VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY
INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

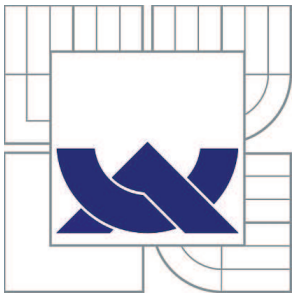
UTILIZATION OF LIGNOCELLULOSE MATERIALS FOR BIOTECHNOLOGICAL PRODUCTION OF POLYHYDROXYALKANOATES

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

AUTOR PRÁCE
AUTHOR

Bc. DAN KUČERA

BRNO 2015



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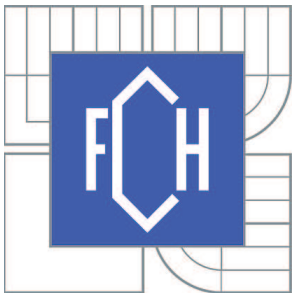

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MASTER'S THESIS

AUTOR PRÁCE — Bc. DAN KUČERA
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SUPERVISOR

BRNO 2015

Brno University of Technology
Faculty of Chemistry
Purkyňova 464/118, 61200 Brno 12

Master's thesis Assignment

Number of master's thesis:	**FCH-DIP0905/2014**	Academic year:	**2014/2015**
Institute:	Institute of Food Science and Biotechnology		
Student:	**Bc. Dan Kučera**		
Study programme:	Chemistry and Technology of Foodstuff (N2901)		
Study field:	Food Science and Biotechnology (2901T010)		
Head of thesis:	**Ing. Stanislav Obruča, Ph.D.**		
Supervisors:			

Title of master's thesis:

Utilization of lignocellulose materials for biotechnological production of polyhydroxyalkanoates

Master's thesis assignment:

1. Literature review on the topic
2. Testing of selected approaches of waste lignocellulose materials hydrolysis
3. Production of hydrolytic enzymes employing selected bacterial strains
4. Utilization of lignocellulose materials hydrolysates for biotechnological production of PHA

Deadline for master's thesis delivery: 11.5.2015

Master's thesis is necessary to deliver to a secreatry of institute in number of copies set by dean and in an electronic way to a head of master's thesis. This assignment is enclosure of master's thesis.

Bc. Dan Kučera Student	Ing. Stanislav Obruča, Ph.D. Head of thesis	prof. RNDr. Ivana Márová, CSc. Head of department

In Brno, 30.1.2015

prof. Ing. Martin Weiter, Ph.D.
Dean

Abstrakt

Tato diplomová práce se zabývala možnostmi utilizace lignocelulosového materiálu jako obnovitelného zdroje k produkci polyhydroxyalkanoátů (PHA) biotechnologickými metodami. Teoretická část práce se zaměřuje na charakterizaci rostlinné odpadní biomasy, její enzymatickou sacharifikaci a možnosti produkce a izolace hydrolytických enzymů. Dále se pak literární rešerše zabývá bakteriální produkcí PHA a možností využití lignocelulosové biomasy pro jejich produkci. V rámci experimentální části byly vybrané odpadní substráty hydrolyzovány chemickou a enzymatickou cestou. Jako odpadní substráty byly použity výlisky z jablek, hroznového vína a řepky olejné a kávová sedlina. Získané hydrolyzáty byly použity k produkci PHA bakteriálním kmenem *Burkholderia cepacia*. Nejslibnějším substrátem se jevily výlisky z jablek. Ukázalo se, že vybraný bakteriální kmen je schopen utilizovat odpadní substráty i bez předchozí úpravy. Supernatant po skončení kultivace jevil následující aktivity: proteasovou, lipasovou (0.47 $nmol \cdot mL^{-1} \cdot min^{-1}$), celulasovou pro CMC (6.05 $nmol \cdot mL^{-1} \cdot min^{-1}$) a filtrační papír (4.63 $nmol \cdot mL^{-1} \cdot min^{-1}$) a xylanasovou (1.71 $nmol \cdot mL^{-1} \cdot min^{-1}$). Tyto enzymy mohou představovat zajímavý vedlejší produkt výroby PHA z odpadních zemědělských materiálů. V rámci této práce byl také posouzen vliv délky kultivace a způsob hydrolýzy na výslednou produkci PHA a enzymatickou aktivitu průmyslově zajímavých enzymů.

Abstract

This diploma thesis describes the possibilities of utilization of lignocellulosic materials as renewable resources to produce polyhydroxyalkanoates (PHA) by biotechnological methods. The theoretical part focuses on the characterization of plant waste biomass, its enzymatic saccharification and the possibilities of production and isolation of hydrolytic enzymes. Furthermore, the literature review deals with bacterial PHA production and the possibility to utilize lignocellulosic biomass for their production. In the experimental part, waste substrates hydrolyzed by chemical and enzymatic pathways were selected. Apple, wine and rapeseed pomace and spent coffee grounds were used as waste substrates. *Burkholderia cepacia* was employed for PHA production from obtained hydrolysates. Apple pomace appears to be the most promising substrate. It turned out that the selected bacterial strain is capable to utilize waste substrates even without previous treatment. The supernatant exhibited following activities: protease, lipase (0.47 $nmol \cdot mL^{-1} \cdot min^{-1}$), cellulase for CMC (6.05 $nmol \cdot mL^{-1} \cdot min^{-1}$) and filter paper (4.63 $nmol \cdot mL^{-1} \cdot min^{-1}$) and xylanase (1.71 $nmol \cdot mL^{-1} \cdot min^{-1}$). These enzymes can represent an interesting byproduct PHA production from agricultural waste materials. The work assesses also the effect of time course of cultivation and method of hydrolysis on the resulting PHA production and enzymatic activity of industrially interesting enzymes.

Klíčová slova

Polyhydroxyalkanoáty, *Burkholderia cepacia*, lignocelulosová biomasa, hydrolýza

Keywords

Polyhydroxyalkanoates, *Burkholderia cepacia*, lignocellulosic biomass, hydrolysis

KUČERA, D. *Využití lignocelulózových materiálů k biotechnologické produkci polyhydroxyalkanoátů*. Brno: Vysoké učení technické v Brně, Fakulta chemická, 2015. 76 s. Vedoucí diplomové práce Ing. Stanislav Obruča, Ph.D..

Prohlášení

Prohlašuji, že jsem diplomovou práci vypracoval samostatně a že všechny použité literární zdroje jsem správně a úplně citoval. Diplomová práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího diplomové práce a děkana FCH VUT.

……
podpis studenta

Poděkování

Na tomto místě bych chtěl poděkovat vedoucímu mojí diplomové práce Ing. Stanislavu Obručovi, Ph.D. za odborné vedení a za ochotu a pomoc při práci v laboratoři.

Dále pak lidem na Ústavu biotechnologií University v Lundu, za to že mi umožnili poznat unikátní multikulturní pracovní prostředí, a zejména pak mým supervisorům Rose Aragäo Börner, PhD. a prof. Rajni Hatti Kaul.

V neposlední řadě bych také rád poděkoval své rodině a přátelům za podporu.

Práce byla podpořena z projektu „Centrum materiálového výzkumu na FCH VUT v Brně-udržitelnost a rozvoj“ financovaného z prostředků Ministerstva školství, mládeže a tělovýchovy ČR v rámci Národního programu udržitelnosti I.

CONTENT

1 INTRODUCTION

With the continued pace of world economic growth, sustainable socio-economic development will depend upon a secure supply of raw material inputs for agriculture, industry, energy, and related sectors [1]. Human society has been largely dependent on petrochemistry in the last few decades. On the other hand, non-renewable resources have been known to the mankind since the dawn of its existence, but their consumption began to grow exponentially with the onset of industrialization. Today's heavy reliance on non-renewable resources – especially fossil fuels – is increasingly constrained by economic, political, and environmental factors [1]. The gradual depletion of fossil resources, fluctuations in oil prices and, last but not least, environmental issues lead to efforts to replace the current non-renewable resources by renewable ones.

Lignocellulose is a renewable resource of organic nature; it includes agricultural and forestry residues, portions of municipal solid waste as well as herbaceous and woody crops. However, the change in raw materials requires a technology shift. Biotechnology, based on transformations of substances by microorganisms is one of the ways to use lignocellulosic biomass to produce fuels (e.g. bioethanol), biodegradable polymers and numerous chemicals in an environmentally sustainable fashion.

In recent years, great efforts have been made to produce suitable alternatives to replace synthetic plastics with bio-based polymers. Conventional synthetic polymers are a problem not only because of high reliance on fossil fuels but they also represent a major portion of non-degradable solid waste material. The accumulation of plastic waste has become a very important environmental issue.

Polyhydroxyalkanoates (PHAs) are generally considered as an alternative to petrochemical-based synthetic polymers. These microbial polyesters are synthesized and accumulated as intracellular granules by some microorganisms belonging to the *Bacteria* and *Archaea* domains of life. This phenomenon has been known since 1921, when microbiologist Lemoigne first isolated poly-3-hydroxybutyrate. Since then, a number of large studies have aimed at reducing production costs of polyhydroxyalkanoates for the purpose of competitiveness with the production of conventional polymers.

A major portion of final cost is represented by price of carbon substrate, but there are also other ways to streamline production of PHAs, besides using inexpensive fermentable raw materials such as lignocellulosic biomass. Simultaneous production of enzymes is one of the possibilities. Some PHA producers are employed to produce commercial enzymes. *Burkholderia cepacia* is an example of such a producer. *B. cepacia* lipases which are commercially available were successfully utilized for production of biodiesel. The combination of PHA production and enzyme production could have a huge impact on the cost of PHA production.

2 THEORETICAL PART

2.1 Lignocellulose Materials

In its broadest definition, biomass can be described as all material that was or is a part of a living organism. For renewable energy applications, however, the definition of biomass is usually limited to include only materials that are plant-derived such as agricultural residues (e.g., wheat straw, corn stover) by-products of industrial processes (e.g., sawdust, sugar cane bagasse, pulp residues, distillers grains), or dedicated energy crops (e.g., switchgrass, sorghum, Miscanthus, short-rotation woody crops) [2].

Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter [3]. Lignocellulose consists of complex structure of interwoven complex of biopolymers such as lignin, hemicellulose and cellulose (Figure 1). The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value [4].

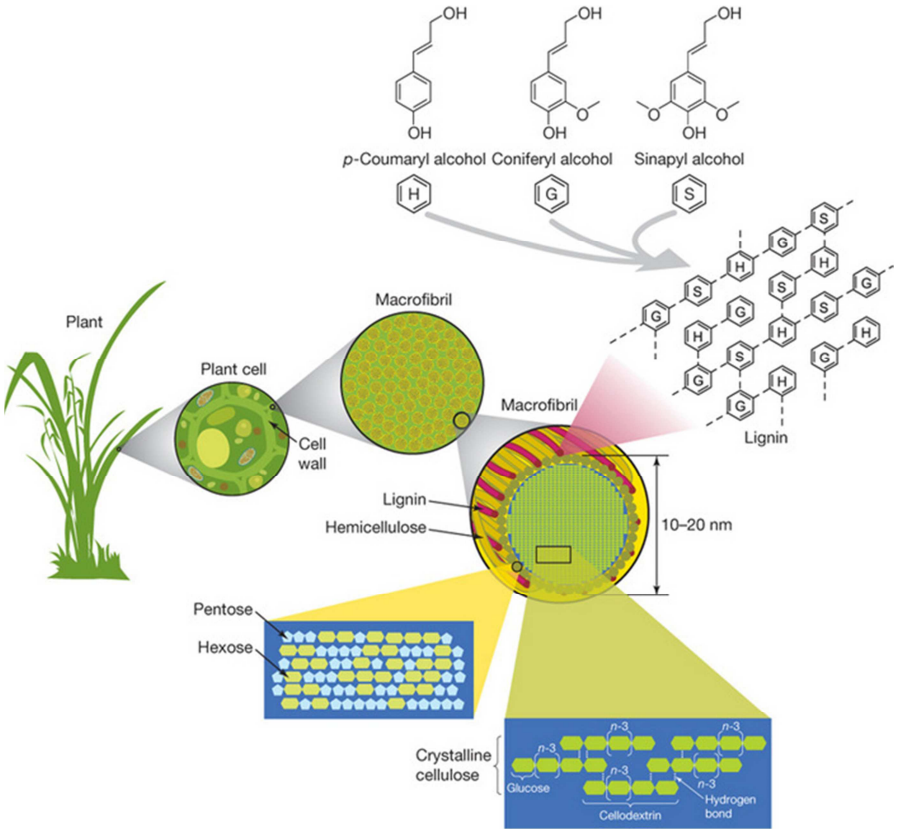


Figure 1 Structure of lignocellulose [5]

Large amounts of lignocellulosic "waste" are generated through forestry and agricultural practices, paper-pulp industries, timber industries, and many agro industries. But, they pose an environmental pollution problem. Sadly, much of the lignocellulose waste is often disposed by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon [6].

However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value added products including bio fuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients. Lignocellulytic enzymes also have significant potential applications in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile, laundry, pulp and paper and agriculture [3].

2.2 The Chemical Composition of Biomass

Plant biomass used as a feedstock for energy applications can take many forms, but all plant materials are comprised of the same basic building blocks, extractives, carbohydrates, lignin, protein, and ash. The relative concentrations of these basic constituents vary, within a plant as a function of tissue type and age, and between plants as a function of genetics and environment [7].

2.2.1 Extractives

The category of extractives in biomass includes all plant materials that are extracellular or not part of the three-dimensional cell wall structure. The definition is behavior-based, in that extractives are all materials that can be easily extracted using water or organic solvents such as ethanol or hexane. Since pretreatment and other conversion processing steps can break down cell-wall components and release soluble products, extractives contents are reported for feedstock samples only [2].

2.2.2 Lignin

Lignin is a constituent of the cell walls of almost all dry land plant cell walls. It is the second most abundant natural polymer in the world, surpassed only by cellulose. Of the polymers found in plant cell walls, lignin and proteins are the only ones that are not composed of carbohydrate monomers [8][9].

Lignin is unique in that it is the only large-scale biomass source of an aromatic functionality. It is composed of up to three different phenyl propane monomers, shown in Figure 2, depending on the species. Coniferyl alcohol occurs in all species and is the dominant monomer in conifers (softwoods). Deciduous (hardwood) species contain up to 40% syringyl alcohol units while grasses and agricultural crops may also contain coumaryl alcohol units.

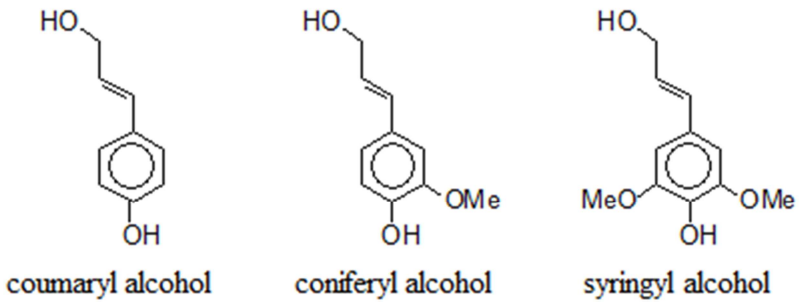


Figure 2 The three phenyl propane monomers in lignin [9]

An additional complexity of lignin is that there are many possible bonding patterns between individual units. Thus our knowledge of lignin chemical structure is less precise than our knowledge of other natural and synthetic polymers. Figure 3 shows a representative lignin fragment containing the most important bonding patterns [9].

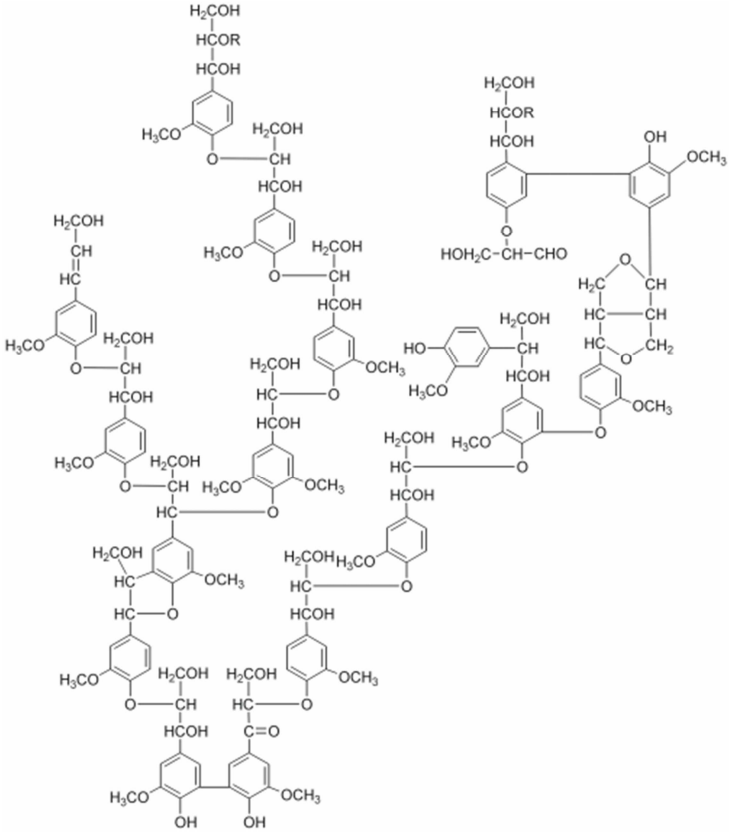


Figure 3 The structure of lignin [10]

2.2.3 Cellulose

Cellulose is the most plentiful of all of the naturally occurring organic compounds. It is a high molecular weight linear polysaccharide composed of two residues (β-1,4-linked glucose, known as cellobiose) in repeated units. Figure 4 shows the structure of cellulose. Complete hydrolysis of cellulose using strong mineral acids yields glucose. The basic building blocks of cellulose are chemically reactive, making it susceptible to chemical modifications. Cellulose has great economical importance; it is processed to produce papers, fibers, and chemical derivatives to yield substances used in the manufacturing of such items as plastics, photographic films, rayon, and so on. Other cellulose derivatives are used as adhesives, explosives, and thickening agents for foods [11][12].

Cellulose exists in nature in two principal types: the pectocelluloses such as flax, hemp, and ramie that contain more than 80% cellulose. As a basic component of plant cell walls, cellulose comprises at least 30% of all plant matters (90% of cotton and up to 50% of wood are cellulose). Because lignocellulosic materials are renewable and abundant, extensive research in recent years has been undertaken to convert cellulose, particularly agricultural residues, forest by-products, and waste cellulose, into food, chemicals, and fuels [12].

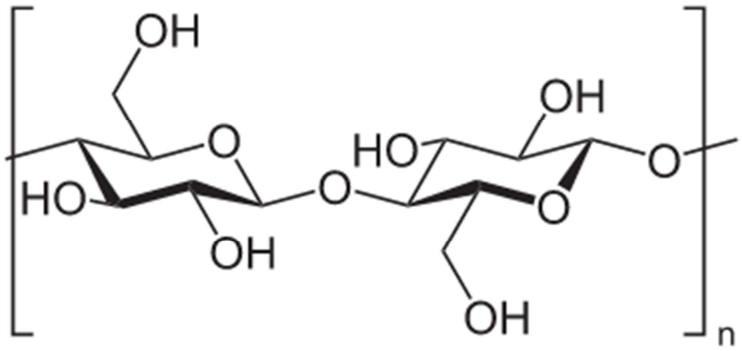


Figure 4 The structure of cellulose

2.2.4 Hemicellulose

Hemicellulose is a plant cell wall polysaccharide that is localized mostly in cell wall middle lamella. It is in close association with cellulose, lignin, and pectic materials. This close association contributes to the rigidity and flexibility of the plant cell wall. Hemicellulose is the third most abundant polymer in nature. In some plants, it comprises up to 40% of the total dry material. Unlike cellulose, hemicellulose exhibits a wide diversity in both structure and constitution. The degree of polymerization of hemicellulose is usually less than 200. The type and amount of hemicellulose vary widely, depending upon plant material, type of tissue, stage of growth, and so on. For these reasons, it is difficult to obtain a typical sugar composition of a typical hemicellulose [13].

Hemicelluloses are heteropolysaccharides that are composed of various hexoses (e.g., glucose, mannose, and galactose), pentoses (D-xylose and L-arabinose), uronic acids, acetic acid, and other minor sugars [14]. Thus, by definition, hemicelluloses are short branched-chain heteropolysaccharides of mixed hexosans and pentosans that are easily hydrolyzed. The most common form of hemicellulose is composed of xylose polymer (xylan) that is found in large quantity in annual plants and deciduous trees and in smaller amounts in conifers. Xylan of grasses and cereals is generally characterized by the presence of L-arabinose, which is linked as a single unit side chain to a D-xylose backbone. Hydrolysis of hemicellulose in annual plants, agricultural wastes, and hardwood yields glucose, D-xylose, L-arabinose, and other minor sugars [13].

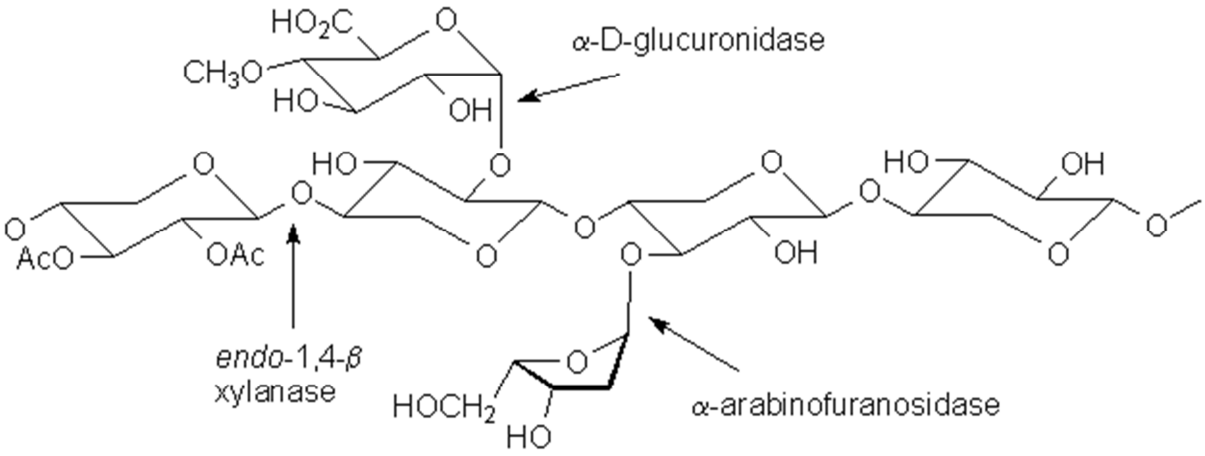


Figure 5 The structure of hemicelluloses [15]

Hemicellulose can be hydrolyzed to its sugar constituents either enzymatically or chemically. Chemical hydrolysis of hemicellulose is much easier to accomplish than the hydrolysis of cellulose due to the heterogeneous nature of hemicellulose, its chemical composition, and its low degree of polymerization. Most acids are good hydrolytic agents. The most common method of acid hydrolysis uses dilute mineral acids. Frequently during acid hydrolysis, xylose is degraded rapidly to furfural and condensation byproducts. Some degradation products are inhibitory to microorganisms [16][17].

2.2.5 Pectin

Pectin is the methylated ester of polygalacturonic acid. It is a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants. The structure of pectin is very difficult to determine because pectin can change during isolation from plants, storage, and processing of plant material. At present, pectin is thought to consist mainly of D-galacturonic acid (GalA) units, joined in chains by means of $\alpha(1,4)$ glycosidic linkage [18].

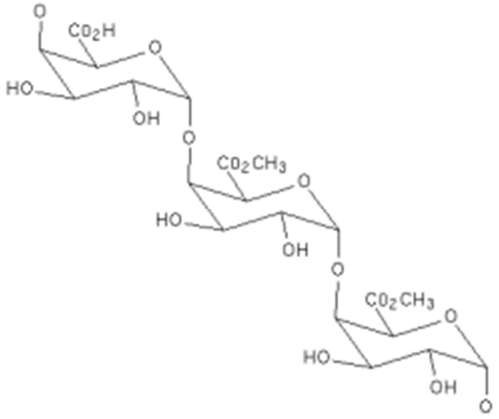


Figure 6 The structure of pectin [19]

These uronic acids have carboxyl groups, some of which are naturally present as methyl esters. This regular structure is interrupted, however, with L-rhamnopyranosyl units and with side chains containing other neutral sugars. The polymer chains may also be partially acetylated. The most important physical property of pectin is its ability to form spreadable gels. Gel formation results when the polymer chains interact over a portion of their length to form a three-dimensional network [20].

Pectin contains from a few hundred to about 1000 saccharide units in a chain-like configuration; this corresponds to average molecular weights from about 50,000 to 150,000 daltons [16].

2.3 Enzymes

Enzymes are protein macromolecules with the catalytic function. They are found in all living systems and it is assumed that even the simplest cell contains over 3000 enzymes which control the speed of almost all reactions in them. The catalytic activity of many enzymes depends on the presence of small non-protein molecules termed cofactors, although the precise role varies with the cofactor and the enzyme. Such an enzyme without its cofactor is

referred to as an apoenzyme; the complete, catalytically active enzyme is called a holoenzyme [21].

Cofactors may be:

- organic groups that are permanently bound to the enzyme (prosthetic groups)
- cations - positively charged metal ions (activators), which temporarily bind to the active site of the enzyme, giving an intense positive charge to the enzyme's protein
- organic molecules, usually vitamins or made from vitamins (coenzymes), which are not permanently bound to the enzyme molecule, but combine with the enzyme-substrate complex temporarily. Enzymes that use the same coenzyme are usually mechanistically similar [22].

The intra- and intermolecular bonds that hold proteins in their secondary and tertiary structures are disrupted by changes in temperature and pH. This affects shapes and so the catalytic activity of an enzyme is pH and temperature sensitive, but it may also be sensitive to ionic strength, detergents, redox potential, and more [22].

Enzymes are highly specific both in the reactions that they catalyze and in their choice of reactants, which are called substrates. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions. Side reactions leading to the wasteful formation of byproducts are rare in enzyme-catalyzed reactions, in contrast with uncatalyzed ones [21].

The speed of the enzyme-catalyzed reaction depends on the substrate concentration, amount of the enzyme, the physicochemical properties of the environment and the presence of effectors. Physico-chemical properties affecting the reaction rate are mainly temperature and pH. With the growth temperature increases the rate of enzyme-catalyzed reactions. There is a certain temperature at which an enzyme's catalytic activity is at its greatest. Above this temperature the enzyme structure begins to break down (denature). This leads to the formation of the temperature optimum, at which the enzymatic reaction is fastest. The catalytic activity of the enzyme is also dependent on pH. Each enzyme works within quite a small pH range. There is a pH at which its activity is greatest (the optimal pH). This is because changes in pH can make and break intra- and intermolecular bonds, changing the shape of the enzyme and, therefore, its effectiveness. The catalytic activity of enzymes also strongly influenced by a number of substances - the effectors [21][22].

If these substances increase the activity of the enzyme it is an activator (ions of certain metals, organic compounds), if the enzyme activity is reduced, they are described as inhibitors (heavy metal ions) [21].

2.3.1 Enzyme Classification

Enzymes can be divided into extracellular and intracellular according to localization, where the catalysation takes place. Intracellular enzymes are the common metabolic enzymes that are responsible for catalyzing all those processes we need in the cells. Extracellular enzymes are released outside of the cell to help break down various things, usually to make it easier for the cell to bring it into the cell for further processing [23].

Furthermore, microbial enzymes can be divided into four groups [23]:

- Constitutive enzymes are present in the cell under any external conditions.

- Inducible enzyme is expressed only under specific conditions.
- Repressible enzyme is an enzyme whose synthesis is inhibited (repressed) when the product that it synthesizes is present in high concentrations.
- Inducible enzymes, which are subject to repression.

Enzymes can be classified into six major groups according to the type of reaction they catalyze [23]:

1. Oxidoreductases: catalyze oxidations and reductions.
2. Transferases: catalyze the transfer of a group of atoms, such as form one molecule to another.
3. Hydrolases: catalyze hydrolysis reactions.
4. Lyases: catalyze the addition of two groups to a double bond or the removal of two groups from adjacent atoms to create a double bond.
5. Isomerases: catalyze isomerization reactions.
6. Ligases or synthetases: catalyze the joining of two molecules.

2.3.2 Lignocellulose-Degrading Enzymes

Lignocellulosic biomass consists of renewable materials and is attractive for the convertion into various different value added products [24]. The ability of microorganisms to degrade lignocellulose is a key factor in the recycling of wastes and in the carbon cycle in nature. Lignocellulose-degrading microorganisms are naturally present in soil and another environment [25].

To realize the effective transformation of lignocellulosic biomass, the key point is breaking the recalcitrant barriers of natural lignocellulose and liberating fermentable sugars. Pretreatment technologies, which reduce the lignin content or cellulose crystallinity and increase the specific surface area of cell wall polysaccharides or the pore size of the materials, could accelerate enzymatic hydrolysis. Biological pretreatment shows great potential because it is environmentally friendly and energy efficient [24].

Fungi seem to dominate over bacteria in lignocellulose degradation, based on their ability to decompose lignin and the fact that most soil cellulolytic activity is of fungal origin. Soil colonized by saprotrophic fungi exhibits significantly higher activity of laccase and manganese peroxidase (MnP) than soil with no apparent fungal colonization and the same is also true for several polysaccharide hydrolases [26].

Although lignocellulolytic fungi such as *Aspergillus, Penicillium, Schizophyllum, Trichoderma, Phanerochaete,* and *Sclerotium* can secrete industrial quantities of extracellular enzymes, bacterial enzyme production can be more cost-efficient. This is because they grow more rapidly, produce multi-enzyme complexes with increased functionality and higher specificity, and can tolerate larger and more diverse environmental stress. Lignocellulolytic bacteria could also potentially allow better separation of lignin from cellulose and thereby increase the value of both lignin, which is currently a waste product, and cellulose. The few bacterial species currently known to degrade cellulose and lignin are within *Pseudomonas, Cellulomonas, Streptomyces,* and other genera which are likely employing extracellular laccases and peroxidases [27].

2.3.2.1 Biodegradation of Cellulose

A typical efficient system for cellulose decomposition includes endo-type hydrolases (endo-1,4-b-glucanases, EC 3.2.1.4), exo-type hydrolases (cellobiohydrolases (CBH) EC 3.2.1.4) and 1,4-b-glucosidases (EC 3.2.1.21); the activities of these enzymes are synergistic. Typical cellulolytic systems of saprotrophic cellulosedegrading fungi (e.g., *Trichoderma* or saprotrophic basidiomycetes) consist of multiple enzymes representing all of these three groups. Cellobiohydrolases are produced with specificity for either the reducing or nonreducing ends of cellulose polymer. The cellulolytic systems of bacteria are different from those of fungi; they are often formed by a complex of enzymes associated to form so-called "cellulosomes" and are frequently associated with bacterial cell walls [26].

2.3.2.2 Biodegradation of Hemicellulose

The hemicellulases most typically assayed in soils are endo-1,4-b-xylanase (EC 3.2.1.8) and 1,4-b-xylosidase (EC 3.2.1.37), but several other enzymes are known to be produced by saprotrophic soil fungi and bacteria, including endomannanases, b-mannosidases, galactosidases, arabinosidases, and acetyl esterases, as well as debranching enzymes. The decomposition of hemicellulose is not limited by its physical structure, but rather by the diversity of its chemical composition and intramolecular bonding. Many cellulases and hemicellulases have recently been demonstrated to have broad substrate specificities and thus, it is not always simple to link a specific enzyme with a target substrate [26].

2.3.2.3 Biodegradation of Lignin

Ligninolytic systems consist of oxidases, peroxidases and hydrogen peroxide-producing enzymes. Ligninolytic oxidase – laccase – oxidizes its substrates using molecular oxygen, while the peroxidases need a supply of extracellular hydrogen peroxide, which is formed by the oxidation of different organic compounds [26].

Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (MnP; EC 1.11.1.13) are able to cleave the lignin polymer and perform lignin mineralization. Laccase (phenoloxidase, polyphenol oxidase, EC 1.10.3.2) can oxidise phenolic compounds, including lignin and its derivatives. However, although it might be involved in some lignin transformation pathways, the enzyme alone cannot cleave or mineralize lignin [26].

2.3.3 Isolation and Purification of Enzymes

Protein purification varies from simple one-step precipitation procedures to large scale validated production processes. Often more than one purification step is necessary to reach the desired purity. The key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required. Most purification schemes involve some form of chromatography. Different chromatography techniques with different selectivities can form powerful combinations for the purification of any biomolecule. Purification can be divided into three steps. The aim of the first step is to isolate, concentrate and stabilize target product. The second step comprises removing most of

impurities such as nucleic acids, carbohydrates, lipids etc. The third step aims to achieve a high purity by removing trace impurities [28].

For purification of the sample are used the following methods [28]:

- Fractional precipitation: precipitation is used to purify the sample, but also can lead to concentration. Precipitation techniques are often affected by temperature, pH and concentration of the sample.
- Centrifugation, filtration: the first step to remove solid particles
- Ultrafiltration: remove salts and concentration of the sample
- Gel filtration: removing salt and ballast proteins

The individual purification steps are based on the use of physical and chemical characteristics of biomacromolecules. The decisive factor in the case is the primary structure of proteins. The order of amino acids in the chain is determines the function and properties of the protein. Secondary and tertiary structure is responsible for their biological activity [28].

Methods of purification [28]:

- separation based on size: gel filtration, SDS-PAGE electrophoresis, ultrafiltration
- separation based on charge: ion-exchange chromatography, electrophoresis
- separation based on hydrophobic interactions: reversed-phase chromatography, hydrophobic interaction chromatography (HIC)
- separation based on biological activity: affinity chromatography
- separation based on the isoelectric point: chromatofocusing, isoelectric focusing

2.3.3.1 Precipitation

After cell lysis, the most often used second step in a protein purification procedure is some sort of a rapid, bulk precipitation step. This is commonly accomplished by altering the solvent conditions and taking advantage of the changes in solubility of your protein of interest relative to those of many of the other proteins and macromolecules in a cell extract [29].

Precipitation is performed using neutral salts, pH changes, organic solvents and temperature. Precipitation methods are divided into negative (precipitates impurity, the enzyme remains in the solution) and positive (precipitates enzyme and impurities remain in solution). Alternating negative and positive precipitation can be achieved high purity enzymes. The most commonly used salt for salting out of ammonium sulfate. Its advantage is the small effect of denaturation and high solubility, which depends only slightly on temperature [29].

Thermal denaturation is mainly used in the isolation of thermostable enzymes in the initial stages of the purification process. The solution is heated to the temperature at which the enzyme still does not denature. The solution is then rapidly cooled and precipitated ballast proteins are removed by centrifugation or filtration [30].

2.3.3.2 Membrane separation processes

Membrane processes cover a group of separation processes in which the characteristics of a membrane (porosity, selectivity, electric charge) are used to separate the components of a solution or a suspension. In these processes the feed stream is separated into two: the fraction that permeates through the membrane, called the permeate, and the fraction containing the

components that have not been transported through the membrane, usually called the retentate. The size of the components to be separated and the nature and magnitude of the driving force provide criteria for a classification of the membrane separation processes, as shown in the Table 1. It should be noted that the boundaries between some of the processes, such as reverse osmosis and ultrafiltration, are arbitrary [31].

Table 1 Classification of membrane separation processes [31]

Name of process	Driving force	Separation size range
Microfiltration	Pressure gradient	10 − 0.1 μm
Ultrafiltration	Pressure gradient	< 0.1 μm − 5 nm
Reverse osmosis (hyperfiltration)	Pressure gradient	< 5 nm
Electrodialysis	Electric field gradient	< 5 nm
Dialysis	Concentration gradient	< 5 nm

Of the processes listed in Table 1, reverse osmosis and ultrafiltration are the most widely used industrially. Membrane processes do not require heating, which makes the process suitable for the treatment of thermolabile products. In addition the relatively low capital and operating costs involved make membrane processes an appealing alternative to more conventional separation processes, particularly when dealing with dilute solutions [31].

2.3.3.2.1. Ultrafiltration

Ultrafiltration is a variety of membrane filtration in which forces like pressure or concentration gradients lead to a separation through a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained in the so-called retentate, while water and low molecular weight solutes pass through the membrane in the permeate. This separation process is used in industry and research for purifying and concentrating macromolecular (10^3 to 10^6 Da) solutions, especially protein solutions. Ultrafiltration is not fundamentally different from microfiltration. Both of these separate based on size exclusion or particle capture. It is fundamentally different from membrane gas separation, which separate based on different amounts of absorption and different rates of diffusion. Ultrafiltration membranes are defined by the molecular weight cut-off (MWCO) of the membrane used. Because the membrane passes molecules of the solvent (water) the volume of solution above the membrane decreases. The concentration of the molecules, which does not pass through the membrane, will increase. Ultrafiltration can therefore be used to remove the solvent and salt from the enzyme solution, the buffer exchange or concentration of enzyme solutions [28][32].

2.3.3.2.2. Dialysis

Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane. A sample and a buffer solution (called the dialysate, usually 200

to 500 times the volume of the sample) are placed on opposite sides of the membrane. Sample molecules that are larger than the membrane-pores are retained on the sample side of the membrane, but small molecules and buffer salts pass freely through the membrane, reducing the concentration of those molecules in the sample. Changing the dialysate buffer removes the small molecules that are no longer in the sample and allows more contaminants to diffuse into the dialysate. In this way, the concentration of small contaminants within the sample can be decreased to acceptable or negligible levels [34].

2.3.3.3 Chromatography

Chromatography involves a sample being dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible stationary phase. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase. According to the arrangement of the stationary phase can be distinguished by column chromatography - stationary phase is positioned in a column, or on the surface - paper chromatography and thin layer chromatography [33].

Among the methods most commonly used for the isolation of enzymes belong a gel filtration, an ion exchange chromatography and the affinity chromatography [33].

2.3.3.4 Electrophoresis

Electrophoresis is a method used to separate charged particles from one another based on differences in their migration speed. In the course of electrophoresis, two electrodes are immersed in two separate buffer chambers. The two chambers are not fully isolated from each other. Charged particles can migrate from one chamber to the other. By using an electric power supply, electric potential is generated between the two electrodes. Due to the electric potential, electrons move by a wire between the two electrodes. More specifically, electrons move from the anode to the cathode. Hence, the anode will be positively charged, while the cathode will be negatively charged. Different ions migrate at different speeds dictated by their sizes and by the number of charges they carry. As a result, different ions can be separated from each other by electrophoresis. SDS-PAGE is most commonly used electrophoresis method to separate proteins [35].

2.4 Poly(3-hydroxyalkanoates) (PHAs)

Polyhydroxyalkanoates (PHAs) are very promising biomaterials in nature, which recently arouse interest in professional and general public [36]. It is a very broad class of polyesters of various hydroxyalkanoates [37].

Just as a human or many other organisms, some bacteria thinks for bad times, but unlike humans do not accumulate triglycerides, but the above mentioned polyesters. These polyesters bacteria can to utilize as a source of energy and carbon, when they come bad times [36].

Bacteria are remarkably well equipped for the use and utilization of a wide range of organic and inorganic compounds, including various types of waste, even under very different conditions would vary depending on the microbial strain. Some bacteria are also able to accumulate in their cells an enormous amount of these polyesters. PHA they may constitute 70-80% of the dry cell weight [37].

PHAs are biodegradable polymers; it means that the polyester may be degraded by a number of microorganisms. In comparison with conventional synthetic polymers made from petroleum if the product made from PHAs is left freely in a suitable biotope will follow its natural and relatively rapid decomposition into organic substances without releasing any toxic components. The majority of the world's plastics are derived from non-biodegradable petroleum-based polymers. The persistence of these materials in the environment has had a profound impact far beyond their functional life in the form of pollution, litter and waste disposal problems [38][39].

Figure 7 shows a general principle of bioproduction PHA, which currently can be divided into two basic groups: production using microorganisms or transgenic plants [40].

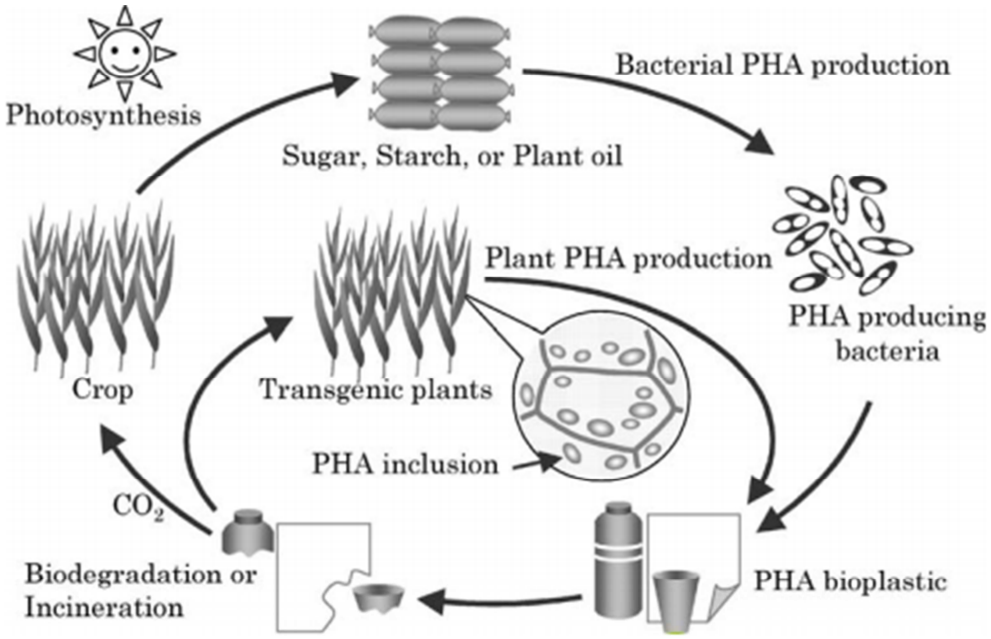


Figure 7 The general principle of bioproduction PHA [40]

Therefore, PHA due to its biodegradability and due to its material properties, which are discussed below, offers a wide range of application possibilities - e.g. the use for the manufacture of certain types of packaging materials, and also in the production of an optically active homopolymer R-3-hydroxybutyrate. This can be used, for example stereoselective organic synthesis, other application possibilities PHA is as a carrier of biologically active substances in human or veterinary medicine or for the preparation or encapsulated granular fertilizers with a gradual release in which the release rate of fertilizer respectively. Encapsulated ingredient will depend on the rate of biodegradation of the polymer, which can be largely controlled [41].

The biggest advantage of producing these materials is the independence of production on oil and other fossil fuels. Reserves of oil and other fossil fuels are not unlimited. Another advantage of the PHAs is mainly the fact that in the manufacture there is no accumulation of large amounts of waste. On the other side, various waste products of food industry and agriculture can be used as substrate for PHA production [42].

PHAs can be classified into two groups depending on the number of carbon atoms in the monomer units: shortchain-length (SCL) PHAs, which consist of 3–5 carbon atoms, and mediumchain-length (MCL) PHAs, which consist of 6–14 carbon atoms. The general structure of PHAs is shown in Figure 8 [42].

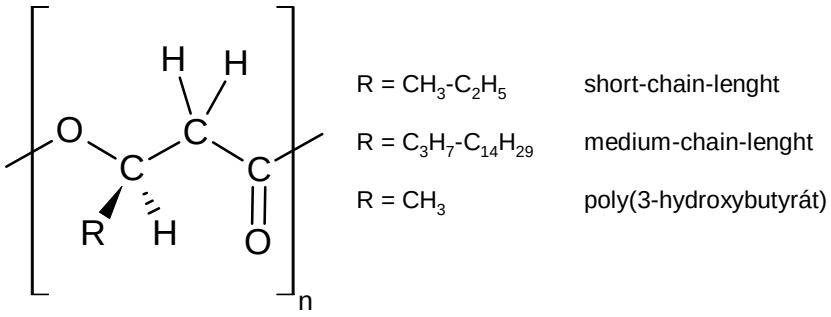


Figure 8 General structure of polyhydroxyalkanoates (PHAs). PHAs are polyesters with (R)-3-hydroxy acids as monomer unit.

The monomer units in these microbial polyesters are all in the R-configuration because of the stereospecificity of the biosynthetic enzymes. The molecular weights of PHAs range from 2×10^5 to 3×10^6, and the number of monomers (n) varies between 1,000 and 30,000. This depends on the specific PHA, the microorganism from which the PHA was obtained, and the growth conditions [42].

2.4.1 SCL PHA

SCL-PHA ("short chain length") is an abbreviation that refers to polymers containing hydroxy acid with a short chain length, ie. 3-5 carbon atoms [42].

PHB, the best-known of the SCL PHAs, not only appears in microorganisms as storage material but is also ubiquitous in nature. A fascinating development in recent years has been the discovery of the very wide distribution of PHB as a low molecular weight oligomer (120-200 monomers) in microorganisms, plants, and animals, including humans. Its ubiquitous expansion, biocompatibility, biodegradability and production from renewable sources makes it very interesting and promising material with a wide application potential [36][42].

2.4.2 MCL PHAs

MCL-PHA ("medium chain length") is an abbreviation that refers to polymers containing hydroxy acid with 6-14 carbon atoms [42].

The ability to accumulate different kind of alkanoate than SCL-PHA is due to the different substrate specificity PHA synthase. Accumulation of MCL-PHA is generally considered to be characteristic for bacteria of the genus Pseudomonas [43].

2.4.3 LCL PHAs

The LCL-PHA, which is uncommon and least studied, consists of monomers with more than 14 carbon atoms [44]. LCL-PHAs are mainly prepared in vitro enzyme-catalyzed polymerization or by chemical synthesis. There are not many bacterial strains which can produce LCL-PHA. This ability to produce copolymer with LCL-PHAs was observed in *Pseudomonas aeruginosa*. *P. aeruginosa* MTCC 7925 accumulated SCL-LCL-PHA co-polymer containing SCL 3HAs (3HB and 3HV) and LCL 3HAs of C16 (3HHD) and C18 (3HOD) units as the constituents [45].

2.4.4 Material Properties

Mechanical properties of the individual polyesters are strongly dependent on the monomer composition and the molecular weight of accumulated polymer. The monomer composition depends essentially on the used production organism wherein further depends on the carbon substrate and media supplementation with suitable precursors [42].

The PHB homopolymer is a completely stereoregular polyester, with all asymmetric carbon atoms in the (R)-configuration, and it is therefore highly crystalline. The high crystallinity (typically 55–80%) makes it relatively stiff and brittle. The glass transition temperature (T_g) of PHB lies between 5 and 9 °C, and the melting point (T_m) lies between 173 and 180 °C. PHB polymer decomposes at approximately 200 °C, which is close to its melting temperature. The mechanical properties of PHB including Young's modulus (3.5 GPa) and the tensile strength (40 MPa) are similar to those of polypropylene (Table 2). However, the elongation to break for PHB is about 3%, which is significantly lower than that of polypropylene (400%) [42].

Table 2 Comparison of properties of PHB and its copolymers with polypropylene [46]

Polymer	**Melting point (°C)**	**Tensile strength (MPa)**	**Young's modulus (GPa)**	**Elongation to break (%)**
P3HB	179	40	4	3
P(3HB/3HV) 90:10	150	25	1	20
P(4HB)	135	20	1	100
P(3HB/3HV) 80:20	53	104	149	1000
P(3HB/4HB) 90:10	159	24	-	242
P(3HB/4HB) 10:90	50	65	100	1080
PP	170	34	2	400

This led to efforts to produce copolymers of PHB with modified properties. There are several ways to achieve changes in the properties produced PHA; supplementation of growth medium by propionyl CoA precursors (salts of propionic or valeric acid) or 1,4-butanediol or a salt of 4-hydroxybutanoic acid. This allows changing the composition of the polymer for example for bacteria *Cupriavidus necator* H16 [42].

2.4.5 PHA Biosynthesis

PHA synthase (PhaC) is the key enzyme responsible for the polymerization of 3HA monomers. Owing to the stereospecificity of this enzyme, all the 3HA monomer units are in the R configuration. PHA synthases are differentiated based on their subunit composition, substrate specificity, and primary structure. The classes of PHA synthases are simplified in Table 3 [47].

Table 3 Classes of PHA synthase [47][48]

Class	Subunits	Representative species	Substrate
I	PhaC ~60 – 73 kDa	*Cupriavidus necator* *Sinorhizobium melioti* *Burkholderia sp.*	$3HA_{scl}$-CoA (~C3–C5) $4HA_{scl}$-CoA, $5HA_{scl}$-CoA,
II	PhaC ~60 – 65 kDa	*Pseudomonas aeruginosa* *P. putida*	$3HA_{mcl}$-CoA (~ ≥C5)
III	PhaC PhaE	*Allochromatium vinosum* *Thiocapsa pfennigii* *Synechocystis sp. PCC6803*	$3HA_{scl}$-CoA ($3HA_{mcl}$-CoA [~C6–C8], $4HA_{scl}$-CoA, $5HA_{scl}$-CoA)
IV	PhaC PhaR ~40 kDa ~22 kDa	*Bacillus megaterium* *Bacillus sp. INT005*	$3HA_{scl}$-CoA

PHA synthases of class I and II are represented by the PHA synthase of *Cupriavidus necator* and *Pseudomonas aeruginosa*, respectively. The class I synthase consists of a single subunit (PhaC) with substrate preference toward SCL-HA monomers. However, the PHA synthases of *Aeromonas caviae* and *Rhodospirullum rubrum* that belong to this class of enzymes do also incorporate 3HHx monomers. The class II synthases also have one subunit which actively polymerizes mcl-HA monomers. The classes III PHA synthases is represented by Allochromatium vinosum and have two different subunits (PhaC/PhaE) which generally prefer to utilize SCL-HA monomers. PHA synthase of *Bacillus megaterium* has two subunits (PhaC/PhaR) which represent class IV of the synthase enzyme and show substrate preference similar to class III PHA synthase. The nature of the enzyme together with the kind of carbon sources fed to the microorganism and its active metabolic pathways determine the type of PHA produced [47].

Bacteria used for the production of PHAs can be divided to two major groups based on the culture conditions required for PHA synthesis. First group of bacteria requires the limitation of an essential nutrient such as nitrogen, phosphorus, magnesium, or sulphur forthe synthesis of PHA from an excess carbon source. This group of bacteria includes e.g. *Cupriavidus necator* or *Burkholderia cepacia*. The second group of bacteria does not require nutrient limitation for PHA synthesis and the polymer is accumulated during growth phase. It is included *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, and recombinant *E. coli*. These characteristics are important to be considered while production of PHA [49].

Figure 9 shows different metabolic pathways leading to formation (R)-3-hydroxyacyl-CoA. PhaC subsequently catalyze the enantio-selective conversion of (R)-3-hydroxyacyl-CoA substrates to PHAs with the concomitant release of CoA [48].

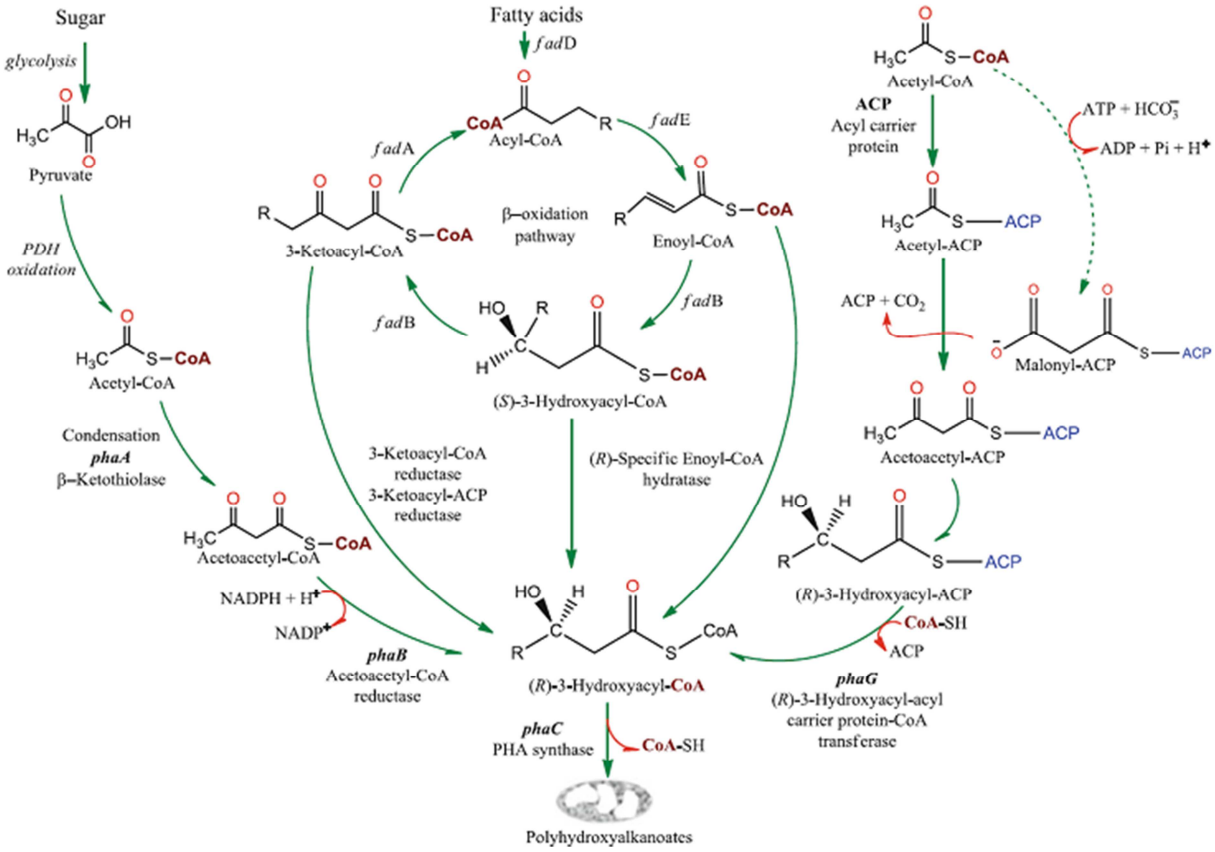


Figure 9 General metabolic pathways leading to PHA [50]

2.4.6 Biosynthesis of PHB

The route for polyhydroxybutyrate PHB) synthesis in *Cupriavidus necator* is one of the simplest and extensively studied PHA biosynthetic pathway. PHB) in *Cupriavidus necator* is synthesized from acetyl-coenzyme A by three successive steps, as shown in the left part Figure 9 [47].

The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by *phbA*). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into poly(3-hydroxybutyrate) by P(3HB) polymerase (encoded by *phbC*) [51].

An important regulatory factor in this pathway is the enzyme 3-ketothiolase. Its activity is influenced mainly by the concentration of acetyl-CoA and free HS-CoA in the cytoplasm, where the path is located. Another regulatory factor is the concentration of intracellular NAD(P)H and the ratio of $NAD(P)H/NAD(P)^+$ [42].

PHB) biosynthesis and accumulation occur in bacteria *C. necator* if a carbon source is provided in excess and if another nutrient, usually nitrogen or phosphate, is limiting. In this

condition occurs, a high ratio of NAD(P)H/NAD(P)$^{+}$. This will cause the inhibition isocitratedehydrogenase and the whole of the citrate cycle. Reduced possibility of utilization acetyl-CoA through the TCA cycle will lead to the use of acetyl-CoA for the synthesis of PHB). Bacteria *C. necator* is able to accumulate PHB from carbohydrate substrates, protein hydrolysates and fat as carbon source [51][52].

2.4.7 PHA Granule Formation

The high molecular weight PHA molecules are hydrophobic but they are synthesized in an aqueous environment under ambient conditions. Nuclear magnetic resonance (NMR) spectroscopy of various bacteria has clearly demonstrated that the polyester in the cells occurs in a metastable amorphous state and that it is stored as a elastomer [42]. Upon synthesis, the bacterial cell has to ensure that the PHA molecules do not crystallize. This is because the crystalline PHA is not recognized by the intracellular PHA depolymerase. In other words, the crystalline PHA will not serve as a carbon and energy storage compound for the bacterial cell. Therefore, the synthesized PHA molecules have to be maintained in an amorphous form. The amorphous PHA granules are approximately 200–500 nm in diameter and exist as membrane enclosed inclusions. [47] The granules are visible by electron microscopy (Figure 10) [48].

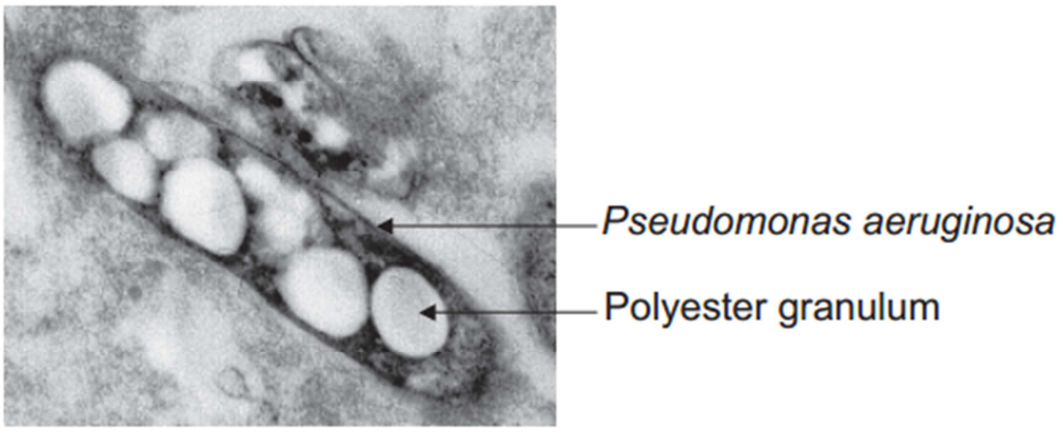


Figure 10 Electron microscopy image of Pseudomonas aeruginosa accumulating PHA granules [48]

The buoyant density of PHB granules is about 1.18–1.24 g·cm^{-3}, in contrast to MCL PHA granules, which have a buoyant density of approximately 1.05 g·cm^{-3}. The isolated granules consist of polyester, proteins, and lipids. The composition was first determined for PHB granules of *Bacillus megaterium*, which consist of 97.7 % polyester, 1.87 % proteins, and 0.46 % lipids or phospholipids [42].

Several different models of granule structure have been proposed. First indications that the native PHA granules in *Pseudomonas oleovorans* might be surrounded by a phospholipid monolayer in which the polymerase enzymes are embedded [53]. In contrast to this model, there is a model in which the hydrophobic PHA core is surrounded by two protein layers with a phospholipid layer in between [54]. In this model, the polymerase is supposed to be embedded only in the inner layer. Recently, determination of the thickness of the boundary

layer surrounding the polymer inclusions of different bacteria confirmed that a lipid monolayer is more likely than two protein layers with a lipid layer between them [42][55].

The membrane of PHA granules is thought to comprise a phospholipid monolayer and four major granule-associated proteins consisting of PhaC, intracellular PHA depolymerases (PhaZi), phasins (PhaP), and regulator protein of the phasin expression (PhaR) [47].

2.4.7.1 Phasins

PHA granules are coated with a layer of phospholipids and proteins. The predominant component of proteins are phasin which may form up to 5% of total proteins in bacteria accumulating PHA [42][47] .

Phasins are in general low molecular weight proteins; their molecular weight is in the range 14-28 kDa. Phasins are assumed to form a close protein layer at the surface of the granules, providing the interface between the hydrophilic cytoplasm and the hydrophobic core of the PHA inclusion. They are often considered a major control factor affecting the size and number of granules in the cytoplasm, but their function is not exactly known yet. PhaP adheres very tightly to native as well as to artificial PHB granules [55][56].

2.5 Use of lignocellulosic materials for PHA production

PHAs have a wide range of potential applications. At the present the cost of production is still the biggest problem. This is the reason for seeking alternative sources for PHA production. One such a cheap source could become the lignocellulosic materials.

To access the polymeric sugars in cellulose and hemicellulose, a pretreatment step is required. Lignocellulosic materials can be converted to fermentable sugars by hydrolysis, which subsequently can be fermented yielding variety of products. Cellulose can be hydrolyzed to yield glucose molecules, while hemicelluloses can be broken up i.e. hydrolyzed to yield molecules such as arabinose, mannose, glucose, galactose, xylose and uronic acid [57][58].

Such pretreatment is a costly and energy demanding process for the industry and includes mechanical, chemical and enzymatic steps. The high cost of biomass conversion is recognized as the major obstacle for its commercialization. The use of microorganisms that are able to degrade complex carbohydrates and simultaneously ferment the released sugars and produce interest compound would reduce the burden of the pretreatment processes. Moreover, enzymes produced from such microorganism can be purified and used as catalyst for the final hydrolytic step of a pretreatment to release sugars [58].

The main part of the experimental part of this thesis is focused on the study of bacteria, which is able to produce PHA, but even it can degrade lignocellulose. Bacteria would therefore not require significant pretreatment of the substrate and the production would not be cost-burdened.

2.5.1 Hydrolysis of lignocellulosic materials

The hydrolysis of lignocellulose waste by chemical or enzymatic treatments or combination of both releases several degradation products that prove being harmful to microbial fermentation. For example, acid hydrolysis of lignocellulose materials produces several inhibitory compounds that inhibit cells, affect specific growth rates and thus considerably decrease the yield of the biotechnological process. Based on origin, inhibitors are usually divided into three major groups, i. weak acids, ii. furan derivatives, iii. phenolic compounds. [59]

Hydrolysis of lignocellulose may also arise volatile fatty acids (VFA), such as acetic acid, formic and levulinic acid. VFA can be further transformed to PHA or can be used for biohydrogenation of fatty acids into alcohols. VFA can serve as precursors PHV and as a result can be obtained polyhydroxyalkanoate with improved material properties due to formation of copolymers [60].

Hydrolysis of lignocellulosic feedstock might also release inorganic ions (K^+, Na^+,Cl^-) that originate from the raw material and chemicals added during pre-treatment and possibly from process equipment. [59]

2.5.2 PHB production from agricultural waste

Since PHAs are intracellular products and; thus, the total PHAs yields are dependent on cell dry weight (CDW). Therefore, reaching high cell density is the first condition of reasonable process of PHAs production. Fed-batch cultivation strategy, which is often in use to achieve high cell densities, can be in case of utilization of lignocellulose hydrolysates complicated by phenomenon called carbon catabolite repression (CCR). By this is meant that in presence of sugar mixtures, a preferential consumption of one of the sugars (usually glucose or other hexoses) is observed while the other sugars (usually pentoses) remain unutilized in the medium. Generally, the rate of feeding during fed-batch cultivation should be controlled to overcome accumulation of slowly assimilated sugars in medium. [59]

Very promising waste substrate for PHAs production is spent coffee grounds. Coffee is one of the world´s most popular beverages and has grown steadily. During the preparation of coffee beverage or the manufacturing of instant coffee solid residues known as spent coffee grounds (SCG) are formed. Spent coffee grounds can be considered as very promising substrate for PHAs production. At first, SCG contain approx. 15 % of oil, which can be simply extracted and converted into PHB by *Cupriavidus necator* [61][62]. The residual solids after oil extraction contain significant portion of hemicelluloses and cellulose. Therefore, they were hydrolyzed (chemical hydrolysis by diluted acid was followed by cellulase treatment) and converted into PHAs employing *Burkholderia cepacia* [63].

Pan et al. employed *Burkholderia cepacia* for PHAs production from maple hemicellulosic hydrolysate obtained by diluted acid treatment as a renewable feedstock. To increase the fermentability of wood hydrolysate, several detoxification methods were tested. Fed-batch fermentation exhibited maximum polyhydroxybutyrate (PHB) production after 96 h - 8.72 g /l broth and 51.4% of dry cell weight [64].

Bagasse is the fibrous matter that remains after sugarcane or sorghum stalks are crushed to extract their juice. Since bagasse is highly abundant and available waste material, there were

several attempts to produce PHAs from acid sugarcane bagasse hydrolysate. Silva et al. employed *Burkholderia cepacia* and *Burkholderia sacchari*. To improve fermentability of the hydrolysate, three step detoxification processes was applied. After the treatment, the highest biomass and PHAs yields were observed in *B. sacchari* 4.4 and 2.7 g/l, respectively [65].

Saraphirom et al. attempted produce PHAs from hydrogenic effluent of sweet sorghum syrup by Cupriavidus sp. KKU38. Bacteria utilized soluble organic compounds in the effluent to produce PHAs with a maximum PHA content of 71.42% and PHA yield of 0.10 g/g COD consumed [66]. Predominant hydrogen producers were *Clostridium sp.* in hydrogen fermentation process [67]. Biological hydrogen (H_2) and polyhydroxybutyrate production from pea-shells biowaste as feed were reported by Patel et al. Defined mixed H_2-producers could evolve 5.2 L H_2 from 4 L pea-shells slurry (PPS). On the other hand, *Bacillus cereus* EGU44 could produce 1685 mg PHB L^{-1} from prehydrolyzed PSS, which was equivalent to 62.5 $g \cdot kg^{-1}$ TS fed [68].

3 EXPERIMENTAL PART

3.1 Material

3.1.1 Used bacteria

In the experimental part of the thesis were used different bacteria strains with the ability to produce PHAs. *Bacillus megaterium* CCM 2037 was acquired from Czech Collection of Microorganisms, Masaryk University in Brno. *Burkholderia cepacia* DSM 7288 was purchased from Leibnitz Institute DSMZ-German Collection of Microorganism and Cell Cultures, Braunschweig, Germany. *Pseudomonas sp.* was isolated in Malaysia by Ms. Wankuson Chanasit.

Furthermore, the subsequent anaerobic strains were used to test ability hydrolyze wheat straw. *Clostridium thermocellum* DMS 1237, *Clostridium cellulolyticum* DSM 5812 and *Clostridium cellulovorans* DSM 3052, *Clostridium butyricum* DSM 2478 from Leibnitz Institute. *Clostridium butyricum* BSF61 and *Clostridium butyricum* BSF59 were isolated from rumen of Llama (*Lama glama*).

3.1.2 Chemicals for cultivation

- Nutrient Broth (Himedia)
- Agar Powder (Himedia)
- Peptone (Himedia)
- Beef extract (Himedia)
- Trypticase (BBL)

All chemicals used were of analytical grade purity and higher.

3.1.3 Used waste lignocellulosic substrates

- Apple pomace
- Spent coffee grounds
- Rapeseed pomace
- Wine pomace
- Wheat straw

3.1.4 Used commercial enzymes

- Cellulase from *Aspergillus niger* CAS Number 9012-54-8 (Sigma Aldrich)
- Hemicellulase from *Aspergillus niger* CAS Number 9025-56-3 (Sigma Aldrich)
- Pectinase from *Aspergillus niger* CAS Number 9032-75-1 (Sigma Aldrich)

3.1.5 Facilities

- GC-FID, Hewlett Packard, Series II 5890, Column - DB-WAX 30 m by 0.25 mm
- GC-TCD, Agilent Technologies, 6890N, Column - HP-5 30 m by 0.25 mm
- HPLC, Jasco, Japan
  Detector - UV/Vis and RI (ERC-inc, Kawaguchi, Japan)
  Column - Aminex HP 87-H
- HPAEC-PAD, DIONEX, California, USA
- TGA Q5000
- AMPTS II, Bioprocess Control Sweden AB
- KrosFlo® Research IIi System, Spectrum® Laboratories, Inc
- Laminar box Aura mini, Bio Air Instruments
- Platform Shaker, Heidolph Unimax 1010, Labicom s.r.o.
- Vortex, Heidolph Reax 2000
- Spectrometer, Biochrom WPA Biowave II
- pH meter, Schott
- Fume cupboard
- Shaker, Hach Lange TOC-X5
- High temperature thermostat, Hach Lange HT 200 S
- Spectrometer with RFID, Hach Lange DR 3900
- Analytical balances, OHAUS PA214C
- Analytical balances, Boeco
- Thermostat, LS-35
- Centrifuge, Hettich MIKRO 200
- Centrifuge, Boeco U-32R
- Centrifuge, Heraeus Biofuge Pico
- Refrigerated Centrifuge SIGMA 3-16PK
- Elisa reader – ELx808, BioTek Instruments, Inc.
- Incubator, IP60
- Incubator, Heraeus B12

3.2 The revitalization of aerobic bacterial cultures

The bacteria were delivered in a lyophilized state. The bacteria have been revived by following the enclosed instructions and cultured on solid medium at 30 ° C. After the cultivation the bacteria were stored at 4 ° C and regularly reinoculated on new petri dishes in an interval of about 30 days.

Inoculum for all bacterial cultures was prepared in Erlenmeyer flasks of 100 ml volume containing 50 ml of medium. Inoculum was inoculated three times from the agar plate by the bacteriological loop. After 24 hours of cultivation at 30 ° C on a shaker at 170 rpm, the

inoculum was used to inoculate the production medium. The production medium was inoculated usually by 3% of the total volume.

3.3 The cultivation of aerobic bacteria

To prepare the inoculum for *Burkholderia cepacia was* used liquid medium; for the preservation of the culture was used solid agar medium Nutrient Broth (NB).

Composition of Nutrient Broth:

- Beef extract 10 g/l
- Peptone 10 g/l
- NaCl 5 g/l
- Agar 20 g/l

To prepare the inoculum for *Bacillus megaterium was* used liquid medium; for the preservation of the culture was used solid agar medium.

Composition of Bacillus medium:

- Beef extract 5 g/l
- Peptone 3 g/l
- $MnSO_4$ 0.1 g/l
- Agar 20 g/l

The production medium for bacteria *Burkholderia cepacia* or *Bacillus megaterium* was used following mineral medium [69]:

- $(NH_4)_2SO_4$ 0.75 g
- $Na_2HPO_4 \cdot 12\ H_2O$ 9.082 g
- KH_2PO_4 1.5 g
- $CaCl_2 \cdot 2\ H_2O$ 0.1 g
- NH_4-FE(III) Citrate 0.06 g
- $MgSO_4 \cdot 7\ H_2O$ 0.2 g
- Carbon source
- Trace element solution* 1 ml
- Distilled water 1000 ml

* Trace element solution

$ZnSO_4 \cdot 7\ H_2O$	0.1g
$MnCl_2 \cdot 4\ H_2O$	0.03 g
H_3BO_3	0.3 g
$CoCl_2 \cdot 6\ H_2O$	0.2 g
$CuSO_4 \cdot 5\ H_2O$	0.02 g
$NiCl_2 \cdot 6\ H_2O$	0.02 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.03 g
Distilled water	1 000 ml

3.4 The cultivation of anaerobic bacteria

Medium 520 was used for the revitalization of bacteria strains *Clostridium cellulolyticum* and *Clostridium cellulovorans*. For the revitalization of *Clostridium thermocellum* was used Medium 122 and for strains *Clostridium butyricum* was used RSM medium. *Clostridium thermocellum* is a thermophile and was cultivated in 55 °C. All other bacteria were cultivated in 30 °C.

The production medium was Medium 520 or with the lower addition of yeast extract (0.5 g/l) or RSM medium. Wheat straw was used as a carbon source instead of cellobiose respective glucose – 8 g/L.

Composition of Medium 520:

- $(NH_4)_2SO_4$ 1.3 g
- $MgCl_2 \cdot 6\ H_2O$ 0.2 g
- KH_2PO_4 1.5 g
- $K_2HPO_4 \cdot 3\ H_2O$ 2.9 g
- $CaCl_2 \cdot 2\ H_2O$ 75.0 mg
- $FeSO4 \cdot 7\ H_2O$ 1.25 mg
- Trace element solution* 1 ml
- Yeast extract 2.0 g
- L-cysteine-$HCl \cdot H_2O$ 0.5 g
- Resazurin 1.0 mg
- Na_2CO_3 2.5 g
- Cellobiose 6.00 g
- Distilled water 1000 ml

Ingredients were dissolved except yeast extract, cysteine, resazurin, carbonate and cellobiose. The medium was boiled in microwave oven for couple of seconds. Medium was cooled below 40 °C by sparging with oxygen-free nitrogen. Then yeast extract, cysteine and resazurin were added. Medium was dispensed into anoxic tubes or vials and autoclaved. After autoclaving, cellobiose from an anoxic stock solution sterilized by filtration was added and pH was adjusted at 7.2 by adding sterile anoxic Na_2CO_3 (10 %) solution.

* Trace element solution Medium 320:

HCl (25%; 7.7 M)	10.0 ml
$FeCl_2 \cdot 4\ H_2O$	1.5 g
$ZnCl_2$	70.0 mg
$MnCl_2 \cdot 4\ H_2O$	0.1 g
H_3BO_3	6.0 mg
$CoCl_2 \cdot 6\ H_2O$	0.19 g
$CuCl_2 \cdot 2\ H_2O$	2.0 mg
$NiCl_2 \cdot 6\ H_2O$	24.0 mg
$Na_2MoO_4 \cdot 2\ H_2O$	36.0 mg
Distilled water	990 ml

Firstly was dissolved $FeCl_2$ in the HCl and then diluted in water.

Composition of Medium 122:

- $(NH_4)_2SO_4$ 1.30 g
- $MgCl_2 \cdot 6\ H_2O$ 2.60 g
- KH_2PO_4 1.43 g
- K_2HPO_4 5.50 g
- $CaCl_2 \cdot 2\ H_2O$ 0.13 g
- Na_2-β-glycerol phosphate$\cdot 4\ H_2O$ 6.00 g
- $FeSO_4 \cdot 7\ H_2O$ 1.1 mg
- L-Glutathione reduced 0.25 g
- Yeast extract 4.50 g
- Resazurin 1.0 mg
- Cellobiose 5.00 g
- Distilled water 1000 ml

Composition of SRSM:

- Trypticase 0.5 g
- Yeast extract 0.5 g
- $Na_2S \cdot 9\ H_2O$ 0.25 g
- K_2HPO_4 0.45 g
- KH_2PO_4 0.45 g
- NaCl 0.9 g
- $(NH_4)_2SO_4$ 0.9 g
- $MgSO_4 \cdot 7\ H_2O$ 0.1 g
- $CaCl_2$ 0.1 g
- Pfenning trace elements* 1 ml
- Vitamin mix** 1 ml
- $NaHCO_3$ 8 g
- Cystein-HCl-H_2O 0.5 g
- Carbon source

*Pfenning trace elements

HCl (0.25 M)	100 ml
H_3BO_3	0.3 g
$ZnSO_4 \cdot 7\ H_2O$	0.1g
$MnCl_2 \cdot 4\ H_2O$	0.03 g
$CoCl_2 \cdot 6\ H_2O$	0.021 g
$CuCl_2 \cdot 2\ H_2O$	0.01 g
$NiCl_2 \cdot 6\ H_2O$	0.0367 g
$Na_2SeO_3 \cdot 5\ H_2O$	0.0134 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.03 g
$FeCl_2 \cdot 4\ H_2O$	0.015 g
distilled water	900 ml

**Vitamin mix [70]

cobalamine	1 mg
biotin	1 mg
PABA	3 mg
folic acid	5 mg
pyridoxamine	15 mg
thiamin	5 µg
riboflavin	5 µg
dist. water	100 ml

3.5 Acid pretreatment

The substrates were hydrolyzed in a 1% sulfuric acid in an autoclave at 121 °C for 20 minutes. The solid substrate concentration was 50 g/L. After hydrolysis the hydrolyzate (liquid portion) was separated from the solid residues of the substrate by Buchner filtration. The hydrolyzate was neutralized with 30% sodium hydroxide to a pH between 6.5 and 7.0.

3.6 Enzymatic pretreatment

Apple pomace as substrate was hydrolyzed enzymatically using commercial enzymes - cellulase, hemicellulase, pectinase and their combinations. Due to different pH optima, enzymes were prepared in citrate buffer pH 4.0; 4.25; 4.5; 4.75 and 5.0. To 1 g of the substrate in the Erlenmeyer flask we added 30 ml of buffer and 50 mg of the enzyme (or 0.6 ml of the liquid enzyme). The substrate was then incubated at optimum temperature for 24 hours. After that the hydrolyzate was separated from the solid residues of the substrate by filtration and the content of reducing carbohydrates was determined by Somogyi-Nelson method.

Table 4 Specification and characterization of the enzymes used for enzymatic hydrolysis of apple pomace

	origin	EC	pH optimum	temperature optimum	state
cellulase	*Aspergillus niger*	3.2.1.4	5	37 °C	solid
hemicellulase	*Aspergillus niger*	1.1.1.48	4.5	40 °C	solid
pectinase	*Aspergillus niger*	3.2.1.15	4	50 °C	solid
pectinase	*Aspergillus aculeatus*	3.2.1.15	4	50 °C	liquid

Table 5 Conditions of enzymatic pretreatment apple pomace with a combination of the enzymes

	pH	t [°C]
control	4.5	45
cellulase + pectinase	4.5	45
pectinase + hemicellulase	4.25	45
cellulase + hemicellulase	4.75	45
cellulase + pectinase + hemicellulase	4.5	45

3.7 Determination of biomass concentration

Biomass was determinated by spectrometric method based on determination of turbidity and gravimetric method. Spectrometric measurements were performed at a wavelength of 630 nm or 600 nm. Distilled water was used as blank.

To determine CDW, 10 ml of cell suspension was used. In order to remove the solid residues of none-utilized substrate from the cell suspension, we carried out filtration. The suspension was centrifuged (8,000 rpm, 5 min.). The supernatant was decanted (and further

used for Determination of enzyme activities 3.9 and the biomass were resuspended in 10 ml of distilled water. The suspension was again centrifuged, the supernatant was decanted and the biomass in 1 ml of distilled water transferred to Eppendorf microtube (weight recorded), which was centrifuged (10,000 rpm, 5 min.). The supernatant was then decanted and the biomass in a microtube dried to constant weight at 105 °C. Weight of the CDW plus Eppendorf microtube was recorded.

3.8 Determination of PHAs in CDW

Approximately 10 mg of dry biomass was weighed into the vials. Furthermore, 1 ml of chloroform and 0.8 ml of 15% sulfuric acid in methanol was added into the vials. Vials were capped and the mixture was maintained at 100 °C for 3 hours. After the esterification, the mixture was cooled and extracted into the 0.5 ml NaOH in concentration of 0.05 M. After the extraction and creation of phase interface lower chloroform fraction was pipetted into the new vials and they were sealed and analyzed by GC-FID.

3.9 Determination of enzyme activities

The supernatant obtained in determination of dry biomass was used in the essays of extracellular enzyme activity.

3.9.1 Assay of proteolytic activity

Azocasein solution was used as a substrate for proteolytic enzymes. Azocasein is chemically modified milk protein casein with bound orange sulfanilamide group. Azocasein is cleaved by enzymatic hydrolysis (37 °C, 60 min) and dye is soluble in trichloroacetic acid. This can be subsequently detected by spectrometer at 440 nm. The unit of activity is defined as the amount of enzyme catalyzing the conversion of the substrate accompanied by an increase of absorbance by 0.001 per 1 minute under the assay conditions [71].

0.1 ml of supernatant was incubated with 0.1 ml azocasein (5 mg/ml) in 37 °C for 60 minutes. The reaction was stopped by adding 0.41 ml of 10% trichloroacetic acid. The solution was centrifuged at 12,000 rpm for 2 minutes. To 0.5 ml of supernatant was added 0.7 ml of 1 M sodium hydroxide and absorbance was measured at 440 nm. The sample in which trichloroacetic acid was added prior to incubation was used as blank. Protease activity was defined as the amount of substrate decomposed by the enzyme relative to the time and quantity of the supernatant [$\mu mol \cdot min^{-1} \cdot mL^{-1}$].

3.9.2 Assay of lipolytic activity

Determination of lipolytic activity using p-nitrofenzylpalmitate is based on the ability of lipolytic enzymes to cleave the substrate to form an amber colored product p-nitrophenol, which is determined by spectrophotometry at 410 nm.

3.9.2.1 Preparation of the substrate

0.0135 g of p-nitrophenylpalmitate was weighed into the 100 ml volumetric flask, dissolved in 10 ml ethanol and made up with distilled water. The solution was stored at 4 °C.

3.9.2.2 Assay procedure

Into the tubes was pipetted 2.5 ml buffer pH 8, 2.5 ml of substrate and 1 ml of supernatant. Tubes were incubated for 30 minutes at room temperature. Subsequent increase in absorbance was measured at 410 nm. Lipase activity was defined as the amount of p-nitrophenol released from p-nitrophenylpalmitate by the enzyme; based on the time and quantity of the supernatant [$nmol \cdot min^{-1} \cdot mL^{-1}$].

3.9.3 Assay of cellulase activity using carboxymethyl cellulose

Cellulase activity was determined by the spectrophotometric method by measuring the increase in reducing groups by the method of Somogyi- Nelson. 1% carboxymethyl cellulose (CMC) was used as a substrate. Samples were incubated at 40 °C for 1 hour.

1 ml of supernatant was added to 0.5 ml of reagent I and 0.5 ml of reagent II (chap. 3.9.4.1).The tubes were immersed in a boiling water bath for 10 minutes which was followed by cooling. After that, 0.5 ml of reagent III was added into the cooled tubes and the contents of the tube were mixed to dissolve the precipitate. The tubes were made up with water to 10 ml. Subsequently, absorbance was measured at 720 nm against the blank. The blank was prepared the same way as a sample but the sample was replaced by distilled water.

In order to be subtracted the amount of glucose released from the substrate from the total amount of reducing sugars in the supernatant was carried out parallel measurements with the non-incubated sample. The activity of the cellulase was defined as the amount of glucose released from the cellulose substrate by the enzyme; based on the time and quantity of the supernatant [$nmol \cdot min^{-1} \cdot mL^{-1}$].

3.9.4 Assay of cellulase activity using filter paper

Cellulase activity was determined by the method of Somogyi-Nelson. The substrate was strip of filter paper of size 1x2 cm.

3.9.4.1 Preparation of Somogyi-Nelson reagents

Reagent I: 24 g $NaCO_3$, 16 g $NaHCO_3$ and 12 g 2 g K-Na tartrate was dissolved in 200 ml distilled water. 144 g Na_2SO_4 was dissolved in 200 ml distilled water. Both solution were mixed together to form reagent I.

Reagent II: 4 g $CuSO_4 \cdot 5\ H_2O$ and 24 Na_2SO_4 dissolved in 200 ml distilled water.

Reagent III: 25 g NH42MoO4 was dissolved in 450 ml distilled water, 21 ml sulphuric acid and 3 g $Na_2HAsO_3 \cdot 7\ H_2O$ was added. Prepared solution was kept 48 h in 37 °C.

3.9.5 Assay of xylanase activity

Xylanase activity was determined by the method of Somogyi-Nelson. The procedure is identical to the determination of cellulase (chap. 3.9.3) only CMC solution was replaced with 1% xylan. The activity of the xylanase was defined as the amount of glucose released from

the cellulose substrate by the enzyme; based on the time and quantity of the supernatant [nmol·min^{-1}·mL^{-1}].

3.9.6 Assay of endo-1,4-β-Mannase

The assay procedure is specific for endo-1,4-β-D-mannanase activity. On incubation of dyed carob galactomannan with β-mannanase, the substrate is depolymerized by an endo-mechanism to produce low-molecular weight dyed fragments which remain in solution on addition of ethanol to the reaction mixture. Precipitated high-molecular weight material is removed by centrifugation, and the color of the supernatant is measured.

3.9.6.1 Preparation of the substrate

Carob galactomannan is dyed with Remazolbrilliant Blue R. To prepare, was added 1 g of powdered substrate to 40 ml of hot (85-90 °C) distilled water and stirred vigorously on a hot-plate stirrer until the substrate completely dissolves (about 10 min). The solution was cooled to room temperature and added 5 ml of 2 M sodium acetate buffer (pH 4.0). Volume was adjusted to 100 ml. Solution was stored at 4 °C.

3.9.6.2 Assay procedure

0.5 ml of supernatant was mixed with 0.5 ml of substrate solution; the mixture was stirred on a vortex stirrer for 5 sec and incubated at 40 °C for 10 min. The reaction was terminated and precipitated high-molecular weight substrate by the addition of 2.5 mL of ethanol (~ 95% v/v) with 1 vigorous stirring for 10 sec on a vortex mixer. The reaction tubes was allowed at the room temperature for 10 min and then centrifuged at 3,000 rpm for 10 min. The supernatant solution was poured directly from the centrifuge tube into a spectrophotometer cuvette and read the absorbance of the blank and reaction solutions at 590 nm.

One Unit of activity is defined as the amount of enzyme required to release one micromole of mannose reducing-sugar equivalents per minute under the defined assay condition.

3.10 Determination of the extracellular proteins

Hartree-Lowry colorimetric assay is based on two-component reagent. The first component is the biuret reagent; the second component is the Folin-Ciocalteu reagent to phenols. These polyacids, phosphomolybdic and phosphotungstic reduce tyrosine residues of proteins and stains blue. The method has been modified many times. One of the most common modifications Hartree drafted in 1972.

3.10.1 Reagents composition

Solution A: 2 g K-Na tartrate · 2 H_2O, 100 g Na_2CO_3, 500 ml 1M NaOH, 1 L distilled water.

Solution B: 2 g K-Na tartrate · 2 H_2O, 1 g $CuSO_4$·5 H_2O, 10 ml 1M NaOH.

Solution B: Folin-Ciocalteu reagent and distilled water (1:15).

3.10.2 Calibration curve

Albumin solution (0.05 to 0.3 mg/ml) was used to construct a calibration curve. To each tube was added 1 ml of albumin solution and 0.9 ml of solution A. Tubes were held for 10 minutes at 50 °C in a water bath. Then the tubes were cooled and 0.1 ml of solution B was added. The tubes were left at the laboratory temperature for 10 minutes. Then 3 ml of solution C were added, and the tubes were again kept for 10 minutes in a water bath at 50 °C. Finally, the absorbance was measured at 550 nm against the blank, wherein the albumin solution was replaced with distilled water.

Extracellular protein concentration was determined in the same way but 1 ml solution of albumin was replaced by 1 ml of sample.

3.11 Purification of enzyme by precipitation

3.11.1 Ammonium sulfate precipitation

Ammonium sulphate precipitation was performed as follow: 10 ml of extracellular secretion was precipitated with solid ammonium sulphate on a magnetic stirrer to 80 % saturation. Whole amount was transferred to 10 ml centrifugation tubes. Samples were centrifuged (12,000 rpm, 15 minutes) and supernatant was discarded and precipitate was suspended in 250 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7.4.

3.11.2 Acetone precipitation

2 ml of extracellular secretion was mixed with 8 ml of cold acetone (-20 °C) in centrifugation tube. Tubes were vortexed and incubated for half hour at -20 °C. The tubes were centrifuged (12,000 rpm, 15 minutes). Supernatant was discarded and acetone was evapored at room temperature for 30 minutes. After that the precipitate was suspended in 50 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7.4.

3.12 SDS PAGE electrophoresis

3.12.1 The preparation of polyacrylamide gel

For electrophoresis of enzyme sample was used a 12% gel. 4 ml stock solution of AA/BIS, 2.5 ml of separation buffer, 3.4 ml MiliQ water and 0.1 ml of 10% SDS, 5 μL TEMED and 50 μL APS was pipetted into a beaker. The mixture was gently stirred and pipetted into the space between two glass pouring dispensers. Plastic comb was inserted between glass to create the holes for sample application. The gel was allowed to polymerize for one hour.

3.12.2 The preparation of samples and electrophoresis performance

Samples were mixed with sample buffer in the ratio 1:1 and boiled for four minutes in a water bath. Precision Plus Protein™ Dual Color Standards has been used as standard.

After solidification of the gel, a plastic comb was removed and the glass with gel was carefully mounted into the apparatus, so that the electrode buffer was not leaking from the electrode buffer space. The electrode buffer was poured, and the samples were loaded into the holes in an amount of 10 to 20 μL. The apparatus was covered with a transparent cover, well-sealed and connected to a voltage source (100 mA) for three hours.

3.12.3 Silver staining

SERVA Silver Staining Kit for SDS PAGE was used for staining. After electrophoresis, the gel was carefully transferred using a squeeze bottle into the bowl with the fixing solution. The gel was left in the fixing solution for at least 20 minutes. After removing the fixative solution, the gel was washed twice with 30% ethanol for 10 minutes. The gel was pre-treated in sodium thiosulfate · pentahydrate (30 mg in 100 ml H_2O dist.) for 1 minute. Then the gel was washed in distilled water three times for 10 second. Then the gel was stained in silver nitrate solution for 15 minutes. In the next step, the gel was rinsed with a small amount of distilled water, and then was added 100 ml of the developer solution (sodium carbonate solution and 50 μL formaldehyde 37 %). Protein image appeared within 2 minutes. After the desired image intensity was reached, the reaction was stopped by adding 100 ml of stop solution (1% w/v glycine) for 5 minutes.

3.13 Waste biomass analysis

3.13.1 Determination of Total Solids (TS)

The substrate was sheared with the aid of a scissor to about 5 mm, washed three times with running water and dried in an oven at 60 °C for 20 h. Aluminum weighing dishes were predried by placing them in a 105 ± 3ºC drying oven for a minimum of four hours. The dishes were cooled in a desiccator. Using gloves or tweezers to handle the dishes, weigh a pre-dried dish to the nearest 0.1 mg. Approximately 1 g dried sample was weighed to the nearest 0.1 mg. The sample was placed into a convection oven at 105 ± 3 °C for a minimum of four hours. The sample was removed from the oven and allowed it to cool to room temperature in a desiccator. The dish was weight containing the oven-dried sample was weight to the nearest 0.1mg and recorded.

3.13.2 Determination of Volatile Solids (VS)

Using a porcelain marker was marked an appropriate number of crucibles with identifiers, and placed in the muffle furnace at 575 ± 25 °C for a minimum of four hours. The crucibles were removed from the furnace directly into a desiccator. The crucibles were weight to the nearest 0.1 mg and recorded. 1 g of sample predried at 105 °C was weight into the tared crucible, to the nearest 0.1 mg. The sample weight was recorded. The crucibles were placed in the muffle furnace at 575 ± 25 °C for 4 hours. The crucible was carefully removed from the furnace directly into a desiccator and cooled for a specific amount of time, equal to the initial cool time of the crucibles. The crucibles and ash was weight to the nearest 0.1 mg.

3.13.3 Determination of Cellulose

Approximately 0.8 g sample was weighed into a 100 ml Erlenmeyer flask to the nearest 0.0001 g. Subsequently was added 30 ml of 80 % acetic acid and concentrated nitric acid (10:1). The flask with suspensions was covered by filter funnel to reduce evaporation, and placed in a boiling water bath for one hour. The suspension was then transferred qualitatively using ethanol into preweighed frit with pore size of 15 μm (designation S4) and filtered with a vacuum pump. The filter cake was washed with ethanol, and then the frit was placed into an oven at 105 °C for at least 4 hours. After drying, the frit was placed in a desiccator. The cooled frit was weighed. Subsequently, the content was quantitatively converted with ethanol to 100 ml Erlenmeyer flask. The ethanol was evaporated using a water bath. After evaporation, 20 ml of 72% sulfuric acid added to the flask. Suspension was hydrolyzed for 1 hour on the shaker (100 rpm). The hydrolyzate was qualitatively transferred using ethanol in frits with a pore size of 30 μm (designation S3) and filtered. The frit with the rest of the sample was placed in an oven and dried under the same conditions. The cellulose content was calculated from the following formulas:

$$\%_{approx.\,cellulose} = \frac{\left(m_{frit\,S4+sample} - m_{frit\,S4}\right)}{m_{sample}} \cdot 100$$

$$\%_{cellulose\ in\ approx.\,cellulose} = 100 - \left(\frac{m_{frit\,S3+sample} - m_{frit\,S3}}{m_{frit\,S4+sample} - m_{frit\,S4}} \cdot 100 \right)$$

$$\%_{cellulose} = \%_{approx.\,cellulose} \cdot \%_{cellulose\ in\ approx.\,cellulose}$$

3.14 Anaerobic supernatant analysis

3.14.1 HPLC

3.14.1.1 Detection of organic acids and alcohols

HPLC instrument (Jasco, Japan) was equipped with a UV/Vis and RI (ERC-inc, Kawaguchi, Japan) detector. Separation was done on an Aminex HP 87-H ion exclusion chromatography column coupled to a guard column (Bio-Rad, CA, USA). The column temperature was maintained at 65 °C and eluted with 0.5 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min.

Sample was diluted by MiliQ water and 20 % H_2SO_4 was added (20 μl/1 ml diluted sample). Mixture was vortexed and transferred through the 0.45-nm pore size membrane filter into the vial.

3.14.1.2 Detection of sugars

Released monosaccharides were analyzed by high-performance anion exchange chromatography with pulsed amperiometric detection (HPAEC-PAD) (DIONEX, Califirnia, USA) using a 250 mm × 4 mm i.d., 8.5 μm CarboPac PA20 column and guard column 50 mm × 4 mm of the same material and an isocratic mobile phase of 1mM NaOH at 1 mL/min and

at the end of each cycle the column was regenerated by washing with 0.6 mole/l NaOH for 4 minutes.

Oligosaccharides were analyzed using a 250 mm × 3 mm i.d., 5.5 μm CarboPac PA200 and a guard column 50 mm × 3 mm of the same material and mobile phase 100 mM NaOH and gradient of sodium acetate of 0-50mmol between 0-20 min at 0.5 mL/min.

Sample was diluted by MiliQ water to volume 2 ml. Mixture was vortexed and transferred through the 0.20-nm pore size membrane filter into the plastic vial.

3.14.2 Determination of Total Organic Carbon (TOC)

For determination was used HACH LANGE cuvette test LCK 385. 2.0 ml sample was pipette into digestion cuvette. Open digestion cuvette was inserted in the TOC-X5 shaker. After 5 minutes blue indicator cuvette was opened and immediately screwed on the membrane double cap tightly. The digestion cuvette was closed tightly with prepared indicator cuvette. Cuvettes were heated in thermostat HT 200 S for 2 hours at 95 °C. Cuvettes were allowed to cool to room temperature. Cuvette combination was inverted; outside of indicator cuvette was cleaned and evaluated by spectrometer DR 3900.

3.14.3 Determination of Total Nitrogen bonded (TNb)

For determination was used HACH LANGE cuvette test LCK 338. 0.2 mL sample, 2.3 mL solution A, 1 tablet B was quickly added to dry reaction tube, which was immediately closed. Tube was placed to the HT 200 S in standard program HT for 15 minutes. After cool down microcap C was added. After dissolution microcap, 0.5 mL digested sample and 0.2 mL solution D was pipetted into the test cuvette. After 15 minutes outside of cuvette was cleaned and evaluated by spectrometer DR 3900.

4 RESULTS AND DISSCUSION

4.1 Chemical hydrolysis of waste materials

4.1.1 Determination of carbohydrates in hydrolysates obtained by chemical hydrolysis

In order to utilize selected agricultural waste streams we performed hydrolysis of the waste materials by diluted acid as described in chapter 3.5 and sugars - desirable products of acid hydrolysis were analyzed by high-performance liquid chromatography with refractometric detector. The samples were diluted ten times.

Table 6 Content of carbohydrates in acid hydrolysates

coffee grounds	**c [g/L]**
glucose	0.081
galactose	13.253
arabinose	1.164
SUM	14.417
yield coefficient[1]	0.288

rapeseed pomace	**c [g/L]**
glucose	2.971
galactose	5.938
arabinose	2.048
SUM	10.957
yield coefficient	0.219

apple pomace	**c [g/L]**
glucose	6.955
galactose	10.497
arabinose	1.651
SUM	19.103
yield coefficient	0.382

wine pomace	**c [g/L]**
glucose	7.417
galactose	6.345
SUM	13.762
yield coefficient	0.275

The measured data show that hydrolysates contain relatively large amounts of simple sugars. Acid pretreatment therefore seems to be very effective for the lignocellulosic material hydrolysis. The largest amount of monosaccharides has been found in the apple pomace hydrolyzate 19.1 g/L. The spent coffee ground (SCG) and the wine pomace amount of sugars does not differ importantly with 14.4 g/L and 13.8 g/L, respectively. Their differences in composition are nevertheless important. While SCG hydrolyzate contains almost only galactose, wine pomace contains the same amount of glucose and galactose. This difference can affect the growth of bacteria producing PHA.

Diluted acid hydrolysis of lignocelluloses is capable of cleaving glycoside bonds of hemicelluloses but is not sufficient for hydrolysis of cellulose. Thus, the hydrolysates were relatively rich in sugars typical for hemicelluloses such as galactose and arabinose which were the dominant sugars in hydrolysates. High content of glucose was observed only in apple

[1] The quantity of monosaccharides (g) obtained from one gram of substrate during the hydrolysis.

pomace and wine pomace. It is likely that the main portion of glucose in these hydrolysates did not release from polysaccharides during hydrolysis but “free” glucose was simply extracted from the materials.

4.1.2 Production PHAs on hydrolysates obtained by chemical hydrolysis

Analyzed hydrolysates were then used as an energy and carbon source in the production medium for PHA production employing two bacterial strains – *Burkholderia cepacia* and *Bacillus megaterium*. The medium was prepared by mixing the hydrolyzate with the same amount of twice-concentrated mineral medium (2xMM). Medium supplemented with glucose (20 g/L), instead of the hydrolyzate, served as a control.

At the end of cultivation (72 h), biomass concentration and PHA content was determined (Table 7). Furthermore, we also performed HPLC analysis of supernatant, however, after the cultivation none hydrolyzate contained significant amount of sugars.

Table 7 Comparison of the ability to produce PHA by bacteria Bacillus megaterium and Burkholderia cepacia

hydrolyzate	***Bacillus megaterium***			***Burkholderia cepacia***		
	CDW [g/L]	PHA in CDW [%]	PHA [g/L]	CDW [g·dm^{-3}]	PHA in CDW [%]	PHA [g/L]
control	1.3±0.0	17.2±2.5	0.2±0.0	4.4±0.0	52.2±4.7	2.3±0.2
coffee grounds	1.2±0.1	0.0±0.0	0.0±0.0	3.2±0.1	19.6±1.8	0.6±0.0
apple pomace	2.0±0.1	41.6±3.1	0.8±0.1	5.0±0.8	34.1±3.1	1.7±0.1
rapeseed pomace	1.7±0.0	8.8±0.3	0.2±0.0	3.9±0.2	5.6±2.4	0.2±0.1
wine pomace	2.1±0.2	31.3±3.5	0.7±0.0	3.7±0.2	23.1±0.7	0.8±0.0

Two bacterial strains *Bacillus megaterium* and *Burkholderia cepacia* were used for PHA production. Both strains were able to grow and produce PHA, but Table 7 shows that the amount of biomass in case of *Bacillus megaterium* is less than half of the biomass of *Burkholderia cepacia*. It indicates that the bacterial strain *Bacillus megaterium* is less suitable for the growth on this type of media. Moreover, also PHA yields obtained with *B. cepacia* are higher than those observed in *B. megaterium*.

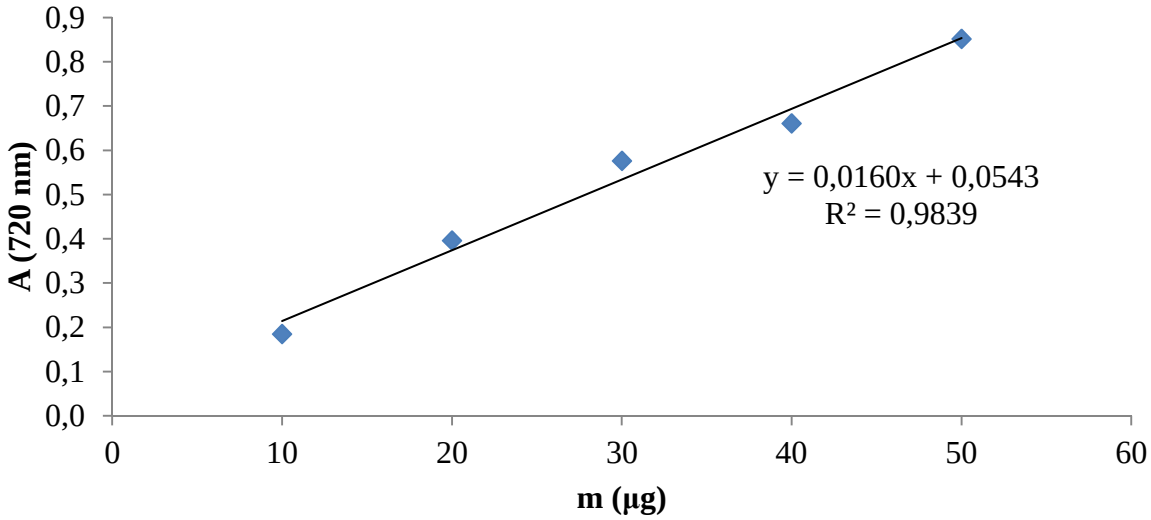
Based on the data, apple pomace seems to be the most promising of the substrates tested. Despite the fact that the amount of sugars contained in this hydrolyzate was not much higher than in the other hydrolysates, just medium containing apple pomace hydrolysate provided environment for the largest increase in biomass and PHA contained in biomass. For these reasons, the following experiments dealing with the production of PHA were focused mainly on apple pomace and the *Burkholderia cepacia* was used as a PHA producer.

The final amount of 1.7 grams of PHA per liter media is not too high yield but we can undoubtedly say that the *Burkholderia cepacia* is a promising bacterial strain in order to produce PHA from lignocellulose-based rest materials.

4.2 Enzymatic hydrolysis of waste materials

4.2.1 Determination of carbohydrates in enzyme hydrolysates

Another strategy how to release fermentable sugars from lignocellulosic waste materials is enzymatic hydrolysis. Therefore, we tested hydrolysis of apple pomace by selected commercially available enzymes (chapter 3.6). Efficiency of the enzymatic pretreatment was determined by Somogyi-Nelson method. It was therefore necessary to construct a calibration curve.



Graph 1 Calibration curve of reducing sugars (glucose) by Somogyi-Nelson

Apple pomaces were subjected to hydrolysis using commercial enzyme preparations. Subsequently, the content of reducing sugars in the hydrolyzate was determined by Somoyi-Nelson.

Table 8 Efficiency of conversion apple pomace into reducing sugars detectably by Somogyi-Nelson method

enzyme	**concentration of sugars after hydrolysis (g/L)**	**yield coefficient[2] (1)**
control	7.38	0.221
cellulase *A. niger*	12.06	0.362
hemicellulase *A. niger*	7.50	0.225
pectinase *A. aculeatus*	7.31	0.219
pectinase *A. niger*	12.38	0.371
controlee	7.94	0.238
cellulase + pectinase	16.50	0.495
pectinase + hemicellulase	13.69	0.411
cellulase + hemicellulase	18.06	0.542
cellulase + pectinase + hemicellulase	20.50	0.615

[2] The quantity of monosaccharides (g) obtained from one gram of substrate during the hydrolysis.

Table 8 shows that about 22 % of apple pomace are reducing sugars which can be simply extracted from the material by water. Enzymatic pre-treatment by hemicellulase from *A. niger* and pectinase from *A. aculeatus* was proved to be ineffective, in contrast cellulase from *A. niger* and pectinase from *A. niger* had some effect on the content of reducing sugars.

The significant increase in the concentration of reducing sugars was recorded in the combination of these enzymes. The highest value reached 61.5% reducing sugars relative to the weight of apple pomace predried. This value was achieved using all three types of enzymes.

4.3 Production PHAs on hydrolysates obtained by enzymatic hydrolysis

Hydrolysates obtained by enzymatic pre-treatment were used for production of PHA in the same way as in chapter 4.1.2. However, the experiment was performed in two different ways. At first we used only supernatant similarly as in the previous experiments with hydrolysates obtained by chemical hydrolysis. Secondly, supernatant was not separated from the residual solid material and bacteria were cultivated in presence of enzymatically pre-treated apple pomace.

Table 9 The yield of PHA production by Burkholderia cepacia

hydrolyzate	**CDW [g/L]**	**PHA in CDW [%]**	**PHA [g/L]**
control	1.96	27.1	0.53
cellulase + pectinase	3.49	66.8	2.33
pectinase + hemicellulase	2.93	78.0	2.29
cellulase + hemicellulase	2.48	45.0	1.12
cellulase + pectinase + hemicellulase	3.02	61.6	1.86

hydrolyzate + residual apple pomace	**CDW [g/L]**	**PHA in CDW [%]**	**PHA [g/L]**
control	1.49	57.9	0.9
cellulase + pectinase	0.85	38.5	0.33
cellulase + hemicellulase	2.68	52.9	1.44
cellulase + pectinase + hemicellulase	0.09	89.8	0.08

Table 9 shows that the best pretreatment combination of enzymes is cellulase + pectinase. Yield of *Burkholderia cepacia* CDW was 3.49 g/L and content of PHA in CDW was 66.8 % Final yield of PHA was 2.33 g/L. In the samples where unhydrolysed apple pomace was left in a production medium, higher production of PHA was not observed. However, *B. cepacia* was able to grow and produce PHA also in presence of none-hydrolyzed apple pomace (control culture). It indicates that *Burkholderia cepacia* is capable of producing enzymes required for hydrolysis of lignocellulose and subsequently the products of the activity of these enzymes can be utilized for PHA production.

4.4 Test of ability to utilize lignocellulosic material without pretreatment

The following experiment was performed to verify the ability of *Burkholderia cepacia* to utilize lignocellulosic material without previous pretreatment. Petri dishes were filled with medium composed of only a lignocellulosic substrate, mineral medium and agar. The culture was spread on the plates. The growth of *Burkholderia cepacia* on these agar-plates proved the presence of an enzyme system allowing the use of lignocellulose as a sole source of nutrients (Figure 11).

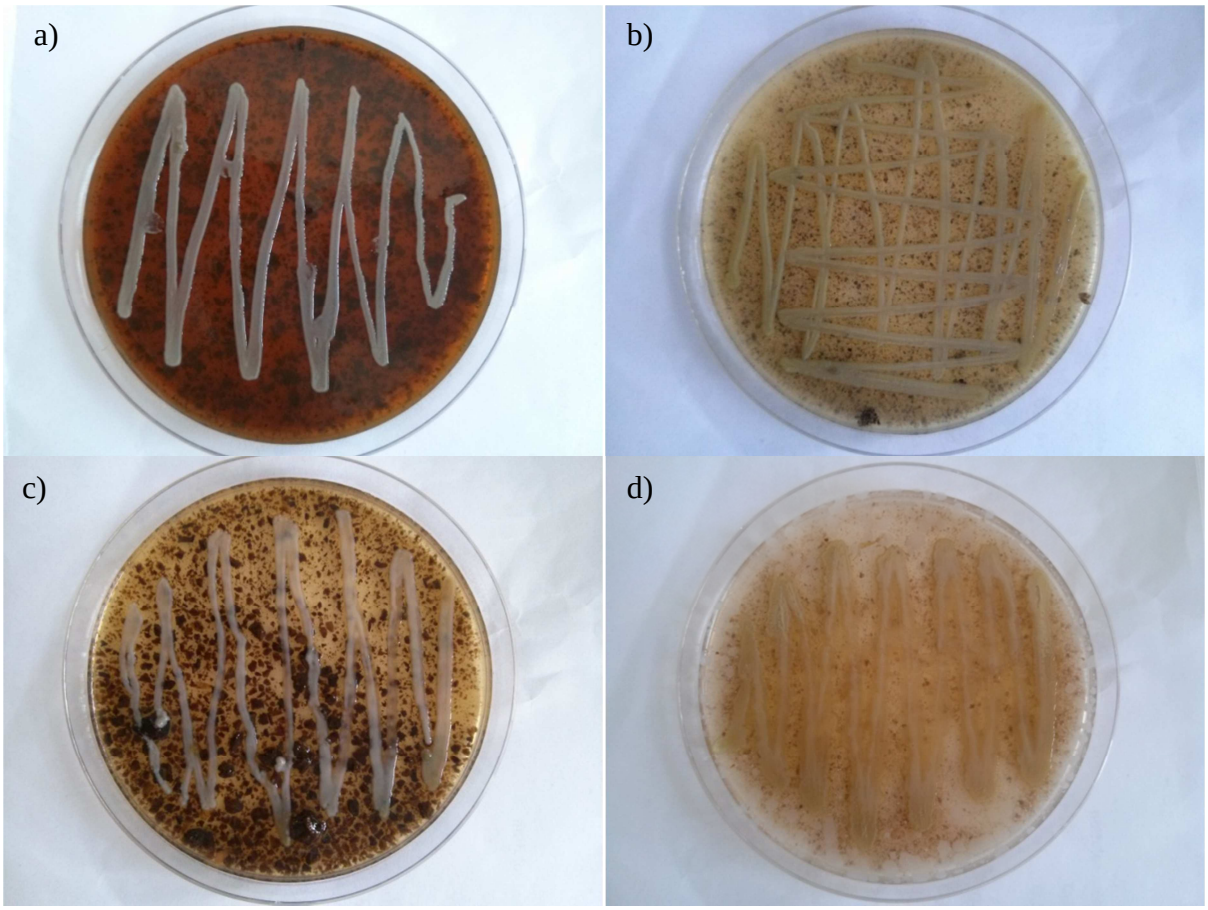
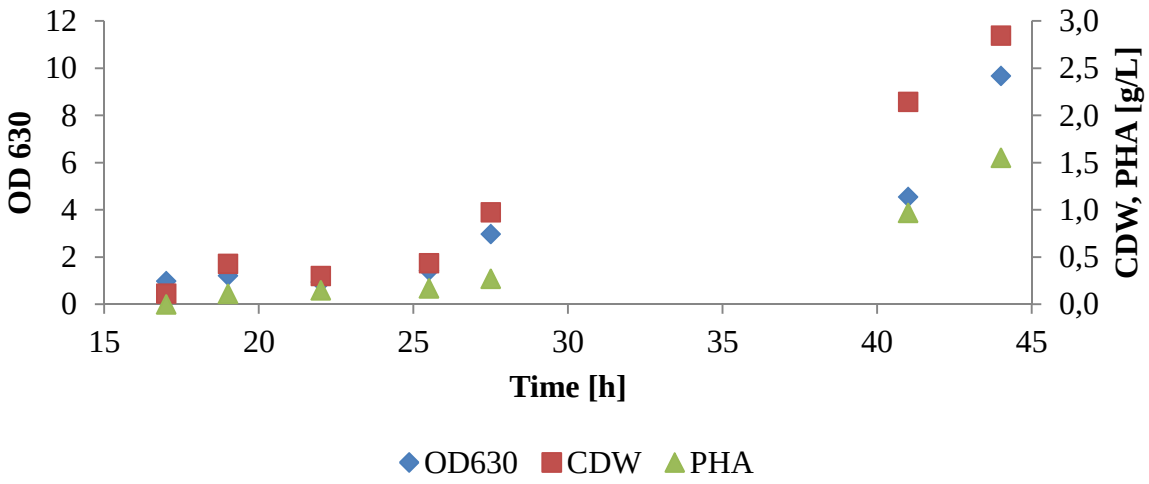


Figure 11 Agar plates with different lignocellulosic substrates as sole nutrient source. Substrates are as follows: a) wine pomace, b) apple pomace, c) rapeseed, d) spent grain

4.5 Production of PHA in bioreactor

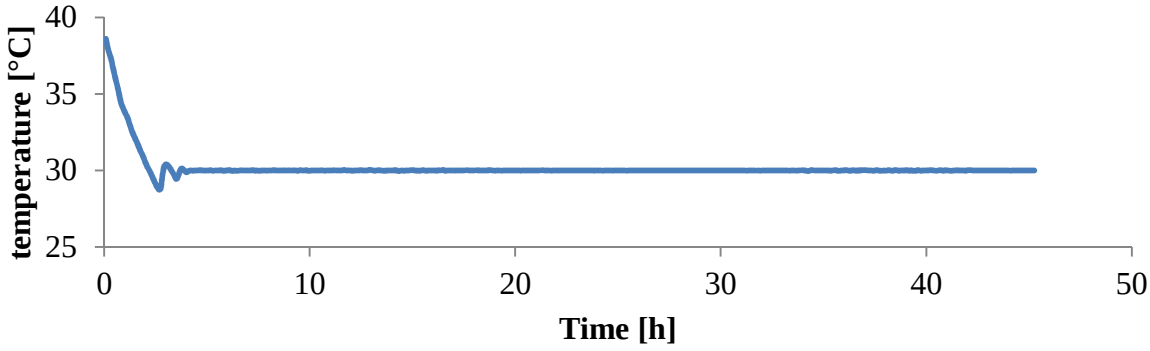
Based on the previous experiments, we carried out batch cultivation in the bioreactor (3 l). Medium was used a mineral medium with finely grounded apple pomace (30 g/L). OD630, CDW and PHA were measured in regular intervals during cultivation. Other parameters such as temperature, redox potential, pH and DO (dissolved oxygen) were measured automatically. Temperature was maintained at 30 °C, pH at 7.0 and DO at 30 %.



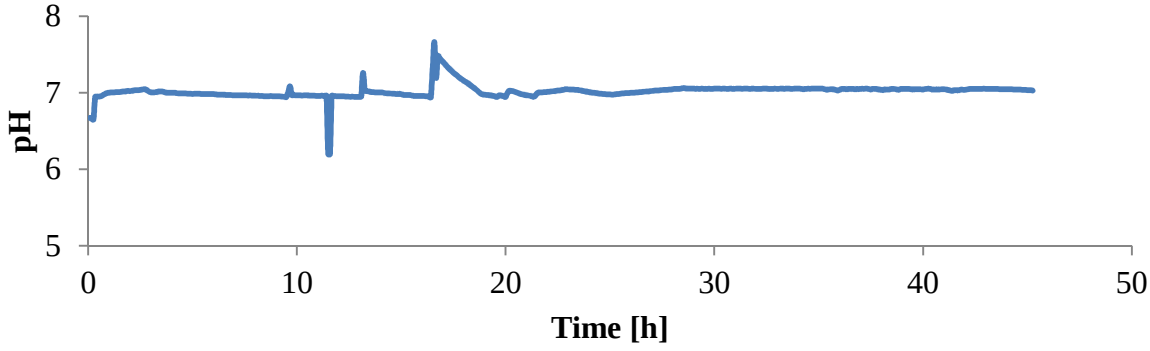
Graph 2 Dependence of optical density, cell dry weight and the content of PHA on the time cultivation

Graph 2 indicates a relatively long lag phase, which ended around the 25th hour of cultivation. The highest PHA and biomas yields were achieved at the end of the cultivation - 2.9 g/L CDW and 1.6 g/L PHA. Cultivation was not long enough to culture reached its stationary phase. We can undoubtedly say that *Burkholderia cepacia* can utilize lignocellulosic material without prior hydrolysis and simultaneously accumulate PHA. Considering the price of the hydrolysis process, it is a finding which could significantly reduce the cost of production of PHA.

Graphs 3-7 show the records of physical quantities during the cultivation. The temperature was stable since 5th hour of cultivation. pH slightly fluctuated between 12th and 20th hours.

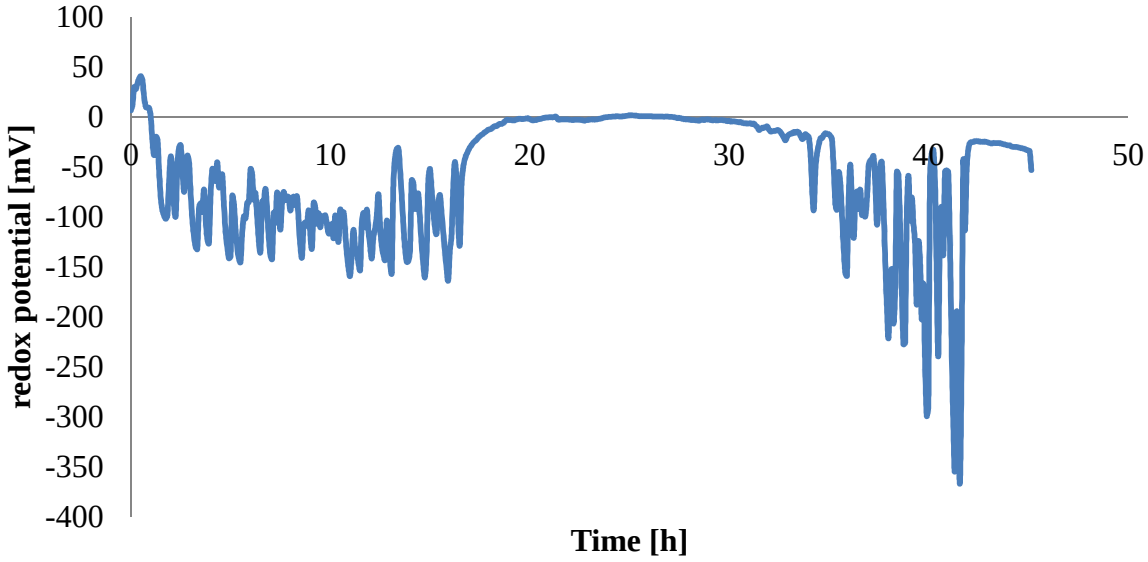


Graph 3 Changes of temperature during batch cultivations

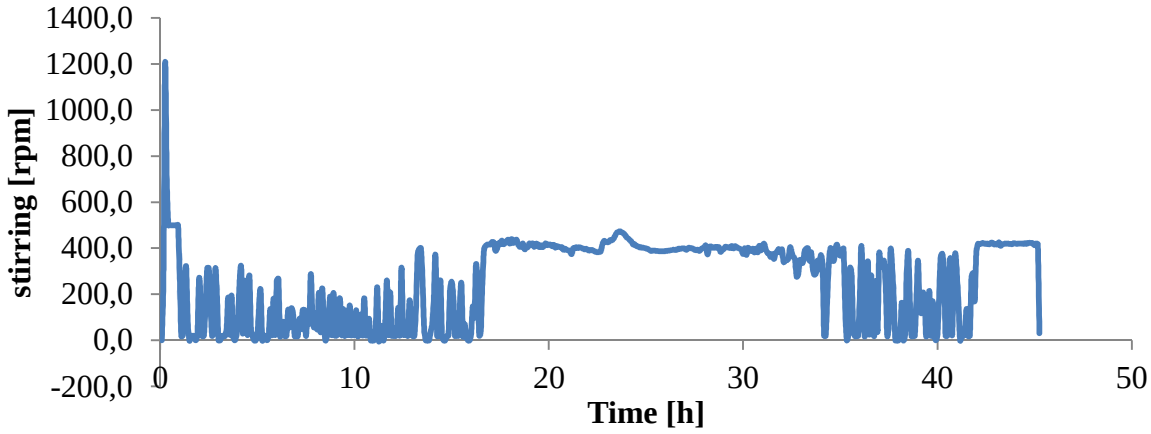


Graph 4 Changes of pH during batch cultivations

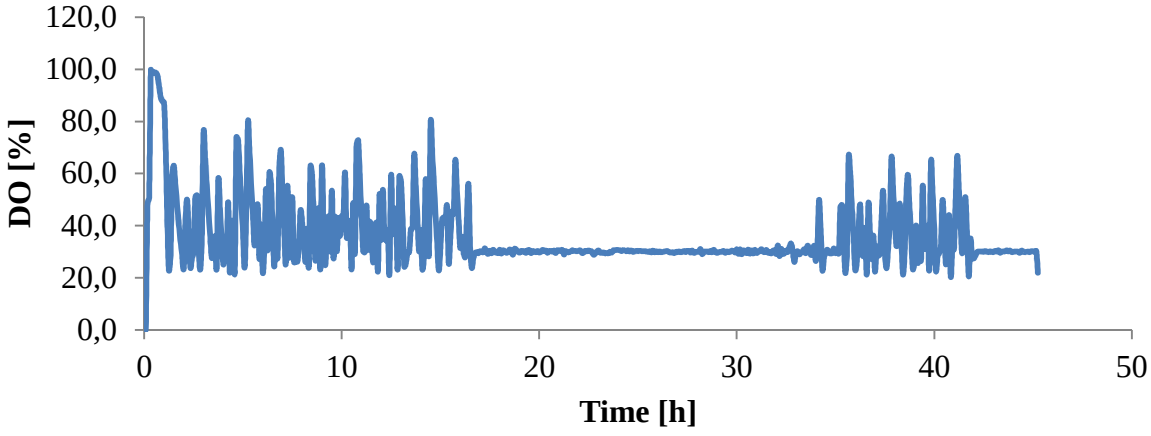
Redox potential, stirring and dissolved oxygen fluctuated significantly. This fluctuation probably could have influenced the culture growth. The growth occurred only at stable values of physical quantities.



Graph 5 Changes in the redox potential during batch cultivations



Graph 6 Changes of the stirring during batch cultivations



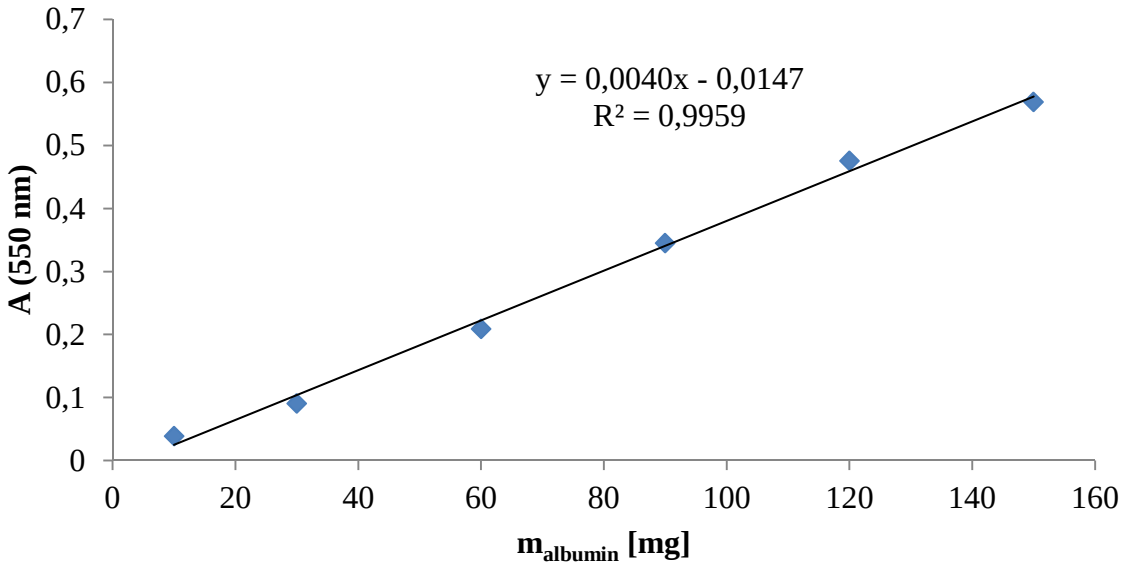
Graph 7 Changes of dissolved oxygen during batch cultivations

4.6 Analysis of the supernatant after the PHA production

The aim of the experiment was to investigate details about processes outside the cell during the utilization of non-pretreated lignocellulosic material. The experiment was designed as follow: 1 g of predried spent coffee grounds was resuspended in 30 ml of mineral medium, which was further inoculated with 3 ml of 24 h culture of *Burkholderia cepacia*. After four days of cultivation, the supernatant was separated from the residual solid material by centrifugation. The supernatant was examined for protein content and enzyme activities of selected hydrolytic enzymes. The supernatant was also used for SDS-PAGE to identify the proteins contained therein.

4.6.1 Determination of the extracellular protein content

To determine the protein concentration was necessary to establish a standard curve for determination according to the Hartree-Lowry. As a standard substance was chosen egg albumin.



Graph 8 Standard calibration curve of Hartree-Lowry protein determination

The content of extracellular proteins was determined based on the standard calibration curve as 2.93 g/L.

4.6.2 Assays of enzymatic activity

Further, all enzymatic assays (chapter 3.9) were performed with the supernatant. A negative response was observed only for endo-1,4-β-D-mannanase activity. Supernatant exhibited positive response for following enzyme activities: protease, lipase (0.47 nmol·mL^{-1}·min^{-1}), cellulase for CMC (6.05 nmol·mL^{-1}·min^{-1}) and filter paper (4.63 nmol·mL^{-1}·min^{-1}) and xylanase (1.71 nmol·mL^{-1}·min^{-1}). All these enzymes represent

important products of biotechnological processes, for instance lipase of *B. cepacia* is commercially available as product of Clea Technologies and this enzymes was successfully utilized for production of biodiesel [72][73]. Therefore, it is very desirable that these enzymes can be produced directly from cheap/waste solid substrate without previous pretreatment. Furthermore, to our knowledge, this is the first report on production of extracellular cellulase and xylanase by *B. cepacia*.

4.6.2.1 The Congo red assay

In addition to spectrophotometric assay, we also performed cultivation assay to confirm ability of *B. cepacia* to extracellularly produce xylanase and cellulose. Figure 12 shows clearing zones around colony of *Burkholderia cepacia*.

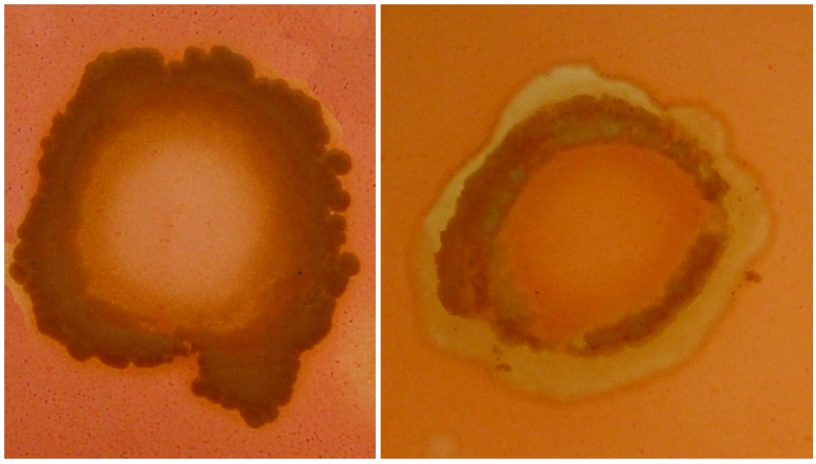


Figure 12 Burkholderia cepacia growing on agar plates containing 1 % CMC (right) and xylan (left). Visible clearing zones indicate CMCase and xylanase activity.

4.6.3 Partial purification and identification of enzymes

Electrophoresis was included as a method for determination of the distribution of proteins in a sample according to their molecular weight. Electrophoretic separation of enzymes was carried out on a 12.5% polyacrylamide gel and Precision Plus Protein™ Dual Color Standards (Bio-Rad) protein mixture was used as a standard.

Initially, electrophoresis was done without preconcentration; stained with Coomassie blue (Figure 13, 1st part). Figure 13, the 2nd part shows supernatant after preconcentration stained by silver kit, from left: standard, ultrafiltration tube 10 kDa, ultrafiltration tube 1 kDa, acetone precipitate (40 times concentrated), ammonium sulphate precipitate (8 times concentrated). 3rd and 4th part of Figure 13 shows supernatant preconcentrated by KrosFlo® ultrafiltration system, 3rd was stained by silver kit and 4th was stained by Coomassie blue.

From the electrophoresis results in Figure 13, we identified four dominant bands in the supernatant of *Burkholderia cepacia* cultivated on non-pretreated spent coffee grounds. These four bands correspond to the protein of size about 60 kDa, 44 kDa, 40 kDa and 30 kDa. Precipitation was proved to be the least suitable of the purification methods. Far preferable method of the purification was an ultrafiltration, either by ultrafiltration tubes or using an ultrafiltration system KrosFlo®. The methods reduced the amount of salt in the samples which decreased background intensity. Precipitated samples were expressed only as a smear on a polyacrylamide gel.

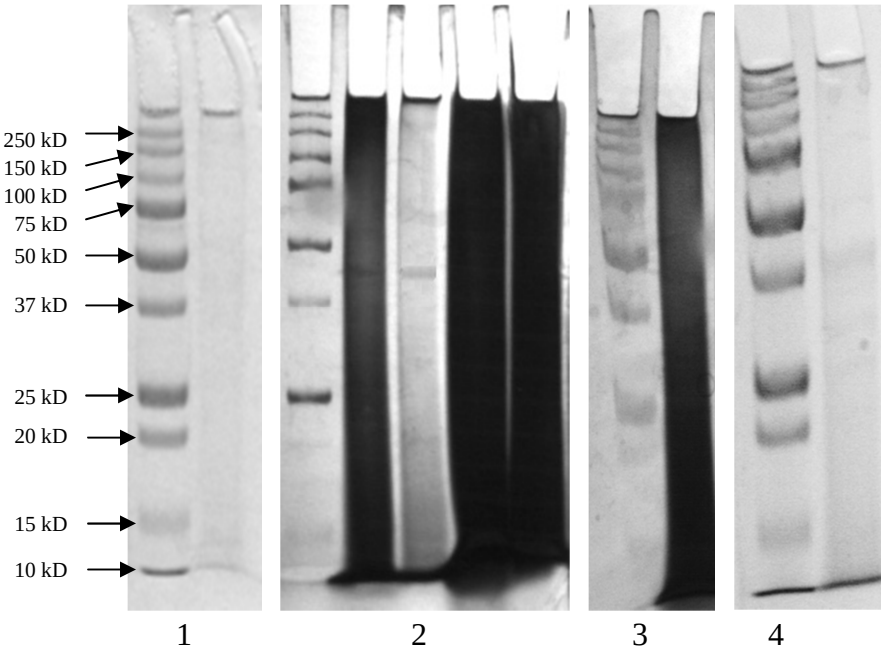


Figure 13 SDS-PAGE electrophoresis. Sample without preconcentration (1). Sample after preconcentration (2, 3 and 4)

The ultrafiltration system KrosFlo® is very effective and fast method for purification of proteins. The system allows to achieve a precise concentration factor and control process of ultrafiltration. It can automatically regulate the pressure in hollow fiber filter module during ultrafiltration in order to reach the best efficiency. Figure 14 shows a record of ultrafiltration the sample used for the SDS-PAGE.

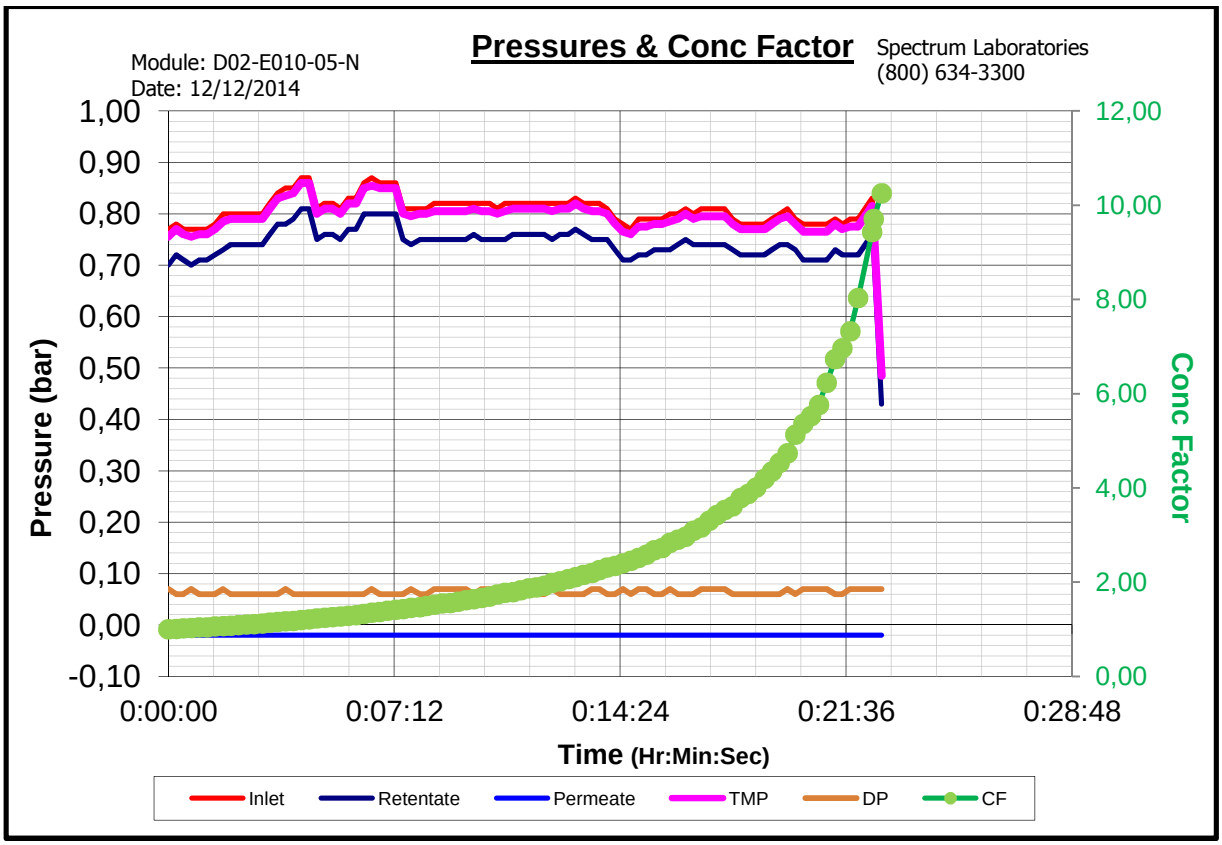


Figure 14 Recorded process data of ultrafiltration

4.7 Monitoring of enzymes and PHA production in time course of cultivation

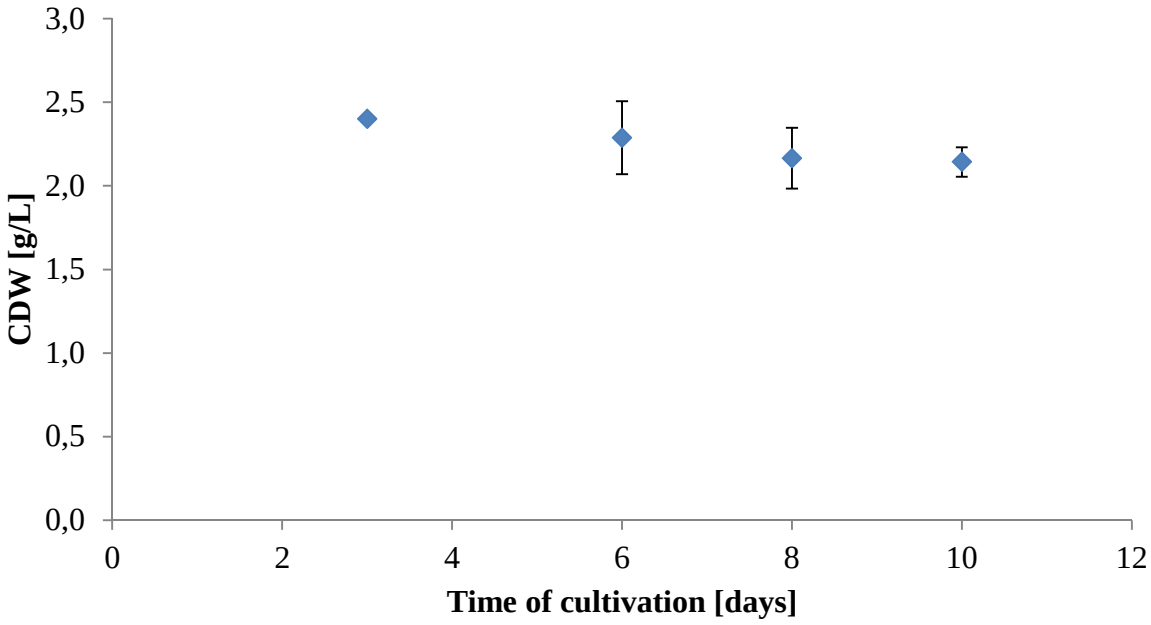
The aim of the experiment was to determine the influence of the length of cultivation on enzymatic activity and also whether the CDW and PHA content will decrease after reaching the stationary phase.

The experiment was performed with the same medium as in the chapter 4.6. The cultivation was terminated after the third, sixth, eighth, and tenth day. Substrate (spent coffee grounds) was removed by filtration. The obtained suspension was centrifuged. CDW and PHA content were determined as described above. The supernatant was used for determination of proteins content by the Hartree-Lowry, polyphenol content and we also carried out the individual enzymatic activities assays.

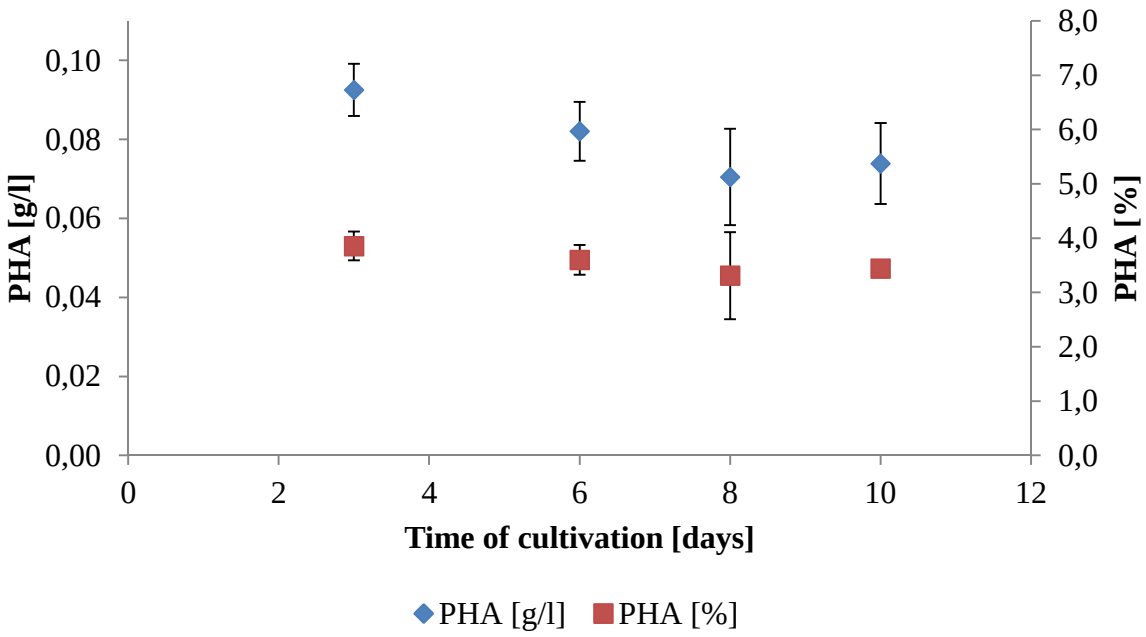
4.7.1 Determination of CDW and PHA

Graph 9 shows influence of length of cultivation on the CDW development. The amount of the cell mass is at its maximum level after three days (2.4 g/L); culture is at this moment at the end of its exponential phase. After reaching this stage there is no further increase in biomass, but there was a slight decline, however this decrease is surprisingly very slow. Cell dry weight was 2.14 g/L after ten days of cultivation.

Graph 10 shows influence of length of cultivation on the content of PHA. This trend is almost identical to the CDW. After three days CDW contains 3.9 % of PHA and total contain of PHA in media is 0.093 g/L. Concentration decreased to 0.074 g/L after next seven days. The decline occurs because the PHA in the cell acts as an energy reserve. Therefore, cell will begin to assimilate accumulated polymer, instead of PHA production, after the reaching stationary phase.

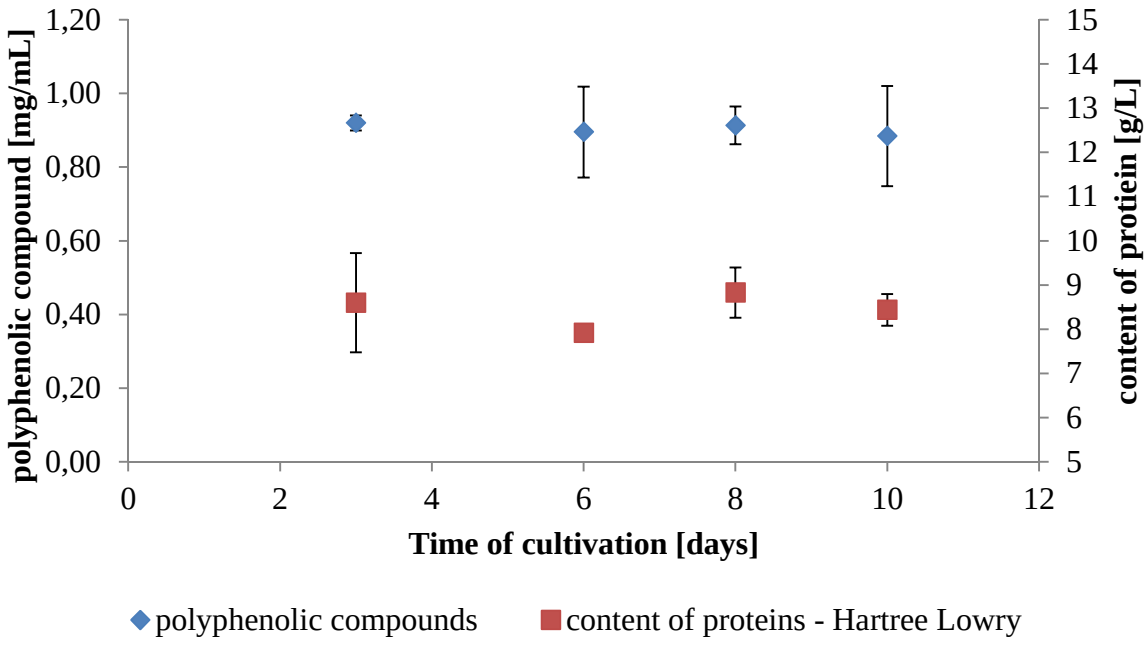


Graph 9 Influence of length of cultivation on the CDW



Graph 10 Influence of length of cultivation on the content of PHA

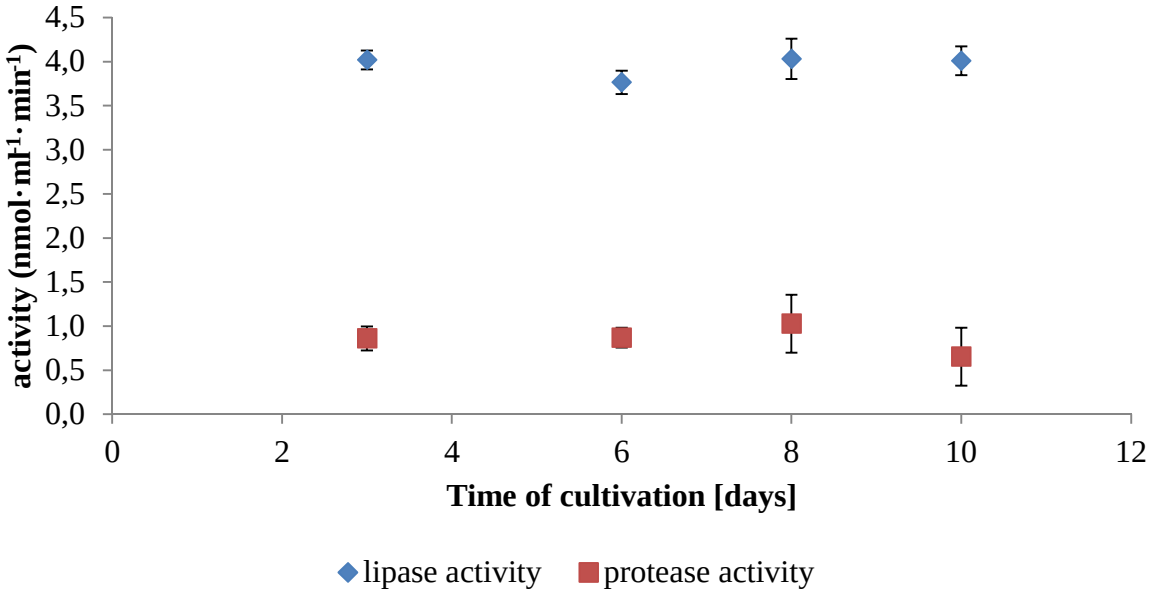
4.7.2 Content of proteins and polyphenolic compounds



Graph 11 Influence of length of cultivation on the content polyphenols and proteins

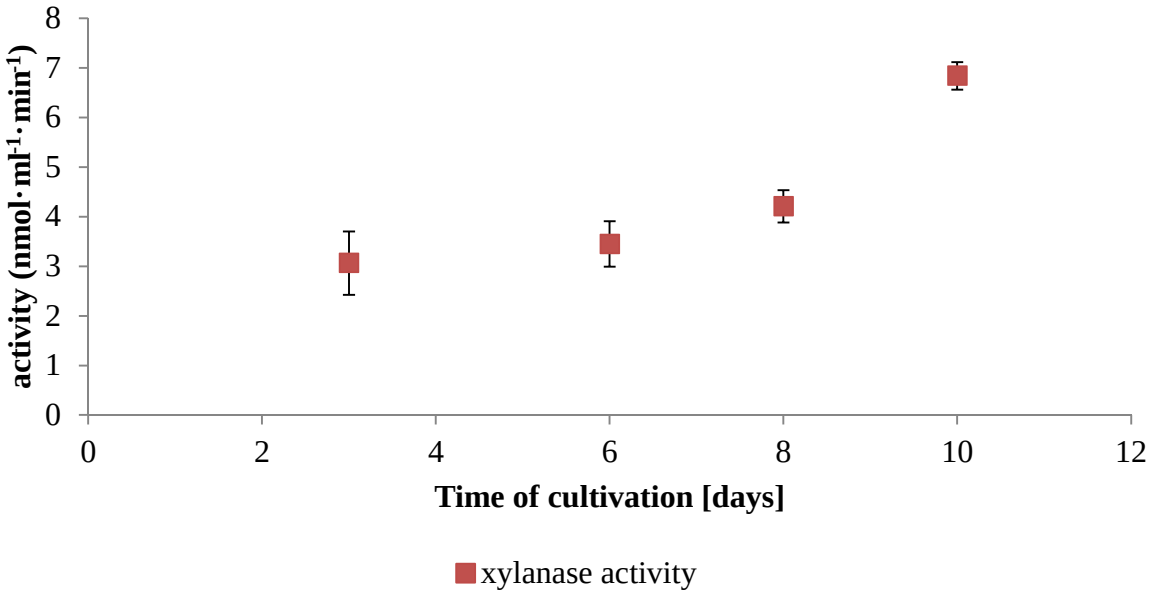
Graph 11 shows that the polyphenol as well as protein content does not change during cultivation after reaching stationary phase. The content of polyphenolic substances held steady at 0.9 mg/mL throughout the cultivation. This probably means that the bacteria can not convert polyphenols into other substances. Protein levels are maintained throughout the cultivation of about 2.8 g/L. This suggests that the bacteria do not begin to degrade proteins after reaching the stationary phase. First, it can utilize the storage polymers such as PHA and second these proteins enables bacteria to process the degradation of SCG.

4.7.3 Enzymatic assays



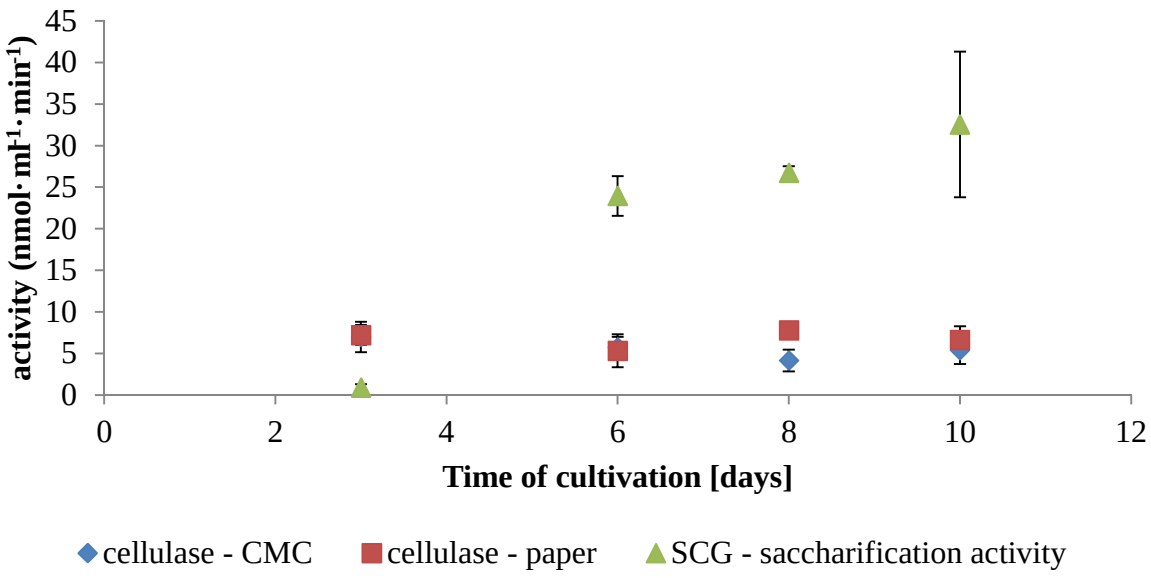
Graph 12 Influence of length of cultivation on the lipase and protease activity

Graph 12 shows that enzymatic activity of lipase and protease is stable over time. Conversely, the Graph 13 shows that the xylanase activity over time is growing steadily.



Graph 13 Influence of length of cultivation on the xylanase activity

Cellulase activity was determined as the ability to release of reducing sugars from the substrate used. The assay used three different substrates - carboxymethyl cellulose, filter paper and SCG. The results are shown in the Graph 14. While the ability to convert paper to CMC and reducing sugars in the course of the cultivation fell slightly, the ability to degrade SCG grew.



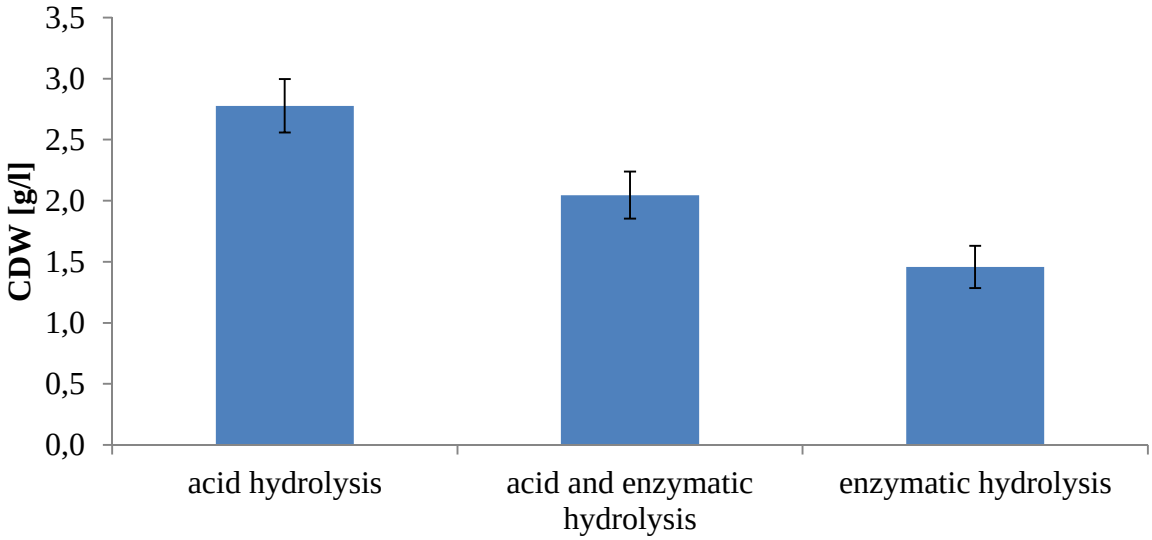
Graph 14 Influence of length of cultivation on the cellulase activity

4.8 Effect of the hydrolysis method

The aim of this experiment was to compare the influence of the method of hydrolysis – chemical hydrolysis by diluted acid, enzymatic hydrolysis by enzymatic system of *B. cepacia* and combination of diluted acid hydrolysis and enzymatic hydrolysis.

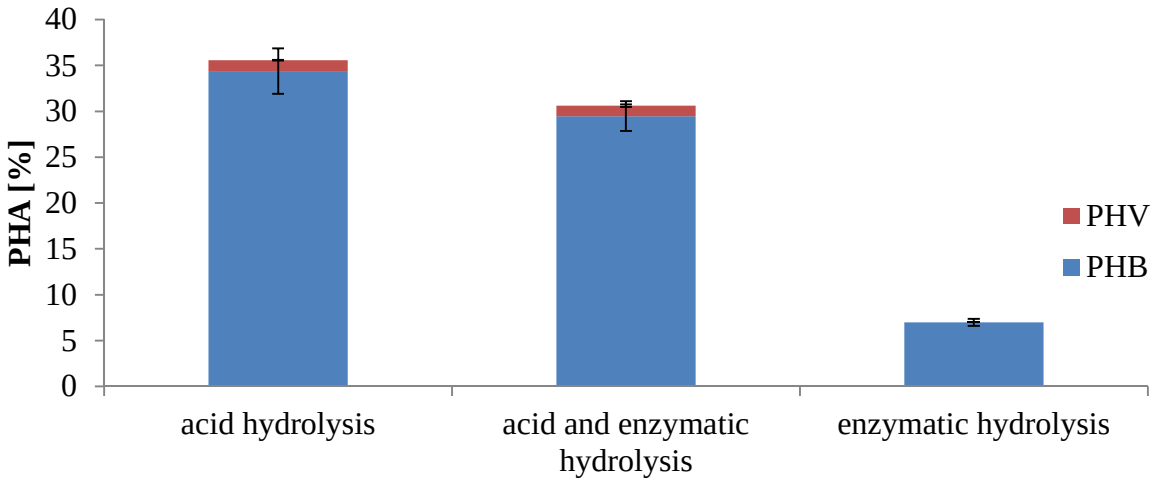
Acid hydrolysis - the effect of 1% sulfuric acid in an autoclave under the conditions, pH was adjusted and subsequent the SCG was separated (chapter 3.5). The second method, acid and enzymatic hydrolysis - the procedure was the same as for acid hydrolysis, but the SCG was left in the hydrolyzate medium during cultivation to be hydrolyzed by extracellular enzymatic system of *B. cepacia*. The last method was only enzymatic hydrolysis - acid hydrolysis was not performed. The medium was identical as described in chapter 4.6 and 4.7.

4.8.1 Effect on the yields of CDW and PHA



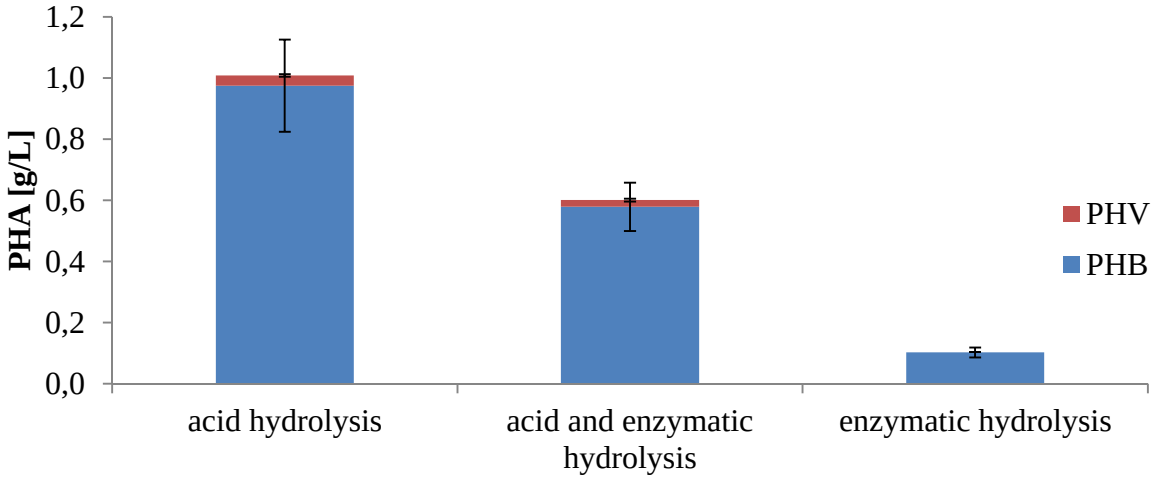
Graph 15 Effect of the hydrolysis method on CDW.

Graphs 15-17 show clearly that the highest yields of PHA as well as biomass were achieved in medium were SCG were hydrolyzed by diluted acid and removed from the medium before cultivation. There is a significant decrease in yields using a combination of acid and consequently the enzymatic hydrolysis. This is likely caused by substances which were released from SCG during cultivation. The lowest yield was observed in the medium, wherein the acid pretreatment was not performed. Simple diluted acid hydrolysis thus appears to be the best step towards achieving not only the highest CDW, but also high intracellular PHA content in the cells.



Graph 16 Effect of the hydrolysis method on content PHA in CDW

Gas chromatography of the sample identified two different types of PHA. Besides the usual PHB; 3-hydroxyvalerate was observed in the samples pretreated by diluted mineral acid. It is very likely that some component which was formed during hydrolysis of SCG by diluted mineral acids served as precursor of 3-hydroxyvalerate. From the spectrum of potential precursors it can be expected that it was levulinic acid – product of degradation of hexoses, which enabled accumulation of PHBV copolymer. However, the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate has better material properties than homopolymer PHB. Its advantage would consist mainly of greater elongation properties.

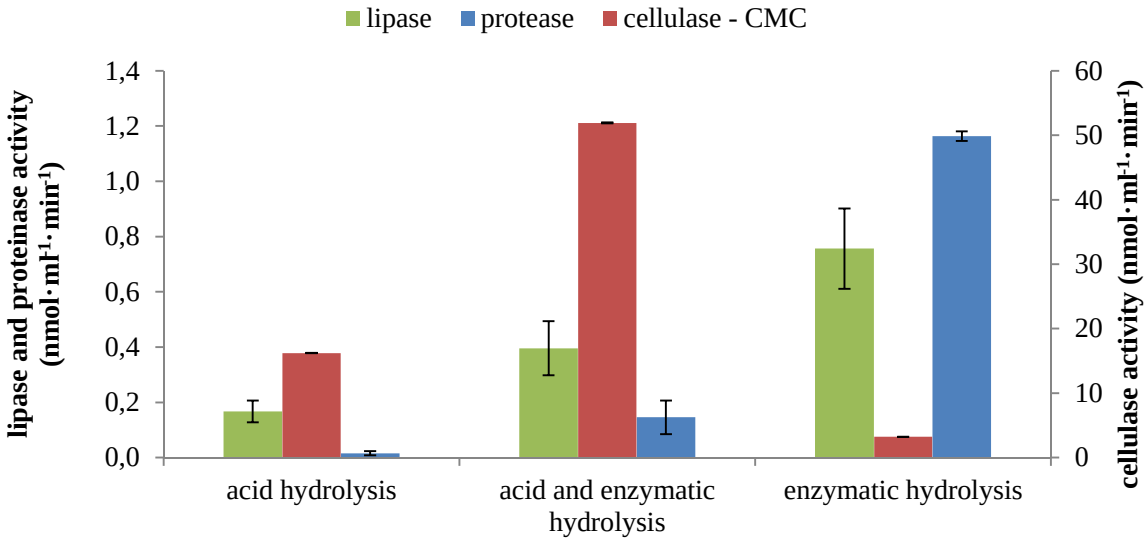


Graph 17 Effect of the hydrolysis method on content PHA in media

4.8.2 Effect on the enzymatic activities

Graph 18 shows effect of the hydrolysis method on the activities of selected biotechnologically important extracellular enzymes of *B. cepacia*. In general, we can say that the enzymatic activity was lowest in the sample, where the medium did not contain solids of SCG. The reason is obvious; the bacteria did not need to produce lignocellulose degrading enzymes. This hypothesis explains the highest biomass yield, the bacteria that can fully "focus" on the production of PHA as a storage polysaccharide utilizing simple sugars.

Furthermore, it is apparent that in the medium after acid hydrolysis with solid SCG, is much greater cellulase activity than in the last sample. Hydrolysis by diluted acid is able to cleavage hemicelluloses but not highly resistant cellulose. On the other side, hydrolysis of hemicelluloses made cellulose much more accessible to action of enzymatic hydrolysis. This may explain why the highest cellulose activity was observed in the sample where SCG were exposed to acid hydrolysis. On the contrary, when SCG was not hydrolyzed prior to cultivation, bacterial culture was able to excrete the highest amount of lipase a protease, which, as has been stated above, represent important industrial enzymes. Therefore, the pretreatment of SCG can be used as a tool to control spectrum of the products of the intended technology. Acid hydrolysis with subsequent removal of SCG prior to cultivation results in the highest PHB yields. On the other side, when SCG are leaved in the cultivation, bacterial culture produces also high amount of PHA, but also high amount of extracellular enzymes, in particular, production of cellulose is enhanced significantly. Finally, when SCG are not hydrolyzed by diluted acid, bacterial culture accumulates only low amount of PHB (up to 10 % of CDW) but it is capable to produce sustainable amount of extracellular hydrolytic enzymes – especially lipase and protease.



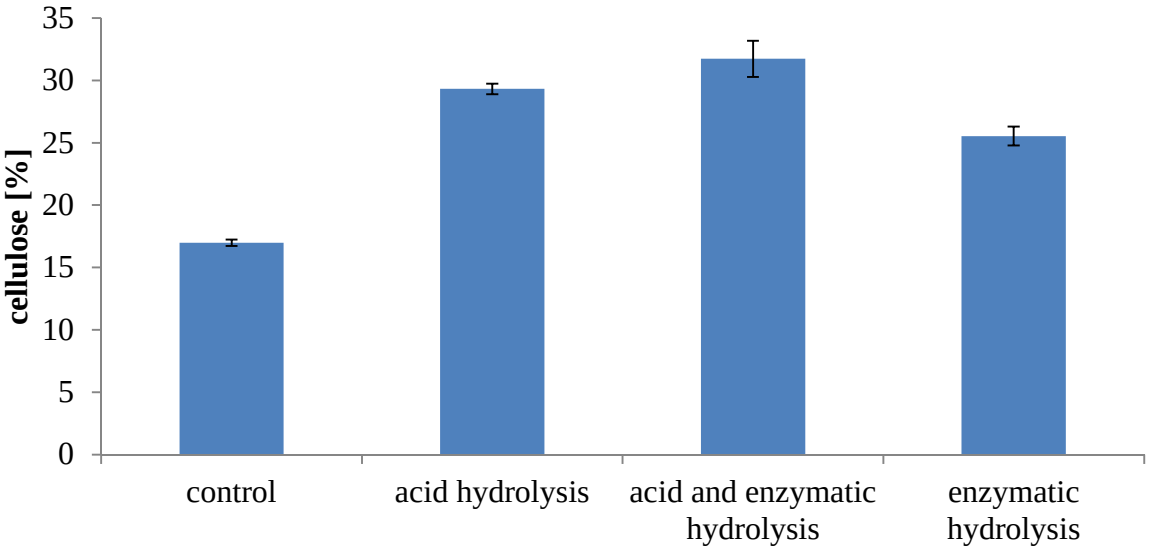
Graph 18 Effect of the hydrolysis method on the activities of selected extracellular enzymes

4.8.3 Effect on the composition SCG

The following chapter continues the previous experiment. SCG was separated by filtration. Subsequently, the cellulose was determined and the individual samples were subjected to TGA analysis.

4.8.3.1 Cellulose content

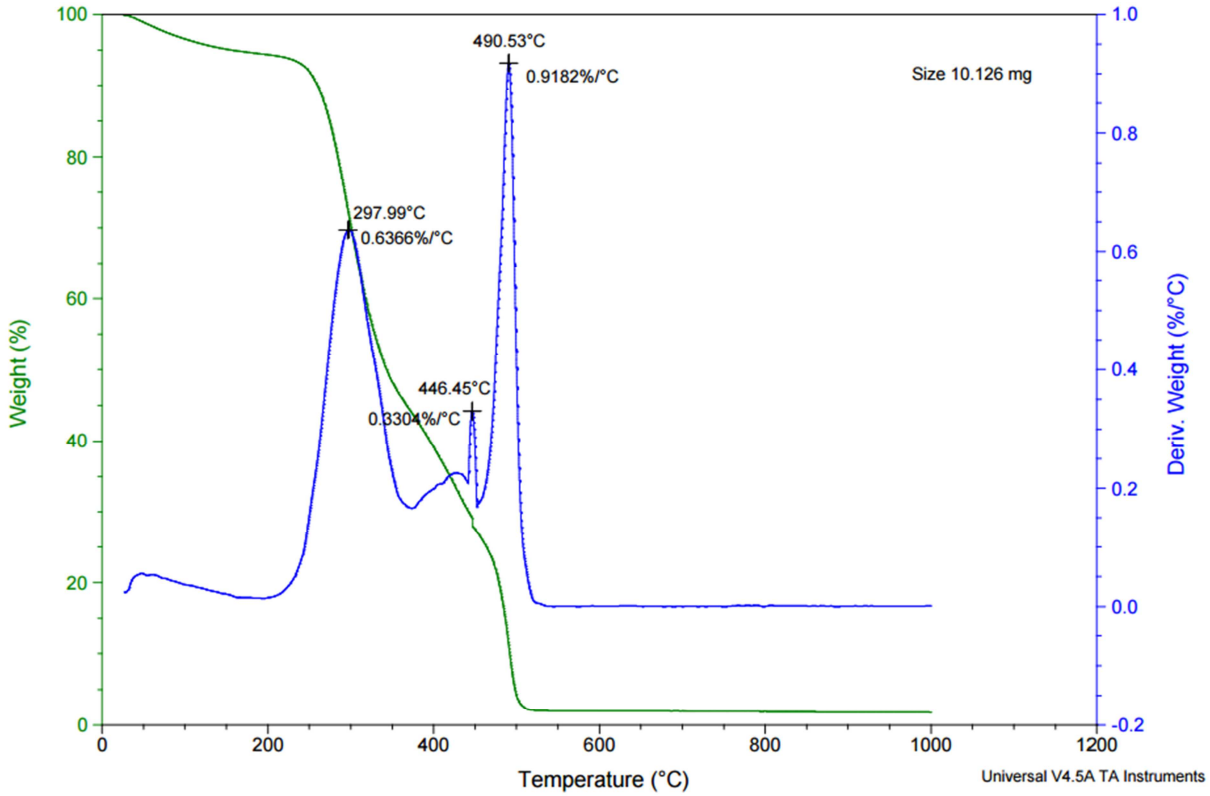
Graph 19 shows content of cellulose in individual pretreated samples. The lowest cellulose content is in control (untreated) sample - about 17.0 %. Conversely, the largest content is in the samples pretreated by acid and enzyme - 31.7%. The data show a growing amount of pretreatment increases cellulose content. It can be concluded that the applied methods of hydrolysis degrade particularly substances other than cellulose.



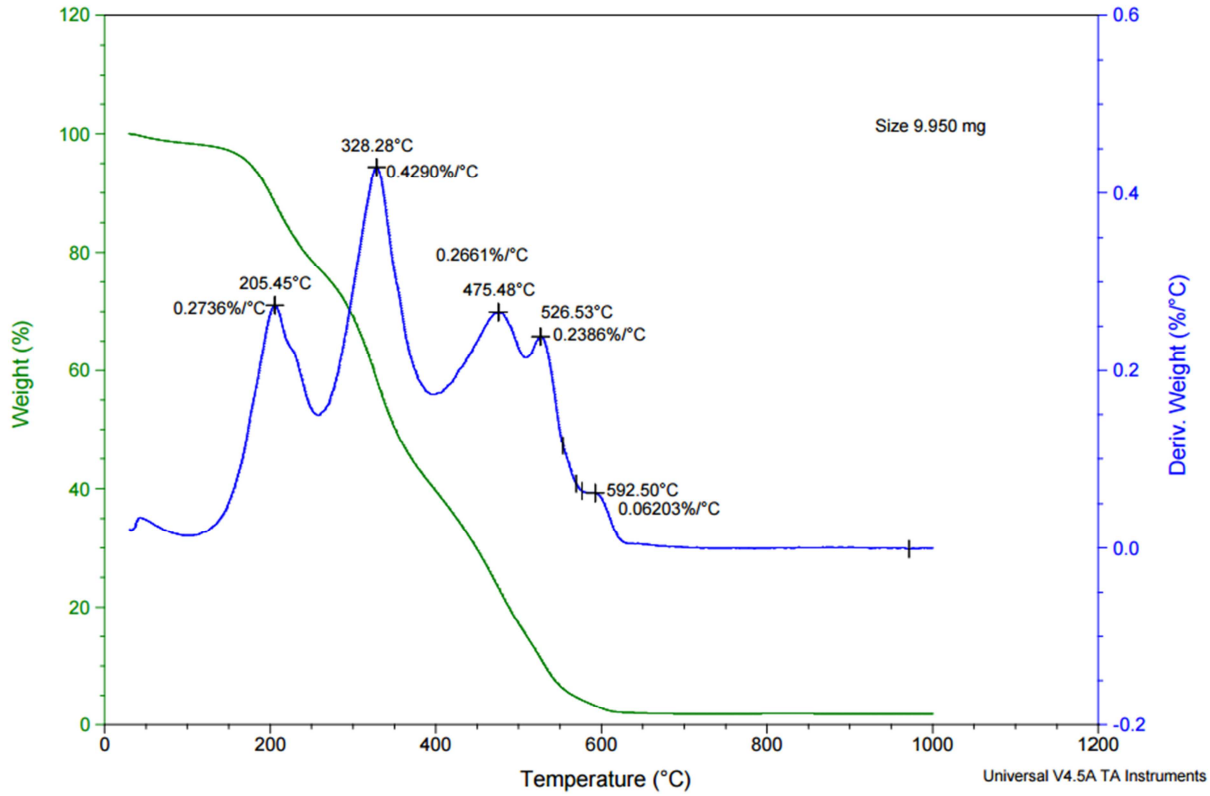
Graph 19 Effect of the hydrolysis method on the cellulose content in SCG

4.8.3.2 TGA analysis

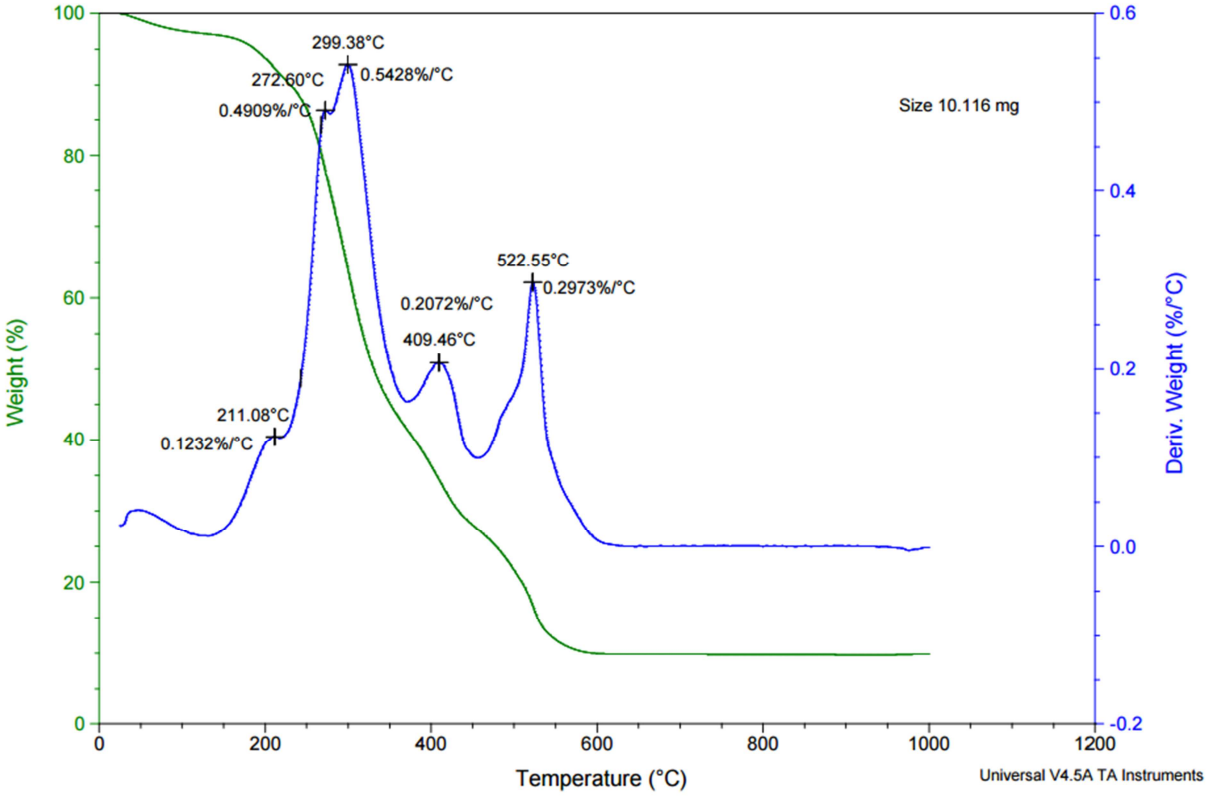
We performed TGA analysis of the SCG to estimate changes caused by their hydrolysis using diluted mineral acid, enzymatic system of *B. cepacia* or their combination. In general, the TGA profile of none-pretreated SCG and SCG hydrolyzed only by enzymes of *B. cepacia* seems to be almost identical, which indicates only negligible influence of enzymes on chemical composition of SCG. However, when SCG are exposed to hydrolysis by diluted mineral acid, their TGA profile changes considerabely. It can be assumed that the first peak in TGA profile (temperature of decomposition about 297 °C) can be attributed to hemicelluloses and cellulose. When the material is hydrolysed only by mineral acid, the peak is decreased significantly and devided into two peaks with lower intensity. This may be caused by hydrolysis of hemicellulose and partial degradation of cellulose. Further, it is interesting that also the most stable components of SCG (peak corresponding to degradation at about 490 °C) is influenced by mineral acid treatment. I can be expected that this peak represent lignin. The fact that lignin is degraded by diluted mineral acid can expleain presence of high amount of phenolic compound in SCG hydrolyzate.



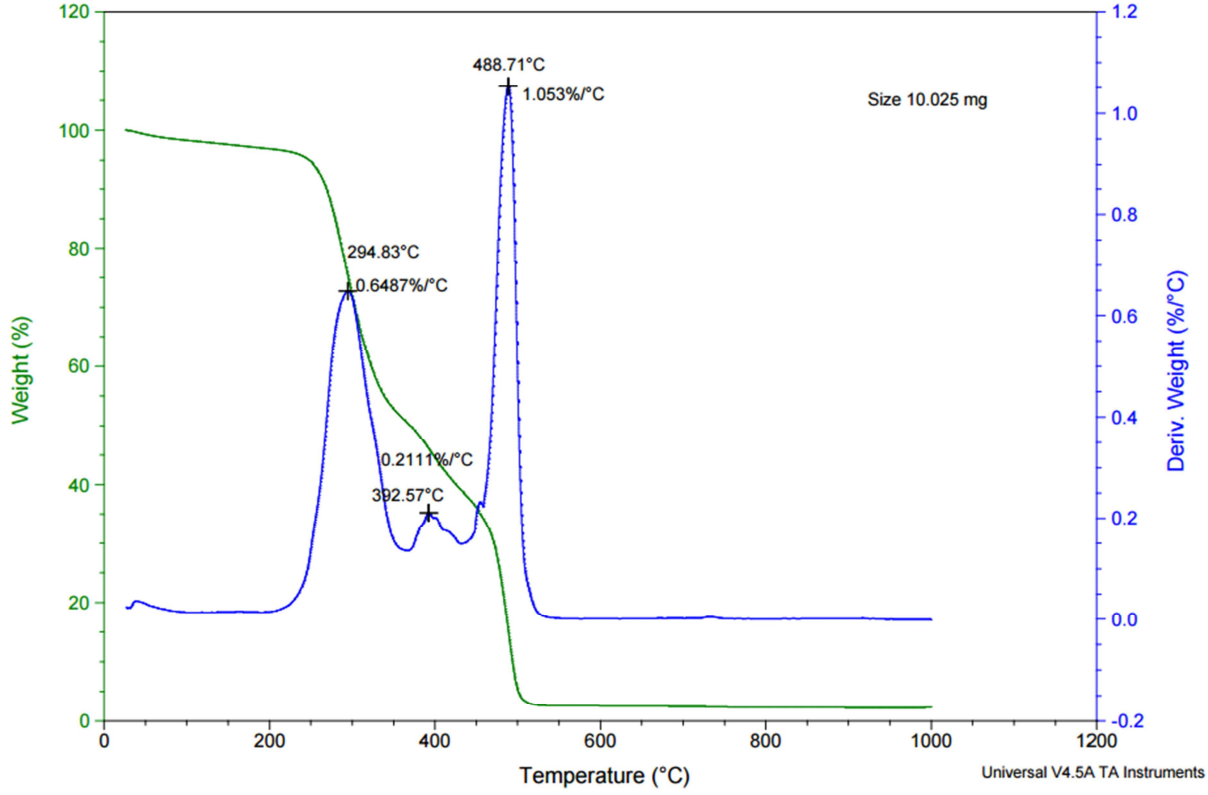
Graph 20 TGA analysis SCG without pretreatment



Graph 21 TGA analysis SCG after acid pretreatment



Graph 22 TGA analysis SCG after acid and enzymatic pretreatment

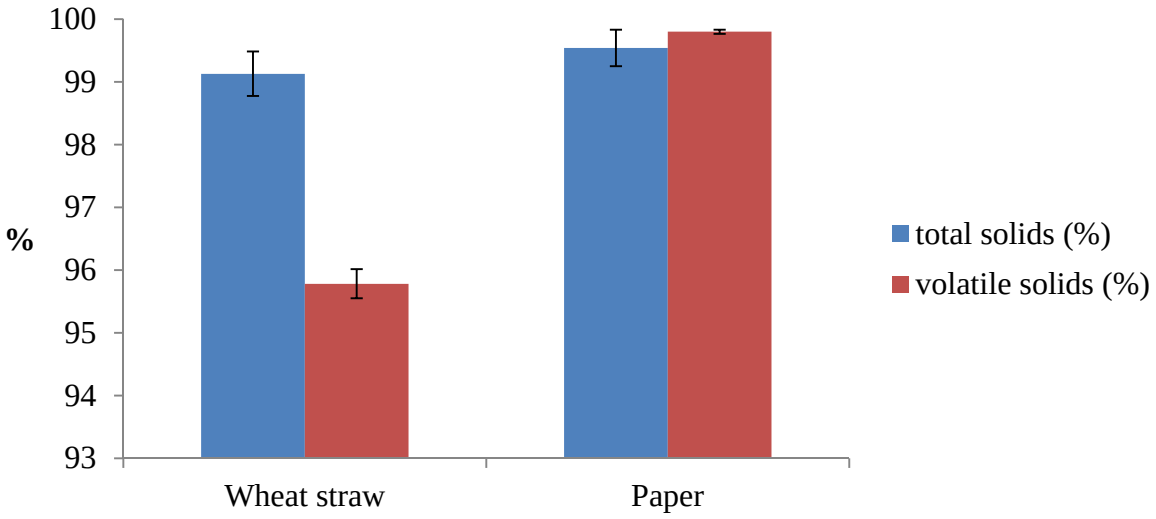


Graph 23 TGA analysis SCG after enzymatic pretreatment

4.9 Saccharification of wheat straw

4.9.1 Determination of total and volatile solids

Wheat straw and filter paper used as a carbon source in the media were characterized for content TS and VS. TS are the amount of solids remaining after all volatile matter has been removed from a biomass sample by heating at 105 °C to constant weight. Volatile solids are those solids that are lost on ignition of dry solids at 550 °C. Wheat straw contained 99.1 % of total solid and 95.8 % volatile solids. Filter paper contained 99.5 % of total solid and 99.8 % volatile solids.



Graph 24 Characteristics of wheat straw and filter paper

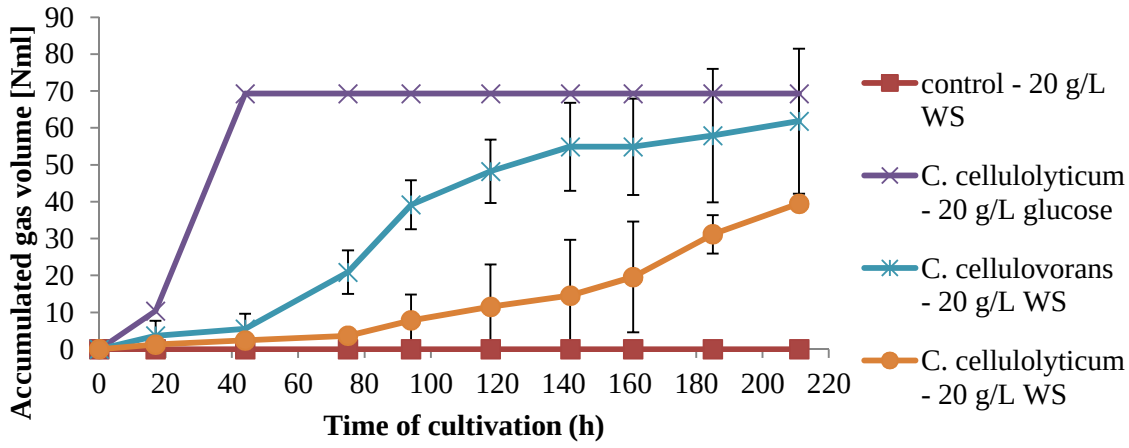
4.9.2 Saccharification in Automatic Methane Potential Test System (AMPTS II)

Strain *Clostridium cellulovorans* and *Clostridium cellulolyticum* were incubated in 37 °C in SRSM medium with 6 g/L cellobiose as a carbon source. These cultures were used as preinoculum for following saccharification.

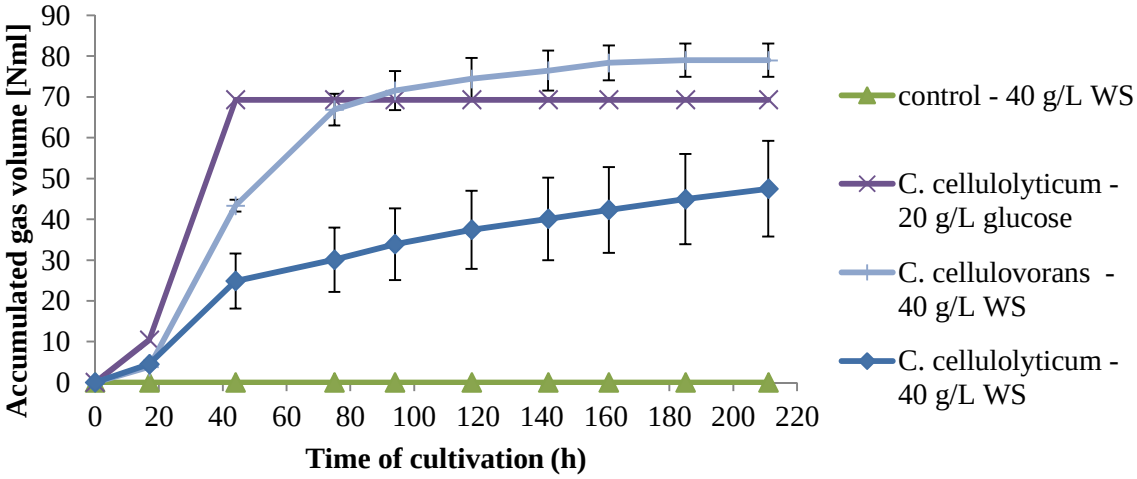
Prepared SRSM medium with 40 and 20 g/L of wheat straw were inoculated (5 % v/v) by culture and tested using AMPTS II, which was modified for real-time measurement of all accumulated gas. Non-inoculated medium and medium with 20 g/L of glucose were used as controls. Samples were taken approximately every 24 hours to measure the OD600 and the carbohydrate content in the supernatant. Furthermore, we also analyzed accumulated gas, and the CO_2 content by GC-TCD.

4.9.2.1 Real-time measurement of accumulated gas

Graphs 25 and 26 shows that bacteria in medium with simple carbon source can start to grow very quickly. Strain *C. cellulovorans* accumulated more gas in both concentrations. Shorter lag phase was achieved at higher concentrations of wheat straw.



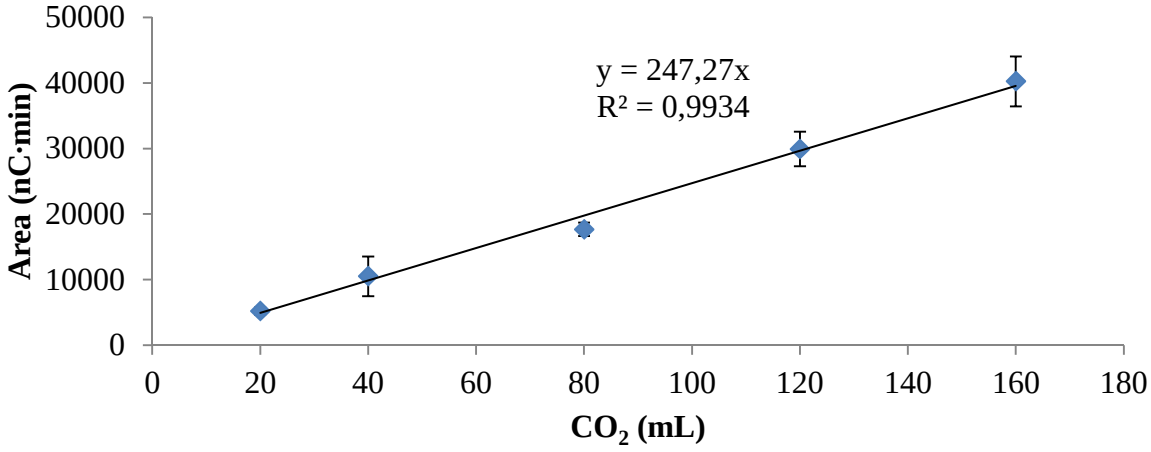
Graph 25 Time course of gas production by C. cellulovorans 3052 and C. cellulolyticum 5812 on SRSM media with 20 g/L wheat straw as a carbon source



Graph 26 Time course of gas production by C. cellulovorans 3052 and C. cellulolyticum 5812 on SRSM media with 40 g/L wheat straw as a carbon source

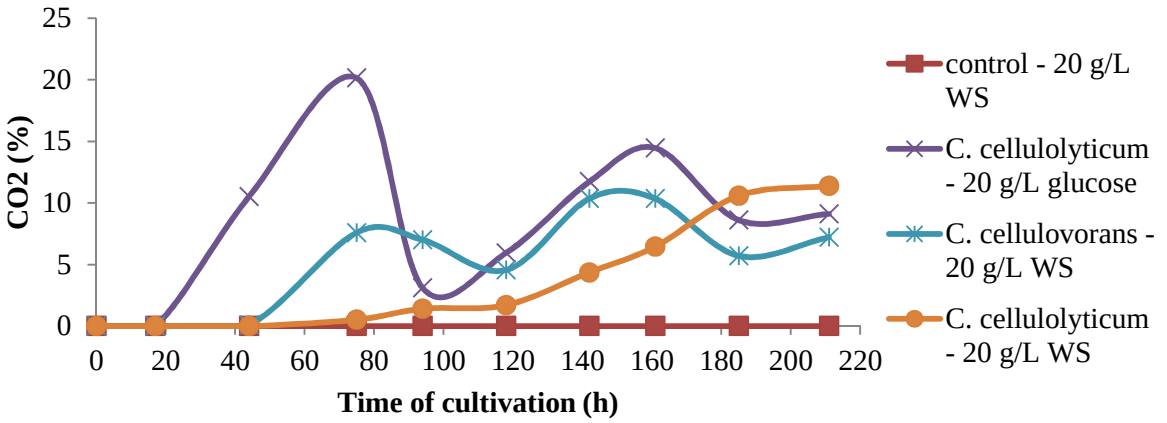
4.9.2.2 Carbon dioxide content in accumulated gas

To determine the CO_2 concentration was necessary to establish a standard curve.

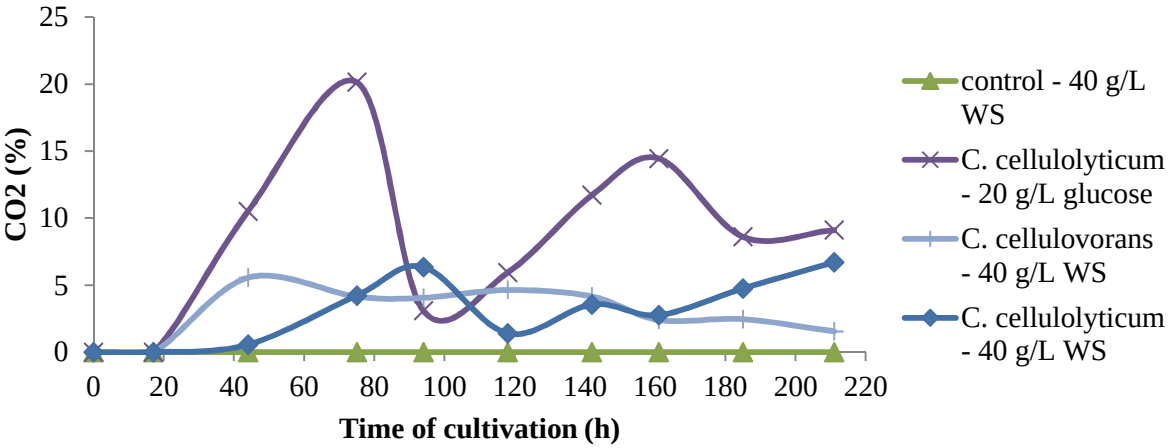


Graph 27 Calibration curve of carbon dioxide on GC-TCD

Graph of the CO_2 content in accumulated gas has very similar course as amount of accumulated gas and optical density. The courses differ by large decline after reaching stationarity of the growth. This decrease is due to the lack of energy and subsequent inhibition of metabolism. Metabolic activation then occurs after the release of further simple sugars from lignocellulosic material.



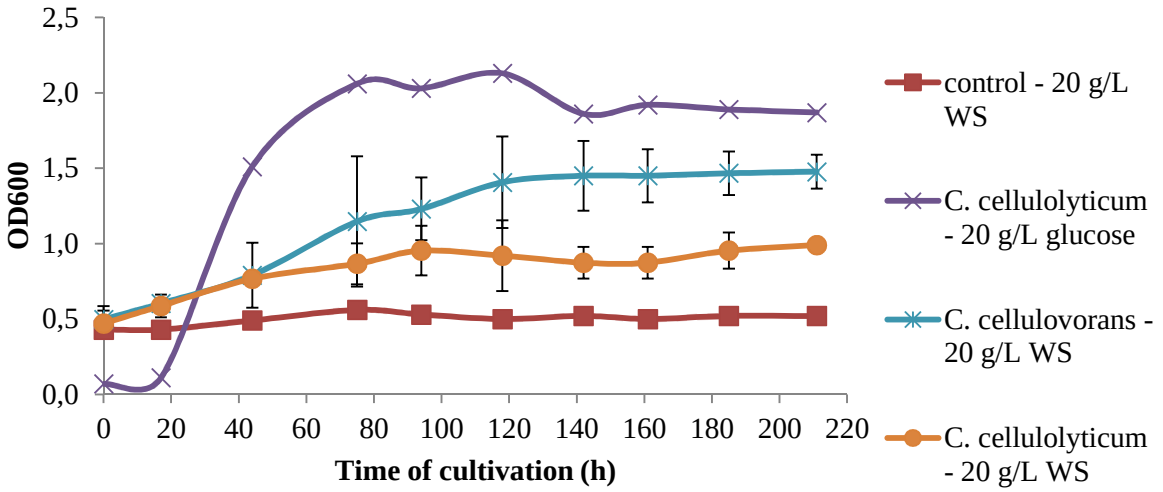
Graph 28 Time course of CO_2 content in accumulated gas by C. cellulovorans 3052 and C. cellulolyticum 5812 on SRSM media with 20 g/L wheat straw as a carbon source



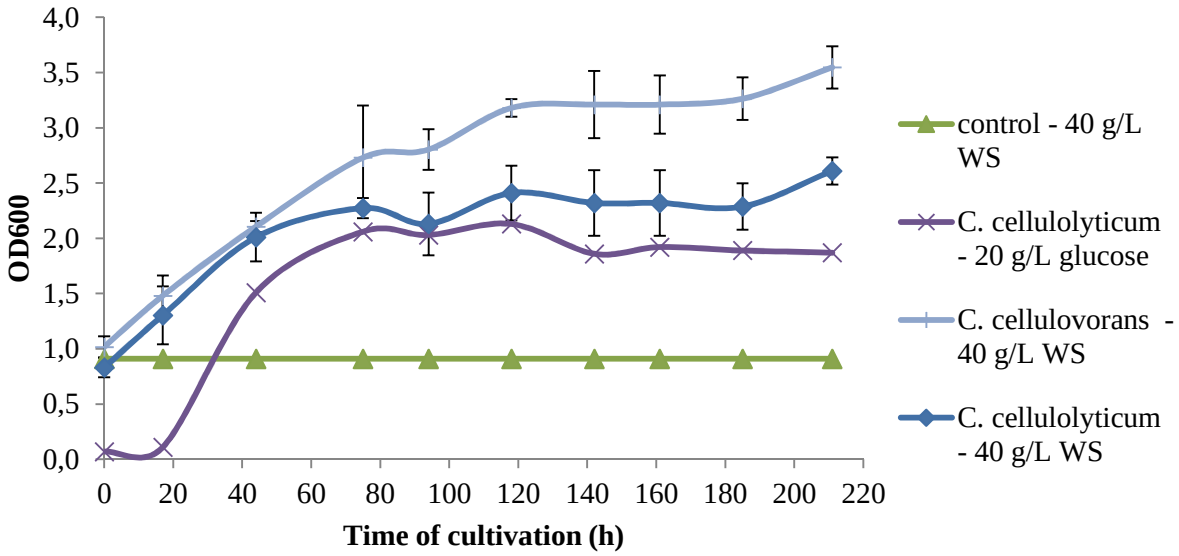
Graph 29 Time course of CO_2 content in accumulated gas by C. cellulovorans 3052 and C. cellulolyticum 5812 on SRSM media with 40 g/L wheat straw as a carbon source

4.9.3 Measurement of optical density at 600 nm (OD600)

The following graph shows that using a higher concentration of substrate will drastically shorten the time between the beginning of cultivation and reaching the stationary phase, from 120 hours to 80 hours. Strain *C. cellulovorans* showed diauxic growth after 80 hours. Likely reason is the change of metabolism on utilization of cleavage products of wheat straw. This subsequently causes a decrease content of released carbohydrates in the supernatant.



Graph 30 Time course of OD600 SRSM media with 20 g/L wheat straw as a carbon source

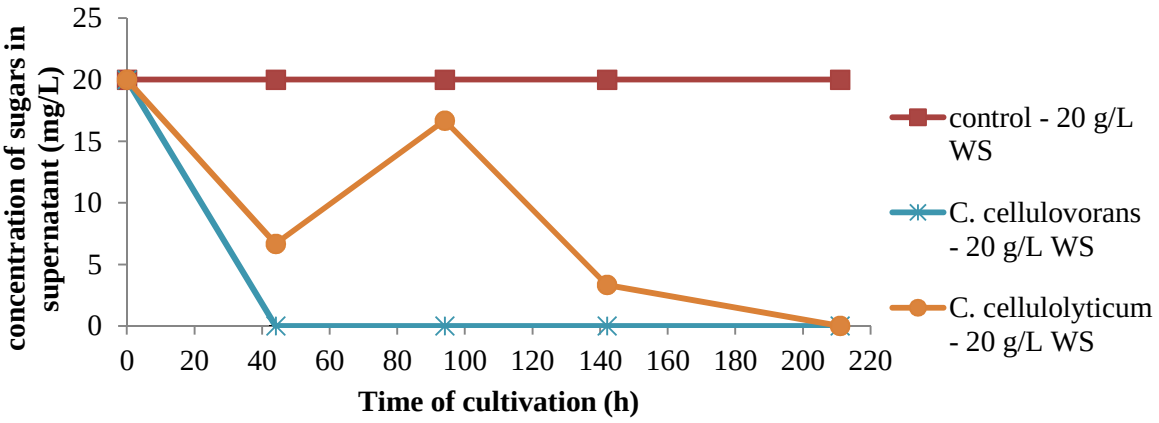


Graph 31 Time course of OD600 SRSM media with 40 g/L wheat straw as a carbon source

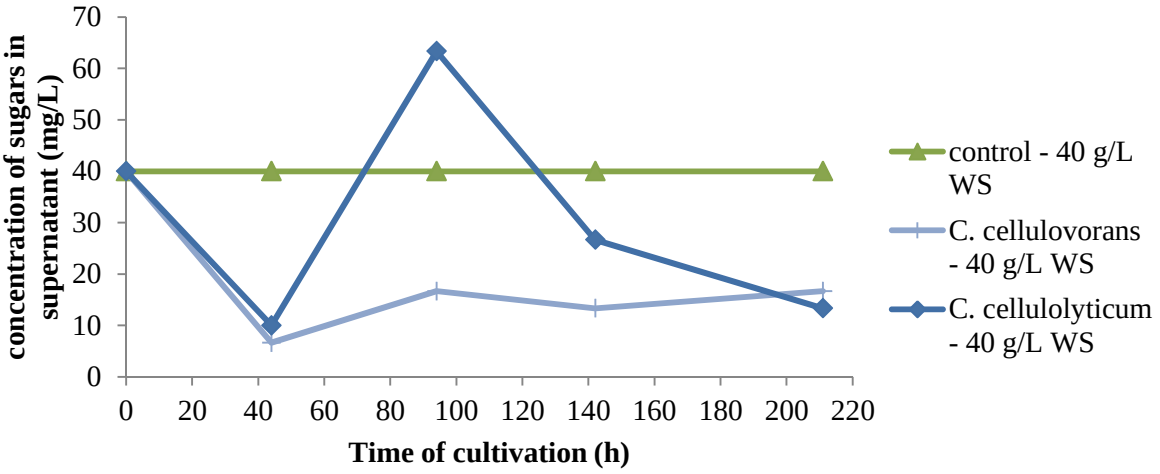
4.9.4 Measurement of sugar content in supernatant

Supernatant was separated from pellets by centrifugation (13,000 rpm; 10 min). Sugar content was measured by Dionex - high-performance anion-exchange chromatography. Standard curves are attached as supplements (8.1).

Graphs 32 and 33 shows the employed cultures are not able to release significant amount of carbohydrates. In medium containing 20 g/l wheat straw, bacterial strains did not release more simple carbohydrate than was contained at the outset. Nevertheless, *C. cellulolyticum* stood the test better than *C. cellulovorans*. Maximum amount of carbohydrate was measured after 100 hours. The analysis also found that the released carbohydrates were mainly mannose, arabinose and galactose. These carbohydrates are typical cleavage products of hemicelluloses.



Graph 32 Time course of sugar content in supernatant of SRSM media with 20 g/L wheat straw as a carbon source



Graph 33 Time course of sugar content in supernatant of SRSM media with 40 g/L wheat straw as a carbon source

The substrate may be made accessible for the activity of enzymes using diluted acid hydrolysis as described in the previous chapter. However, each step can reduce the effectiveness and thus increase the financial cost of pretreatment of lignocellulosic material. Therefore, the conclusion is the saccharification of wheat straw by *C. cellulovorans* and *C. cellulolyticum* does not benefit when processing wheat straw for PHA production.

5 CONCLUSIONS

- In the context of this thesis, the aim was to test the possibility of using selected waste lignocellulosic materials (spent coffee grounds, apple pomace, rapeseed pomace, and wine pomace) for the production of PHA. Lignocellulosic materials were hydrolyzed by diluted mineral acid and the hydrolysates were used as the main component of the production medium. The selected bacterial strains and their ability to accumulate PHA was described in the literature - *Burkholderia cepacia* and *Bacillus megaterium* were subsequently employed for the production of PHA.
- Bacterial strains were compared in two parameters at the end of cultivation - biomass yields and PHA content. *Burkholderia cepacia* seems to be better producer in both aspects. For this reason, only this strain was used for the production PHA in further experiments.
- HPLC analysis of the individual hydrolysates showed that the highest content of monosaccharide was present in the hydrolyzate of apple pomace (19.1 g/L), which also provided the best production media. Spent coffee grounds, rapeseed pomace and wine pomace contained 14.4, 11.0 and 13.8 g/L respectively.
- The highest biomass (5.0 g/L) and PHA yields (34.1 %) as well as PHA content (1.7 g/L) was obtained on hydrolyzate of apple pomace, which seems to be the most promising substrate for biotechnological production of PHA among the tested lignocellosic materials.
- As a further method of processing of the raw lignocellulosic material, hydrolysis by commercial enzymes was tested. The experiment was carried out with apple pomace. The highest degree of hydrolysis was achieved using a combination of commercial cellulases, pectinases and hemicellulase of *A. niger* - 62% of the original weight of the substrate was released into the hydrolyzate as reducing sugars.
- A production medium was subsequently prepared from the enzymatic hydrolysates. The highest yield was achieved on hydrolyzate obtained by treatment with a combination of cellulase and pectinase – 67 % of CDW and a resulting yield 2.3 g of PHA per liter of medium.
- It was found that *Burkholderia cepacia* can utilize lignocellulosic material without prior hydrolysis and simultaneously accumulate PHA. Considering the price of the hydrolysis process, it is a finding which could significantly reduce the cost of production of PHA. Furthermore, extracellular hydrolytic enzymes can be considered interesting side products of the technology.
- A secondary task of the thesis was to study enzyme activities in the supernatant after cultivation. The objective was to determine whether the extracellular hydrolytic enzymes could be produced simultaneously with the production of PHA. The supernatant exhibited positive response for following activities: protease, lipase, CMC and filter paper cellulase and xylanase.
- SDS-PAGE revealed four proteins of approximately 60 kDa, 44 kDa, 40 kDa and 30 kDa in the supernatant.

- The KrosFlo® is a very effective ultrafiltration system for purification and preconcentration of extracellular proteins.
- In further experiments, the goal was to monitor the influence of the length of culturing on PHA production and enzymatic activities in the supernatant. The content of dry biomass and PHA contained in it started to decrease slightly after reaching stationary phase after 72 hours. Enzymatic assays showed no significant changes in enzymatic activities of proteases, lipases and cellulases. However, there was a significant increase in xylanase activity as well as an increase in its ability to release reducing sugars from untreated spent coffee grounds. The amount of extracellular proteins and polyphenols in the supernatant did not change during the time course of cultivation.
- Another part of the experiment then focused on comparing the three methods for the production PHA from SCG. The highest yield of PHA production was obtained from hydrolyzate pretreated by mineral acid. Using this method, however, the production of extracellular enzymes achieved the lowest values. Conversely, the lowest yield of PHA but the highest enzyme activities were observed during production directly from untreated SCG, where hydrolysis was provided by the hydrolytic enzyme system of *Burkholderia cepacia*. In the third method, where the SCG was hydrolyzed by acid and then by extracellular enzyme, the yields of PHA and selected enzymes were average, but cellulase activity was unexpectedly high.
- Hydrolysis by diluted acid is able to cleave hemicelluloses but not highly resistant cellulose. On the other hand, hydrolysis of hemicelluloses made cellulose much more accessible to enzymatic hydrolysis. This may explain why a high cellulose activity was observed in samples pretreated by diluted mineral acid.
- The analysis of the content of cellulose in SCG after each method of hydrolysis also showed that the treatment increased cellulose content, i.e. physicochemical exposure degraded mainly other than cellulose component of the substrate. The used hydrolytic procedures degraded probably mainly hemicellulose. Another method used to detect changes in the composition of SCG before and after hydrolysis was thermogravimetric analysis

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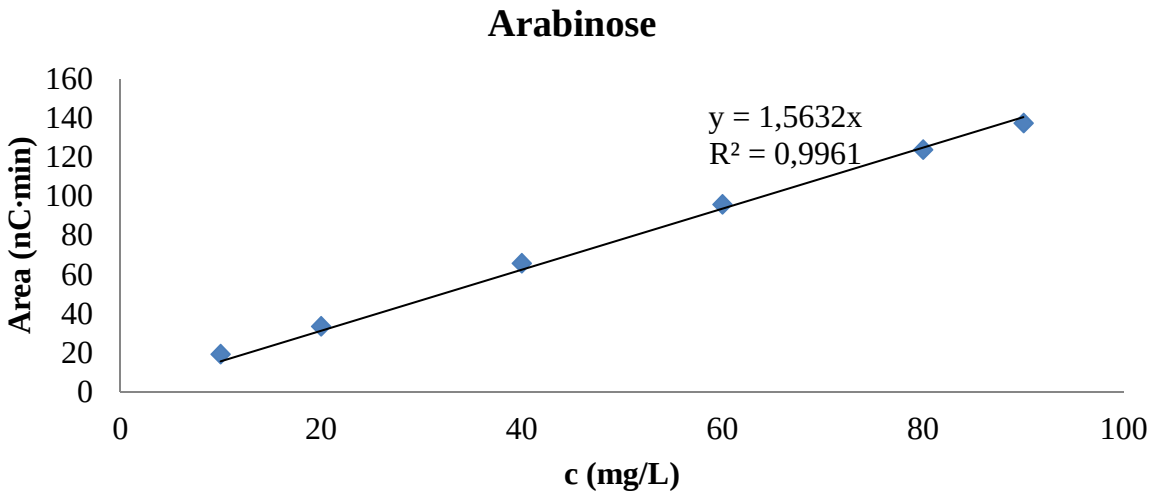
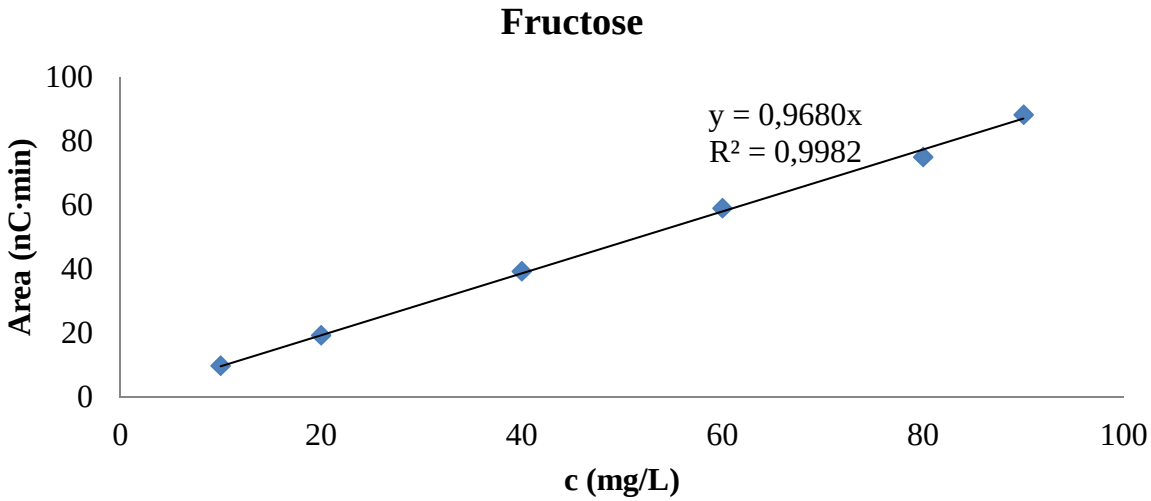
7 LIST OF ABBREVIANTIONS

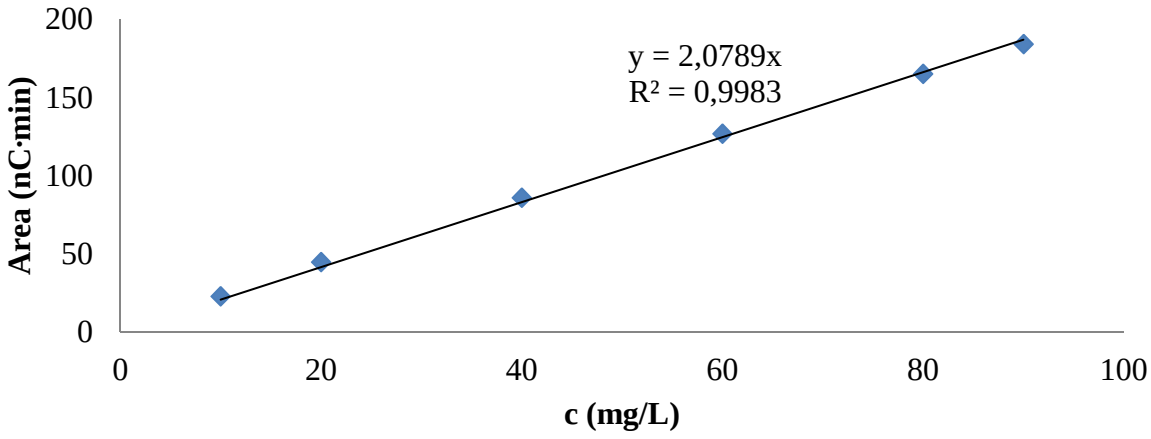
3HAs	3-hydroxyalkanoates
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
3HHD	3-hydroxyhexadecanoate
3HOD	3-hydroxyoctadecanoate
A	absorbance
AA	acrylamide
APS	ammonium persulfate
CBH	cellobiohydrolases
CCM	Czech collection of Microorganisms
CDW	cell dry weight
CMC	carboxymethyl cellulose
CoA	coenzyme A
DO	dissolved oxygen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
FID	flame ionization detector
GalA	D-galacturonic acid
HPAEC	high-performance anion exchange chromatography
HPLC	high-performance liquid chromatography
LCL	longchain-length
MCL	mediumchain-length
MnP	manganese peroxidase
NB	Nutrient Broth
PSS	pea-shells slurry
PHB	polyhydroxybutyrate
PHV	polyhydroxyvalerate
PAD	pusled amperiometric detection
PAGE	polyacrylamide gel electrophoresis
PHAs	polyhydroxyalkanoates
PhaC	PHA synthase
PhaP	phasins
SCG	Spent coffee grounds
SCL	shortchain-length
SDS	sodium dodecyl sulfate
TEMED	tetramethylethylenediamine
TNb	total nitrogen bonded
TOC	total organic carbon
Tris	tris(hydroxymethyl)aminomethane
TS	total solids
VFA	volatile fatty acids
VS	volatile solids

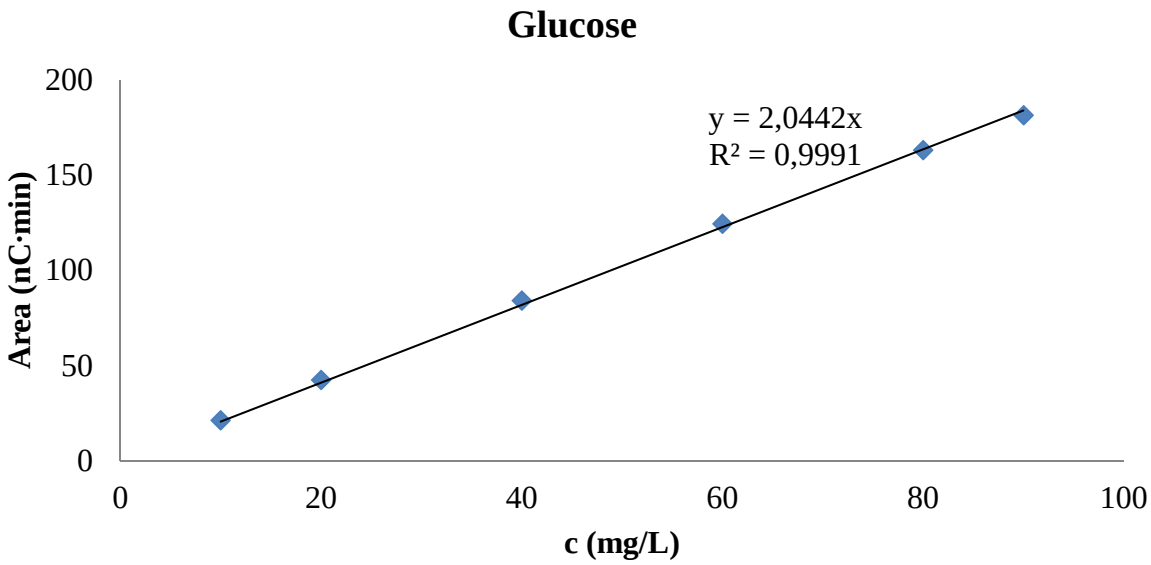
8 SUPPLEMENTS

8.1 Standard curves for HPLC








Glucose
y = 2,0442x
R² = 0,9991
Area (nC·min)
c (mg/L)
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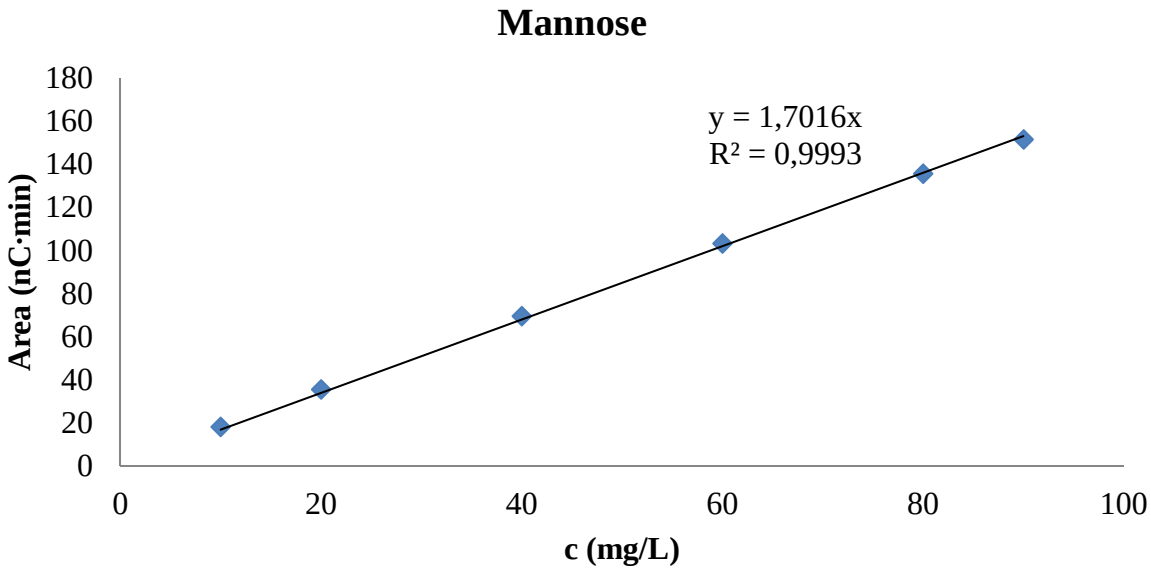
50
100
150
200
0
20
40
60
80
100


Mannose
y = 1,7016x
R² = 0,9993
Area (nC·min)
c (mg/L)
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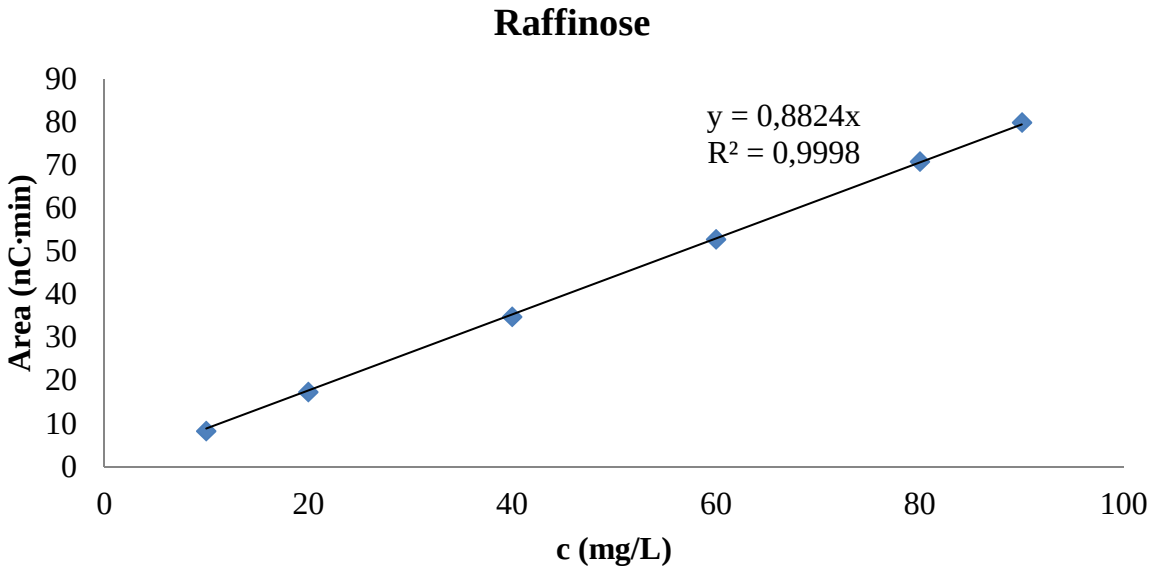
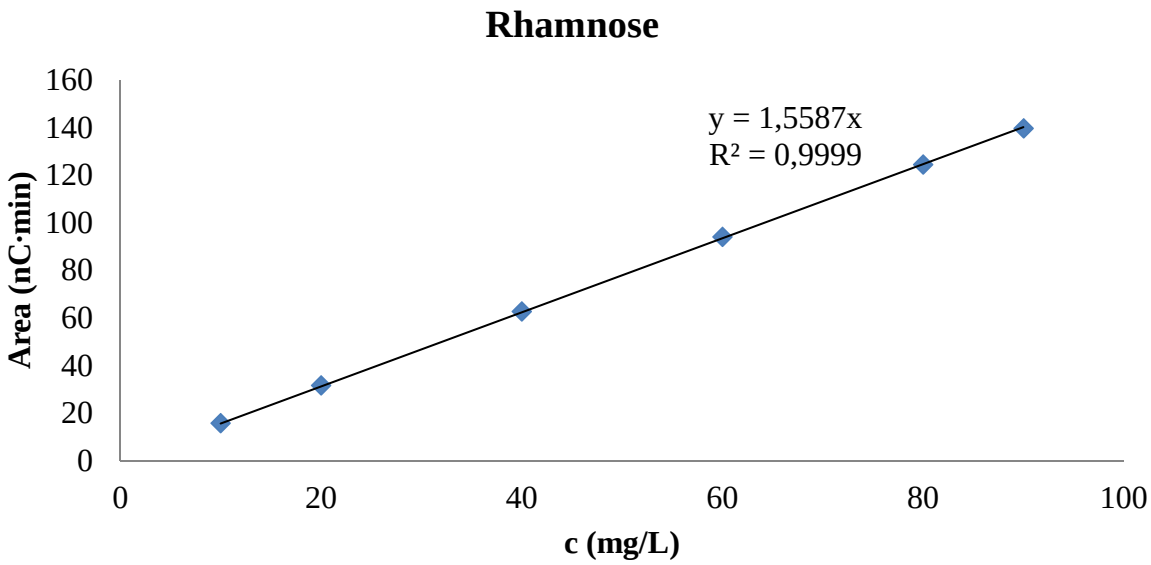
20
40
60
80
100
120
140
160
180
0
20
40
60
80
100


Raffinose
y = 0,8824x
R² = 0,9998
Area (nC·min)
c (mg/L)
0
10
20
30
40
50
60
70
80
90
0
20
40
60
80
100


Rhamnose
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R² = 0,9999
Area (nC·min)
c (mg/L)
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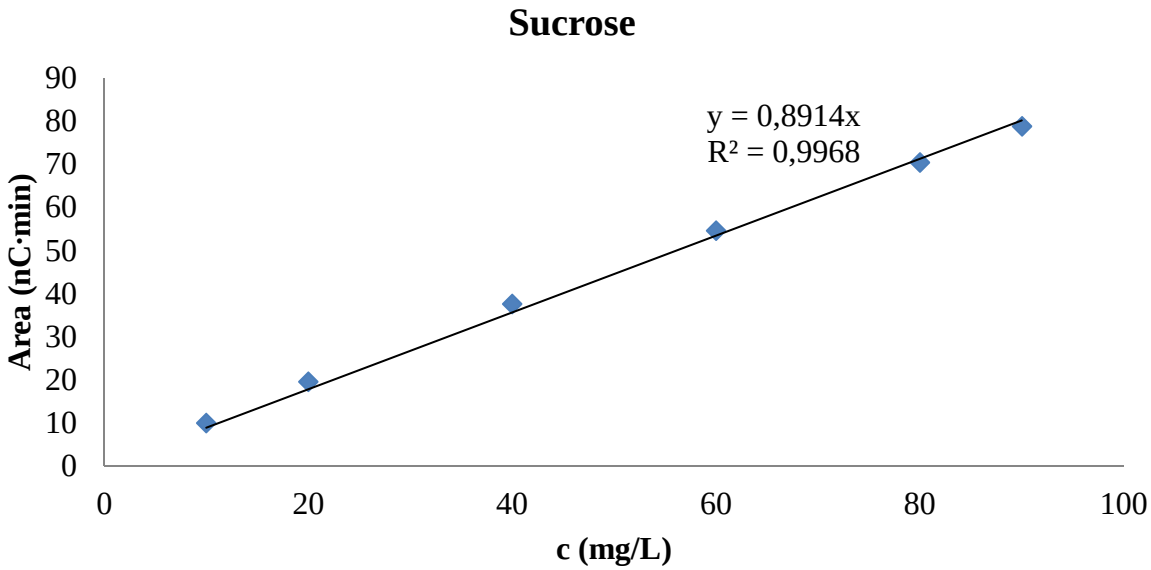
20
40
60
80
100
120
140
160


Sucrose
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R² = 0,9968
Area (nC·min)
c (mg/L)
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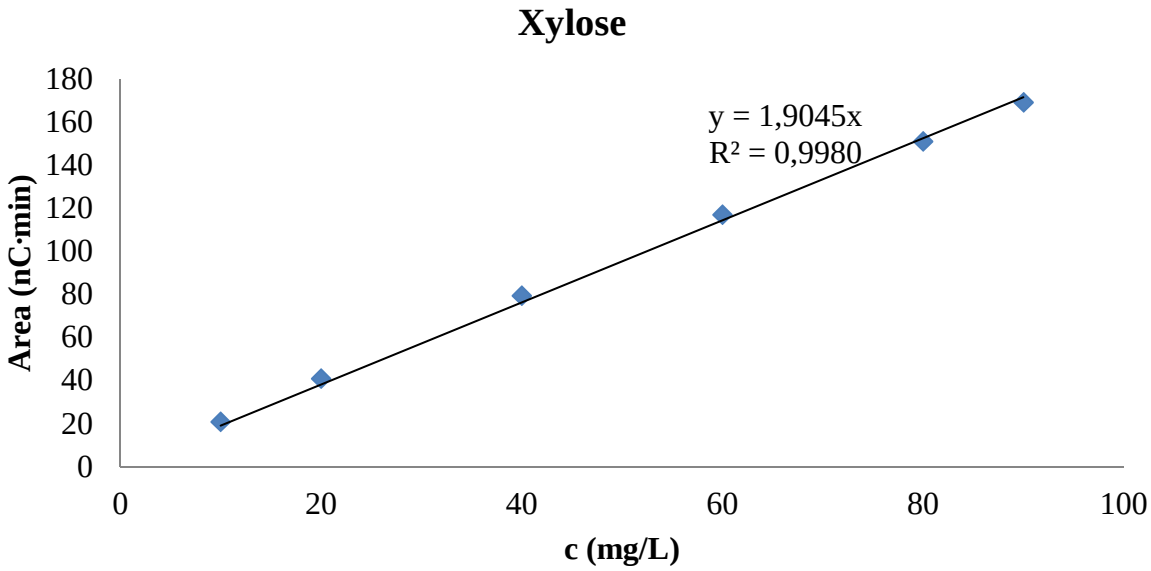
20
30
40
50
60
70
80
90
100


Xylose
y = 1,9045x
R² = 0,9980
Area (nC·min)
c (mg/L)
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20
40
60
80
100
120
140
160
180