case, these microorganisms are known as polyextremophiles [3]. In addition, some of them may lose gained tolerance to previously adapted extremal factors of the environment during the bacterial life cycle. The bacterium *Deinococcus radiodurans* serves as an example. This extremophilic bacterium is reputed to be a radiation-resistant microorganism. However, radiation resistance is considerably diminished in stationary compared to logarithmic phase growth, under the increased concentration of Mn^{2+} and under nutrient-limited conditions, and with freezing or desiccation conditions [1].

2.1.1.1. Adaptive mechanisms of microorganism to extreme environmental conditions

The adaptation of microorganisms to the environment involves the development of evolutionary protective mechanisms such as alteration of physiology and enhancement of repair capabilities in extreme conditions. In general, all evolved protective mechanisms based on the adjustment of three key classes of biomolecules: nucleic acids, membrane lipids, and proteins. For instance, to maintain increased membrane fluidity, the cell has to adjust its membrane composition, including the types and amount of lipids in it; and to maintain the structure and function of proteins, it has evolved a mechanism to increase the content of ion pairs, forming higher-order oligomers and decreasing flexibility at room temperature [1; 5].

The vulnerability of DNA might be decreased with the occurrence of monovalent and divalent salts such as KCl and $MgCl_2$. These salts might enhance the stability of nucleic acids to protect DNA from depurination and hydrolysis [1]. With increased osmolarity, some microorganisms are reputed to accumulate osmolytes in the cytosol to protect themselves from cytoplasmic dehydration and desiccation in high-salt environments. The osmotic concentration increases during desiccation, and as a result, the cell might demonstrate similar responses that occur in terms of high-salt environments. Compounds such as K^+, glutamate, glutamine, proline, glycine betaine, and sucrose tend to accumulate away from proteins by forcing water nearby and, consequently, stabilizing the dry membrane [1; 3; 5].

2.1.2. Thermophiles and hyperthermophiles

Thermophiles and hyperthermophiles are defined as organisms that thrive at relatively high temperature The growth optimum of hyperthermophiles is ≥ 80 °C, and thermophiles is above 45 °C. Thermophiles and hyperthermophiles could be found in various geothermally heated regions of the Earth, such as deep sea hydrothermal vents and hot springs, as well as decaying plant matter, such as peat bogs and compost. The main interest of thermophilic microorganisms lies in their ability to produce enzymes known as thermozymes. They have considerable applications in pharmaceutical, pulp and paper, textile, animals feed industries, and biofuel production. Consequently, thermostability may be a crucial factor that helps to withstand industrial conditions [3; 4].

2.1.2.1. *Brevibacillus borstelensis* – selected representative

In 1995, 26 putative *Bacillus brevis* strains were placed in five distinct groups based on the result of DNA base composition, cellular fatty acids, and isoprenoid quinone composition analyzes. However, one of the groups was distinguished phenotypically and genetically. Among the representatives of this group were strains *Bacillus reuszeri*, *Bacillus formosus*, and *Bacillus borstelensis* [6]. Later in 1996, draft genome sequences of *Brevibacillus* species were established, where subsequent findings represented the difference between both genus: *Brevibacillus* and *Bacillus*. The difference was based on the revelation of the presence of many ABC transporters, amino acid permeases, and phosphotransferase sugar transporter, which indicates uptake into the cell; additionally, *B. brevis* was found to be unable to ferment various carbohydrates. Subsequently, the genus *Brevibacillus* was included in the *Paenibacillaceae* family [7].

Brevibacillus is a Gram-positive bacterium, possesses a motile with peritrichous flagella, and is able to form ellipsoidal spores in swollen sporangia. Cell structure is characterized with the presence of specific S-layer proteins; the main cellular fatty acids such as iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The bacterium is forming flat, smooth, circular, and entire colonies. The growth of the bacterium occurs under strictly aerobic conditions and at a temperature of 50 – 55 °C [6; 7]. Currently, the *Brevibacillus* genus includes 20 species which have a great capacity of adaptation to diverse environments.

Among them are mainly introduced representatives of extremophiles such as thermophilic, psychrophilic, acidophilic, alkalophilic, and halophilic [7; 8].

Today *Brevibacillus borstelensis* was found to be a bacterium capable of producing potential metabolites of great industrial interest, such as extracellular hydrolytic enzymes [9] and polyhydroxyalkanoates (PHA) [10]; and recently it has been discovered as a microorganism capable of degrading low-density polyethylene (LD-PE) [11] and antibiotic residues: tylosin [12]; and is considered an excellent laboratory model due to its high growth rate, electroporation transformation efficiency, availability of shuttle vectors, and constitutive expression of heterologous proteins [6; 7].

Both strains 707 and TYL2 of *Brevibacillus borstelensis* are known for their ability to degrade pollutants. The first was discovered by D. Hadad et al. due to its potential to degrade photo-oxidated and non-irradiated LD-PE. After 30 days of cultivation, the activity and viability of the bacterial biofilm formed on the film was determined. Additionally, to identify changes in the LD-PE structure, flourier transform infrared spectroscopy was used. After one month, incubated photo-oxidized LD-PE with strain 707 showed a reduction in the amount of carbonyl residues and in the carbonyl index of approximately 70% in its structure [11]. The second mentioned strain *Brevibacillus borstelensis* TYL 2 was discovered for its ability to degrade tylosin in natural sewage with a degradation rate of 65% in 7 days. This result is valuable, as the accumulation of antibiotic residues in the environment can cause serious environmental problems. Therefore, this capability of strain TYL2 is helpful in providing guidance for the degradation of other antibiotics [12]. Therefore, this bacterium is known to use a variety of carbon autotrophic and heterotrophic growth [7].

Iva Pernicová was devoted to the identification and isolation of PHA producing bacteria within the scope of the dissertation. There was isolated from the composting plant *Brevibacillus borstelensis* BZ and subjected to determination of PHA synthase. The results proved the presence of PHA synthases of two first classes, which in turn are responsible for the biosynthesis of scl-PHA and mcl-PHA [10].

2.2. Enzymes

Enzymes are protein catalysts that promote the conversion of substrates into products. Based on the reactions they catalyze, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) classifies enzymes into six main families: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [13].

Regardless of the 3000 different enzymes, only a few hundred are being used in industries. This underutilization is caused by the inability of many enzymes to withstand harsh industrial conditions. Indeed, enzymes are the main metabolic products of microorganisms, and extremophiles are no exception. Hence, they and their derived extremozymes are in high demand to be identified and characterized. Extremozymes possess protective mechanisms that have been developed due to the adjustment of microorganisms to physical extremes (*e.g.*, temperature, pressure, radiation, etc.) geochemical extremes such as salinity and pH [1; 2].

2.2.1. Extremozymes

Extremophiles play an important role in the discovery of extremozymes which maintain functional stability in economically preferable conditions for industrial applications [1; 2]. Extremozymes obtained from acidophilic and acidothermophilic microorganisms may remain stable at low pH and high temperatures. Additionally, they have been shown to be functionally active under harsh industrial conditions. Among the most frequent extremozymes produced by extremophiles belong amylases, proteases, cellulases, xylanases, lipases, oxidases, and esterases. To withstand severe environmental and

later industrial conditions, enzymes had to develop adaptive mechanisms. They mainly occur to some extent at the molecular level. For instance, the activity and stability of xylanases at extremal pH and temperature are the result of the ionization state of two glutamic acids, namely Glu102 and Glu192. During catalysis, Glu102 behaves as a nucleophile, while Glu192 behaves as an acid or a base. The network of hydrogen bonds is around these residues, which also may affect the catalytic ability of acids [14; 15].

2.2.1.1. Thermozymes

The majority of the current available commercial enzymes are of mesophilic origin. They are obtained from microorganisms that support an optimal growth range of 20 − 45 °C. Therefore, these enzymes introduce low stability at high temperatures and at extreme pH. Enymes with such characteristics cannot be used in industry because they cannot cross the boundaries in biocatalysis. Therefore, there is a high demand to incorporate enzymes of thermophilic origin, namely thermozymes [16].

The thermozyme is a representative of the thermostable. and thermoactive group of biocatalysts. Such features as tolerance against proteolysis and harsh conditions allow them to overcome destructive barriers present in the industry by implementing processes in optimal, for them high temperature; and this group of enzymes is resistant to high pressure and denaturing solvents. These characteristics make thermozymes desirable as biocatalysts in implementation [16].

2.2.2. Molecular mechanisms involved in protein thermostability

Thermostability and thermophilicity of thermostable enzymes are intended to be encoded in the peptide sequence, where they are not consequences of post-translational modifications or non-covalent interactions with the cellular component [17]. Molecular mechanisms responsible for protein thermostabilization are divided into two categories: (1) intrinsic factors (specific amino acid replacement, altered unfolding entropy, hydrophobic core packing, loop region engineering, etc.); and (2) extrinsic factors (glycosylation, immobilization, stabilization by salts, pressure effect, etc.) [14].

2.2.2.1. Intrinsic mechanisms

According to the comparison of thermozymes and mesozymes, thermostability and thermophilicity properties are known to be due to subtle changes in the whole amino acid sequence present in thermophilic enzymes [14]. In the research provided by Argos et al. [18] was made the conclusion that thermal stability could be related to: (1) replacement of Gly, Ser, Ser, Lys and Asp by Ala, Thr, Arg, and Glu, respectively; (2) decreased external hydrophobic amino acids and increased internal hydrophobic amino acids; and (3) stabilization of the helix by more exclusive use of amino acids commonly found in the helices.

The proline-zipper model plays a pivotal role for loop regions in protein thermostability. This model is analogous to the stop on a zipper, where prolines are used to control loop regions to prevent the sequential dissociation of numerous coulombic stabilizing interactions between the two adjacent core elements [14]. Salt bridges are also responsible for the stabilization of proteins. They might be very stable even at the surface of a protein in a highly polar solvent environment. The interactions of salt bridges lead to stabilization of the secondary structure, thus subsequently increasing the strength and rigidity of the structure [14; 19].

Hydrophobic interactions and core packing are other significant factors that play a role in stabilization. To fold proteins in an aqueous solution, hydrophobic interactions have to provide the energy needed for that. It was shown that residue water/ethanol free energy of transfer from residue water/ethanol had contributed roughly 80% of the difference in protein stability [14]. X-ray crystallographic studies stated that thermostable enzymes contain cavities, where some molecules

are filled by water and others not. In addition, the folded structure can be stabilized with aromatic-aromatic interactions in the hydrophobic core environment [14; 20].

2.2.2.2. Extrinsic factors

In vitro thermostability and activity are known to be affected by the nature and concentration of effector molecules in solution, and by some environmental factors [14]. Proteins might be stabilized by inorganic salts in two ways: (1) through a specific effect, where a metal ion interacts with the protein in a conformational manner; and (2) through a general salt effect, which affects the wat activity. Among all ions, K^+, NH_4^+, SO_4^- and HPO_2^{2-} are superior, because they might stabilize proteins more effectively [19]. In addition, it was previously proved that high pressure might stabilize proteins. This ability occurs because of the knowledge that folded proteins have densities similar to those of crystalline solids that, as follows, might serve as a result of general stabilizing force. At increased pressure, unfolded proteins are less stable than folded ones, hence protein remains more compact in the presence of high pressure [14; 17].

2.2.3. The industrial applications of thermolyzes

The molecular mechanisms made thermozymes applicable in harsh industrial conditions. Many of them have been used or proposed for implementation in biotechnological applications such as the production of biofuels, pharmaceutical and food and textile industries, the production of laundry detergents, paper manufacturing, and the production of purified enzymes for use in molecular biology [21; 20].

For instance, thermostable cellulases have broad commercial applications, because they depolymerize complex lignocellulose polymers into simple monosaccharides. As a result, this the simplest form of carbohydrate could be used in manufacturing of other valuable products: bioethanol, biobutanol, and 2,3-butanediol. The derivation of bioethanol from lignocellulose is an environmentally friendly process. Therefore, the enzymatic saccharification of this biomass as well as thermostability is beneficial in industry [20]. Table 2 shows examples of thermophilic enzymes and their potential applications.

Table 2: *Examples of thermophilic enzymes and their potential application [16; 21]*

Type of Enzyme	Organism	Applications
Glutamate dehydrogenase	*Pyrococcus furiosus* *Thermotoga maritima* *Thermococcus litoralis*	Research and diagnostics, aroma and flavour development in cheese, analysis in wine production
Lipase	*Pyrococcus furiosus* *Bacillus* sp. HT19 *Archaeoglobus fulgidus*	Stereoselective hydrolysis of esters, transesterification and dynamic kinetic resolution of alcohols. Backing, food processing, flavours
Laccase	*Thermus thermophilus* *Aquifex aeolicus* *Thermobaculum terrenum* *Bacillus* sp. FNT	Textile. Precipitation of phenolic substrates, enzymatic browning of food, glueing of flake boards, modification of elasticity and consistency of pastes, gums, production of microbicides
Protease	*Bacillus thermoproteolyticus*	Synthesis of aspartame precursor. Cleanup of DNA before PCR. Meat tenderization Peptide synthesis. Detergents. Leather soaking

Table 2: *Examples of thermophilic enzymes and their potential application [16; 21] (continued)*

Nitrilase	*Bacillus pallidus* *Geobacillus pallidus* *Pyrococcus abyssi*	Synthesis of polymers such as polyacrylonitrile, pharmaceuticals and pesticides
Xylanase	*Dictyoglomus thermophilum* *Nonomuraea flexuosa*	Feed, food processin, silage additives. Biofuel production. Research and diagnostic analysis. Pharmaceutical industry. Papermaking process
DNA polymerase	*Thermus thermophilus* HB8 *Thermus aquaticus*	DNA amplification, reverse transcription. DNA labelling. DNA sequencing, cycle sequencing method
Pullulanases	*Bacilus acidopullulyticus* *Thermus caldophilus* *Pyrococcus furiosus*	High-glucose syrup production. Saccharification of starch into maltotetraose, maltotriose, and maltobiose

2.3. Biodegradation

Biodegradation is the process at which both low- and high-molecular-weight substrates of natural and synthetic origin might be degraded. Living organisms, mainly microorganisms, are involved in the decomposition. They utilize substrates as a sole of carbon and energy. In the natural environment, biodegradation begins with chain cleavage by enzymes secreted by microorganisms. They convert the substrate by their extracellular or intracellular enzymes into smaller oligomers and eventually monomers that may pass through the cell membrane followed by assimilation and mineralization in their intracellular metabolism (Figure 1). This kind of cleavage is named as endolytic (Route A). However, there is exolytic cleavage, which directly leads to monomers (Route B). The cell of the microorganism gains energy from the mineralization process by producing environmentally acceptable products, such as carbon dioxide, water, methane, dinitrogen, and biomass [23; 24].

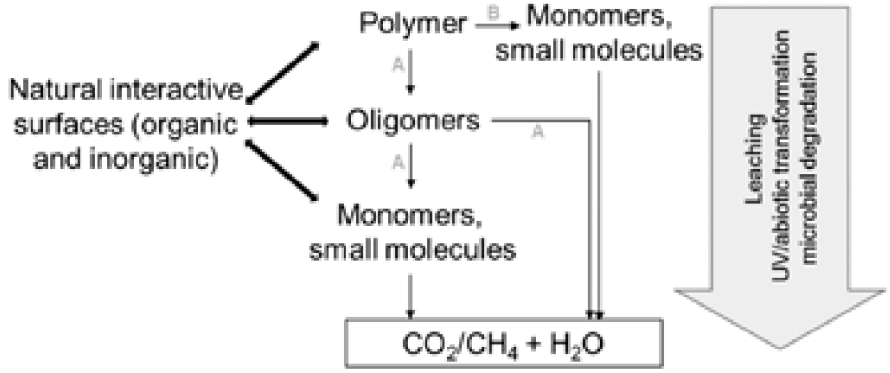

Figure 1: *Mechanism of biodegradation [24]*

Mineralization of organic compounds is a highly beneficial process in nature. Because by the oxidization of primer organic compounds into carbon dioxide and water, the cell gains energy and carbon for its growth. However, microbial degradation may be influenced by several factors such as humidity, oxygen level, light, and temperature [24].

Depending on the oxygen level, the biodegradation of the organic compound could be conducted under either aerobic or anaerobic conditions. In the presence of oxygen, microorganisms use it as an electron acceptor and break down organic chemicals into smaller organic compounds, like carbon dioxide and water, which are the by-products of this process. If oxygen is present, some anaerobic microorganisms use nitrate, sulphate, iron, manganese, and carbon dioxide as their electron acceptors, to break down into smaller compounds, where the result among the by-products will be methane in addition to carbo dioxide and water [25; 26].

Biotic and abiotic factors are further essential factors involved in microbial biodegradation. Abiotic factors include parameters of mechanical, thermal, light stressful effects, and chemical transformations, where the organic compound is exposed to the weathering condition in natural environments (*e.g.*, soil, composting plant, etc.). The biotic factors explain the metabolic abilities of the microorganism that includes the capability of microorganism to interact with the surface of natural or synthetic material; and in consequence assess its ability to decompose it. Such characteristics are based on the ability of the microorganism to secrete the proper enzymes. The rate of biodegradation might be affected by the temperature, pH, and moisture of the environment [25].

2.3.1. Biodegradation of waste substrates

Waste substrates might cause a troublesome environmental pollutant that is generated from the agricultural sector, the petrochemical sector, petroleum industry, etc. Mechanical and chemical methods generally used for treating waste substrates and their possible released contaminants have limited effectiveness and cost; and soon direct burial in landfills might become prohibitively expensive. When considering possible benefits of using waste substrates obtained in the agricultural sector, there are many advantageous ways how they may be applied and used in favor of industry. Some examples of waste substrates and their prospective applications are discussed in the following paragraphs.

2.3.1.1. Spent coffee ground

Coffee is one of the most widely consumed beverages by millions of people around the world and is the second most traded commodity after oil. However, the coffee industry is generating an immense amount of biobased waste, such as silver coffee skin and spent coffee grounds (SCG) [27; 28; 29]. The annual worldwide SCG generation rate exceeds 6 million tons. Today, this biomass residue is underutilized. In addition, the majority of waste is dumped in landfills or incinerated [28]. SCG is considered as a hazardous waste substrate due to its chemical composition that is represented by caffeine, tannins, and phenolic compounds. These chemical compounds might cause toxicity towards natural environments if their treatment with them is not proper. In addition, SCG might spontaneously combust and, consequently, generate hazardous gases. Therefore, these waste substrates require appropriate process technologies and regulations in accordance with the use of harmless methods for the environment and the provision of its biotechnological value in industry. Controversially, SCG maintains a highly beneficial rich chemical composition that is renewable and inexpensive. Therefore, SCG may be used in the flavour of the biotechnological industry [27; 29]. Figure 2 represents which value-added products might be obtained by using SCG [27; 30].

The chemical composition of dry SCG is composed of polysaccharides, lignin, proteins, and lipids. The composition of SCG can vary, it depends on the variety of coffee trees, the growing conditions, and the brewing methods [27]. Fatty acids such as linoleic, palmitic, and oleic present in SCG, make this waste substrate interesting for the development of biodegradable packaging materials. The oil from SCGs can be cultivated with microorganisms to produce biopolymers such as PHAs. These polymers could be an environmentally friendly alternative to petroleum-based plastics. The production of such biopolymers is more advantageous than the production of biodiesel due to the high content of free fatty acids, which conform for standard specifications to the production of biodiesel [27; 31]. The oil content

might be varied and characterized by high stability against the antioxidant content. This may depend on the origin of the coffee and the extraction method used extraction method [29; 30].

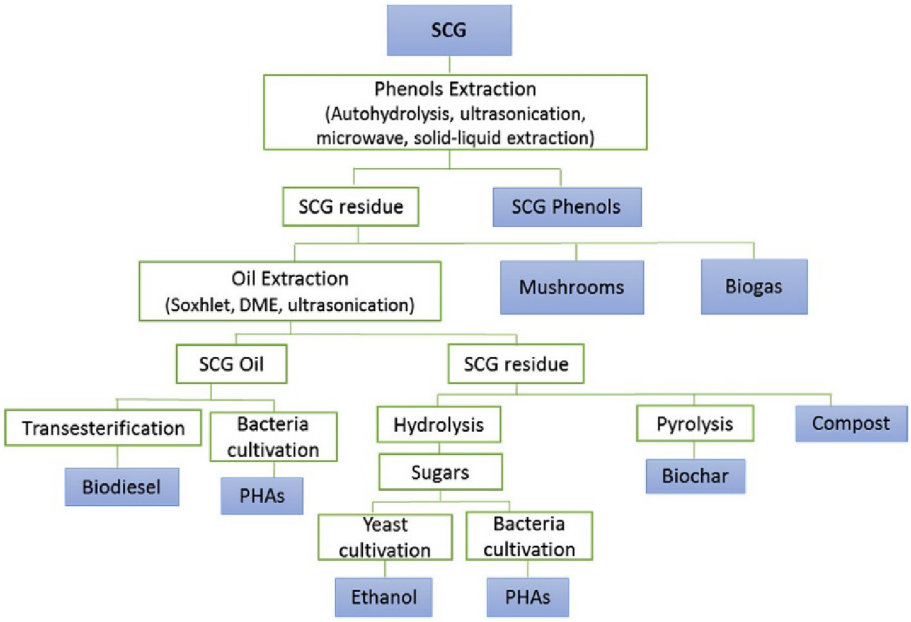

Figure 2: *Scheme illustrating and integrated biorefinery approach for the valorisation of SCG [30]*

Recently, the generation of electricity by using SCGs and carbon black with the implementation of high-temperature carbon fuel cell technology has been examined. The chemical structure of SCG contains heteroatoms and hydrogen, which are crucial for gasification and the formation of oxidizable compounds (*e.g.*, H_2, CO_2). Therefore, SCG is a carbon neutral fuel and is important source for sustainable electricity generation [30].

Jooste T. et al. in their research isolated the range of fungal strains that were cultivated with SCG. As a result, weight loss of substrate was observed, and hence it was proposed that fungal strains use it solely as carbon and energy sources for its growth and division. Subsequently, the researchers identified prospective hydrolytic enzymes that are involved in the degradation of SCG. Among them, mannanase, endoglucanase, and xylanase belong to these three enzymes. Potentially, these biocatalysts might be purified and used in the decomposition of other possible polysaccharides [32].

2.3.1.2. Sawdust

Sawdust is a by-product derived from wood processing such as drilling, milling, sawing, planning, routing, and sanding. These operations shatter wood cells and lead to the formation of wood debris known as sawdust. In general, this by-product is used as blotting material, hand cleaner, wood filler, compost, and packing [33]. However, sawdust may cause health hazards. For instance, it may develop cancer of the nasal cavity and paranasal sinuses [33; 34].

Sawdust consists of three major components: cellulose, hemicellulose, and lignin, and the importance of its biodegradation in the environment has received attention for several years. However, microbial degradation of raw sawdust is very difficult to be decomposed in the soil because of the lack of nitrogen and the presence of the lignin. Therefore, the process of sawdust biodegradation in environments could last for 180 days. Lignin is a highly recalcitrant component and has a nitrogen lack that is required for the microbial metabolism [33; 35; 36]. However, Lennox et al. reported the ability of indigenous microorganisms to degrade wood sawdust. Isolated from the soil, bacteria and fungi were able to utilize

sawdust as the sole source of carbon and energy source. To degrade waste substrate, these microorganisms secreted hydrolytic enzymes, such as carboxymethyl cellulose, lignin peroxidase, lipase, and xylanase. The biodegradation of sawdust leads to the generation of economically important and useful products such as glycine, N-cyclopropylcarbonylmethyl ester, and acetic acid. Both the first and the second are applicable in the cosmetic industry, in pharmaceuticals, and food processing. Acetic acid benefits in lowering blood pressure and reducing inflammation [33].

2.3.1.3. Wheat bran

Wheat bran (WB) is a by-product of the wheat milling process. The annual worldwide production rate of WB reaches roughly 150 million tons. However, only a small percentage of this waste product is used, despite its cheapness and potential wide applicability in industry [37]. WB is considered to be a valuable substrate for the cultivation of microorganisms. The properly selected microbial strain is an important factor in producing organic acids such as lactic and ferulic acids. They are using WB as a source of carbon, nitrogen, and energy for growth and division. Microorganisms might supply the industry with other relevant metabolites (*e.g.*, enzymes, pigments, food additives, antibiotics, etc.) [38]. The dry matter of WB is composed of non-starch carbohydrates, starch, proteins, and the smallest amount pertains to minerals and fats. The chemical composition of WB is represented in Figure 3 [38].

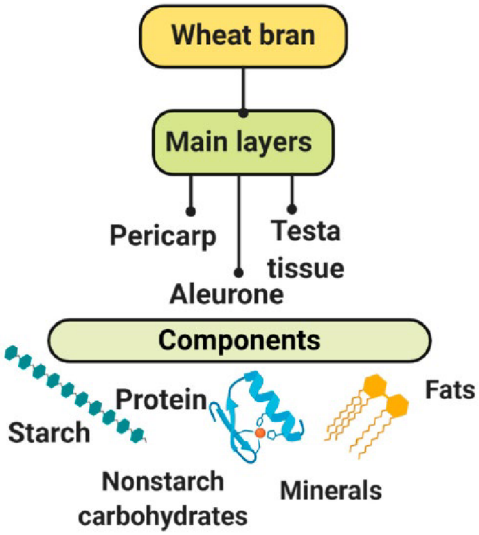

Figure 3: *Wheat bran and components distribution. These constitute 55-60% of non-starch carbohydrates, 14-25% of starch, 13-18 % of protein, 3-8% of minerals, and 3-4% of fat calculated on a dry matter [38]*

The major component of non-starch carbohydrates is arabinoxylan. Xylan could be degraded into xylooligosaccharides which may benefit the prevention of atherosclerosis, inhibition of melanoma cell proliferation, treatment of atopic dermatitis, enhancement of collagen production, antihyperlipidemic, etc. [37]. WG is a rich source of phenolic compounds including hydroxycinnamic acid, ferulic acid, sinapic acid, and p-coumaric acid; and hydroxybenzoic acid, whose derivatives include vanillic acid, syringic acid, and gallic acid. These acids have wide applications in the pharmaceutical, cosmetic, and food industry [37-39]. Table 3 summarizes the role of WB in various fields.

Table 3: *The role of wheat bran in various fields [38]*

Field	Application/Product	Role as/in
Enzymes	Enzyme production by solid-state fermentation and submerged fermentation	Substrates for enzymes production
	As an inducer for enzymes	Complex substrate
	Production of protease, amylase, and glucoamylase	Nitrogen source
Metabolites	Bacitracin Cyclosporine-A Gibberellic acid	Cheap raw material
Biofuel	Bioethanol Biobutanol Biohydrogen	Lignocellulosic material
Heavy metals removal	Removal, Hg^{2+}, Cd^{+2}, Pb^{+2}, Cu^{+2}, Cr^{+4}, Ni^{+2}	Biosorbent material, lignocellulosic substrate
Health	Minimizes the risk factor for various illness: diabetes, colon cancer, hypertension, coronary heart disease	Fiber source, strong antioxidant activity, bioactive agents that inhibit colon carcinogenesis
Food	Enrich the nutritional and physical properties of bread and baked products	Nutritional and physical properties
Feed additive	The stock material for animal feed preparations	High starch content, indispensable amino acids, high content of non-starch polysaccharides

2.3.1.4. Brewer's spent grain

Brewer's spent grain (BSG) or draff is the insoluble part of the barley grain that is generated in the beer-brewing process. Brewing industries generate 85% of brewing waste. The number of by-products depends on the efficiency of mashing on the quantity of starchy endosperm and walls of aleurone cells and thirdly on used regime conditions in brewing processes. Figure 3 represents the process of obtaining BSG from natural barley [40].

The chemical composition of BSG can vary depending on the barley cultivator, the malting process, and the quality and formation of the brewing cereals. However, BSG always consists of high levels of dietary fiber, protein, and essential acids, polyphenols, vitamins, and lipids. Among the main components are xylan, cellulose, arabinan, lignin, etc. Due to the high polysaccharides and moisture content of BSG, it makes the waste substrate susceptible to microbial decomposition over a short period of time (*e.g.*, 2-7 days) [41]. Despite the vast annual generation of BSG and its prospective applicability in various industries, this by-product is used mainly (70%) as animal feed due to its high content of dietary fiber, protein and essential amino acids. The combination of BSG with inexpensive nitrogen sources, such as urea, may provide the waste substrate with all essential amino acids for nutrition. Recently, the presence of BSG in cow's diets was reported to increase milk production and decrease fat content in milk [41; 42].

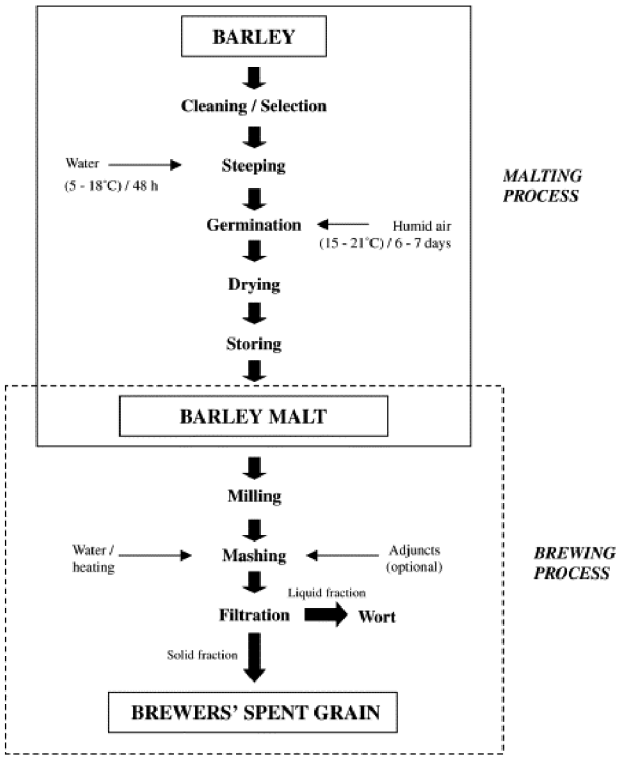

Figure 4: *Schematic representation of the process to obtain brewers' spent grain from natural barley [40]*

Today, BSG has received much attention for its incorporation as a food supplement for the fortification of human food products, because BSG is considered an antioxidant, antiallergenic, anti-inflammatory ingredient and, therefore, may reduce the incidences of diabetes, cancer, and the risk factors for cardiovascular diseases [42; 43]. BSG as an adjunct added to food and is evaluated in manufacturing numerous bakery products such as bread, cookies, breadsticks, and snacks [41; 43]. Supplementation with 10% BSG could increase the content of protein and essential amino acids; hence, its addition improves the nutritional value of the product and, subsequently, contributes to countries, where food is scarce [41].

2.3.1.5. Apple pomace

The fruit processing industry generates a large number of by-products annually that could be exploited to manufacture other value-added goods. One of these by-products is apple pomace (AP). AP is a rich natural source of pectin substances, dietary fibers, minerals, vitamins, and carbohydrates, although the main part of this waste substrate is underestimated and subsequently ends up in landfills [44; 45]. The biodegradation of AP in nature might cause environmental concern since its complete decomposition requires a high biochemical oxygen process [45].

AP is considered an important raw material for the production of pectin. By microbial fermentation, other valuable products might be obtained, such as bioethanol and organic acids (*e.g.*, citric acid). The demand for citric acid increases each year by 5%. This acid may be applied in the food and pharmaceutical industries [44; 45]. Furthermore, the use of AP by microorganisms can yield pectolytic enzymes. They have a wide range of applications in the food and textile industries. For example, produced pectolytic enzyme – β-fructofuranosidase might be applied in the clarification of apple juice [46]. AP is also used to produce aroma compounds and natural antioxidants. Currently, it is estimated that almost 100 different natural aroma compounds might be produced by microbial fermentation using medium containing AP [46; 47].

2.3.1.6. Waste feathers

In poultry processing plants, the generation of waste feathers reaches five million tons worldwide annually. Feathers are mainly composed of valuable pure keratin protein, which represents a potential alternative to more expensive ingredients and food additives. However, the process of converting feathers into animal feed requires a large amount of energy and processing technologies, which may result in amino acids [48]. Furthermore, despite all potential benefits in feather applications, a major part of this waste substrate is not properly utilized. Utilization includes either incineration or disposal in landfills. However, when it comes to the decomposition of chicken feathers, there are physical and chemical methods. These methods are observed to have high energy consumption during processing and may bring substantial damage to prospective products. Therefore, there is a growing demand in the development of new feather processing technologies that would be environmentally friendly [49; 50].

Chicken feathers contain more than 90% keratin protein, which is rich in cysteine, arginine, threonine, hydrophobic amino acids, and has high nutrient potential. However, because of the rigid stable structure, where protein chains are tightly packed and stabilized through hydrophobic interactions and disulphide bonds, the protein is resistant to hydrolysation by common proteolytic enzymes, such as pepsin, papain, or trypsin. Unhydrolyzed milled feathers may have low biodegradability efficiency and nutritional potential, when converted to feed supplements. Therefore, the waste substate might have some limitations in food applications. However, as mentioned earlier, the keratin obtained from feathers might be used as in animal feedstuffs, fertilizers, and feed supplements. However, using feathers as feedstock is a rather expensive process because it also includes processes, such as sterilization and conversion, into material that will be digestible for animals. Therefore, it is considered that the decomposition of feathers in nature is a more cost-effective way of utilization, although soil microorganisms are not capable of biodegrading such a large number of feathers that are produced annually [48; 49]. Nevertheless, microbial degradation of keratin has recently been discovered. The study showed that while natural biodegradation occurs, the original keratin structure remains stable and the activity of other products, such as amino acids and peptides, is maintained. Keratinolytic microorganisms may produce keratinase, protease, and disulphide reductase. Keratinolytic enzymes have a potential role in keratin biodegradation in the poultry and leather industries [48; 49; 50]. Figure 5 represents the degradation of feathers by keratinolytic bacteria.

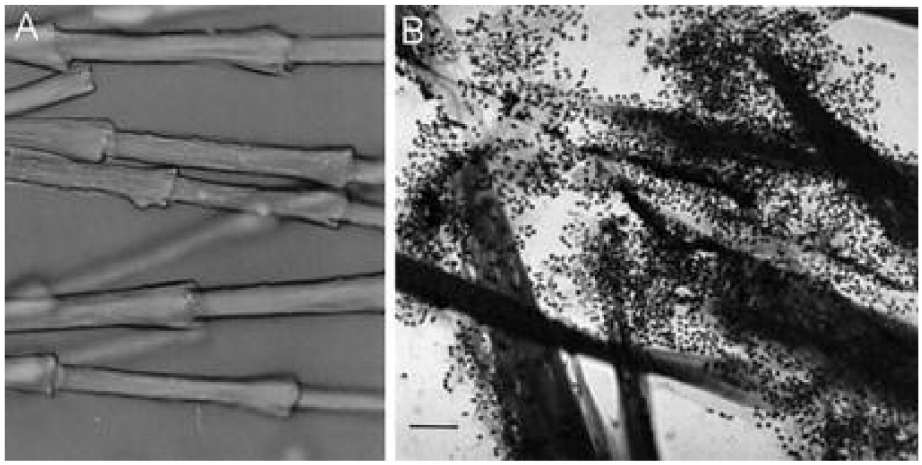

Figure 5: *Feather degradation by keratinolytic bacteria. Native feathers were autoclaved and incubated at 30 °C for 3 days in A the absence or B in the presence of Chryseobacterium sp. strain kr6 [48]*

Keratinase belongs to a class of proteases that has keratolytic activities. Serine/metalloprotease are identified as keratinases that possess the ability to degrade keratinous proteins [49; 50]. To decompose feathers, microorganisms produce enzymes that selectively degrade β-keratin. Through enzymatic hydrolysis, bacteria obtain carbon, sulphur, and energy for their growth and division [48].

2.3.2. Biodegradation of biobased and fossil-based polymers

Synthetic polymers are widely used in all aspects of human life and in industries such as packaging, building and construction, mobility and transport, electronics, agriculture, and healthcare. They are derived from non-renewable resources; therefore, in the environment, they are degrading extremely slowly. Their reclamation and incineration could release dangerous pollutants into the atmosphere, soil, and groundwater [51; 52]. The replacement of fossil-based polymers with well-known renewable alternatives is appealing, and hence a solution to the current concern could be an increase in the production of biobased polymers. They are made partially or completely from renewable raw materials. Their biodegradability gives additional value. Today, among widely produced biobased materials belong polylactic acid (PLA) and PHA. For instance, the basis for obtaining PLA is the fermentation of sugars derived from renewable sources such as corn and sugarcane. It makes PLA an eco-friendly non-toxic polymer that, furthermore, may demonstrate similarity to that of polystyrene (PS). Because of its high stiffness and transparency in its amorphous state, it is a suitable material for application. Regarding biodegradation, the main degradation route of PLA is chemical hydrolysis. This process occurs at high humidity and at elevated temperatures. The release fragments (*e.g.*, oligomers and monomers) might be consumed by microorganisms [24; 53].

PHA is a class of thermoplastic polyesters derived from hydroxyalkanoic acid. PHA may be obtained from renewable resources such as sugars and starch, or from fatty acids through a single fermentation step. Furthermore, for the fermentative synthesis of poly(3-hydroxylbutyrate) by methanotrophic bacteria, hydrocarbons such as methane, which is the most common representative of PHA, could be reached. The polymer itself might be obtained by microorganisms, where the polymer in the microorganism might reach up to 90% of the dry weight of the cell mass. Since PHAs are found as metabolites of microorganisms, many abundant microbe species could accept them as food sources [24].

3. EXPERIMENTAL PART

3.1. Materials and instruments

3.1.1. Model Organism

The experimental part of this study was conducted on the bacterial strain *Brevibacillus borstelensis* BZ that was obtained from Blansko composting plant within the dissertation of Iva Pernicová [10].

3.1.2. Chemicals

Table 4: *Utilized chemicals*

Compound	Supplier
4-Nitrophenyl acetate	Sigma-Aldrich, DEU
4-Nitrophenyl palmitate	Sigma-Aldrich, DEU
Ammonium chloride	Lach-Ner, CZE
Ammonium nitrate	Lach-Ner, CZE
Apple pectin	Sigma-Aldrich, DEU
Azocasein	Sigma-Aldrich, DEU
Calcium chloride dihydrate	Lachema, CZE
Carboxymethylcellulose	Sigma-Aldrich, DEU
Citric acid	Lach-Ner, CZE
Dipotassium phosphate	Lach-Ner, CZE
Dried skimmed milk	Bohemik, CZE
Ethanol 99,8%	Lach-Ner, CZE
Ferric ammonium citrate	Fluka, CHE
Ferrous sulphate heptahydrate	Lach-Ner, CZE
Glycerol	Lach-Ner, CZE
Magnesium sulphate heptahydrate	Lach-Ner, CZE
Manganese sulphate	Sigma-Aldrich, DEU
Nutrient broth	HiMedia, IND
P-Nitrophenol	Sigma-Aldrich, DEU
Potassium chloride	Lach-Ner, CZE
Potassium dihydrogen phosphate	Lach-Ner, CZE
Sodium citrate dihydrate	Lach-Ner, CZE
Sodium dodecyl sulphate	Lach-Ner, CZE
Sodium hydrogen phosphate dodecahydrate	Lach-Ner, CZE
Sodium hydroxide	Lach-Ner, CZE
Sodium nitrate	Lach-Ner, CZE
Soluble starch	Penta, CZE
Sunflower oil	Olitalia, CZE
Trichloroacetic acid	Lach-Ner, CZE
Tris base	Lach-Ner, CZE
Xylan from beechwood	Apollo Scientific, UK
Yeast extract	HiMedia, IND
Zinc sulphate heptahydrate	Sigma-Aldrich, DEU

3.1.3. Apparatus and instruments

Table 5: Utilized apparatus

Specification	Apparatus
Analytical balances	Ohaus Pioneer PA224C
Balance	Kern EW 620-3NM
Centrifuge	Hettich EBA 200
Centrifuge	Sartorius Sigma 1-15
Incubator shaker	IKA KS4000i control
Laminar flow box	Euroclone BioAir Auro mini
pH meter	Thermo Scientific pHTestr 30
Thermostat	LTE IP100U
Thermostat	LTE IP60
Vortex	Benchmark BenchMixer V2

Table 6: Utilized instruments

Specifiction	Instrument
ELISA reader	BioTek
Scanning electron microscope, energy dispersive analyzer	Zeiss EVO LS -10, analyser OXFORD X-Max 80 mm^2
Stereomicroscope with ZEN core software	Zeiss Stemi 2000-C
UV-VIS spectrophotometer	Implen P300

3.2. Cultivation

3.2.1. Inoculation

The bacteria were stored in cryotubes in 10% glycerol at − 80 °C. Inoculation was performed in a laminar flow box under sterile conditions. Nutrient Broth bacterial growth medium was prepared at the concentration of 25 g/l in a volume of 50 ml, in 100 ml Erlenmeyer flask. All prepared flasks were sterilized. After being cooled, they were transferred to the sterile laminar flow box, where each was inoculated with 0.5 ml of strain BZ from a cryotube. Subsequently, the inoculums were incubated for 24 hours on a rotary shaker (170 rpm) at 50 °C.

3.2.2. Production medium preparation and culture conditions

Brevibacillus borstelensis BZ assayed for its ability to secrete hydrolytic enzymes on original sources and the waste substrates were grown in M1 mineral medium. The composition of this medium is shown in Table 7. Each 250 ml Erlenmeyer flask contains 100 ml of the corresponding medium. All original sources and waste substrates were prepared at the concentration of 20 g/l per flask.

The study of the BZ strain biodegradation capacity towards selected biobased and fossil-based polymers, the BZ strain was grown in synthetic medium (SM) (Table 7).

The identification of the optimal pH and temperature to produce selected hydrolytic enzyme, in the beginning stage its secretion was tested on three different media, such as M1, SM, and VB medium (Vogel and Bonner) (Table 7). SM was chosen for xylanase production. The subsequent identification of pH and temperature was carried out in 100 ml Erlenmeyer flask containing 50 ml of the corresponding medium with the supplementation 20 g/l of xylan from beechwood.

Bacterial strains assayed for their ability to utilize biobased and fossil-based polymers as the sole source of carbon and energy were grown in SM medium for 24 days. The cultivation of the BZ strain with polymers was carried out in 250 ml Erlenmeyer flasks, containing 100 ml of the corresponding medium.

All prepared flasks were sterilized and kept overnight at the thermostat. The following day 10% of the inoculum was transferred to flasks with 250 ml volume containing mineral medium in a laminar flow box.

Table 7: *List of mineral media*

Medium	**Composition of the mineral medium [g/l]**		**Trace elements solution**	**Composition of trace elements solution [g/l]**	
M1	$Na_2HPO_4 \cdot 12\ H_2O$	9	**TES II**	EDTA	50
	KH_2PO_4	1.5		$FeCl_3$	8.3
	NH_4Cl	1		$ZnCl_2$	0.84
	$MgSO_4 \cdot 7\ H2O$	0.2		$CuCl_2 \cdot 2\ H_2O$	0.13
	$CaCl_2 \cdot 2\ H_2O$	0.02		$CoCl_2 \cdot 6\ H_2O$	0.1
	NH_4Fe^{III}citrate	0.0012		$MnCl_2 \cdot 6\ H_2O$	0.016
	Yeast extract	0.5		H_3BO_3	0.1
	After sterilization				
	TES II	1 ml/l			
	NH_4NO_3	1			
SM	$MgSO_4 \cdot 7H_2O$	0.2			
	K_2HPO_4	1			
	$CaCl_2 \cdot 2\ H_2O$	0.1			
	KCl	0.15			
	Yeast extract	0.1			
	$FeSO_4 \cdot 6\ H_2O$	0.001			
	$ZnSO_4 \cdot 7\ H_2O$	0.001			
	$MnSO_4$	0.001			
VB	KNO_3	2	**MES**	$FeSO_4 \cdot 7H_2O$	2.78
	KH_2PO_4	5.8		$MnCl_2 \cdot 4H_2O$	1.98
	K_2HPO_4	3.7		$CoSO_4 \cdot 7H_2O$	2.81
	$MgSO_4 \cdot 7\ H_2O$	0.25		$CaCl_2 \cdot 2H_2O$	1.67
	Yeast extract	0.1		$CuCl_2 \cdot 2H_2O$	0.16
	After sterilization			$ZnSO_4 \cdot 7H_2O$	0.29
	MES	1 ml/l			

3.2.2.1. Biodegradation of original sources

Starch, xylan from beechwood, carboxymethylcellulose, apple pectin, dried skimmed milk, sunflower oil were used as original sources. They were prepared at a concentration of 20 g/l and added directly to M1 mineral medium. The cultivation was carried out at 50 °C, 3 and 7 days, in an incubator shaker (170 rpm).

3.2.2.2. Biodegradation of waste substrates

The production of hydrolytic enzymes by strain BZ on various waste substrates was studied. Waste substrates which were used in the thesis were: sawdust, feathers, spent coffee grounds, brewer's spent grains, apple pomace, wheat bran. Two methods for waste substrates preparation were tested. In the first, each substrate was washed, dried, and added to M1 medium. In the second, substrate was directly added to M1 medium. The cultivation was carried out at 50 °C, 3 days, in an incubator shaker (170 rpm).

3.2.2.3. Biodegradation of biobased and fossil-based polymers

Before cultivation, all biobased and fossil-based polymers had to be properly prepared. The biodegradation of nine different polymers was performed. The list of used polymers and their images may be seen in Figure 6.

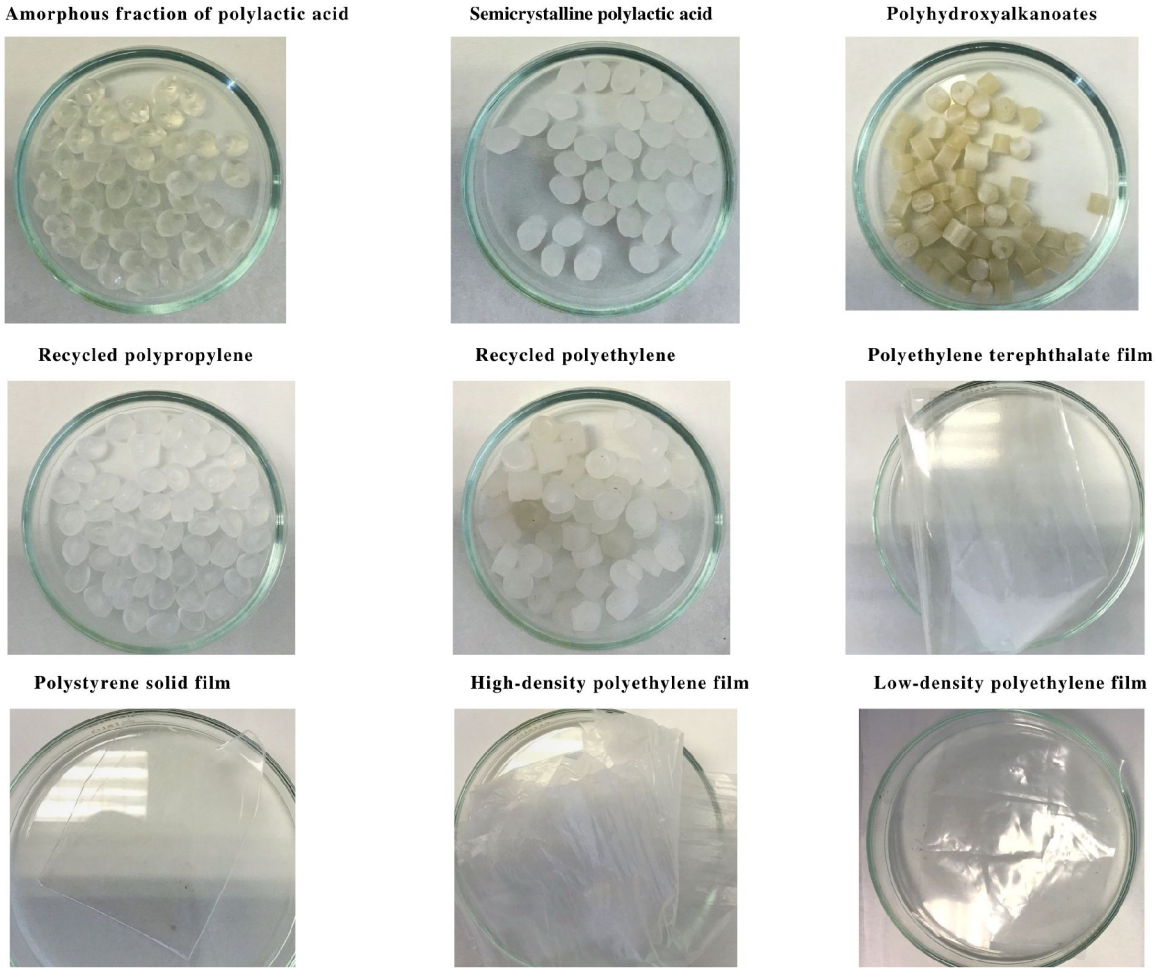

Figure 6: *Used biobased and fossil-based polymers*

Before cultivation, each polymer was weighted at approximately 300 mg, washed with 70% ethanol, and dried overnight at the thermostat at 50 °C. The following day, all polymers were reweighted under sterile conditions, transferred aseptically to a 70% (v/v) ethanol solution for 30 min, then in laminar flow box under sterile conditions, they were again rewashed with distilled water and replaced in the flasks.

In general, 18 flasks containing SM medium were prepared, whereas 9 were incubated with bacterial strain BZ and respective polymer; and 9 others had merely contained polymers without inoculum. The cultivation was carried out 24 days. During this period, the optical density (OD_{640}) was measured regularly.

To facilitate accurate measurement of the weight of the residual polymers, after the cultivation period, the residual bacteria were completely removed from the films and granules with a 2% (w/v) aqueous sodium dodecyl sulfate solution. After polymers were rewashed with 70% (v/v) ethanol solution, and later with distilled water. The washed polymers were placed on Petri dishes and dried overnight at 50 °C. The following day, polymers were weighted, and the weight loss was calculated.

3.2.3. Measurement of biomass and supernatant preparation

To measure biomass, bacteria culture was removed 2x10 ml from each flask and placed in centrifuge tubes. Tubes with bacterial culture were centrifuged (6000 rpm, 5 min). After centrifugation, the supernatants were taken, frozen, and stored for the subsequent enzymatic activity measurements. In turn, the precipitates were washed with distilled water and centrifuged (6000 rpm, 5 min). The prepared biomasses were dried to a constant. The resulting amount of biomass was defined as the average weight of both measured samples.

3.2.4. Biodegradation of waste substrates

After 3 days of cultivation, the loss of waste substrates was determined. Each Erlenmeyer flask was prefiltered using water vacuum filtration. The waste substrates were dried at a thermostat at 50 °C. After drying, the substrates were weighted, and the weight loss was calculated.

3.3. Parametric optimization for xylanase cocktail production

The effect of pH and temperature on the production of xylanase cocktail was assessed by cultivating the BZ strain in non-buffered growth SM medium of pH 4.0 – 10.0 at 50 °C for 3 and 7 days. The pH of the medium was adjusted using 0.5 M NaOH or 1 M H_2SO_4. The effect of temperature was studied by growing the bacterium at different temperatures (40 °C; 50 °C; 60 °C), pH 7.0 for 3 and 7 days. After the third and seventh days of cultivation, samples from each flask were taken in order to measure possible altered pH; and to prepare supernatants for enzymatic activity measurements.

3.4. Measurement of enzymatic activity

3.4.1. Measurement of the activity amylases, xylanases, cellulases and pectinases

The supernatants obtained from production media were used to measure the enzymatic activities of amylases, xylanases, cellulases, and pectinases. In Bachelor's thesis [54], the appropriate solvent for the dissolution of the substrate and the incubational period of the supernatant, containing a pivotal enzyme substrate solution was optimized. In Table 8 may be observed the summery of an appropriate incubation period and solvent for each enzyme.

Table 8: Summarising of composition of substrate solutions, its solvents, and incubation period

Enzyme	Solvent		Substrate, g/l		Incubation period, hours
	Citrate Buffer*	Water			
Amylase	+		Starch	10	24
Xylanase		+	Xylan	10	24
Cellulase		+	Carboxymethylcellulose	10	4
Pectinase		+	Apple pectin	10	4

Enzyme activities were determined in doublet reactions using the 3,5-dinitrosalicylic acid (DNSA) method. The initial reaction mixture for each enzyme consisted of 250 µl of supernatant and 250 µl a corresponding substrate. The corresponding substrate was previously dissolved in water or buffer (Table 8). Additionally, samples containing commercial enzymes were prepared. The samples with commercial enzymes were prepared in the same way as substrates, although instead of supernatant, the commercial enzyme was used and weight directly to tubes. The prepared blank contained merely distilled water and substrate.

The incubation of enzyme-substrate was carried out at 50 °C in accordance with the appropriate time for the production of certain enzyme (Table 8). The incubational period was stopped with 500 µl DNSA and then boiled at 70 °C for 10 minutes. After cooling, 9 ml of distilled water was pipetted to the tubes and afterwards the absorbance at 540 nm against the blank was measured.

* Citrate buffer composition:

Component	Amount
Sodium citrate dihydrate	25.703 g/l
Citric acid	2.421 g/l

800 ml of distilled water was added to recently mentioned weighed chemicals, the solution was adjusted to final pH 5 by using HCl or NaOH and after then the distilled water was refilled to final volume (1 liter).

3.4.1.1. Calibration curve of glucose

Glucose stock solution at the concentration of 3.5 g/l was prepared; and, subsequently, was diluted to concentrations 1; 1.5; 2; 2.5; 3; 3.5 g/l with distilled water to the final volume 0.5 ml. Volume 0.5 ml of DNSA was pipetted into tubes, and subsequently the tubes were boiled at 70 °C for 10 minutes. After cooling, 9 ml of distilled water was added into the tubes and the absorbance at 540 nm was measured. The calibration curve for glucose concentration is shown in Figure 7.

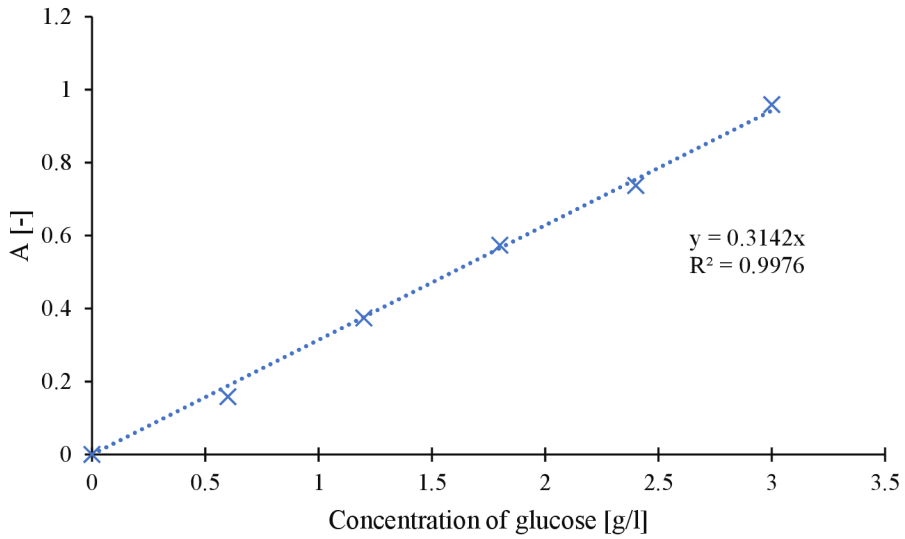

Figure 7: *The calibration curve for glucose concentration*

3.4.2. Measurement of protease activity

In the beginning, the method for measuring protease activity was optimized. In total, two methods were tested. In both methods, azocasein was used as the substrate. The principle for measuring protease activity is the enzymatic cleavage of azocasein into azooligopeptides. Colored peptides are released by enzymatic hydrolysis and are soluble in trichloroacetic acid. Subsequently, these peptides might be detected at 440 nm.

In both methods stored supernatants were used. The first method included the following steps: 100 µl of azocaseine solution at 5 mg/ml concentration was mixed with 100 µl of enzyme-containing supernatant, after which the samples were incubated at 50°C for 30 and 60 minutes. The reaction was stopped by 410 µl 10% trichloracetic acid, microtubes were centrifuged at 14 000 rpm for 2 minutes. 500 µl of supernatant was added to the cuvette that contained 700 µl 1 M NaOH. Subsequently, the absorbance was measured at 440 nm against the blank. The blank was prepared in the same way as the samples, although instead of supernatant distilled water was used.

The second enzyme assay method included further steps: in 2 ml microtubes were mixed 450 µl Tris-HCl buffer, azocasein solution at concentration of 5 mg/ml, and 300 µl of supernatant containing enzyme. Microtubes were incubated at 50 °C for 30 and 60 minutes. After incubation, a 1 ml aliquot was removed from the solution and placed in 2 ml microtubes that were containing 1000 µl of 110 mM trichloracetic acid and the samples were centrifugated at 14 000 rpm for 10 minutes. After centrifugation, 1 ml of supernatant aliquot was removed in 2 ml microtubes and mixed with 1000 µl 500 mM NaOH. Subsequently, the absorbance was measured at 440 nm against the blank. The blank was prepared in the same way as samples, although instead of supernatant, was pipetted 750 µl of 100 mM Tris-HCl buffer*. The sample with commercial enzyme was prepared the same, although, instead of supernatant the commercial enzyme was added at the concentration 2 g/l.

*100 mM Tris-HCl buffer composition:

Component	Amount
Tris base	12.11 g/l
HCl (36%)	4.8 ml

800 ml of distilled water was added to recently mentioned weighed chemicals, the solution was adjusted to final pH 8 by using HCl or NaOH and after the distilled water was refilled to final volume (1 liter).

3.4.3. Measurement of lipase enzymatic activity

To measure the lipolytic activity, supernatants obtained from the production media were used. As substrates were used: 4-nitrophenyl acetate and 4-nitrophenyl palmitate. Both substrates were prepared at the concentration of 15 mg per 10 ml of solvent. As a solvent, 96% ethanol was used. For the measurement, PBS buffer* with a pH of 7,5 was used.

Measurement was carried out in a 96-well microtiter microplate, where in the individual holes pipetted 230 µl PBS buffer and 25 µl supernatant containing the enzyme. The microplate was incubated in an autoclave at 50 °C for 10 minutes. After incubation, both substrates were added to the separate holes. Subsequently, using the ELISA reader, the absorbance was measured at 405 nm for 16 minutes with a two-minute interval. The resulting absorbance value was obtained by subtracting the values at time 0 and at a given time.

* PBS buffer composition:

Component	Amount
NaCl	8 g/l
KCl	0,2 g/l
KH2PO4·2H2O	0,24 g/l
Na2HPO4 ·2H2O	1,44 g/l

800 ml of distilled water was added to shown in the Table above weighed chemicals, the solution was adjusted to final pH 7,5 by using HCl or NaOH, and after the distilled water was refilled to final volume (1 liter).

3.4.3.1. Calibration curve of p-nitrophenol

15 mg of p-nitrophenol was weighted and dissolved in 10 ml of water. Subsequently, the tube was incubated at 50 °C for 24 hours in the incubator shaker. After cooling, this solution was pipetted inv quantities of 166, 333, 667, 800 and 1000 µl to prepared microtubes; while the distilled water was added to reach the final volume 1000 µl.

The measurement was carried out in a 96-well microtiter microplate, where 230 µl PBS buffer and 25 µl of each prepared mixture were pipetted into the individual holes. Using an ELISA reader, the absorbance was measured at 405 nm. The final concentration of lipolytic activity was determined according to the calibration curve of p-nitrophenol that can be found in Figure 8.

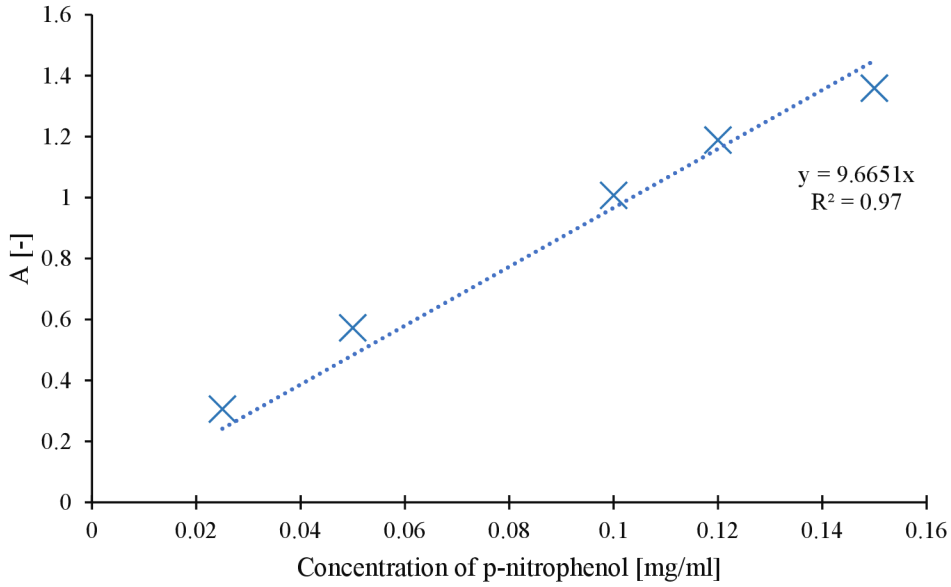

Figure 8: *The calibration curve of p-nitrophenol*

3.5. Stereomicroscopy

Macroscopic images of the polymers were taken using a Zeiss Stemi 200-C stereomicroscope with ZEN core software.

3.6. Scanning Electron Microscopy

Detailed images of polymer surfaces were taken using a Zeiss Evo LS-10 scanning electron microscope (SEM) with energy-dispersive (EDS) analyzer OXFORD X-Max 80 mm^2.
The analyses parameters:

- accelerating voltage 5 kV.
- work distance approx. 8-12 mm.
- the death time approx. 50%.
- beam current 100-200 pA.
- magnitude 50-10 000×.

4. RESULTS AND DISCUSSION

The initial part of the experimental work of this master's thesis is focused on the production of hydrolytic enzymes by *Brevibacillus borstelensis* BZ on various waste substrates and original sources. Within the scope of Iva Pernicová's dissertation, strain BZ was isolated from a composting plant and identified as a bacterium capable of producing PHA [10]. This strain gained additional attention during the bachelor's thesis [54] due to the production of extremozymes such as proteases, lipases, xylanases, cellulases, amylases, and pectinases. This interesting finding led to a study that *Brevibacillus borstelensis* BZ may be capable of producing these hydrolytic enzymes on other sources. Therefore, this thesis focuses on the production of hydrolytic enzymes on various original sources and waste substrates; and the characterization of selected enzymes, *e.g.*, the identification of optimal pH and temperature. In addition, the biodegradation capacity of the BZ strain toward selected biobased and fossil-based polymers was discovered.

4.1. Production of hydrolytic enzymes

4.1.1. Production of hydrolytic enzymes on original sources

The initial part of the experimental work started with the production of hydrolytic enzymes by *Brevibacillus borstelensis* BZ on original sources. The strain BZ was cultivated for a period of 1 week on five different original sources, such as xylan from beechwood, carboxymethylcellulose, starch, apple pectin, dried skim milk, and sunflower oil. The supernatants obtained after the third and seventh days of cultivation were used to measure the enzymatic activities of further enzymes: xylanases, cellulases, amylases, pectinases, proteases, and lipases; and the precipitates were dried and weighted, and thus the results were evaluated as the ability of the strain BZ to grow on a certain original source.

The incubation period of xylanases and amylases was carried out for 24 hours, while the incubations of cellulases and pectinases were performed for 4 hours. The optimization of protease production was performed. Table 9 summarizes the results obtained from measurements of the enzymatic activities of amylases, pectinases, and absorbance values of proteases on original sources after the 3rd and 7th day of the cultivation period. In addition, in the lower part of the table may be found the results of the measurements of enzymatic activities of commercial enzymes.

Table 9: *Summarizing of measurements of enzymatic activities on original sources (above) and commercial enzymes (below). The results of amylases and pectinases are introduced at the concentration (g/l of glucose released for a certain period of time), while proteolytic activities are introduced in their absorbance values*

A day of cultivation	Amylase, g/l	Pectinase, g/l	Protease, A [-]
The determination of enzymatic activities on original sources			
3rd day	4.233±0.019	1.015±0.012	0.139±0.006
7th day	5.175±0.017	2.447±0.012	0.177±0.003
The determination of enzymatic activities of commercial enzymes			
	10.489±0.008	9.098±0.008	0.231±0.008

According to Table 9, it may be observed that *Brevibacillus borstelensis* BZ was capable of the production of all hydrolytic enzymes on different original sources; additionally, it should be mentioned that the enzymatic activities increased with increasing cultivation period. However, among all these measured enzymes, xylanase and cellulase achieved the highest enzymatic activities, while the production of pectinases the bacterium produced at least. In addition, taking into consideration that xylanase and cellulase had different incubation periods, they introduce relatively similar values of enzymatic activities. According to the incubation period for cellulolytic activity, the results of a previous study [54] indicated that if the incubation period increases to 24 hours, it will cause a decrease in cellulolytic activity. Regarding xylanase production for 4 and 24 hours, a significant increase in the enzyme activity of the mentioned enzyme was observed after the 24 hours incubation period. Therefore, the selected incubation periods for both enzymes are optimal to moderate a sufficient amount of product (xylanases and cellulases). Figure 9 and Figure 10 represent determination of xylanolytic and cellulolytic activities, respectively.

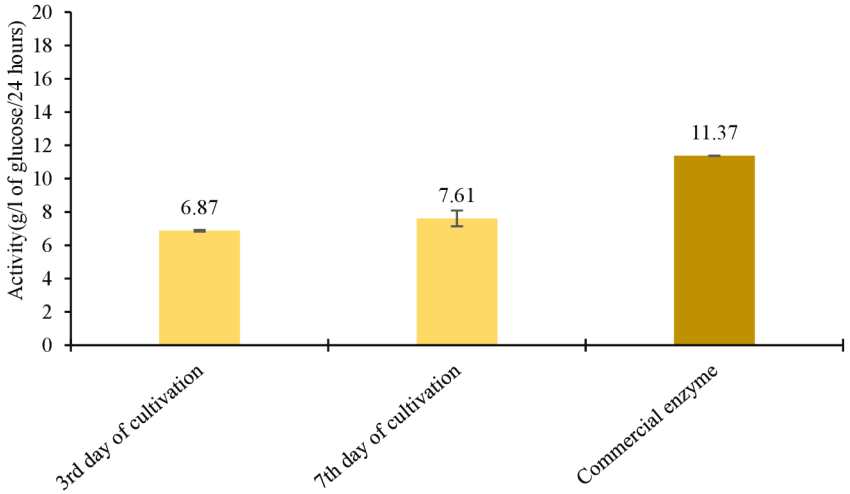

Figure 9: *The determination of enzymatic activity of xylanase*

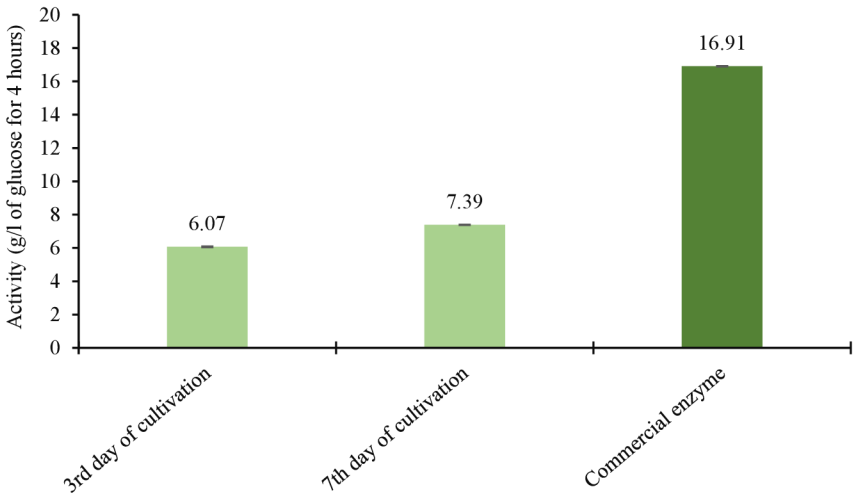

Figure 10: *The determination of enzymatic activity of cellulase*

As it can be observed in both figures, xylanase and cellulase represent relatively the same values after the 3rd and 7th day of the cultivation. First column introduces the value of enzyme obtained after the 3rd day of cultivation, where xylanase activity is around 6.870±0.050 g/l of glucose for 24 hours, and cellulase activity is 6.065±0.018 g/l of glucose for 4 hours. On the 7th day of cultivation, the enzymatic activities of xylanase increased to a value 7.612±0.054 g/l of glucose for 24 hours, while cellulolytic activity increased to 7.391±0.011 g/l of glucose for 4 hours.

Furthermore, as mentioned earlier, the enzymatic activities of commercial enzymes were also measured. The third column in both figures represents the activities of commercial xylanase and cellulase; and the results of the rest enzymes can be seen in Table 9 in the lowest row. Commercial enzymes possess higher values than enzymes produced by *Brevibacillus borstelensis* BZ, which is not odd due to the crude form of enzymes produced by strain BZ. Hence enzymes in a crude form have to be purified before their activities might be properly assessed and compared with commercial ones.

Brevibacillus borstelensis BZ has proven as promising producer of xylanases and cellulases simultaneously. Both enzymes are known to be the most widely used in industries, and very often a synergistic action of these enzymes is required. For instance, they might be applicable in bioethanol production, deinking of waste paper, animal feed processing, food processing, paper and pulp production, and pharmaceuticals. Therefore, microorganisms capable to produce xylanase and cellulase simultaneously are in high demand [55].

Setting aside the results obtained from measurements of the enzymatic activities of xylanase and cellulase, the growth of the BZ strain on xylan and carboxymethylcellulose was on average level in comparison with other original sources, such as starch and dried skim milk. According to Figure 11, where is presented the dependence of biomass on the cultivation period and original source, strain BZ showed a small increase in biomass between 3rd and 7th day of the cultivation on xylan, where on the 3rd day the biomass is 1.061±0.353 g/l and on the 7th – 1.279±0.424 g/l.

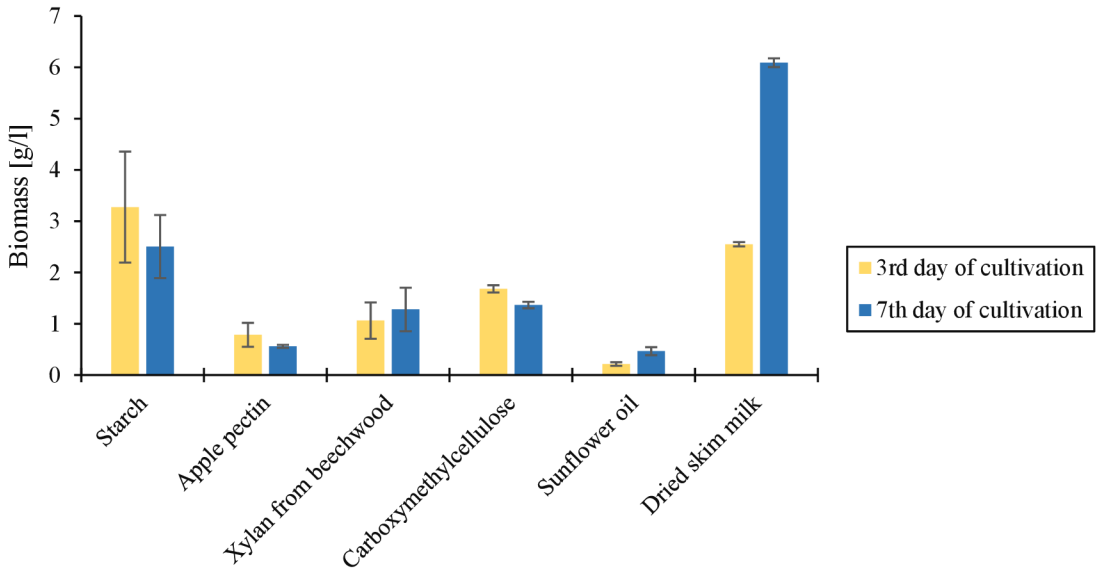

Figure 11: *Dependence of biomass on the cultivation period and on original sources in Brevibacillus sp. BZ*

The same situation occurs with use of the sunflower oil, although, as may be observed, the strain was capable of its growth at least from among all used waste substrates. The highest biomass value was reached on dried skim milk, and at the same time, a significant increase in biomass was demonstrated with an increase in the incubation periods. Despite the fact that all enzyme activities were increasing, there is an occurrence of a biomass decrease on the 7th day on further original sources: starch, apple pectin, and carboxymethylcellulose.

Taking into consideration such high increase in biomass obtained on dried skim milk, it is interesting to mention the ability of strain BZ to produce protease as well. Regarding the measurement of proteolytic enzymes, two methods were tested, where azocasein was used as a substrate in both. The principle for measuring protease activity is the enzymatic cleavage of azocasein into azooligopeptides. Colored peptides by enzymatic hydrolysis are soluble in trichloroacetic acid and could be detected at 440 nm [56]. For the measurement of protease activities, there were two incubation periods: 30 and 60 minutes, although the values obtained after both incubation periods did not demonstrate significant difference. Therefore, a 30-minute incubation time was chosen as a sufficient time to measure the absorbance. The calibration curve to calculate protease activity was not performed; thus, the results are represented in absorbance values which may be observed in Table 9. It is interesting to mention that such double absorbance values between both cultivation periods are not indicated as it is with biomass.

As was mentioned, the lowest bacterial biomass was observed on the sunflower oil as the carbon substrate. The measurement of lipolytic activity was performed for 16 minutes with a two-minute interval. The activity was calculated according to the calibration curve of p-nitrophenol. The highest values were obtained on substrate 4-nitrophenyl acetate. By comparing both cultivation periods, the highest enzymatic values of lipolytic activities were obtained using the supernatant gained after the 7th day was 0.0200±0.0003 mg/ml p-nitrophenol for 16 minutes. The following Figure 12 shows the gradual growth of lipolytic activity with increasing time.

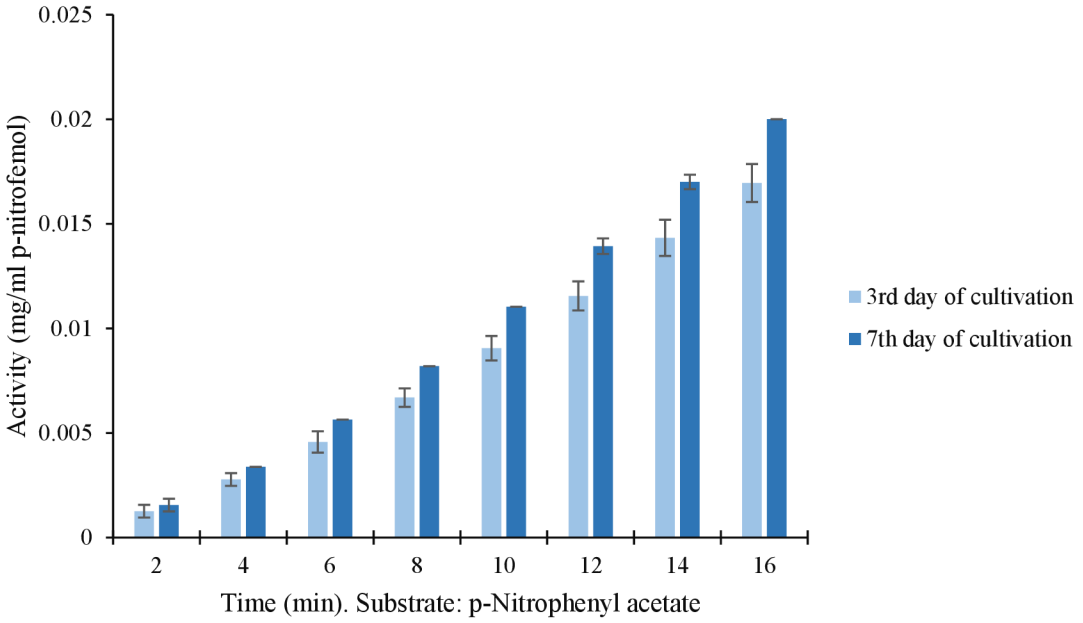

Figure 12: *The determination of enzymatic activity of lipase*

In order to explain why 4-nitrophenyl acetate as a substrate is more efficient for lipolytic production on sunflower oil, it should be mentioned the difference between two substrates: 4-nitrophenyl acetate and 4-nitrophenyl palmitate. The difference lies in the structure of both substrates. Less energy is used for lipolytic cleavage in the case of 4-nitrophenyl acetate. The ester bond in 4-nitrophenyl acetate is located between the phenolic and acetal fatty acid residue, which is shorter than the palmitic fatty acid residue that is in the case of 4-nitrophenyl palmitate.

4.1.2. Production of hydrolytic enzymes on waste substrates

The capability of *Brevibacillus borstelensis* BZ to grow and produce hydrolytic enzymes on original sources led to the further study that this strain might degrade various waste substrates to produce these hydrolytic enzymes. This might be beneficial in both cases, whereas the use of waste substrates is not commonly applicated in the industry, thus waste substrates are often disposed in landfills or incinerated. According to the structure, waste substrates can be exploited to manufacture the additional value-added goods. For instance, they might be used as substrates for the cultivation of microorganisms in order to produce biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, etc.

The production of hydrolytic enzymes was studied on the waste substrates such as sawdust, feathers, spent coffee ground (SCG), spent grain (SG), apple pomace (AP), and wheat bran (WB). In this experimental part, two methods of handling with waste substrates were chosen. In the first, they were washed, dried, and weighted before cultivation; however, in the second approach the waste substrates directly added to the medium. This kind of procedure was managed in order to compare how the production of hydrolytic enzymes may vary and how the presence of contaminants or the absence of important ingredients from substrates may affect its production. The cultivation on the waste substrates lasted for 3 days. According to Table 10 representing the summarizing of the measurements of enzymatic activities on a waste substrate, it is interesting to mention that in almost all cases the washing procedure of waste substrates was beneficial for the increase of enzymatic activities, but with the exception of AP. This brings us to a conclusion that washing process made waste substrates more accessible for enzymatic cleavage.

Table 10: *Summarizing of measurements of enzymatic activities on a waste substrate. The results of amylases, cellulases, and pectinases are introduced at concentration (g/l of glucose released for a certain period of time), while proteolytic activities are introduced in their absorbace values*

Substrate	Amylase, g/l	Pectinase, g/l	Cellulase, g/l	Protease, A [-]	Amylase, g/l	Pectinase, g/l	Cellulase, g/l	Protease, A [-]
The determination of enzymatic activity on washed waste substrates					**The determination of enzymatic activity on non-washed waste substrates**			
Sawdust	0.612±0.005	0.363±0.014	0.445±0.003	0.100±0.005	0.165±0.003	0.319±0.014	0	0.128±0.015
Waste feathers	1.304±0.007	0.121±0.006	0.081±0.013	0.040±0.001	0.176±0.007	0.405±0.005	0.638±0.019	0.045±0.004
Spent coffee ground	1.689±0.017	1.690±0.017	0.580±0.005	0.118±0.013	0.628±0.014	1.755±0.021	0.515±0.009	0.073±0.016
Brewer's spent grain	1.942±0.021	1.362±0.014	0.481±0.004	0.099±0.008	0.264±0.007	0.321±0.006	0.883±0.024	0.142±0.004
Apple pomace	3.920±0.010	0.679±0.024	0.618±0.010	0.115±0.016	2.877±0.078	2.835±0.032	2.184±0.044	0.038±0.012
Wheat bran	2.377±0.052	0.319±0.024	0.538±0.006	0.203±0.024	0.997±0.049	1.616±0.041	0.588±0.037	0.244±0.003

Xylanases achieved the highest enzymatic values on all waste substrates among all other measured enzymes. Figure 13 represents the dependence of xylanase production on various waste substrates.

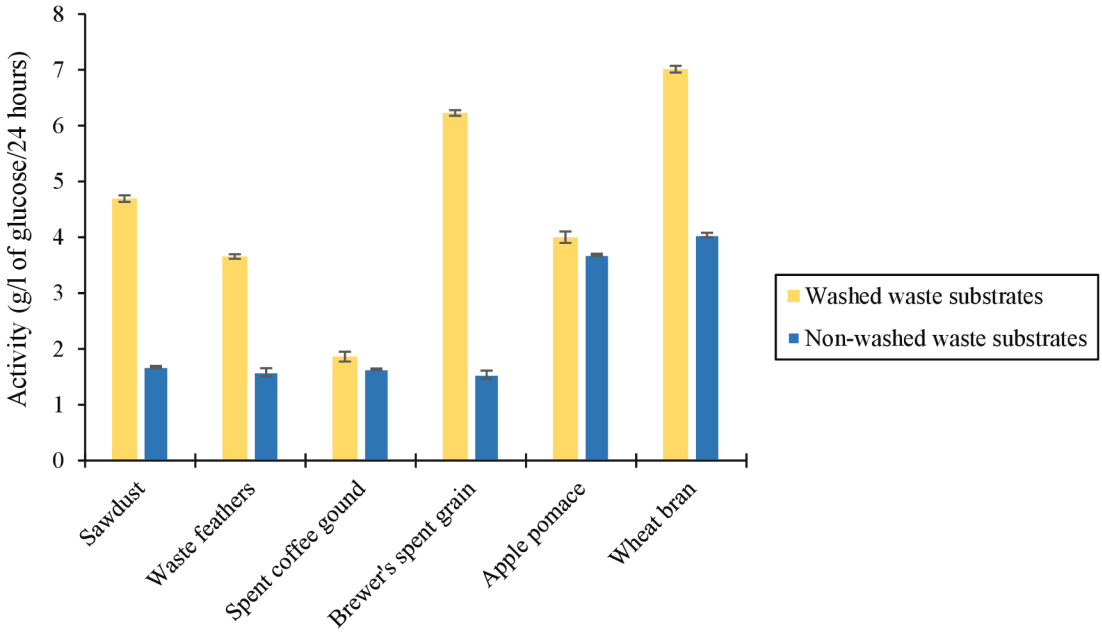

Figure 13: *The determination of enzymatic activity of xylanase on waste substrates*

According to Figure 13, the highest enzymatic values of xylanases are detected on washed WB, where the enzymatic activity of xylanase is 7.011±0.060 g/l of glucose for 24 hours, while on the non-washed substrate − 4.040±0.040 g/l of glucose for 24 hours. These values can be compared with amylase production on the same washed substrate – 2.377±0.052 g/l of glucose for 24 hours. The high enzymatic values of xylanase on WB may explain the structure of the substrate. This substrate consists of 55 – 60% non-starch carbohydrates, 14 – 28% starch, and 13 – 18% protein. The main component of non-starch carbohydrates is xylan [38]. Therefore, it could be concluded that WB is a rich source of xylan and, subsequently, a valuable source for xylanase production. However, WB is also considered to be a rich source of fatty acids, such as linoleic, oleic, palmitic, linolenic, stearic [57]. However, the lipolytic activity value measured on 4-nitrophenyl acetate was merely 0.105±0.0098 mg/ml of p-nitrophenol for 16 minutes, which is lower in the comparison with the values obtained on washed BSG – 0.193±0.006 mg/mg p-nitrophenol for 16 minutes; and washed feathers – 0.226±0.008 mg/ml p-nitrophenol for16 minutes. Figure 14 represents the results obtained from the measurement of lipase activity on washed waste substrates.

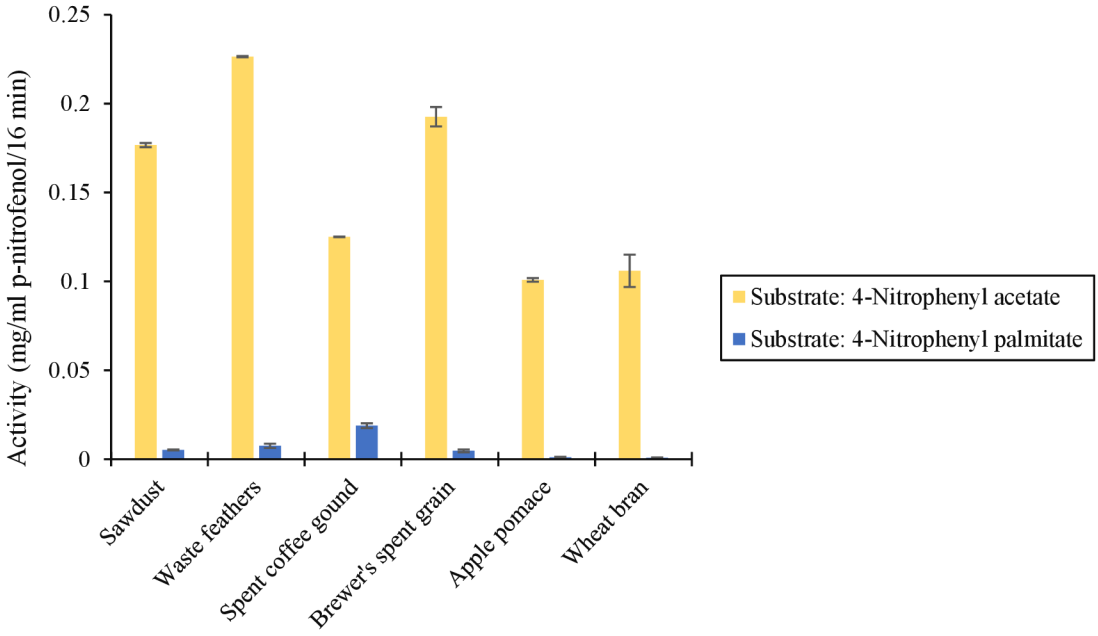

Figure 14: *The determination of enzymatic activity of lipase on washed waste substrates*

BSG is the second waste substrate, where the high enzymatic activity of xylanase is recorded. The value of xylanolytic activity on washed BSG is 6.227±0.050 g/l of glucose for 24 hours. Mostly, BSG consists of cellulosic and non-cellulosic polysaccharides; however, the composition of this substrate may vary depending on the quality and formation of the brewing cereals, the chosen malting process, or even the barley cultivator [41]. Therefore, if we do not take the capability of strain to produce some enzymes, then these conditions may explain, for instance, the possible losses in the enzymatic activities of cellulase. For comparison, the cellulolytic activity produced on washed BSG is 0.481±0.004 g/l of glucose for 4 hours; while on non-washed − 0.883±0.024 g/l of glucose for 4 hours. The following Figure 15 represents BSG before (a) and after (b) degradation. In these images may be observed that the structure of substrate did not considerably change, despite the high production of hydrolytic enzymes on this substrate.

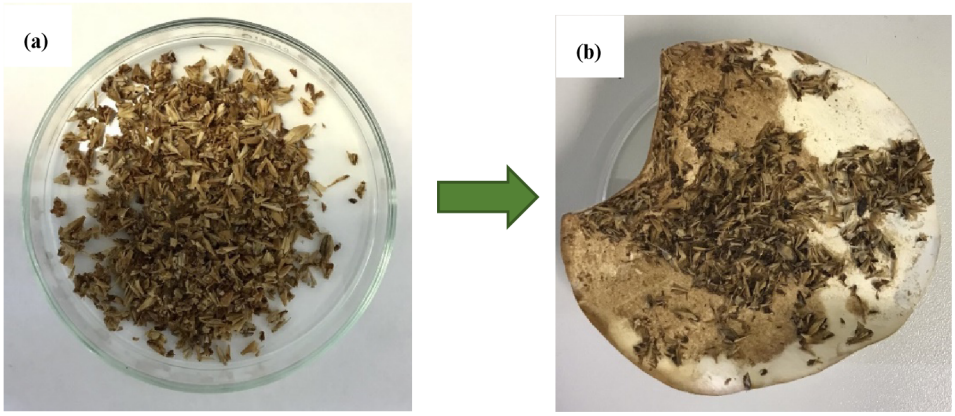

Figure 15: *The demonstration of BSG biodegradation. The first image (a) shows washed and dried substrate before cultivation, while the second (b) represents substrate after biodegradation by Brevibacillus borstelensis BZ*

It is interesting to mention the ability of *Brevibacillus borstelensis* BZ to degrade and produce hydrolytic enzymes on a sawdust substrate. As can be observed in Table 10 and Figure 13, xylanase and amylase achieved the highest values on washed sawdust, where the xylanolytic activity is 4.693±0.058 g/l of glucose for 24 hours; and the amylolytic activity is 0.612±0.004 g/l of glucose for 24 hours. As is known, sawdust consists of cellulose, hemicellulose, and lignin. Lignin occupies up to 30% of woody plant tissue. Lignin is an aromatic polymer and provides rigidity and resistance to biological attack [33]. Due to the lack of lignin hydrolyzable linkages, it is difficult to enzymatically depolymerize lignin. There are only several microorganisms capable of degrading sawdust due to the presence of lignin, although *Brevibacillus borstelensis* BZ may be considered as the strain capable of degrading the sawdust and use it to produce hydrolytic enzymes.

The xylanolytic activity produced on washed and non-washed SCG slightly changes the same as the enzymatic activities of pectinase and cellulase. However, a considerable difference can be observed in amylases (Table 10). The amylolytic activity on washed SCG is 1.689±0.017 g/l of glucose for 24 hours, while on non-washed – 0.628±0.014 g/l of glucose for 24 hours. When it comes to lipolytic activity, SCG is a very interesting substrate for lipase production because of the presence of fatty acids such as linoleic, palmitic, and oleic acids. The capability of the strain BZ to produce lipases on washed SCG may be observed in Figure 14. The highest lipolytic activity was produced on the waste feathers. This may explain the presence of waxes in the feathers. According to the obtained results of lipolytic activities produced on a non-washed substrate, it should be mentioned that the preparatory washing procedure of the substrates was beneficial to produce lipases. However, by comparing the strain capability to produce lipases with other enzymes, *Brevibacillus borstelensis* BZ produces lipases at least.

The following Figure 16 represents the growth of biomass on waste substrates in *Brevibacillus borstelensis* BZ. Returning to the enzymatic activities, where the highest values were measured on WB, BSG and sawdust, it should be noted that the high values of biomass was also observed in washed BSG and WB. While comparing the growth of strain BZ on washed BSG the biomass is 2,585±0,035 g/l, while on the non-washed substrate is 1.47±0.113 g/l. Considering the ability of the strain to produce hydrolytic enzymes on sawdust, the biomass value is relatively small in comparison with other substrates, where on washed substrate the value reached 0.270±0.056 g/l, on non-washed – 0.441±0.169 g/l.

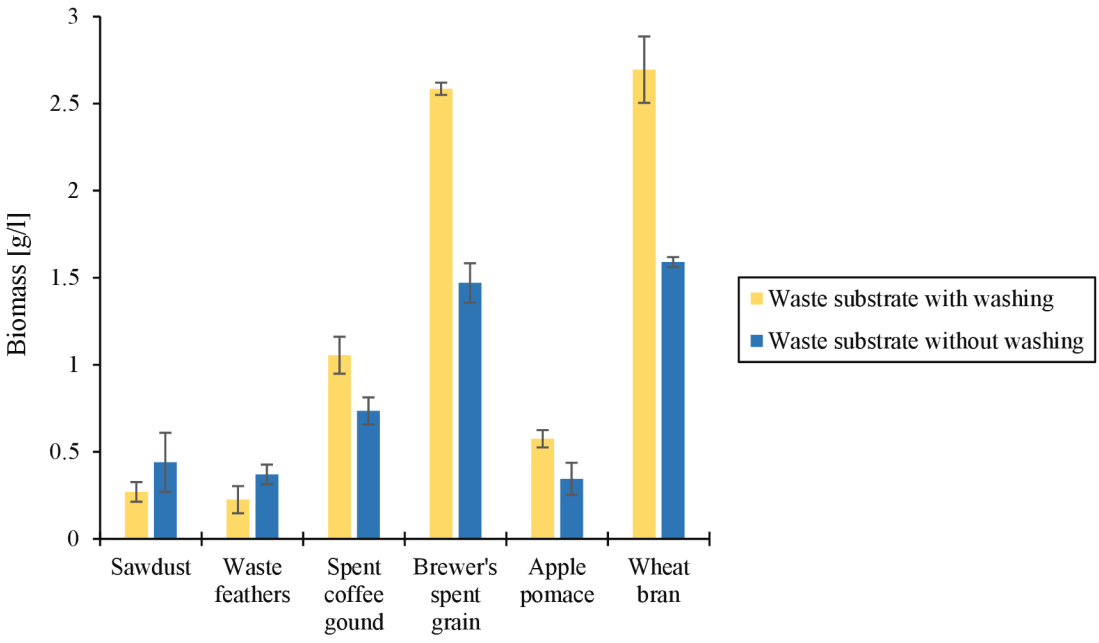

Figure 16: *The dependence of biomass on waste substrates in Brevibacillus borstelensis BZ*

Additionally, it is intreating to mention that the bacterium was growing better on washed AP than on non-washed AP, even though the enzymatic activities of all enzymes reached much higher values on non-washed AP. The ability of the bacterium producing hydrolytic enzymes and growing on the certain type of substrate, has to be corresponded also with the total weight loss of waste substrates. Table 11 represents the total weight loss of all substrates after 3rd day of cultivation in percentage (%).

Table 11: *The total weight loss of waste substrates after 3rd day of cultivation, in percentage (%)*

Sawdust	Feathers	Spent coffee ground	Brewer's spent grain	Apple pomace	Wheat bran
The weight loss of washed waste substrates (%)					
5.76	9.97	12.50	35.04	31.22	42.48
The weight loss of not washed waste substrates (%)					
3.6	19.17	14.05	48.41	56.12	46.80

All weight substrates were prepared at the same concentration 20 g/l either for washed substrates or non-washed. From Table 11 a significant weight loss after cultivation may be observed in WB, BSG, and AP. The results conclude that bacterium prefer to grow on these substrates rather than on sawdust, feathers, or SCG. Additionally, a slightly higher value is noticeable in the total weight loss on non-washed substrates than on washed substrates.

Moreover, the biodegradation of AP is another intreating result to mention. Each substrate was prepared in doublet. In the case of washed AP, the substrate was washed, dried and reweighted, and it was confirmed that from 6 g/l the initial weight of AP after washing left merely 2.769 g/l. As a result, weight loss of AP may be explained by the fact that the substrate was dissolved; in addition, we have to keep in mind the fact that AP consists of 15% soluble fiber [56]. According to feather biodegradation, the lowest biomass concentration was confirmed on this substrate (Figure 16); and strain BZ did not prefer feather biodegradation in comparison with other waste substrates. Figure 17 represents feathers before (a) and after (b) biodegradation.

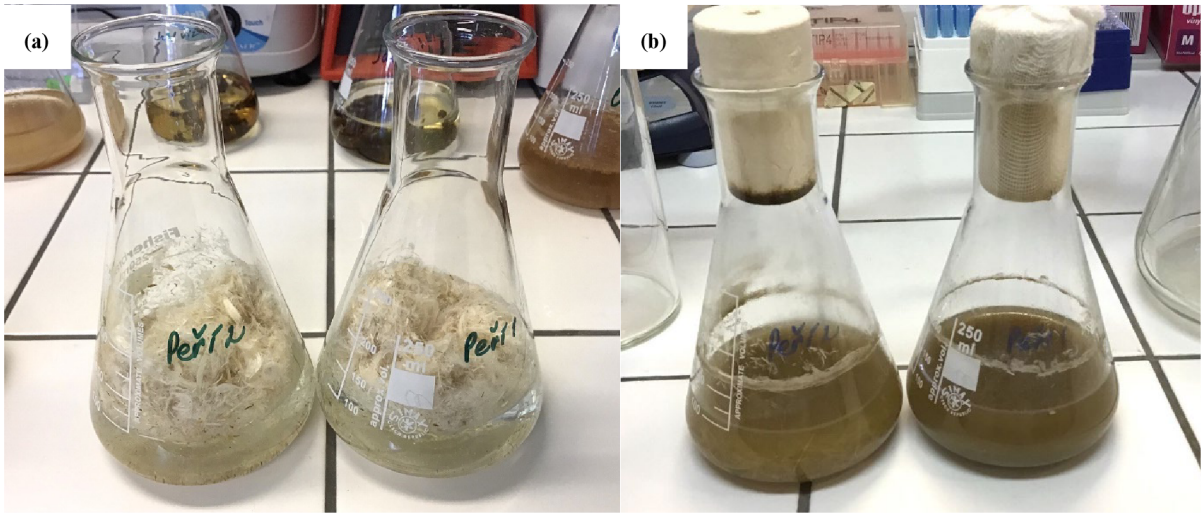

Figure 17: *Demonstration of feathers biodegradation before (a) and after (b) three days period of cultivation*

According to the proteolytic activity, protease is a group of enzymes catalyze the hydrolysis of proteins to peptides and oligopeptides to amino acids. Since proteins have a high molecular weight, it carries out the first enzymatic stage of protein degradation on the outside of the microbial cell. By specific transport systems, compounds with low molecular weight (*e.g.*, amino acids) and here the amino acids then deaminated. There are several substrates for determining protease activity. Among them belong gelatin, casein, azocazein, peptides and albumins [58]. As was mentioned earlier, in this study was used azocasein as a substrate. Figure 18 represents the proteolytic activity determination on washed and non-washed waste substrates.

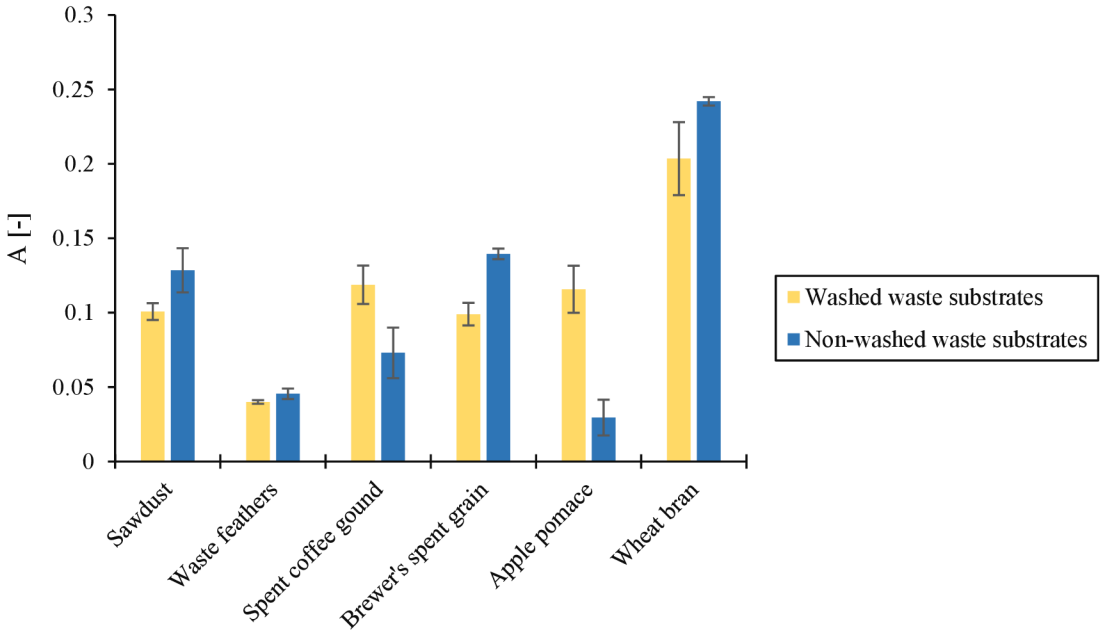

Figure 18: *The optimalization of the proteolytic activity determination on waste substrates*

The highest absorbance values were expected to be measured on waste feathers, although the results obtained from the experiment came out that the absorvance values were the lowest and did not change considerably neither on washed, nor non-washed substrate. The absorbance value on washed feathers is 0.040±0.001, while on non-washed is 0.045±0.004. Feather is a generally difficult degradable substrate, thus there are known merely several microorganisms capable of degrading it [56]. However, WB, BSG, and sawdust substrates were recommended as substrates, where the proteolytic activity may be measured.

According to the measurements of hydrolytic enzymes on AP, higher values of pectinases, xylanases, cellulases, and proteases might be observed on non-washed substrate (Table 11). For the better comparison, obtained values of mentioned enzymes were pulled down to the same time – 1 hour. Actually, Figure 19 represents the enzymatic activities of amylase, xylanase, pectinase and cellulase in g/l of glucose for 1 hour.

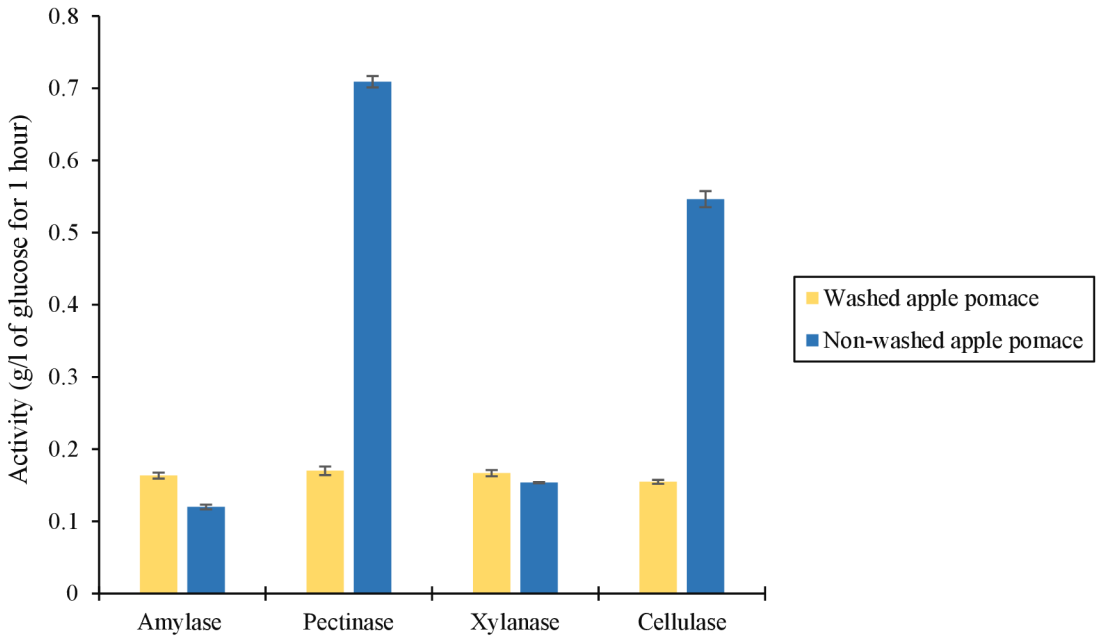

Figure 19: *The determination of the enzymatic activities of amylases, pectinases, xylanases and cellulases on apple pomace*

Upon the enzymatic values obtained comparing visually in Figure 19 on washed and non-washed AP, it might be concluded that the method of non-washing substrates is more efficient to produce a hydrolytic enzyme on this substrate. For instance, the value of pectinase on non-washed AP is 0.708±0.007 g/l of glucose for 1 hour, while on washed − 0.169±0.005 g/l of glucose for 1 hour. The same situation that may be observed was by using the cellulolytic activity. The enzymatic activities of amylase and xylanase on either washed substrate showed relatively the same values. Furthermore, a higher weight loss of AP was observed in the method, where the substrate was not defeated in the preliminary washing (Table 11), thus higher enzymatic values. It may be said that AP consists of some valuable compounds for the growth of *Brevibacillus borstelensis* BZ and its efficiency in producing valuable metabolites.

Differences in the enzymatic values of amylase, pectinase, xylanase, and cellulase might explain by several factors, including the proportion of the compounds present in the apple, the ripeness of the apple, and the AP drying method. Regarding the composition of AP, plant cell wall polymers consist of cellulose (44%), hemicellulose (24%), pectin (12%) and lignin (20%). All these compounds are defined mainly as insoluble fibers, occurring around 36.5% of biomass. The remaining pectin and hemicellulose are defined as soluble fiber (14.6%) [59]. This may be explained by the difference in activities of washed and non-washed AP. The second factor that could considerably affect the production of enzymes, specifically pectinases, is the drying method. In one study, it was shown that AP drying by drum drying or freeze drying showed no difference in the hemicellulose, cellulose, and lignin content, although pectin was significantly lower in drum drying than in freeze drying [60]. Therefore, for the future this factor

may be also be considered before pectinolytic activity measurement. Based on the obtained results from the production of hydrolytic enzymes on original sources and waste substrates, *Brevibacillus bortselensis* BZ may be a highly promising source for the production of thermostable xylanases due to its ability to grow on xylan and various waste substrates. As a result, we reached the conclusion to optimize pH and temperature to produce xylanase.

4.2. Optimization of temperature and pH to produce xylanase

Lignocellulose consists of lignin, hemicellulose, and cellulose. Hemicellulose is the second most abundant polysaccharide after cellulose and consists of β-1, 4 linked D-xylopyranosyl units linked with branches of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues. To decompose this component, there is a demand in the synergic action of several enzymes such as endoxylanases (endo-β-1,4-xylanase), β-xylosidases (xylan 1,4-β-xylosidase), and α- glucuronidases (α-glucosiduronase). Therefore, xylanase plays a significant role in the degradation of hemicellulose, therefore, it has many industrial applications, for example, bioconversion of lignocellulosic materials to fermentable substrates in biofuel industries [61; 62]. Therefore, there is a necessity to increase xylanase production remaining stable in industrial processes. As a result, in this experimental part, the hydrolytic activity of the crude xylanase cocktail produced by *Brevibacillus borstelensis* BZ was studied.

In the initial stage of this experimental part, the optimization of the medium for the maximum xylanase production was managed. Three media with different chemical compositions were tested. Including M1, SM and VB medium (Table 7). All mentioned media were supplemented with 2% xylan from beechwood, inoculated with the BZ strain and incubated at 50 °C for 3 and 7 days. Obtained supernatants were prepared for the measurement of xylanolytic enzymatic activity. Figure 20 represents the enzymatic activities of xylanases measured in three different media.

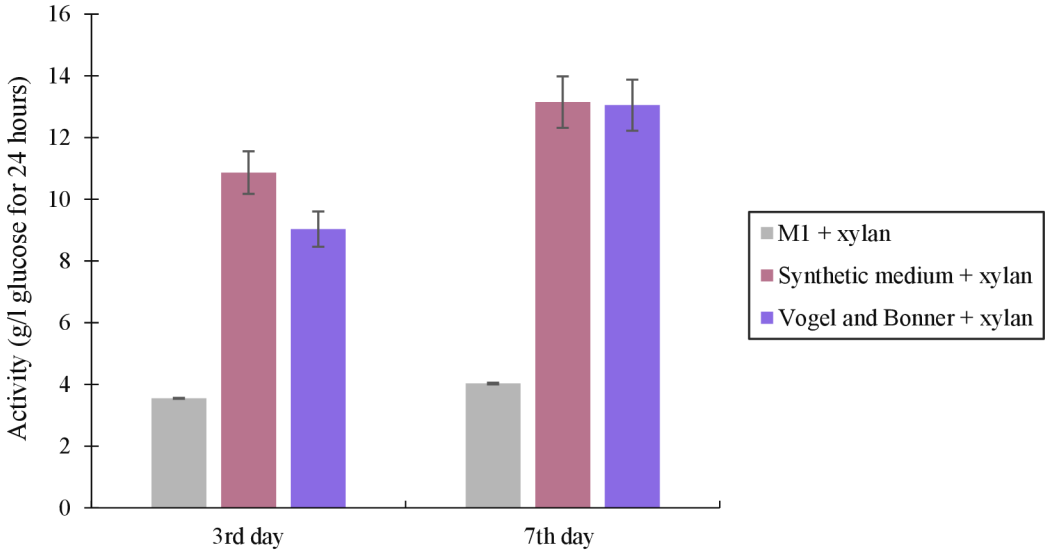

Figure 20: *The enzymatic activities of xylanases on three different mineral media*

According to Figure 20, it can be visually compared the highest enzymatic values, which were obtained in the SM and VB media. Taking into account the fact that the greatest growth of the strain was observed in M1 media with the demonstration of double growth of the bacterium, the activity of xylanases was much lower on M1 medium than in comparison with both other media. Furthermore, in Figure 20 is observed xylanase activity measured on M1 medium, what does not show any considerable difference with increasing time of the cultivation. In the case of SM and VB media, the difference in the production of xylanase is proved with the increasing time of cultivation is proved. The xylanase activities produced in SM medium after 3rd day of cultivation is 10.864±0.014 g/l of glucose for 24 hours, while after the 7th day of cultivation the value increased to 13.149±0.017 g/l. Consequently, the SM medium was chosen for further parametric optimization for the xylanase cocktail production.

4.2.1. Effect of growth-medium temperature on crude xylanase activity

The effect of temperature on crude xylanase activity was studied by growing *Brevibacillus borstelensis* BZ at different temperatures: 40 °C, 50 °C, 60 °C, at pH 7.05 for 3 and 7 days. Figure 21 represents results from the temperature of the growth medium that affected the production of xylanase activity. The BZ strain showed a maximum amount of xylanase activity at 50 °C on the 7th day of the cultivation and in the same period the activity decreased drastically when the temperature was decreased to 40 °C and increased to 60 °C. However, it is interesting to mention that on 3rd day of the cultivation the highest value of xylanase activity was observed exactly at 40 °C, while the activity measured at the following temperatures decreased smoothly.

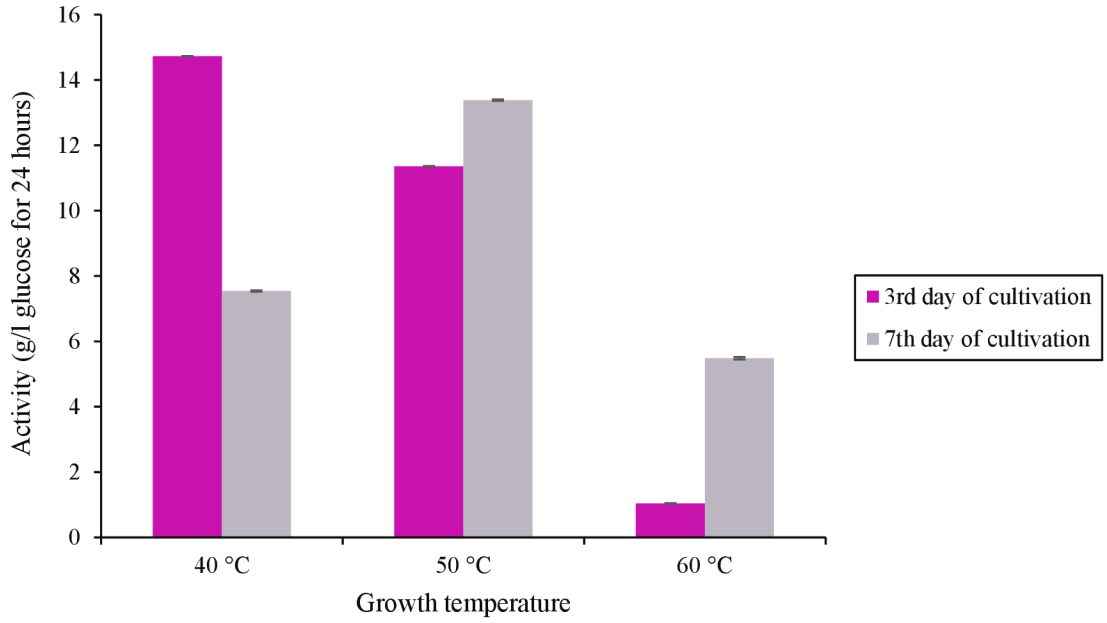

***Figure 21**: Effect of growth temperature on the production of xylanase*

4.2.2. Effect of growth-medium pH on crude xylanase activity

The next task was to study the effect of growth-medium pH on the crude xylanase activity. While discovering the impact of temperature on the production of xylanase, changes in pH were monitored simultaneously during the cultivation period. The initial pH was 7.05. In case the of 40 and 50 °C, gradual decrease in pH occurred to the final value 5.6, whereas the value of pH at 60 °C is increased in contrast to pH 8.17.

Considering the discovered fact that 50 °C was an optimal temperature for the BZ strain to produce xylanase, consequently, it was interesting to find out the effects of the growth medium pH on the crude xylanase activity at this temperature. The pH of the medium was adjusted using 0.5 M NaOH and 1 M H_2SO_4 in the pH range of 4.0 – 10.0. The cultivation period in the adjusted media lasted 3 and 7 days. After each period pH was measured. The results obtained from the measurement of pH changes during the 3rd and 7th day of cultivation at 50 °C can be observed in the following Table 12.

Table 12: *Change in pH values over 7 days during xylanase production at 50 °C*

A day of cultivation	pH						
0	10.04	9.06	8.09	7.05	6.13	5.03	3.95
3	7.68	8.23	7	5.71	6.03	5.46	5.21
7	4.97	4.91	5.46	5.66	5.62	5.56	5.72

From the results presented in Table 12, it could be observed that the pH of 6.0 – 10.0 gradually decrease to the pH 5.0 – 5.6 with an increase on the day of cultivation, while in the pH range of 4.0 – 5.0 there is shown increase to the value of pH 5.6 during the cultivation period.

The following Figure 22 represents the growth medium pH impacted the production of xylanase activity. The pH 6.0 and 7.0 adequately supported the xylanase activity with the maximum at pH 7.0. Without a doubt, the highest xylanase activities are introduced after the 7th day of cultivation. Regarding the results of xylanases activity obtained after the 3rd day, it might be said that the pH 6.0 – 8.0 supported the xylanase activity, furthermore, no significant increase or decrease in xylanase activities in pH region 7.0 and 8.0 has not been observed. However, on the 7th day has been shown splendid increase between the mentioned pH values.

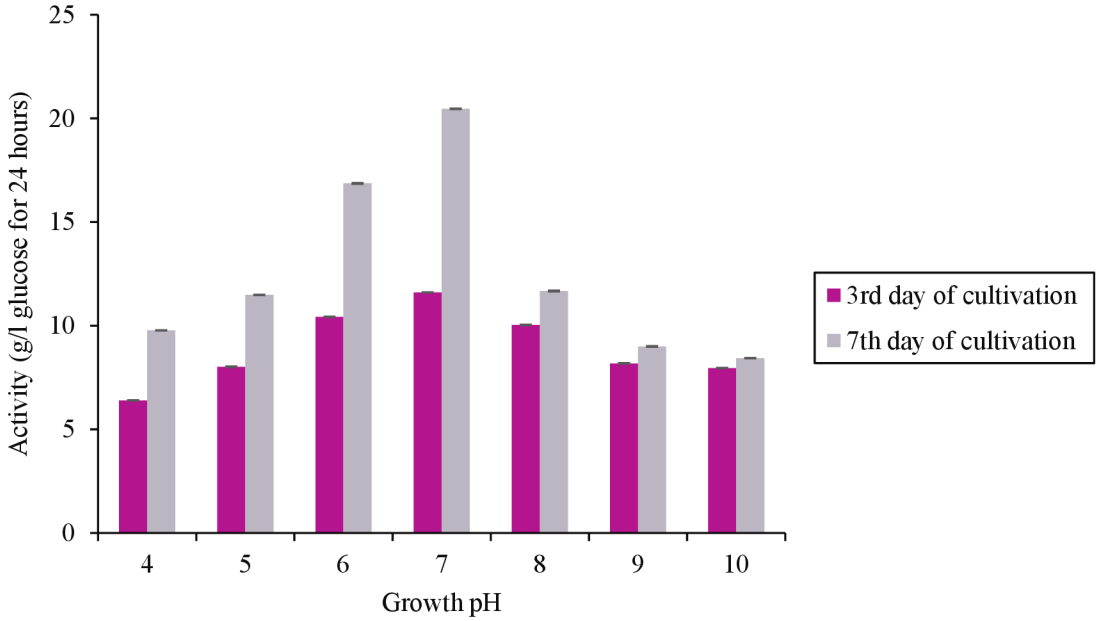

Figure 22: *Effect of growth pH on the production of xylanase*

The production of xylanase was impacted by pH whereas pH 7.0 supported the production of xylanase, where the value is 20.461±0.013 g/l of glucose released for 24 hours. The xylanase production decreased sharply when the pH was either decreased or increased around optima. At pH 6.0, the relative activity of xylanase was 83% and it decreased to 57% at pH 8.0, while pH 4.0, 5.0 and 9.0, 10.0 supported the production as well, although the activities of xylanase with the increase and decrease of pH was steadily decreasing. For example, at 4.0 relative xylanase activity was 47% and at pH 10.0 it decreased to 41%.

It has been shown that the pH of the growth medium strongly influences many enzymatic reactions by affecting the transport of a number of chemical productions and enzymes across the cell membrane. The results confirm that growth medium was an important factor affecting the crude xylanase activity in BZ. Furthermore, to the best knowledge of xylanase production by microorganisms, the optimum growth pH is between 5.5 and 9.5 [62]. Regarding temperature, higher growth temperatures are industrially desirable due to temperatures above 50 °C, which could lead to significantly reduced risks of mesophilic microbial contamination [63]. As a result, *Brevibacillus borstelensis* BZ in this study recommended itself as the strain capable to grow and perform its metabolic role at 50 °C and at pH 7.0. Such a thermostable enzyme has an obvious advantage as a catalyst in the lignocellulose conversion processes due to the better enzyme accessibility and cell wall disorganization achieved at high-temperature reaction conditions. High temperatures might allow for better solubility of reactants and products by lowering the viscosities, leading to faster hydrolysis. A longer active life under high temperature conditions would make these enzymes favourable for enhanced and efficient biomass conversion. Therefore, to be an effective enzyme, thermostability is the most important attribute of the enzyme utilization under extreme bioprocessing conditions [62; 63].

4.3. Biodegradation of biobased and fossil-based polymers

Synthetic polymeric materials have gained wide influence due to their high durability and stability, excellent mechanical, and thermal properties. They possess a unique chemical composition and physical forms. However, reclamation and incineration of synthetic polymers such as polystyrene (PS), polyurethane, polyethylene, etc. might release dangerous pollutants into the atmosphere, to the soil,

and groundwater. Therefore, it is necessary to develop efficient methods for plastic degradation. However, in recent years, low-density polyethylene (LD-PE) and high-density polyethylene (HD-PE) have proved their biodegradability, and their biodegradation mechanisms have become clearer [64]. Based on the research provided by Hadad D. et al. [11], where the biodegradation of LD-PE was demonstrated by the act of *Brevibacillus borstelensis* strain 707, we came to the idea of discovering the ability of *Brevibacillus borstelensis* strain BZ to decompose biobased and fossil-based polymers. The ability of the BZ strain to degrade polymers was proved by gravimetric measurements and by studying surface morphology of the biodegraded polymers by scanning electron microscope (SEM).

4.3.1. Optical density measurements

In this experimental part, the cultivation period on biobased and fossil-based polymers lasted for 24 days. There were prepared flasks which were incubated with strain BZ and a certain type of polymer; and, in addition, there were prepared control flasks containing merely polymers without inoculum. This approach was managed in order to discover how the medium itself may affect a certain type of polymer or, on the contrary, how the polymer might influence the medium. To observe possible changes during this cultivation period, optical density (OD_{630}) measurements were carried out on a regular basis every third day. The OD measurements of the control samples incubated with the polymer and samples incubated with the inoculum did not introduce turbidity during the cultivation period, with the exception of the sample that contained HD-PE films. Throughout the whole cultivation period, the OD measurement of the control samples was 0, while the sample contained HD-PE films the OD value was around the 0.100±0.130. However, the later studying of the morphology with SEM did not represent any changes in the physicochemical surface properties of polymers incubated without inoculum despite the presence of turbidity. In this experimental part, the presence of turbidity in the case of HD-PE may explain several factors. Presumably, the initial weight of the HD-PE film was too large for the volume of the flask, because the presence of many HD-PE films cased the formation of bubbles and films were sticking to the walls of the flask. The same occurrence was noticed with HD-PE film treated with strain, subsequently, such factors may also affect the possible formation of biofilm on the film's surface and its biodegradation. However, according to the results obtained from SEM observation, changes in the morphological structure were observed only on the PET film among all fossil-based polymers used in this work. Therefore, it is interesting to mention the bacterial growth on the PET film. The gradual growth of the strain on this polymer was confirmed. Figure 23 represents the growth curve of *Brevibacillus borstelensis* BZ on the PET film.

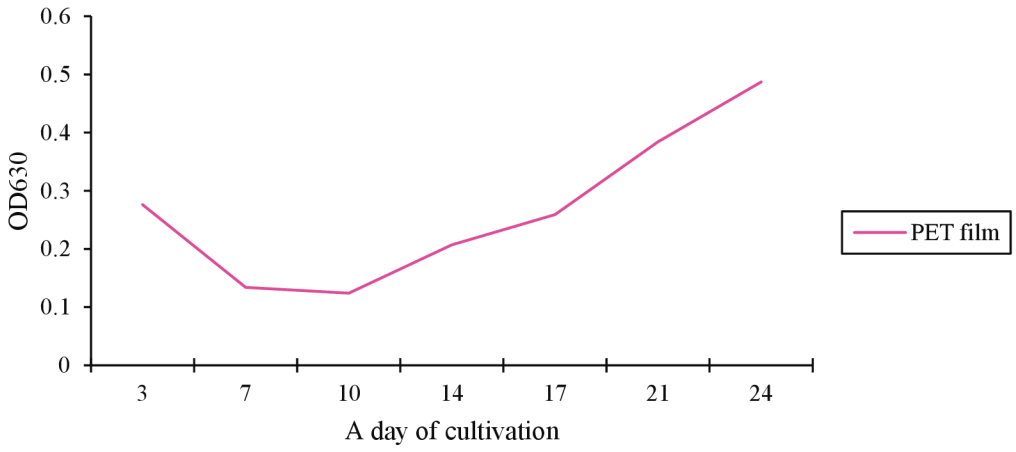

Figure 23: *The growth curve of Brevibacillus borstelensis BZ on the PET film*

In Figure 23 the OD curve of the growth of the BZ strain on PET film indicates that the OD value on the 3rd day was the highest in the range of 3 – 17 days of cultivation. The OD value decreased sharply after the 3rd day, but on the 10th day the gradual growth of the strain started to return, and the maximum growth is stated on the last day of cultivation – the 24th day.

In this experimental part, the biodegradation of biobased polymers was also tested. Among the used polymers belong amorphous fractions of PLA, semicrystalline PLA, and PHA granule. As was mentioned earlier, the OD measurements of samples containing granules incubated without the inoculum did not indicate any changes. In the granules treated with strain BZ occurred an increase in OD values throughout the cultivation period. Figure 24 represents the growth curve of *Brevibacillus borstelesnis* BZ on three biobased polymers. The growth of bacteria on the amorphous fraction of PLA was gradually increasing, while the highest OD value is stated on 17th day. In the same period, this sharp peak in growth is noticeable during the cultivation on PHA granules. Regarding the growth curve of strain on PHA granules, the OD measurements on the 3rd day introduced the highest value among other biobased polymers, although the growth after the 3rd day decreased and began returning on the 10th day of cultivation. After the occurrence of sharp peak on the 17th day, on the 21st day the peak decreases and remains relatively stable until the end of the cultivation period. The growth curve of strain on semicrystalline PLA was linearly increasing, and the highest value is on the last day of the cultivation.

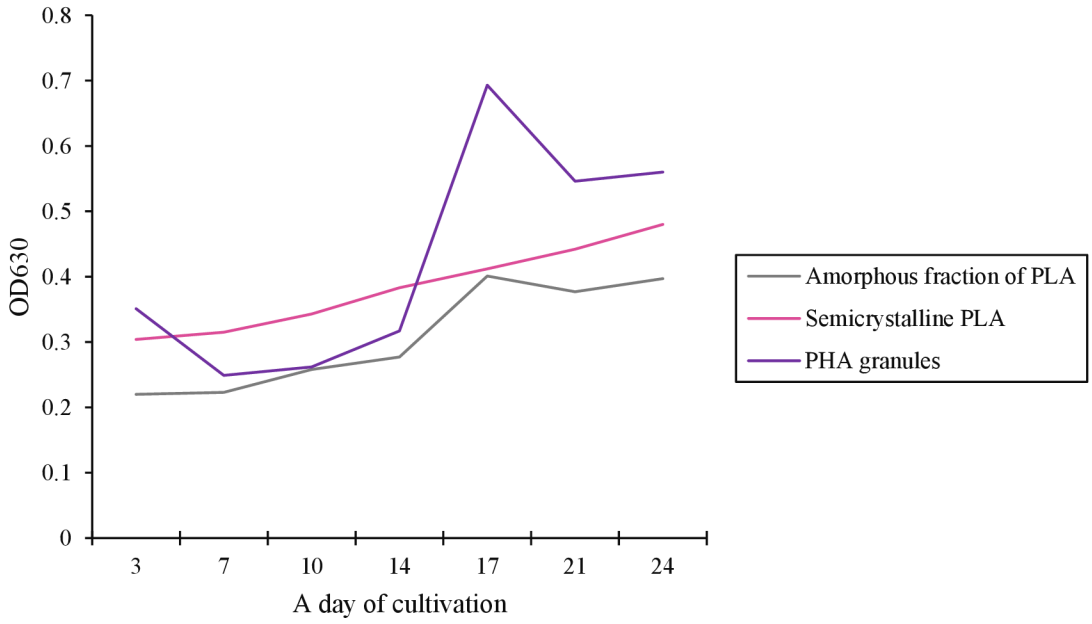

Figure 24: *The growth curve of Brevibacillus borstelensis BZ on biobased polymers*

4.3.2. The total weight loss of biobased and fossil-based polymers

The mechanism and the biodegradation process depend on the molecular weight of the polymer and/or its composition. Table 13 represents the total weight loss of biobased and fossil-based polymers treated and noon-treated with strain after 24 days of the cultivation, in percentage (%). According to these results, it might be said that the medium did not influence the polymers that were incubated without the inoculum, due to the non-significant weight loss of these polymers. Comparing these results with those ones, where polymers were treated with strain, the weight loss of polymers is indicated.

However, the lowest weight loss is noticed in treated HD-PE and PS films, PP granules. These findings are not unusual because polymers such as PE, PP, PS, PET demonstrate extremely low biodegradability. There are many factors that restrict the microbial attack against them: *e.g.*, minimally reactive functional groups in the backbone, chain mobility, crystallinity, surface hydrophobicity, and the lack of enzymes directly cleaving the C−C bonds [65; 66].

Table 13: *The total weight loss of biobased and fossil-based polymers treated and non-treated with strain after 24 days of cultivation, in percentage (%)*

PS film	LD-PE film	HD-PE film	PET film	Recycled PE granules	PP granules	Amorphous fraction PLA	Semicrystalline PLA granules	PHA granules
The total weight loss of treated polymers								
2.33	**13.33**	**2.09**	**9.02**	**4.29**	**1.33**	**19.22**	**6.12**	**92.27**
The total weight loss of non-treated polymers								
0.44	**0.29**	**0.43**	**0.16**	**0.72**	**0.24**	**0.17**	**0.03**	**0.24**

However, the ability to biodegrade some polymers may be enhanced by addition of UV. irradiation, as it was shown in the study of LD-PE biodegradation [11], where the treated LD-PE with UV irradiation showed the reduction in the weight loss of polymer from 11 to 30%. According to Table 13, the weight loss of LD-PE was approximately 13.22%, nevertheless no changes in the physiochemical surface properties of this polymer were confirmed. The same as with the PS solid film – no changes in surface morphology were confirmed with SEM, despite the weight loss of treated polymer. Moreover, it should be mentioned that PS possesses the most difficult structure among other tested polymers. Due to its high crystallinity, high molecular weight, and highly stable structure, the presence of a linear carbon backbone with the alternating backbone atoms attached to phenyl moieties, makes PS a non-biodegradable durable thermoplastic. While the possible pathway to metabolize the rest of the polymer is relatively undebatable. As a result of the aromatic structure of the PS complex with a C-C backbone, enzymes that could depolymerize this complex structure were not yet precisely defined. Therefore, it is not surprising that any changes were found in morphology; and no significant mass loss was noted [67]. According to recycled PE and PP granules, despite the small weight loss was noted, although they did not possess any changes in morphology of polymers.

Only PET, amorphous fractions of PLA, semicrystalline PLA, and PHA granules confirmed their total weight loss by changes in morphology. Meanwhile, the greatest weight loss was indicated in the PHA granules, where the value in percentage is around 92.27%. Sample containing PHA granules treated with strain BZ, during the cultivation period, granules were decomposed. The decomposition of the granules is supposed to have occurred under the influence of the bacterium, because in the control sample where the PHA granules were incubated without the inoculum, the granules remained intact until the end of the cultivation period. Figure 25 demonstrates non-treated PHA (a) and treated (b) granules with strain after 24 days of cultivation period. From these figures, we might conclude that granules were decomposed by strain

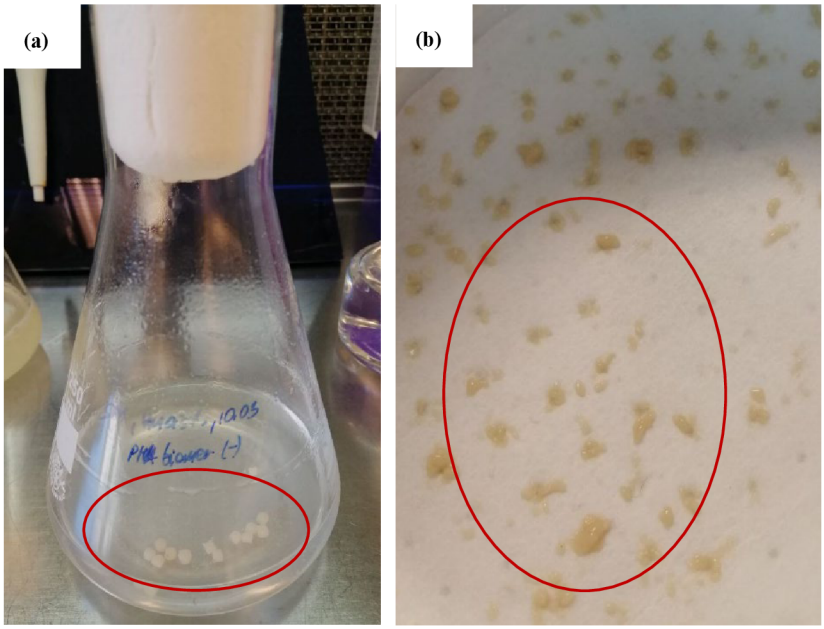

Figure 25: *Demonstration of PHA granules (a) the control sample containing PHA granules (highlighted); (b) shows the rest of granules after cultivation with strain BZ*

Amorphous fraction of PLA was a second favorable polymer for biodegradation by the strain BZ; and the second polymer demonstrated the highest weigh loss that was around 19.22%. Semicrystalline PLA and PET also confirmed weigh loss 6.12% and 9.02%, respectively.

4.3.3. Results of Scanning Electron Microscope

Scanning electron microscope (SEM) is considered one of the common methods for imaging the microstructure and morphology of materials. The main principle of SEM is an electron beam with low energy is radiated to the material and scans the surface of the sample; and several different interactions occur as the beam reaches and enters the material, which lead to the emission of photons from or near the sample of surface. The output of the analysis is an image of surface which introduces the ability to study the morphology of the selected object [68]. This experiment was carried out with all polymers that were treated with strain; and polymers contained in control samples; and the surfaces of polymers such as amorphous fractions of PLA, semicrystalline PLA, and PET were also studied before cultivation.

Above all, the changes in the physicochemical surface properties of HD-PE; LD-PE; PS films, recycled PE; and PP granules were not confirmed by SEM neither in the control sample containing film/granules, nor in polymers treated with strain BZ. Figure 26 represents SEM observation of changes in the surface of HD-PE film incubates with strain BZ after 24 days. Based on images we can concluded that there is no significant difference between treated and non-treated films.

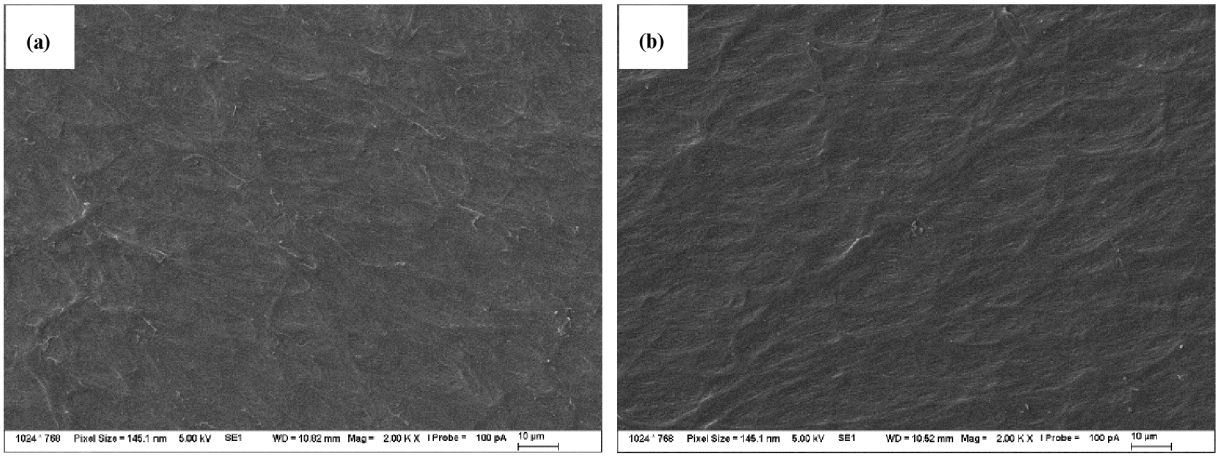

Figure 26: *SEM observation of the changes in the surface of HD-PE film incubated with strain BZ after 24 days. (a) HD-PE film treated with strain BZ. (b) HD-PE film incubated without inoculum*

In fact, it is worth to mention that among all treated petrochemical plastics, merely PET film contained changes in its structure. However, PET has a compact chemical structure that is chemically inactive and highly resistant to microbial attack. This high-molecular weight thermoplastic polymer made of dimethyl terephthalate monomers comprising terephthalic acid and ethylene glycol. In natural soil, microbial metabolism is the major degradation pathway responsible for diethyl terephthalate decomposition [65; 69]. The surface of PET film treated with strain BZ and the sample incubated without inoculum were subjected to SEM control. The observation of the PET surface after (treated and non-treated polymers) and before cultivation was provided several times, and every time the low morphological heterogeneity, roughness, and the presence of obvious pits and cavities were confirmed on the treated polymer after 24 days of the cultivation period. Additionally, it should be mentioned that while washing treated PET film on its surface was noticed a fixed thin bacterial film in contrast this was not observed in HD-PE film. Figure 27 demonstrates SEM observation of the changes in the surface of PET film incubated with strain BZ after 24 days.

SEM observations of the changes in the surface of PET film treated with strain BZ revealed a number of whitish cracks, grooves and pits; and in Figure 27 (c) is demonstrated the obvious roughness surface after 24 days, while (d) shows decomposition and penetration of bacterium through film that is highlighted. Importantly, there was no changes in the surface of non-treated PET film (b); and the surface is opaque and very smooth. To the better understanding how the medium may affect the polymer, there may be compared non-treated film with this one (a) which has been defeated to the observation before cultivation. Non-treated film (b) looks cleaner than in (a), where may be found the presence of some dirties. In this study, although the degradation of PET by strain BZ was observed using SEM, the degradation ability was not determined quantitively. Therefore, it would be intreating to attempt an analysis of the PET degradation mechanism by strain BZ; and systematically evaluate its degradation ability. However, presented finding provided with SEM might conclude the fact that *Brevibacillus borstelensis* BZ shows a significant interaction and effect on the PET surface, where the ability of strain to react on film was confirmed.

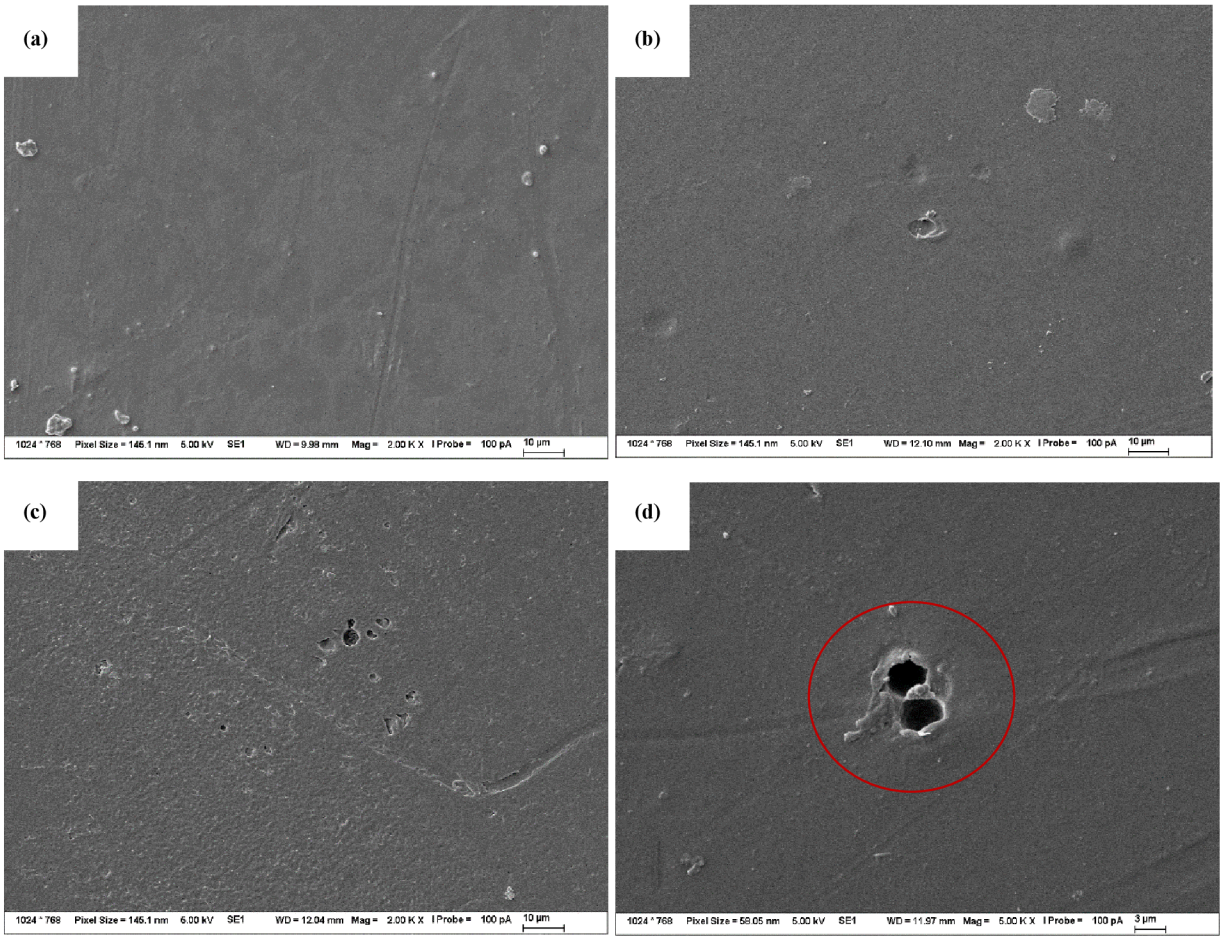

Figure 27: *SEM observation of the changes in the surface of PET film incubated with strain BZ after 24 days. (a) PET film before cultivation. (b) PET film incubated without inoculum. (c) and (d) PET film treated with strain BZ*

Regarding biobased polymers, *Brevibacillus borstelensis* BZ demonstrated its biodegradation capacity towards all selected polymers: PHA granules that were at least decomposed during the cultivation period, and changes in morphology structure amorphous fraction of PLA and semicrystalline PLA were also presented.

Macroscopic images of both amorphous fraction of PLA and semicrystalline PLA were taken using stereomicroscope. Figure 28 demonstrates images of amorphous fraction of PLA taken with stereomicroscope.

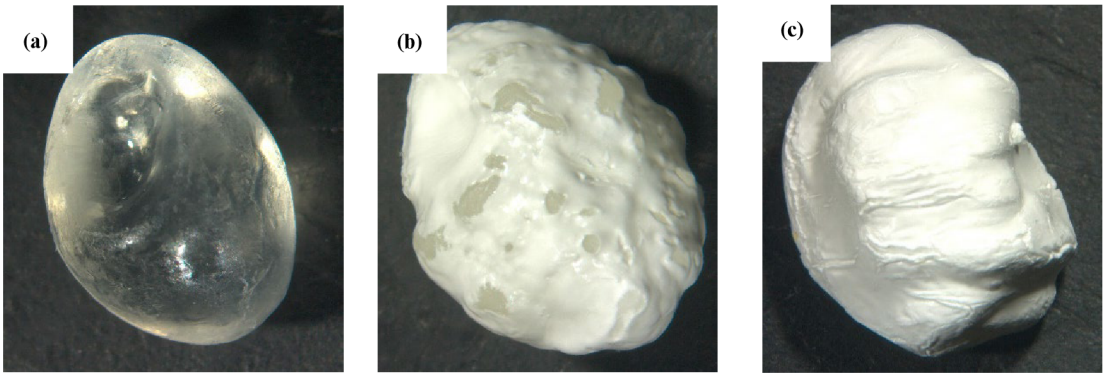

Figure 28: *Stereomicroscope observation of the changes in the surface of the amorphous fraction of PLA granule incubated with strain BZ after 24 days. (a) PLA granule before biodegradation. (b) PLA granule incubated without inoculum. (c) PLA granule treated with strain BZ*

Even without studying the detail morphological structure with SEM, all three images could be visually compared. The first image (a) introduces a smooth surface without cracks, cavities, or porousness. The only thing looking as a defect is a small dent on the left side, however, it is considered as a manufacturing flaw. While comparing this granule to the second one (b) that was incubated without inoculum, some changes in structure are present. The surface is rough and easily hilly. When comparing these both granules with the last one (c), which was inoculated with strain BZ, on the surface are already visible cavities, deeper dents, and voids. Moreover, two factors that unify the last two granules (b, c) and differ from the untreated granule (a), are different color and size of granule. The last two granules are swollen and whitened. The medium is supposed to influence the changes in color and size. According to the last image (c), it may be observed that the strain BZ interacted with the surface by producing morphological heterogeneity and disruptions in the structure. Therefore, it was interesting to observe prospective morphological changes in detail using SEM. Furthermore, Figure 29 represents changes physiochemical surface properties in amorphous fraction of PLA. These findings confirm the hypothesis mentioned above regarding the surface and the presence of cracks, dents, voids, etc.

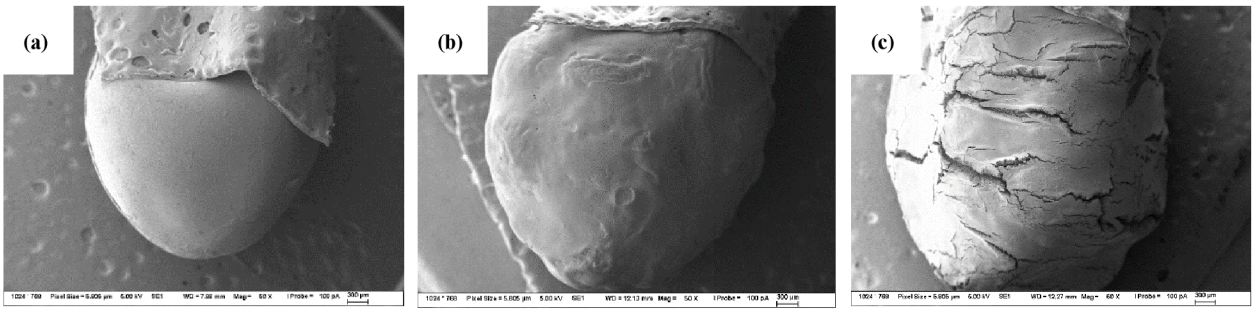

Figure 29: *SEM observation of the changes in the surface of the amorphous fraction of PLA granule incubates with strain BZ after 24 days. (a) PLA granule before biodegradation. (b) PLA granule incubated without inoculum. (c) PLA granule treated with strain BZ*

Importantly, granules before cultivation Figure 29 (a) and non-treated with strain (b) represent a smooth surface, although in case of (b) image there may be observed a little hilly surface that might provoked by mineral medium. Taking into consideration these results, where the deterioration of structure of PLA was stated and the presented changes in color and size (Figure 28 (b), (c)), it would be interesting to examine both granules, namely treated and non-treated with strain, at greater magnification. Figure 30 represents SEM observation of the changes in the surface of the amorphous fraction of PLA, where (a) is an image taken of PLA granule incubated without inoculum, while (b) shows an image taken of the PLA granule treated with strain.

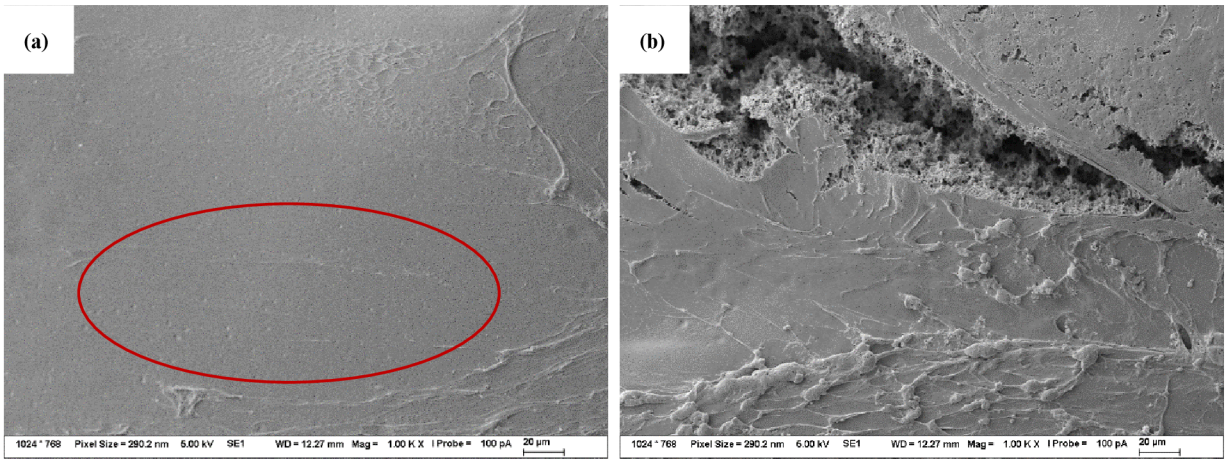

Figure 30: *SEM observation of the changes in the surface of the amorphous fraction PLA granule incubated with strain BZ after 24 days. (a) PLA granule incubated without inoculum. (b) PLA granule treated with strain BZ*

Comparing both images present in Figure 30, where the second one (b) treated with strain confirms clear deterioration of structure and the presence of huge pits, holes and voids. Consequently, the first image (a), even though the fact that in Figure 28 (b) there and Figure 29 (b) were some changes in structure is presented, although here it seems like no significant changes are proved; and merely what should be mentioned it is a mildly porous surface (highlighted).

The biodegradation capacity of *Brevibacillus borstelensis* BZ toward semicrystalline PLA was also proved. However, the total weight loss of this polymer was 6.12%, so that the value has not been as high as in the case of PHA granules and the amorphous fraction of PLA, but the gradual growth of strain was confirmed and, following Figure 31 and Figure 32 demonstrate changes in physicochemical surface properties are demonstrated. While Figure 31 shows of crystalline PLA taken with stereomicroscope.

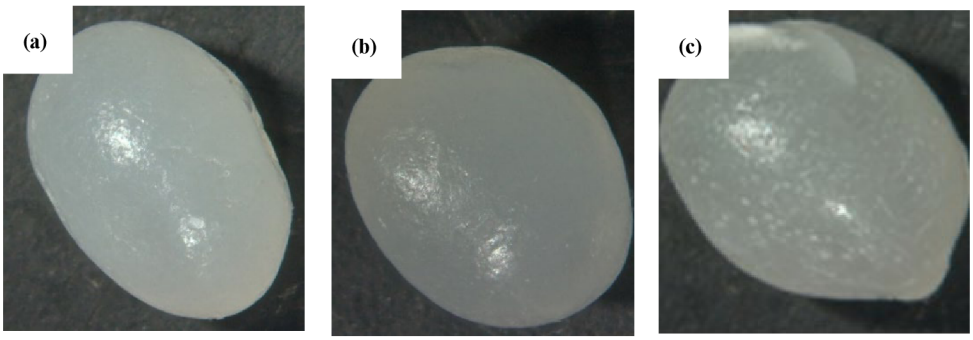

Figure 31: *Stereoscope observation of the changes in the surface of semicrystalline PLA granule incubated with strain BZ after 24 days. (a) PLA granule before biodegradation. (b) PLA granule incubated with strain. (c) PLA granule treated with strain BZ*

All three granules represent a smooth surface and the absence of visible changes in the structure. However, the same as with amorphous fraction of PLA, the semicrystalline PLA demonstrates a slight change in color of incubated granules (b, c); what is interesting that the middle one that was incubated without inoculum is the whitest one. Such a change of color is supposed to occur under the influence of the mineral medium. However, the presence of small white spots on the last granule (c) visually differs from those of the other ones. Probably, such spots may be affected by the erosion of strain on the surface, although this should be confirmed using at least SEM. Consequently, Figure 32 demonstrates SEM observation of the changes in the surface of semicrystalline PLA granules, namely, treated one (b) with strain and non-treated (a).

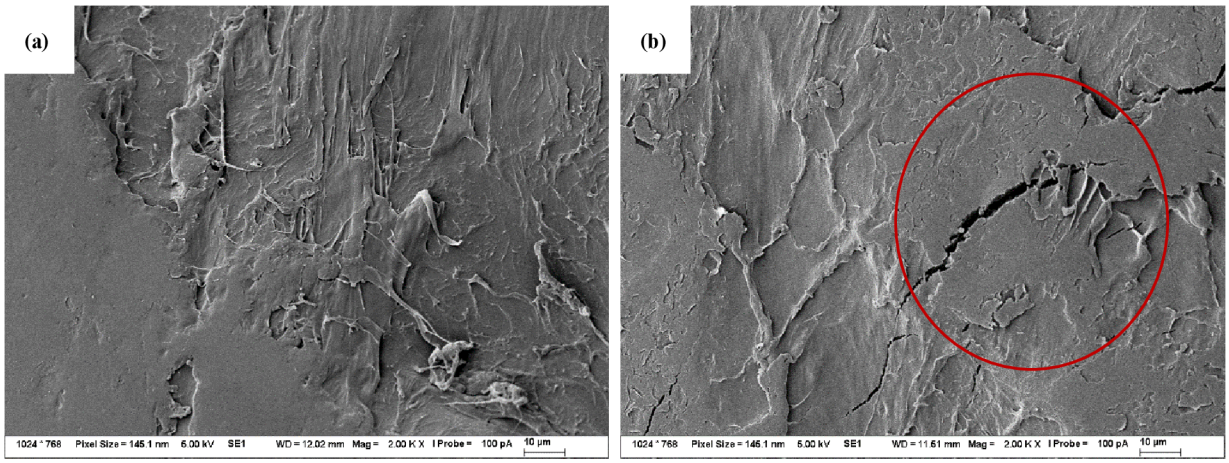

Figure 32: *SEM observation of the changes in the surface of semicrystalline PLA granule incubated with strain BZ after 24 days. (a) PLA granule incubated without inoculum. (b) PLA granule treated with strain BZ*

The SEM observation of the treated granule (b) confirms the presence of deep cavities and voids (highlighted with the red circle); and in general, the effort of strain to decompose the semicrystalline PLA polymer for 24 days. To compare these findings, the image (a), where polymer was incubated without inoculum, such significant changes were not proved.

5. CONCLUSION

The aim of the Master's thesis was to study the biotechnological potential of the thermophilic Gram-positive bacterium *Brevibacillus borstelensis* BZ. The first part of this work was focused on the production of thermostable hydrolytic enzymes on original sources and various waste substrates. Consequently, the enzyme with the highest enzymatic values was selected for further characterization, namely the identification of optimal pH and temperature. The next part aimed to study the biodegradation capacity of strain BZ toward selected biobased and fossil-based polymers.

- *Brevibacillus borstelesnsis* strain BZ is reputed to be a highly promising source of thermostable enzymes. The capacity of the strain to grow and produce such enzymes as xylanases, cellulases, amylases, pectinases, proteases, and lipases was confirmed on original sources and various waste substrates. According to the cultivation on original sources, it was discovered that the increase of microbial cultivation period results in the rising of enzymatic activities. Among all enzymes measured, xylanases and cellulases achieved the highest enzymatic values on the 7th day of cultivation. The xylanolytic value on the 7th day reaches the value of 7.612±0.474 g/l of glucose for 24 hours, while the cellulolytic was 3.385±0.008 g/l of glucose for 4 hours.

- The strain BZ was assayed for its ability to degrade various waste substrates in order to moderate thermostable enzymes. After the 3rd day of cultivation on washed and non-washed substrates, the total weight loss of the substrates was determined. Maximal biodegradation was obtained either on washed or non-washed WB, AP, and BSG. Regarding the production of hydrolytic enzymes, the same as it was with original sources, the strain prioritized the production of xylanases over other measured enzymes. The highest xylanolytic activities were obtained on WB and BCG. The xylanolytic values on these substrates were 7.011±0.060 g/l of glucose for 24 hours and 6.227±0.050 g/l of glucose for 24 hours, respectively.

- Subsequently, the identification of the optimal pH and temperature for the production of xylanase was carried out. The optimal pH and temperature for the crude xylanase cocktail were 7.0 and 50 °C, where xylanolytic activity in optimized parameters achieved the value 20.461±0.013 g/l of glucose for 24 hours.

- *Brevibacillus borstelensis* strain BZ demonstrated biodegradation capacity toward PET and all selected biobased polymers, such as the amorphous fraction of PLA, semicrystalline PLA, and PHA. The ability of the BZ strain to degrade polymers was proved by gravimetric measurements and by observing the surface morphology using SEM. After 24 days of the cultivation period, the PHA granules treated with strain BZ were completely degraded that, subsequently, the analysis with SEM was made impossible. The total weight loss of the PHA granules in a percentage was approximately 92.27%.

- SEM provided some insights about biodegradation of PET and PLA. The observation of the changes in the surface of the PET film treated with strain BZ confirmed an occurrence of roughness surface, a number of grooves, and pits; additionally, some images even demonstrated an utter penetration of the bacterium through the film. Importantly, the surface of the non-treated film was opaque and very smooth. Regarding the amorphous fractions of PLA, there was confirmed a clear deterioration of the structure was confirmed by the presence of huge pits and voids. The same changes occurred with semicrystalline PLA, where numerous cracks were demonstrated. Such findings may conclude the fact that bacterium interacted with the surface by producing morphological heterogeneity and influenced the degradation both biobased polymers.

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7. ATTACHMENTS

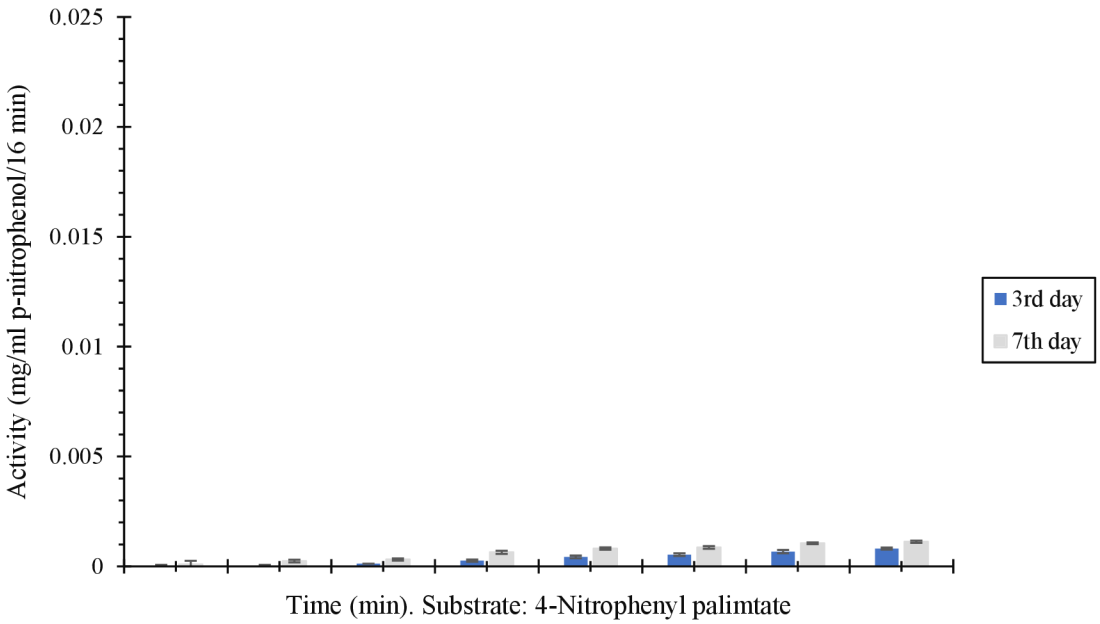

Figure attached 1: *The determination of enzymatic activity of lipase on substrate: 4-Nitrophenyl palmitate*

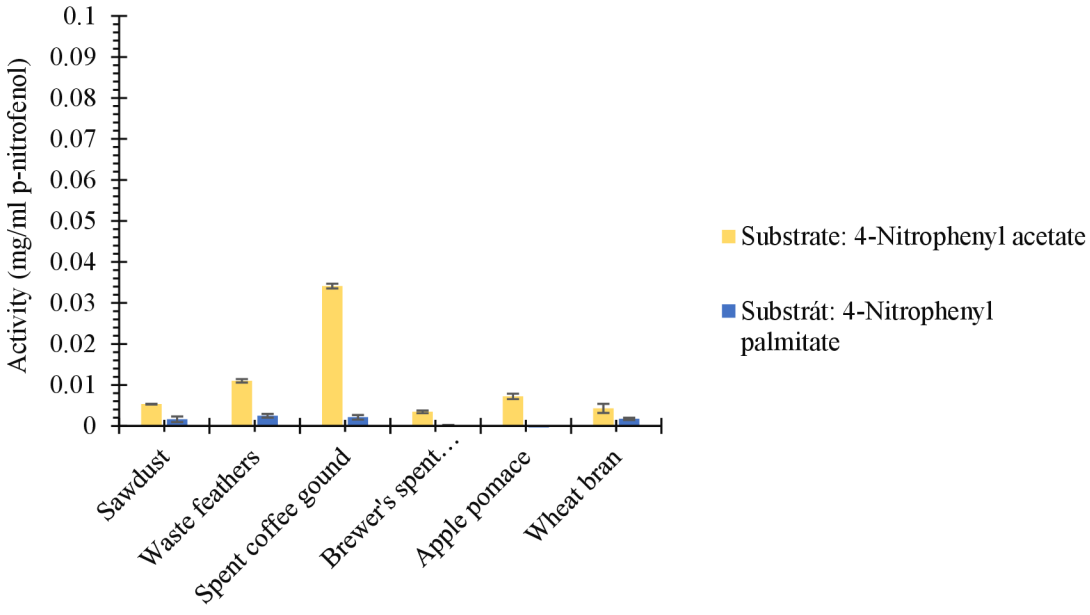

Figure attached 2: *The determination of enzymatic activity of lipase on washed waste substrates*

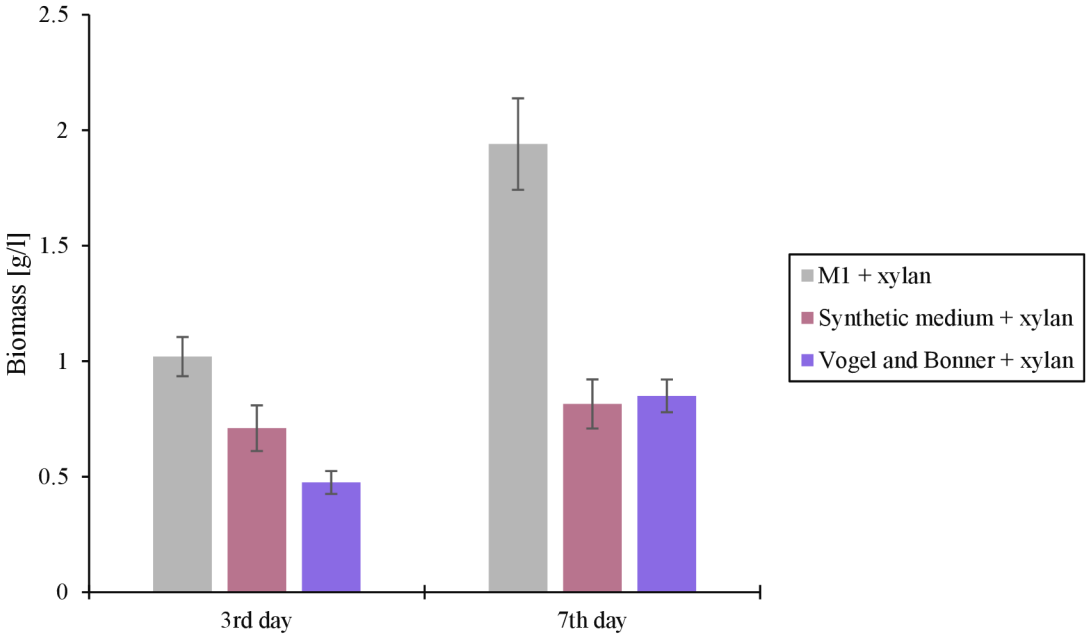

Figure attached 3: *Dependence of biomass on the cultivation time and type of mineral medium in Brevibacillus borstelensis BZ*

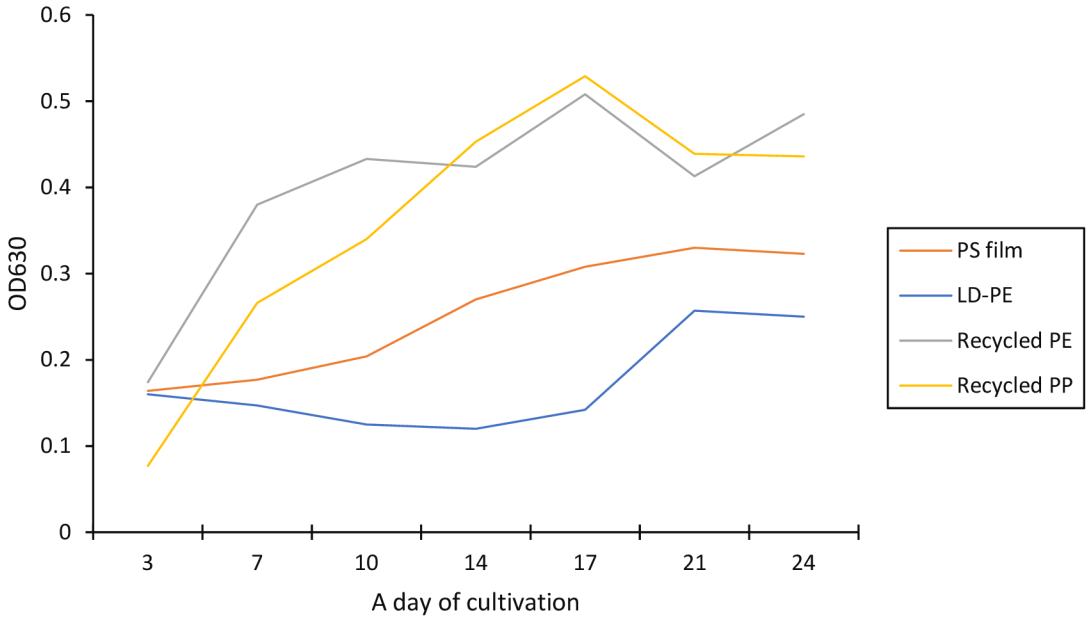

Figure attached 4: *The growth curve of Brevibacillus borstelensis on selected synthetic polymers*

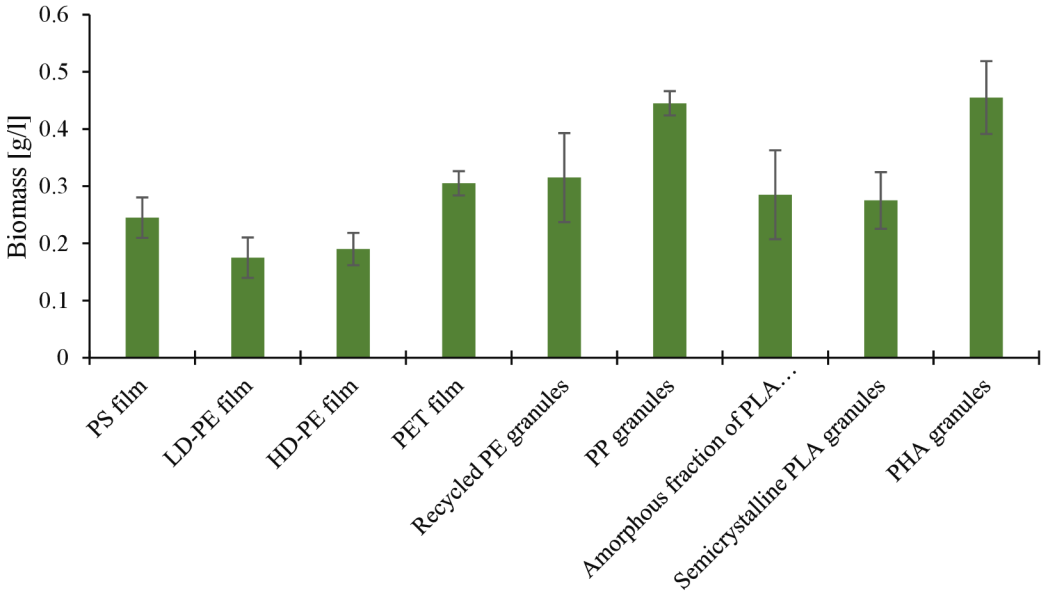

***Figure attached 5**: Dependence of biomass on biobased on fossil-based polymer in Brevibacillus borstelensis BZ*

8. LIST OF SYMBOLS AND ABBREVIATIONS

AP	apple pomace
BSG	brewer's spent grains
DNSA	3,5-dinitrosalicylic acid
HD-PE	high-density polyethylene
LD-PE	low-density polyethylene
OD	optical density
PET	polyethylene terephthalate
PHA	polyhydroxyalkanoates
PLA	polylactic acid
PP	polypropylene
PS	polystyrene
SCG	spent coffee grounds
SEM	scanning electron microscope
SM	synthetic medium
VB	Vogel and Bonner medium
WB	wheat bran