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FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

Zagreb, October 2016 Dina Kačar, 627/MB

BIOETHANOL PRODUCTION FROM LIGNOCELLULOSE WASTE USING FUNGAL CO-CULTURES

Experimental part of Graduate thesis was done at the Department of Food and Environmental Sciences, Division Microbiology and Biotechnology, University of Helsinki under the supervision of PhD Taina Lundell, University Lecturer, Docent, and the assistance of Hans Mattila, PhD student.

Theoretical part of Graduate thesis was done in the Laboratory of Biochemical Engineering, Industrial Microbiology, Malting and Brewing Technology at the Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb under the supervision of Anita Slavica, Associate Professor, University of Zagreb.

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Proizvodnja bioetanola iz lignoceluloznog otpada s pomoću fungalnih ko-kultura

Dina Kačar, 627/MB

Abstract: Dva filamentozna funga, *Phlebia acerina* FBCC4 i *Phlebia radiata* FBCC43, odabrani su kao biokatalizatori za hidrolizu lignoceluloze do reducirajućih šećera i fermentaciju ovako proizvedenih reducirajućih šećera do etanola. Bioproces direktne konverzije lignoceluloze u etanol proveden je s pomoću monokultura dvaju funga kao i ko-kulture ovih dvaju funga FBCC4 i FBCC43. Eksperimenti su provedeni u modificiranoj Okamoto podlozi u kojoj je dodan reciklirani karton (50 g L^{-1}) kao glavni izvor ugljika i energije. Bioproces je poboljšan korištenjem ko-kulture *P. acerina* FBCC4, *P. radiata* FBCC43 i odabranog kvasca *Saccharomyces cerevisiae* HAMBI1164 (FBCC4 + FBCC43 + HAMBI1164). Ovaj kvasac dobar je kandidat za ko-kulturu FBCC4 + FBCC43 + HAMBI1164 jer je fermentirao glukozu i vrlo učinkovito proizveo etanol. Nadalje, ovaj je kvasac dobro rastao kao član ko-kulture FBCC4 + FBCC43 + HAMBI1164 i na njegov rast skoro da nisu utjecali oksalna kiselina ili proizvodi metabolizma FBCC4. Ko-kultura FBCC4 + FBCC43 + HAMBI1164 proizvela je najveću koncentraciju etanola (redom 0,34 g L^{-1} i 1,89 g L^{-1}) nakon 9 dana bioprocesa pri mikroaerofilnim i skoro anaerobnim uvjetima. Tijekom ovog perioda bioprocesa masa kartona se smanjila za 27,1 % od početne mase, dok je masa micelija porasla do 0,19 g u jednoj Erlenmeyer tikvici.

Ključne riječi: *lignoceluloza, fungi, etanol, ko-kulture*

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Bioethanol production from lignocellulose waste using fungal co-cultures

Dina Kačar, 627/MB

Abstract: Two filamentous white-rot fungi, *Phlebia acerina* FBCC4 and *Phlebia radiata* FBCC43, were employed as biocatalysts for hydrolysis of lignocellulose to reducing sugars and fermentation of reducing sugars therefrom to ethanol. Bioprocess of direct conversion of lignocellulose to ethanol was carried out by using monocultures of the two fungi as well as two-member co-culture of FBCC4 and FBCC43. Experiments were performed in modified Okamoto medium with core board (50 g L^{-1}) as main carbon and energy source. The bioprocess was improved by employing three-species co-culture of *P. acerina* FBCC4, *P. radiata* FBCC43 and selected yeast *Saccharomyces cerevisiae* HAMBI1164 (FBCC4 + FBCC43 + HAMBI1164). The yeast was a good candidate for the three-species co-culture because it was able to ferment glucose and produce ethanol very efficiently. In addition, the yeast grew well as a member of FBCC4 + FBCC43 + HAMBI1164 and its growth was almost not affected by oxalic acid or products of metabolism of FBCC4. Three-species co-culture FBCC4 + FBCC43 + HAMBI1164 produced the highest concentration of ethanol (0.34 g L^{-1} and 1.89 g L^{-1}) on the 9th day of the bioprocess under microaerophilic and almost anaerobic conditions, respectively. During this period weight of core board was reduced for 27.1 % of the initial weight while weight of dry mycelial biomass increased to 0.19 g in one Erlenmeyer flask.

Keywords: *lignocellulose, fungi, ethanol, co-cultures*

Thesis contains: 70 pages, 20 figures, 04 tables, 56 references, no supplements

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1. INTRODUCTION

Prediction is that there will be over 2 billion different vehicles worldwide by 2030 (Ali et al., 2016). Raising concerns over environmental pollution, high emission of CO_2 and scarce of fossil fuels have brought high interest in production of alternative, more sustainable biofuels from renewable sources, mostly agricultural residues. Among other biofuels used for transportation, bioethanol is being the most produced on the industrial scale. In order not to compete with food production and animal feed, abundant lignocellulosic biomass materials available at relatively low cost make considerable renewable substrates for bioethanol production. Due to high production costs of converting lignocellulose into ethanol, there have been made significant advances in bioprocessing technologies lately, such as developing simultaneous saccharification and fermentation (SSF) and a single step processes, referred to as consolidated processing. Both approaches are mostly relying on genetically improved microorganisms with a few reports on the production in co-cultures.

Recently, filamentous wood decaying fungi have become interesting candidates for SSF due to their great ability to completely degrade all components of lignocellulosic materials and they do not require strict anaerobic conditions for the ethanol fermentation. They are being used for biopulping in pulp and paper industry and for cost-effective biological pre-treatment of lignocellulose raw-materials in bioethanol production. Although ethanol yields from integrated fungal fermentation are still quite low, changes in culture conditions or addition of chemicals have been suggested to increase the production of ethanol and shorten the incubation time.

Main goal of this Thesis was to improve bioprocess of lignocellulose conversion into ethanol by employing co-culture consisted of white-rot filamentous fungi *Phlebia radiata*, *Phlebia acerina* and yeast. Improvement of state-of-the-art process comprised cultivation of the filamentous fungi on core board as only carbon source, characterisation and selection of yeast candidate, regarding its fermentative efficiency and ability to grow in the co-culture, and finding optimal conditions for the ethanol production. After selection of yeast, conditions for the ethanol production have to be optimised in order to define inoculation time point for selected yeast and produce as high as possible ethanol concentration over reasonable duration of the bioprocess. Appropriate set of analytical methods for determination of reducing sugars concentration and ethanol concentration and growth of selected yeast were applied for monitoring of the bioprocess for direct conversion of lignocellulose to ethanol. In such semi-solid system it would be of great importance to define the core board decay as well as portion of synthesized fungal mycelial biomass.

2.THEORETICAL PART

2.1. Role of wood decay fungi in environment

One of the most important parameters in regulating ecosystem productivity and climate is global carbon cycle. The short-term C-cyle consists of carbon exchange in terms of terrestrial and marine photosynthesis, respiration, and organic matter formation (Horwath, 2007). In order to keep the carbon cycling between atmosphere, soil, water and living beings (see Fig.1), as well as other nutrients, the carbon exchange must be in balance. Two-thirds of the terrestrial organic carbon is fixed in organic compounds of nonliving biomass, mainly plant lignocelluloses. Complete degradation of dead wood components and humified material in forest ecosystems can reach up to thousands years and that is why wood decay fungi play important role in the short term C-cycle. Wood decay fungi are the only species in the world known to be able to completely degrade wood.

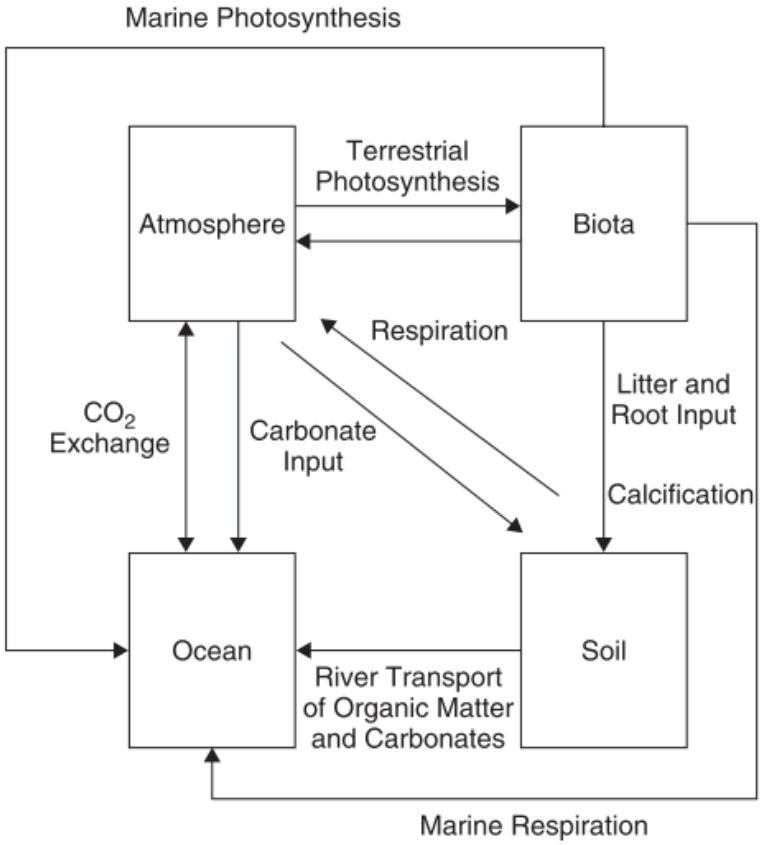

Figure 1. The short-term C cycle (Horwath, 2007)

In terms of waste recycling, fungi as well have a great importance. Nowadays, agricultural industry produce huge amount of wastes by throwing away more than 70% of what is grown. Cereal straw or sugarcane bagasse is a typical agricultural residue containing 30-40% of

cellulose, 20-30 % of hemicellulose and 15-35 % of lignin (Moore et al., 2011). Decomposition of acumulated plant material and thrown agricultural waste by fungi allow the release of carbon dioxide back into the atmosphere as well as release of essential nutrients as nitrogen and phosphorus to sustain plant biomass and other organisms (Horwath, 2007).

2.2. Main biopolymers in wooden materials

The most abundant biopolymers in nature as components of plant cell walls are cellulose, hemicellulose and lignin. Cellulose is the most abundant organic compound in the world, accounting for over 50% of organic carbon and 10^{11} tonnes of cellulose is synthesised each year (Moore et al., 2011). It is a linear polymer consisted of repeating glucose units linked together by β-1,4-glycosidic bonds. The degree of polymerisation can reach from 8000 up to 12000 of glucose units per one polymeric chain (Souza, 2013). The major product of cellulose breakdown by microorganisms and macrofungi, though, is cellobiose which is further hydrolysed to glucose (Eriksson et al., 1990). Cellulose chains are stabilised by hydrogen bonds into microfibrils and further into cellulose fibers. It can exist in two forms, highly organised crystalline cellulose which is harder to degrade and amorphous non-organised more degradable cellulose.

Hemicellulose is comprised of linear and branched heteropolysaccharides consisting of mostly 5-C sugars D-xylose and L-arabinose, and hexoses D-mannose, D-glucose, D-galactose, and D-glucuronic acid which can also be acetylated or methylated (Eriksson et al., 1990). Galactoglucomannans and arabinoglucuronoxylan hemicelluloses dominate in softwood while glucouronoxylan dominates in hardwood. Due to its moderate degree of polymerisation (100-200 units) and amorphous form, hemicellulose is more degradable than cellulose (Mäkelä, 2009).

Lignin serves as mechanical support to plants and aids in water transportation. It as well protects cellulose from enzymatic attack by different microorganisms and pathogens due to its dense nature, hydrophobicity and non-specific structure (Horwath, 2007). It is a heterogenic aromatic polymer composed of *p*-hydroxyphenil, guaiacyl and syringyl type of monolignol subunits joined together by different carbon-carbon and ether bonds (Mäkelä, 2009). Similar to hemicellulose, composition and amount of lignin varies between softwood and hardwood. It makes 15-36 % of wood dry weight and along with cellulose is the most important renewable material (Eriksson et al., 1990).

2.3.Wood degradation by white-rot fungi

Based on macroscopic characteristics of wood decay by fungi, four different patterns can be visually distinguished in forest ecosystems: white-rot, brown-rot, soft-rot and blue stain (Lundell et al. 2014). Common characteristic of white-rot decayed wood is white to yellow colour with dark manganese deposits (Lundell et al., 2014). The decayed wood may be brittle, soft, and spongy or fragmented into strings (Eriksson et al. 1990). White-rot fungi belong to phylum Basidiomycota class Agaricomycetes and attack dead coniferous wood, fallen trunks or burned wood (Lundell et al., 2014). Specific feature of *Phlebia* sp. white-rot fungi is selective lignin-degradation (Hatakka, 1994). Although they degrade cellulose and hemicellulose as well, excessive amount of lignin is being degraded from cell walls of softwood and hardwood by *Phlebia* sp. The order and the proportion in which lignin, hemicellulose and cellulose are being decomposed vary. The enzymatic attack can result in simultaneous degradation of all three components or in a preferential removal of one or more components (Eriksson et al., 1990). The lignocellulose decomposition as well depends on a type of wood substrate and its composition.

2.3.1. Enzymatic strategy in wood decomposition

Wood decomposition by fungi is possible due to their lignin-degrading machinery consisting of broad range of oxidative enzymes such as laccase, lignin peroxidase, manganese peroxidase and other versatile peroxidases. In lignocellulose degradation H_2O_2-generating enzymes also have an important role: aryl-alcohol oxidase, glyoxal oxidase and pyranose 2-oxidase (Mäkelä, 2009). Chemical linkage between lignin and hemicellulose can be a possible reason for similar regulation of their breakdown (Eriksson et al. 1990). In order to completely hydrolase cellulose, several enzymes are needed: 1) β-1,4-endoglucanase, which hydrolyses amorphous regions of cellulose microfibrils; 2) cellobiohydrolase, which attacks ends of cellulose chains, both reducing and non-reducing, releasing disaccharide cellobiose, 3) β-1,4-glucosidases, which cleave cellobiose to glucose units (Lundell et al., 2014). The three types of enzymes act synergistically. The activity exhibited by the mixture of three enzymes is higher than the sum of the individual activities (Zhang and Lynd, 2004). Complex chemical linkages between lignin, hemicellulose and cellulose make lignocellulolysis a difficult process that requires powerful enzymatic machinery working in synergy. During oxidation of lignin, highly-reactive free radicals are being generated and they affect other polysaccharide components. Big group of so called Carbohydrate-Active enzymes (CAZymes) is as well

aiding the lignocellulose decomposition. There have been detected numerous carbohydrate oxidases and other alternative enzymes such as copper-dependent lytic polysaccharide monooxygenases that enhance cellulose degradation (Levasseur, 2013).

2.3.2. Non-enzymatic factors in wood decomposition

Besides mostly extracellular and oxidative enzymatic reactions involved in lignocellulose degradation, secreted fungal metabolites such as phenolic and other aromatic compounds, smaller peptides, lignocellulose-derived compounds, metal ions and organic acids play an important role in wood decomposition (Lundell et al. 2010). Out of all organic acids produced by different species of all classes of fungi, oxalic acid is being produced in the largest quantities. It has an important role in pathogenesis, ecology and lignocellulose degradation (Dutton and Evans, 1996). Oxalate production by fungi provides them many competitive advantages, as being toxic for many microbes. In pathogenesis, due to its chelating properties, sequestration of calcium ions from host cell walls and forming of calcium oxalate crystals weakens the cell wall of the host. Therefore, oxalic acid is aiding the access of fungal cell wall degrading enzymes and initiating wood decay (Dutton and Evans, 1996). Oxalic acid also could have a synergistic role in lignin degradation acting as an electron donor in multiple redox reactions and a source of creating radicals by acting as a chelator in Fe(II)/H_2O_2 system (Singh and Rajini, 2004). Oxalic acid production varies between fungal species and depending on the carbon and nitrogen sources and the pH of the environment. Although brown-rot fungi in general produce more oxalic acid than white-rot fungi, in some white-rot fungi significant amounts of oxalic acid were detected during secondary metabolism when nutrients were depleted (Dutton and Evans, 1996.)

Other factors such as oxygen, glucose concentration or presence of aromatic compounds can effect decomposition of lignocellulose. While the lignin and hemicellulose degradation is strongly enhanced, degradation of cellulose is being repressed under oxygen atmosphere. Aromatic compounds like vanillic acid and veratryl alcohol stimulate lignin degradation in the oxygen atmosphere, but veratraldehyde, ferulic acid or veratric acid repress it (Cho et al., 2009). Aromatic compounds as well stimulate hemicellulose degradation under oxygen atmosphere, but repress cellulose degradation. Interestingly, small amounts of glucose (0.05% w/v) can strongly increase lignin degradation and decrease cellulose breakdown (Cho et al., 2009).

2.4. *Phlebia radiata* and *Phlebia acerina*

White-rot *Phlebia* genus belongs to the *Polyporales phlebioid* clade and to the family *Meruliaceae* and includes up to 220 species worldwide (Kuuskeri et al., 2015). They are often genetically distinct but phenotypically similar to each other. Cultivated *Phlebia acerina* was described as having wooly subicular mycelia and yellowish-brown hymenia with continuous but dried specimens frequently interrupted by areas of white (Nakasone, 1993). *Phlebia radiata* develops persistent, conspicuous, thick walled marginal hyphae. *P. radiata* can be identified by its narrow, cylindrical basidiospores and folded hymenial surface (Nakasone, 1990). Seven days old fungal mycelia of University of Helsinki Fungal Biotechnology Culture Collection (FBCC, Helsinki, Finland) isolates on malt extract agar are shown in Fig.2. *P. radiata* FBCC43 and *P. acerina* FBCC4, that were used in this Thesis are two different species, yet molecular systematics demonstrates a close evolutionary relationship between them (Kuuskeri et al., 2015). Enzyme activity profiling of laccase, manganese peroxidase, cellobiohydrolase, *β*-glucosidase and endoglucanase after 14-days long cultivations on a milled spruce wood also showed a big similarity between these two species (Kuuskeri et al., 2015). For *P. radiata* it has been reported that is able of efficiently degrading hardwood and softwood, as well as milled pine wood (Cho et al., 2009).

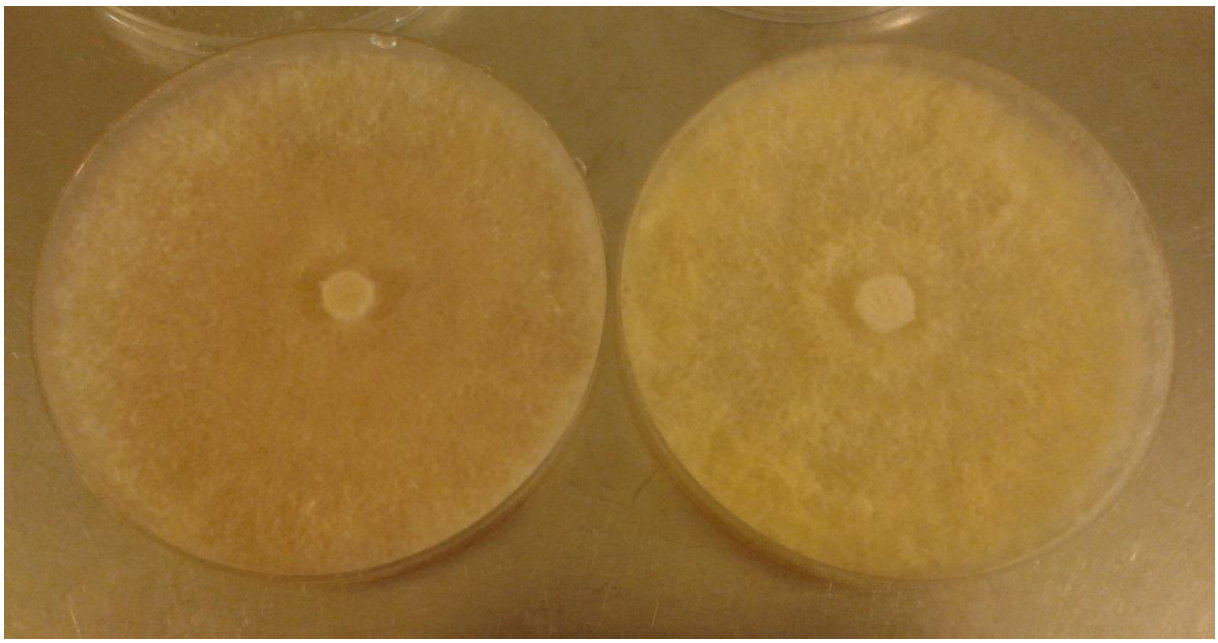

Figure 2. Mycelia of *Phlebia radiata* FBCC43 (left) and *Phlebia acerina* FBCC4 (right) after seven days cultivation on a malt extract agar at 25°C.

2.5. Potential of biofuels

Nowadays, the world is facing rapid climate changes caused by excessive release of CO_2 due to human activities during this and past century. CO_2 concentrations in the atmosphere are increasing faster than ever before. Followed by rising of the oil prices on a global scale because of the depletion of the energy sources and the political instability of the producing countries, sustainable energy production and renewable energy sources are becoming major driving forces in planning the global sustainable development. The fast growing human population is demanding more food and places to inhabit and the fuel production from abundant waste lignocellulosic biomass has recently become an interesting replacement for other agricultural resources which are competing for human food and animal feed. Biofuels produced today refer to bioethanol, bio-methanol, vegetable oils, biodiesel, biogas, biosynthetic gas (bio-syngas) and bio-hydrogen (Balat, 2011). In terms of producing fuels from resources which do not pollute environment, along with biodiesel, bioethanol is the only biofuel for transportation produced on an industrial scale today. Biofuels have been used by men since nineteenth century: it has been used in Germany and France already from 1894 starting the industry of internal combustion engines (ICEs) (Demirbas et al., 2007). Nikolaus August Otto developed his prototype of a spark ignition engine in the 1860s using ethanol and Deutz Gas EngineWorks designed one third of their heavy locomotives to run on pure ethanol in 1902. (Antoni et al., 2007).

There are many benefits of biofuels production. From the economic point of view, it is sustainable, offers fuels diversity, increases competiveness, reduces the dependency on imported petroleum, increases number of rural manufacturing jobs, *etc*. It also has an environmental impact through reducing greenhouse gases and air pollution. It is biodegradable, renewable and improves land and water use (Balat, 2011). The European Union (EU) intended that biofuels will account for 10% by 2020 and in 2003 EU directive has implemented a tax exemption on biofuels of up to 100%. From the countries in EU utilising ethanol, Sweden is the one producing the most, using substrates as crops, sugar cane and wood waste. In addition, the largest single ethanol production facility in EU is located in Zeitz in Germany (Antoni et al., 2007). On the other hand, the world's first commercial ethanol plant using cellulose as a substrate is considered to be in Crescentino in Italy with a full capacity of 75 million litres a year (Dionisi et al., 2014). Another plant in Strážske in Slovak Republic is currently under construction while in Fuyang in China a biorefinery four times the capacity of the Crescentino plant is under development (Ali et al., 2016). Besides the

combustion, ethanol has been used as solvent and basic chemical for medical and pharmaceutical purposes as well as in food and beverage industry and households.

2.6. Ethanol production from lignocellulosic raw materials

Biofuels produced from sugar based materials as corn or sugarcane, are called first-generation biofuels (Soccol et al., 2011). The most common way of producing bioethanol nowadays is by microbial fermentation of simple sugars using bacteria like *Zymomonas mobilis* or other engineered bacteria containing its enzymatic systems, and fungi, mostly yeast *Saccharomyces cerevisiae* (Antoni et al., 2007). Ethanol production by yeast is the most efficient, technologically and economically, allowing high ethanol yields in short time, but its disadvantage is limitation in substrate variations. For example, wild type *S. cerevisiae* is able to ferment only hexoses and cannot metabolise xylose, the main component of hemicellulose. Also, the most of the microbes used for fermentation are not able to hydrolyse cellulose. When talking about ethanol production from lignocellulose materials, second-generation biofuels, it is far more technically and economically challenging. Although lignocellulose raw materials serve as abundant feedstock at low cost, it still has not reached its full industrial potential (Balat, 2011).

The production of second-generation biofuels includes several biological processes that can be performed separately or combined in a single process: 1) breaking the lignin structure and making cellulose and hemicellulose available for hydrolysis, 2) further hydrolysis of polymers to simple sugars, 3) fermenting simple sugars to ethanol and 4) biomass separation and ethanol purification (Dionisi et al., 2014). Chemical pre-treatments like acid or alkaline hydrolysis or physical pre-treatments of lignocellulose, such as steam explosion or milling, result in accumulation of unwanted or toxic chemicals, which are later needed to be removed and deposited. Also, high pressure and high temperature conditions and the usage of commercially available expensive hydrolytic enzymes for a hemicellulose and cellulose treatment are making the process economically unattractive. Only pre-treatment of lignocellulosic feedstock contributes to 33% of the overall costs for ethanol production (Behera et el., 2014). All these steps performed separately are cause for current high costs of ethanol production from lignocellulose materials. Additionally, separation, washing and polishing between each step also contributes to low cost-effectiveness (Brethauer and Studer, 2015). For example, in 2012 biofuels production from lignocellulosic feedstock accounted for only 0.15% of the total production (HLPE, 2013). Therefore, usage of biological pre-treatment by fungi, co-cultivation of different microorganisms, making simultaneous

saccharification and fermentation (SSF) processes or entirely microbial processes in one step, so-called consolidated bioprocessing (CBP), brings cost advantages and is becoming more industrially interesting. In the following chapters different steps and latest advances in bioprocessing of lignocellulose to ethanol are described and the respective scheme is presented (see Fig. 3 below).

2.6.1. Simultaneous saccharification and fermentation (SSF)

Comparing to traditional separate hydrolysis and fermentation (SHF) process with little cost-effectiveness, new more economical approaches have been made. Combining enzymatic hydrolysis and the fermentation of sugars in one step is termed as simultaneous saccharification and fermentation (SSF). In this process, pre-treatment of lignocellulose is done separately and cellulolytic enzymes are added into single reactor where the hydrolysis and fermentation of hexoses is taking place. In SSF the fermentation of pentoses needs to be performed separately. Research group from United Kingdom performed SSF process of steam exploded duckweed using commercially available cellulases and β-glucosidase and has successfully reached 80% (w/w) of the maximum theoretical ethanol yield (Zhao et al., 2015). In addition, when fermenting high concentrations of duckweed substrate, higher ethanol yields were reached by increasing the yeast titre in inoculum or preconditioning yeast on steam exploded liquor so that the yeast could metabolise fermentation inhibitors (Zhao et al., 2015). In bioethanol production from banana pseudostem, two fungal strains *Aspergillus ellipticus* and *Aspergillus fumigatus* were used in co-culture to produce cellulases on-site and allow release of reducing sugars. The obtained hydroxylate was further used for ethanol fermentation by *S. cerevisiae* (Ingale et al., 2014).

Main advantages of SSF processes are the avoidance of end-product inhibition and prevention of hexose losses. Also, decrease in capital investment has been estimated to be larger than 20% (Olofsson et al., 2008). On the other hand, the optimal temperature for hydrolysis is much higher than the optimal temperature for the fermentation, therefore there must be found a compromise in SSF process with the consequence of affecting the process productivity. The ideal microorganism for SSF processes should have high temperature tolerance, inhibitor tolerance and the ability to use many kinds of simple sugars.

2.6.2. Simultaneous saccharification and co-fermentation (SScF)

The microorganisms used for fermentations are not efficiently co-fermenting variety of sugars released from lignocellulosic materials. That is why it is considered to co-cultivate microorganisms with different substrate preferences. As an upgraded version of previously described SSF, simultaneous saccharification and co-fermentation does not require separate hexose sugars and pentose sugars fermentations. One of attempts was done by group of researchers in Zürich where filamentous fungus *Trichoderma reesei* has been used for production of cellulolytic enzymes in a co-culture with *S. cerevisiae* and *Scheffersomyces stipitis* that were fermenting simple sugars into ethanol. For that purpose bioreactor system enabling simultaneous aerobic and anaerobic conditions was created. A dense oxygen permeable membrane served as a growth support for fungus *T. reesei*. Oxygen necessary for the growth of *T. reesei* and the secretion of cellulolytic enzymes was delivered from the gas-phase through the membrane at the bottom of the bioreactor and was consumed by fungus and an oxygen gradient was created within the bioreactor. The released enzymes hydrolysed the lignocellulosic fractions to soluble sugars, which were metabolized by the ethanol fermenting microorganisms in the upper, anaerobic parts of the bioreactor (Brethauer and Studer, 2014). Compared to commercial enzyme based SScF, using the co-culture with *S. cerevisiae* and *S. stipitis*, ethanol yields and xylose consumption were higher in those processes using the membrane reactor system. Another successful work has been done by scaling-up SScF process from laboratory scale to 10 m^3 demonstration scale plant (Koppram et al., 2013). In work described by Koppram et al. (2013) recombinant *S. cerevisiae* carrying genes from xylose fermenting *S. stipitis* was used in the process reaching 4% (w/v) ethanol using xylose rich corncobs. Despite of the technical advances in SScF processes and the efforts to optimise culture conditions, the main disadvantage is still present inhibitory effect of hydroxylate on some microorganisms, like *S. stipitis*, yeast strain important for pentose sugars fermentation.

2.6.3. Consolidated bioprocessing (CBP)

The most advanced processing approach which reduces process costs to up to 40% compared to SSF is consolidated bioprocessing (CBP) that integrates cellulolytic enzymes production, cellulose and hemicellulose hydrolysis and fermentation of simple sugars into ethanol in a single step (Brethauer and Studer, 2015). There are two possible suggestions of doing it and both are scientifically challenging and highly demanding. One way is to genetically engineer an organism, which is naturally capable of producing ethanol, to gain

new abilities for hydrolysing cellulose. Throughout last 10 years, there have been engineered different *S. cerevisiae* yeast strains carrying genes from *T. reesei* coding for cellulolytic enzymes endoglucanase and cellobiohydrolase (Yamada et al., 2013). In order to achieve enzyme synergy, cellulosome strategy is applied in yeast cells, where cellulolytic enzymes are being expressed and transported to the cell surface where they are able to attach to α-agglutinin anchor protein (Tsai et al., 2012). Another approach is to genetically engineer organisms which can naturally hydrolyse cellulose and ferment sugars therefrom into ethanol. Recently, advances are made in the development of genetic systems for several cellulolytic bacteria, where a thermophilic bacterium was engineered to produce ethanol at commercially interesting yields (Olson et al., 2012). *Clostridium thermocellum* and *Clostridium cellulolyticum*, being cellulosome-forming bacteria, are the best-developed as potential CBP biocatalysts. Although there have not been many reports of using fungi as CBS candidate organisms, group of researchers have developed strains of fungi *Fusarium oxysporum* and *T. reesei* with a great CBS potential (Ali et al., 2016.). Main characteristics of CBS organism should be: tolerance of relatively high temperature and ethanol concentration, then high tolerance toward inhibitors released as by-products during process, huge variety of monosaccharides that can be used in fermentation, *etc*. Disadvantage that consolidated bioprocessing is facing is ensuring culture conditions optimal for all types of processes performed simultaneously; and by using genetically engineered microorganisms: negative effects of the co-expression of multiple heterologous genes on the cell capacity, simultaneous expression of multiple genes and improper folding of secretion proteins (Ali et al., 2016).

2.6.4. Microbial consortia

An alternative for a single genetically engineered CBP organism which may face losses of overall performance, robustness and capacity due to importation of multiple gene systems, is utilization of naturally occurring mixed cultures. The application of mixed cultures is relatively rare and it has been reported to be used in traditional food and beverage industries as well as wastewater treatment, biogas production and biological soil remediation (Sabra et al., 2010). Industrial processes involving the use of mixed cultures, where many different naturally-occurring microorganisms co-exist, to produce biodegradable plastics, methane or hydrogen is under investigation (Dionisi et al., 2014). The advantages for the usage of naturally occurring microbial consortia in ethanol production from lignocellulose is wide variety of lignocellulose substrates, high self-stability and the reducing of pre-treatment of lignocellulose and sterilisation requirements (Du et al., 2015). So far, studies on mixed

cultures have reported only 0.8 mol of ethanol per mol^{-1} of glucose, compared with the maximum theoretical yield which is 2 mol of ethanol per mol^{-1} of glucose (Dionisi et al., 2014). Besides low ethanol yield, the main challenges that mixed cultures will have to overcome are: low rates of lignin and cellulose hydrolysis, control of the fermentation of sugars to ethanol and co-existence of different microbial populations. Also, the synchronisation of grow rates of co-cultured species and the competiveness for the nutrients is quite a challenge. Fungi like *T. reesei* and *Phlebia* sp. are great candidates for cellulases production, but their growth is slow compared to yeast or bacteria species. Successful results in ethanol production from cellulosic materials have been obtained with natural bacterial cosortia from a variety of habitats in China where *Pseudoxanthomonas taiwanensis* has been introduced (Du et al., 2015). It was shown that *P. taiwanensis*, which lacks the capability for ethanol fermentation, can increase the ethanol production in bacterial consortia as well *β*-glucosidase activity. In microbial consortia, existing synergies may results in more efficient substrate utilization and increased product yield (Brethauer and Studer, 2014). Investigating complex microbial consortia occurring in nature could lead to better understanding of specific interaction between organisms and in future creating artificial co-cultures in order to reach high ethanol yields from lignocellulosic raw materials.

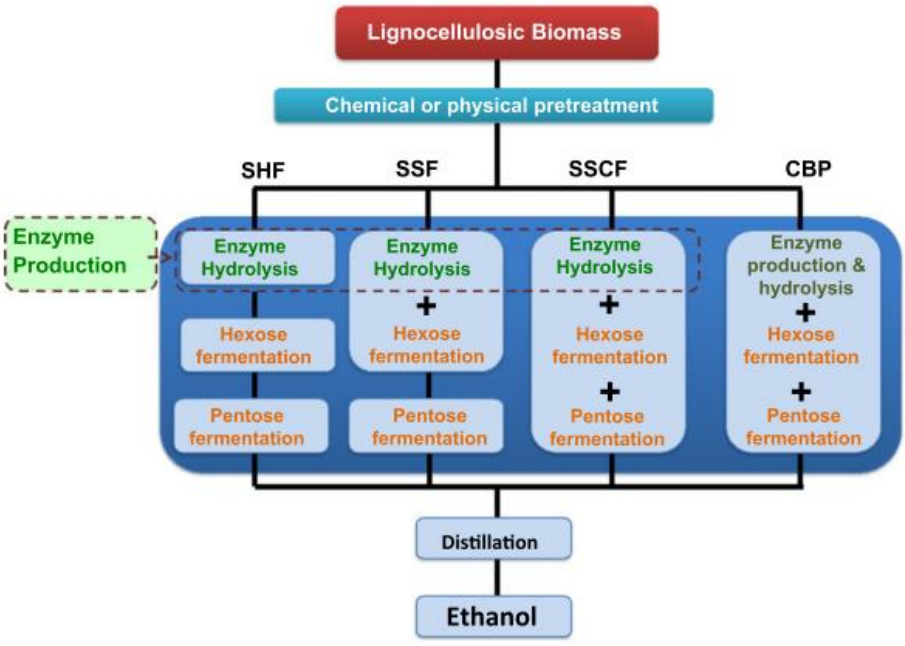

Figure 3. Scheme represents different steps in the bioprocessing of lignocellulose to ethanol. SHF - separate hydrolysis and fermentation, SSF – simultaneous saccharification and fermentation, SSCF – simultaneous saccharification and co-fermentation, CBP – consolidated bioprocessing (Ali et al., 2016).

2.7. Ethanol production by white-rot fungi

Despite of raised interest in using filamentous fungi in conducting SSF processes when producing bioethanol from lignocellulosic feedstock, there are still very few results from direct ethanol production from lignocellulosic raw material. Researchers mostly rely on modifying non-cellulolytic organisms that are able to produce high amounts of ethanol in order to gain cellulolytic abilities, as mentioned in previous chapters. There have been reported several fungal species for ethanol production from cellulose such as *Aspergillus* sp. (Ingale et al., 2014), *Rhisopus* sp. (Skory et al., 1997; Millati et al., 2005), *Neurospora* sp. (Rao et al., 1983), *Fusarium* sp. (Ali et al., 2016), and *Trichoderma* sp. (Stevenson and Weimer, 2002; Brethauer and Studer, 2014; Ali et al., 2016). Since white-rot fungi are able to degrade all the components of plant cell-walls and do not require strict anaerobic conditions for fermentation, they have become interesting microorganisms for consolidated bioprocessing. There have been few reports of enzymatic saccharification using white-rot fungi *Phanerochaete chrysosporium* (Zeng et al., 2011), *Echinodontium taxodii* (Yu et al., 2009), *Ceriporiopsis subvermispora* (Wan and Li, 2011), *Irpex lacteus* (Xu et al., 2010), *Trametes hirsuta* (Saritha et al., 2012), and *Cyathus stercoreus* (Yamagishi et al., 2011).

Japanese research group performed screening of ethanol-producing basidiomycetes and have found species *Peniophora cinerea* and *Trametes suaveolens* as potential candidates for application in SFF processes (Okamoto et al., 2010). More efficient *P. cinera* was able to assimilate glucose, mannose, fructose, galactose, sucrose, maltose and cellobiose and produce ethanol under both aerobic and micro-aerobic conditions. Initial glucose concentration was 20.0 g L^{-1} and maximal concentration of produced ethanol after 16 days of cultivation under micro-aerobic conditions was 8.1 g L^{-1}. On the other hand, when amorphous cellulose previously treated with phosphorus acid, was used as an only carbon source, maximal concentration of produced ethanol after 18 days of cultivation was 3.0 g L^{-1}. In addition, by HPLC analysis of products of cellulose hydrolysis showed that released sugars are being rapidly consumed for growth of biocatalyst and ethanol production.

The latest investigations on lignocellulose conversion using *Phlebia* sp. are made on several wood waste materials: sugarcane bagasse, unbleached hardwood kraft pulp, hardwood, cellulose materials and recent finding are described in the following chapters.

2.7.1. Sugarcane bagasse conversion to ethanol by *Phlebia* sp. MG-60

Every year sugarcane industry throws away large quantities of agro-waste causing environmental problems. One way to deal with it is to screen for efficient fungal species whose delignifying capabilities could be introduced for different industrial applications. The great ability of white-rot fungus *Phlebia* sp. MG-60 to efficiently degrade lignin was tested using whole sugarcane bagasse (WSB) (Li et al., 2002). It was showed that more than 50% of lignin in WSB was degraded, while less than 10% of cellulose was lost after 30 days of cultivation. These results suggested possible employment of *Phlebia* sp. MG-60 in biopulping in the pulp and paper industry as well as in animal feed production because lignin degradation in lignocellulosic agro-residues improves their digestibility by cattle. White-rot fungus *Phlebia* sp. was as well proposed for bioethanol production by delignification and fermentation of sugarcane bagasse and it was suggested that suitable culture conditions could improve fermentation efficiency (Kondo et al., 2014). The effect of initial moisture content for the fermentation was tested and 75% of initial moisture showed to be the most beneficial for the substrate availability. With this initial moisture content, the highest ethanol yields achieved after 2 and 4 weeks of incubation were 44.2 and 64.2 mg of ethanol per g^{-1} of bagasse powder, respectively, which was 16.1% and 20.4% of the theoretical maximum. It was also suggested that the addition of some chemical additives such as basal media could improve fermentation and shorten the process time.

2.7.2. Ethanol production from cellulose materials using *Phlebia* sp. MG-60

Phlebia sp. MG-60 has combined abilities of the lignin degradation, cellulose saccharification, and ethanol fermentation, as showed in direct ethanol production from cellulosic materials (Kamei et al., 2012a). Microcrystalline cellulose (Avicel PH-101), unbleached hardwood kraft pulp (UHKP) and waste newspapers were used as substrates for integrated fungal fermentation. When *Phlebia* sp. MG-60 was cultured for 168.0 h with UHKP, 8.4 g L^{-1} of ethanol was produced with 0.42 g of ethanol per g^{-1} of UHKP or 71.8% of theoretical yield. It was also showed that the change in inoculum preparation, like dispersion of mycelia, affects ethanol production and could shorten incubation period. From waste newspaper, 4.2 g L^{-1} of ethanol was produced after 216.0 h of incubation giving yields of 0.20 g of ethanol per g^{-1} of newspaper, which was 51.1% of theoretical yield. In cultivation with 20.0 g L^{-1} microcrystalline cellulose, 2.8 g L^{-1} of ethanol, or 25.3% of the theoretical yield was detected after 480.0 h of incubation.

Another study of fermentation of the UHKP was performed with the aim to evaluate the effects of initial concentration of the UHKP on ethanol production by *Phlebia* sp. MG-60 (Kamei et al., 2014). Direct fermentation of the UHKP was carried out at several initial concentrations: 2.0, 4.7, 9.1, and 16.5% w/w and the highest ethanol concentration of 25.9 g L^{-1}, or 46.7% of the theoretical yield was observed in the culture containing 9.1% of UHKP after 13.0 days of incubation. On the other hand, the highest cellulase activity detected using filter paper activity assay for cellulases (FPase) was observed in the 4.7% UHKP culture which was explained by possibility that synthesis of cellulases might be induced by water-soluble depolymerisation products such as cellobiose. In this study (Kamei et al., 2014) it was also indicated that the integrated fungal fermentation by *Phlebia* sp. MG-60 may be affected by water content and benefits from a small amount of aeration.

2.7.3. Ethanol production from hardwood using *Phlebia* sp. MG-60

Based on previous findings that *Phlebia* sp. MG-60 was able to degrade lignin under aerobic conditions and produce ethanol by fermenting simple sugars therefrom, Japanese research group has manage to produce ethanol from woody biomass in a single step (Kamei et al., 2012b). After 56.0 days of incubation on oak wood, 40.7 % of initial lignin was degraded. Right after the delignification, fermentation was performed under semi-aerobic conditions reaching 43.9% of the theoretical yield of ethanol after 20.0 days. Ethanol concentrations were estimated to be 311.3, 301.6, 353.7, 377.6, and 359.7 mg of ethanol per g^{-1} of wood, that was previously aerobically treated for 0.0, 14.0, 28.0, 42.0, and 56.0 days, respectively. Although the whole process lasted 76.0 days, it was the first report of the use of a single organism for ethanol production directly from wood without any additional chemicals or enzymes. Also, analysis of cellulase activities and xylanase activities indicated switching of metabolism from selective lignin degradation to polysaccharide degradation by changing from aerobic condition to semi-aerobic condition.

2.7.4. Ethanol production using wood-decaying fungal mix in co-culture with *Saccharomyces cerevisiae*

An interesting study was performed using samples from decomposing wood collected in the field to find strains that can exploit a broad spectrum of components from substrate, and have faster metabolism (Holmgren and Sellstedt, 2008). The fungal isolates obtained from the samples, capable of fermenting hexoses and pentoses, were identified by ITS-sequence

analysis as soft rot fungus *Chalara parvispora* and white rot fungi, *Trametes hirsuta* and *T. versicolor*. The substrate used was spent sulphite liquor obtained from treated wood in pulp industry and it consisted of lignosulfonates, hexoses and pentoses. Using the co-cultures with *C. parvispora, T. versicolor* and *S. cerevisiae* it was showed three times increase in ethanol production compared to fermentation using only *S. cerevisiae.* The amount of ethanol produced by *S. cerevisiae* in spent sulphite liquor was 8.64 g L^{-1}, fungal co-culture consisted of *C. parvispora* and *T. versicolor* could produce 11.64 g L^{-1} of ethanol, while 24.61 g L^{-1} of ethanol was produced by using *S. cerevisiae* together with the fungal co-culture. Similar study was reported in India where fungal mycelium from different species and waste mycelium from antibiotic industry increased ethanol production from cane molasses batch fermentation by *S. cerevisiae* (Patil and Patil, 1990). In the presence of mycelium, ethanol production was 30% to 65% higher at 30°C.

2.8. Future prospects for bioethanol production from lignocellulose

Despite the significant advances so far in lignocellulose processing for bioethanol production, there are still efforts and steps left to develop for the production on a large-scale. Some of the ideas for future biofuels development are: breeding of climate adapted energy plants for each area, utilisation of the whole plant as a substrate and simultaneous fermentation of pentoses and hexoses, either by introducing genetically modified organisms or creating artificial mixed cultures. Also, optimisation of the use of biofuels, standardisation of new types of biofuels and fuel mixes and adaptation to present day engines with the emphasis on development of new engines should be considered (Antoni et al., 2007).

From the biological point of view, future advances should rely on improvement of different microorganisms as cell factories; designing new strains or enzymes for biofuel fermentation, strains with higher fermentation temperature and better tolerance towards product, introduction of the lignin hydrolysis capability into microorganisms that are naturally able to hydrolyse cellulose, or introduction of the cellulose hydrolysis capability into microorganisms that are naturally able to hydrolyse lignin (Dionisi et al., 2014). By doing directed mutagenesis and using recombinant DNA technology, microorganisms that are native ethanol producers could be engineered to be able to degrade cellulose. On the other hand, microorganisms that are naturally able to hydrolyse cellulose could be engineered to produce ethanol. When discussing lignocellulose pre-treatment, the most costly step, besides expected low energy, capital and operating costs, the future effort should be put on maximising the

sugar yields in the hydrolysate, while minimizing the degradation of sugars caused by pre-treatment method and the formation of inhibitory compounds (Brethauer and Studer, 2015).

Applications of white-rot fungi in development of future bioprocessing systems are promising due to its 1) possession of lignocellulolytic enzymes and 2) production of various industrially interesting by-products like organic acids and ethanol. Combining delignification and consolidated bioprocessing has advantages related to its low cost for ethanol production because of the need for only one strain in a single bioreactor. On the other hand, integrated fungal fermentation has longer time requirements and lower ethanol yields (Kondo et al., 2014). One suggestion to increase ethanol yields is to improve culture conditions (Okamoto et al., 2010) and the other is to introduce co-cultures for SSF whose various interactions such as symbiosis, cooperation and competition could synergistically affect both delignification and ethanol production (Brethauer and Studer, 2015).

3. EXPERIMENTAL PART

3.1. MATERIALS

3.1.1. (Micro)organisms

In this Thesis two filamentous fungi, *Phlebia acerina* 4, University of Helsinki Fungal Biotechnology Culture Collection (FBCC, Helsinki, Finland) and *Phlebia radiata* 43 (FBCC), and selected yeast, *Saccharomyces cerevisiae* 1164, University of Helsinki, Faculty of Agriculture and Forestry, Division of Microbiology and Biotechnology (HAMBI, Helsinki, Finland) were used as working (micro)organisms in lignocellulose conversion to ethanol.

The yeast *Saccharomyces cerevisiae* HAMBI1164 was selected among 12 yeast species, as listed in Table 1., all from HAMBI. The selection was based on several criteria: ability to ferment glucose into ethanol and efficiency of the fermentation (Fig. 7), sensitivity to oxalic acid (Fig. 8) and sensitivity to ingredients of filtrate that was obtained after cultivation of *Phlebia acerina* FBCC4 (FBCC FG; Fig. 9). All 12 yeast species were as well systematically compared (Fig. 13) by their internal transcribed spacer (ITS) DNA sequences, previously amplified using polymerase chain reaction (PCR) method (see Chapter 3.2.14.) and sequenced (see Chapter 3.2.15.).

Table 1. List of 12 yeast species used in selection of single yeast strain for bioprocess catalysed by three-species co-culture.

yeast	HAMBI culture collection number
Saccharomyces cerevisiae	1165
Saccharomyces cerevisiae	1164
Saccharomyces cerevisiae	2108
Saccharomyces cerevisiae	10
Saccharomyces cerevisiae	785
Saccharomyces cerevisiae	1459
Kluyveromyces lactis	2238

Table 1. List of 12 yeast species used in selection of single yeast strain for bioprocess catalysed by three-species co-culture (continued).

strain	HAMBI culture collection number
Candida humilis	1169
Wickerhamomyces anomalus	811
Rhodosporidium toruloides	2246
Candida albicans	261
Zygosaccharomyces rouxii	2239

3.1.2. Chemicals

3.1.2.1. Modified Okamoto media

Media used in this Thesis are modifications of T medium (Okamoto et al., 2010), further called modified Okamoto medium (see Table 2). Original T medium consisted of: yeast extract, 10.0 g L^{-1}; potassium phosphate, 10.0 g L^{-1}; ammonium sulphate, 2.0 g L^{-1}; and magnesium sulphate heptahydrate, 0.5 g L^{-1}; and it had pH value of 6.0.

In two modified Okamoto media two different carbon sources were used - glucose, 20.0 g L^{-1} (modified Okamoto medium 1) or core board, 50.0 g L^{-1} (modified Okamoto medium 2).

Table 2. Purity and manufacturer of chemicals used in preparation of modified Okamoto media.

Chemical	Purity grade	Manufacturer
glucose	Pharmaceutical Applications (Ph.Eur)	Sigma Aldrich, St.Louis, MO, USA
core board	/	Metsä Tissue, Mänttä, Finland
yeast extract	/	LabM, Lancashire, United Kingdom
potassium phosphate monobasic (KH_2PO_4)	analytical reagent (Analar reagent)	Sigma-Aldrich, Munich, Germany
ammonium sulphate [$(NH_4)_2SO_4$]	general purpose reagent (puriss)	Merck, Darmstadt, Germany
magnesium sulphate heptahydrate ($MgSO_4\ 7H_2O$)	analytical reagent (p.a.)	J.T.Baker, Deventer, Holland

3.1.2.2. Malt extract agar (MEA)

Malt extract agar was used for maintenance and determination of growth of *Phlebia acerina* FBCC4, *Phlebia radiata* FBCC43 and *Saccharomyces cerevisiae* HAMBI1164 monocultures. Three monocultures were cultivated in malt extract agar (pH=5.5) which contained malt extract, 20.0 g L^{-1} (Biokar Diagnostics, Beuvais Cedex, France) and agar-agar, 20.0 g L^{-1} (Amresco, Solon, Ohio, USA).

3.1.2.3. Other chemicals

Chemicals used in screening studies for the selection of yeast species as a candidate for ethanol production (see Chapter 4.2.), are listed in Table 3.

Table 3. Purity and manufacturer of other chemicals used in research.

Chemical	Purity grade	Manufacturer
glucose	p.a.	Merck, Darmstadt, Germany
Oxalic acid	anhydrous puriss p.a.	Sigma Aldrich, St.Louis, MO, USA
Ethanol	96.1%	Altia Oyj, Helsinki, Finland
Ringer tablets 115525	-	Merck KgaA, Darmstadt, Germany
NaOH	p.a.	Merck, Darmstadt, Germany
HCl	p.a.	Merck, Darmstadt, Germany

3.1.2.4. Primers used in PCR-amplification of Internal Transcribes Spacer (ITS) of the rDNA region of yeast species

Primers used in amplification of the yeast rDNA region consisted of the conserved 18S (partially), 5.8S and 28S (partially), and variable ITS1 and ITS2 rDNA regions were **ITS1** 5'-TCCGTAGGTGAACCTGCG-3' and **ITS4** 5'-TCCTCCGCTTATTGATAT-3' (White et al. 1990), synthesised by Tag Copenhagen A/S, Copenhagen, Denmark.

3.1.3. Equipment

3.1.3.1. Multiwell spectrophotometer Tecan

Spectrophotometer Tecan 811001737 (Tecan Austria GmbH, Grödig, Austria) was used allowing measurements at wavelength (λ) of 540 nm for determination of reducing sugars concentration by DNS method (see Chapter 3.2.6.), and at λ of 600 nm for measuring optical density (OD_{600}) of yeast cells (see Chapter 3.2.5.).
It was equipped with UV-lamp (Tecan Austria GmbH, Grödig, Austria) for measuring of NADH concentration at λ of 340 nm (see Chapter 3.2.7.)

3.1.3.2. Other equipment

- Arktik Thermal Cycler TCA4848 (Thermo Fisher Scientific, Vantaa, Finland);
- Gel imaging system Universal Hood II.,SN:720BR/01908 (BioRad, Hercules, CA, USA);
- Drying oven 40050-IP-20 (Memmert GmbH & Co. KG, Schwabach, Germany);
- Incubator Innova 4230 (New Brunswick Scientific Co., Edison, New Jersey, USA);
- Centrifuge ScanSpeed Mini (LaboGene ApS, Lynge, Denmark);
- pH-meter Orion SA720 (Merck, Darmstadt, Germany);
- Lab balance Precisa junior 3100C (Precisa Gravimetrics AG, Dietikon, Switzerland);
- Water filtration system (Millipore, Bangalore, India);
- 96-well plates Costar 3635 (Corning, Vordingborg, Denmark);
- Optical microscope Leica DM 750 (Leica, Wetzlar, Germany);
- Leica E3 camera (Leica, Wetzlar, Germany);
- Rubber plugs with hole (top diameter 29 mm, height 35 mmm, bottom diameter 30 mm), and rubber plugs without hole (top diameter 38 mm, height 35 mmm, bottom diameter 31 mm) (VWR, Radnor, Pennsylvania, SAD);
- Lab glassware: graduated cylinder, Erlenmeyer flask, beakers (Schott Duran, Wertheim, Germany);
- Magnetic stirrer;
- Vortexmixer;
- Pipettmans;
- Tips, filter tips.

3.2. METHODS

3.2.1. Preparation of modified Okamoto medium 1

For the preparation of 1.0 L of basic Okamoto medium 10.526 g of yeast extract (LabM, Lancashire, United Kingdom), 10.526 g of KH_2PO_4 (Sigma-Aldrich, Munich, Germany), 2.105 g of $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany) and 0.526 g of $MgSO_4 \cdot 7 H_2O$ (J.T.Baker, Deventer, Holland) were weighed using laboratory balance Precisa junior 3100C (Precisa Gravimetrics AG, Dietikon, Switzerland), passed into 1 000.0 mL beaker and to this mixture 700.0 mL of water [obtained from the filtration by reverse osmosis (RO water) using reverse osmosis system (Millipore, Bangalore, India)] was added. Magnetic stirrer was used to dissolve the mixture. The measurement of pH was done by pH-meter Orion model SA720 (Merck, Darmstadt, Germany) and pH was adjusted to pH 6.0 by adding 4.0 M NaOH or 4.0 M HCl (Merck, Darmstadt, Germany). After setting the pH, the solution was passed into volumetric flask, filled up to 1 000.0 mL with the RO water and closed with plastic plug. The flask was inverted 3-4 times. Finally, the total volume was divided into 1.0 L bottles and autoclaved (121°C, 1 atm, 15 min). Sterile medium was stored in cold room at 4°C. Modified Okamoto medium supplied with 2% D-(+)-glucose (Sigma-Aldrich, St.Luis, MO, USA) was further called modified Okamoto medium 1 and the procedure for preparation od modified Okamoto medium 2 is described in Chapter 3.2.2.

3.2.2. Preparation of the substrate for modified Okamoto medium 2

The substrate for lignocellulose conversion into ethanol as the only carbon source used was core board roll from the toilet paper roll (Metsä Tissue, Mänttä, Finland). The exact composition of the core board is under determination, but it is assumed to be mainly lignocellulose. The core board was soaked in RO water in a plastic container for 5-10 minutes and milled using IKA A11 Basic Analytical Mill 230V (Sigma-Aldrich, Munich, Germany). The milling was performed 3 times for 2-3 seconds. Each core board roll was milled separately. Afterwards, the milled core board was left to dry in a drying oven 40050-IP-20 (Memmert, Schwabach, Germany) at 105°C for 24.0 h. Dried core board was milled once again, as described before, and the final result was core board of dust-like texture. Core board prepared as described was then weighed using lab balance Precisa junior 3100C (Precisa Gravimetrics AG, Dietikon, Switzerland) and 1.0 g was transferred by forceps into 100.0 mL Erlenmeyer flask. By using graduated cylinder the basic Okamoto medium (19.0 mL; see

Chapter 3.2.1.) was added to the prepared core board in Erlenmeyer flask and 1.0 mL of the RO water was pipetted to make a total volume of modified Okamoto medium 2 of 20.0 mL. The Erlenmeyer flask was covered by aluminium foil and autoclaved (121°C, 1 atm, 15 min).

3.2.3. Inoculation of one and two discs portion of filamentous fungi and cultivation in modified Okamoto medium 2

Monocultures of filamentous fungi *Phlebia radiata* FBCC43 and *Phlebia acerina* FBCC4 were cultivated on a malt extract agar (MEA) in Petri dish for one week at 25°C in incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) before inoculating one disc (see below) into modified Okamoto medium 2 (see Chapter 3.2.2.). By using sterile metal cylinder every inoculum-disc (0.6 cm in diameter) was cut out from mycelium pre-grown on MEA under described conditions. The disc was carefully transferred with sterile forceps into 20.0 mL of sterile modified Okamoto medium 2. Double portion of inoculum (two discs) was prepared in the same way, as described above. The whole procedure of inoculation was accomplished in laminar flow hood. The Erlenmeyer flask with inoculated medium was closed with previously autoclaved rubber plugs with a hole in the middle (VWR, Radnor, Pennsylvania, USA) carrying filter-tip and then placed in the incubator at 25°C.

3.2.4. Preparation of overnight culture of yeast cells for selection experiments and bioprocesses for ethanol production

By using inoculating microbiological loop one colony of pre-grown yeast was aseptically transferred to 5.0 mL of sterile modified Okamoto medium 1 (see Chapter 3.2.1.) in test tube. Inoculated medium was incubated overnight in the incubator at 25°C with constant shaking at 160 rpm.

3.2.5. Determination of optical density of yeast cells suspension

Optical density (OD_{600}) of yeast cells suspension was determined by using multiwell spectrophotometer Tecan 811001737 (GmbH, Grödig Austria) at λ of 600 nm. The volume of 150.0 μL of sample or blank was added into each well of 96-well plate (Costar 3635, Corning, Vordingborg, Denmark) in three technical replicates. The blank used for measuring OD_{600} was sterile corresponding medium.

3.2.6. Determination of reducing sugars by DNS assay

3,5- dinitrosalicylic acid (DNS) assay was used for determination of concentration of reducing sugars. It was assumed that glucose, which was released after core board lignocellulose hydrolysis, was main reducing sugar in liquid phase of the medium. The method is based on a redox-reaction between 3,5- dinitrosalicylic acid and reducing sugars. In the presence of reducing sugars, 3,5- dinitrosalicylic acid in alkaline medium reduces to 3-amino-5-nitrosalicylic acid while aldehyde group of reducing sugar oxidise to carboxyl group and, due to described reaction, colour of mixture will be changed from bright yellow to brown-red while heated. Described procedure was, as follows: 50.0 μL of sample was mixed with 75.0 μL of DNS reagent (Miller, 1959) in a plastic 96-well plate. Obtained mixture was then boiled in vigorously boiling water for 5.0 min and left to cool down in cold water. Tecan multiwell spectrophotometer 811001737 (GmbH, Grödig Austria) was used for measurement of absorbance at λ of 540 nm. The obtained absorbance value was converted to concentration of reducing sugars expressed in g L^{-1} by using standard curve and resulting equation. The standard was glucose (Merck, Darmstadt, Germany) and stock solution containing 0.01M of glucose in a 0.05 M Na-citrate buffer, pH 5.0, was prepared, aliquoted and freezed. After unfreezing, dilution series was prepared by using Milli-Q water obtained by filtration system (Millipore, Bangalore, India) in concentrations from 0.00 mM to 10.00 mM, as follows: 0.00 mM, 1.25 mM, 5.00 mM and 10.00 mM. Three technical replicates were performed. Blank for absorbance measurement was 50.0 μL of Milli-Q water mixed with 75.0 μL of DNS reagent.

Each set of reducing sugars determination was done with new curve and corresponding equation. One of equations is presented below.

$$y = 0.8988x - 0.0264 \qquad R^2 = 0.9990 \qquad [1]$$

y= determined absorbance at λ of 540 nm

x= concentration of reducing sugars in g L^{-1}

3.2.7. Enzymatic assay for determination of ethanol concentration

Concentration of ethanol (g L^{-1}) was determined by enzymatic method. Megazyme ethanol kit (Megazyme International, Wicklow, Ireland) was used. Priciple of the determination is described briefly below. Ethanol was converted into acetaldehyde in reaction catalysed by alcohol dehydrogenase and then resulting acetaldehyde was converted by aldehyde dehydrogenase to acetic acid. In both reactions one mole NADH was formed per mole of substrate. The change in NADH concentration was determined spectrophotometrically at λ of 340 nm by using UV-lamp of multiwell platereader Tecan 811001737 (Tecan GmbH, Grödig Austria).

Protocol for a full 96-well plate Costar 3635 (Corning, Vordingborg, Denmark) consisted of several steps starting with preparing standards solutions from Megazyme ethanol standard stock (5.0 μg μL^{-1}). The dilutions were prepared by pipetting 40.0 μL of standard stock into 960.0 μL of Milli-Q water in an Eppendorf tube and vortexed to prepare so called Standard 3 (dilution 1:24, concentration of ethanol of 0.20 μg μL^{-1}). Then, 500.0 μL of Standard 3 was mixed with 500.0 μL of Milli-Q water in a new Eppendorf tube to prepare Standard 2 (dilution 1:1, concentration of ethanol of 0.10 μg μL^{-1}). Afterwards, 500.0 μL of Standard 2 was mixed with 500.0 μL of Milli-Q water in another Eppendorf tube to make Standard 1 (dilution 1:1, concentration of ethanol of 0.05 μg μL^{-1}). Standard zero (0) was pure Milli-Q water. To prepare alcohol dehydrogenase (ADH) dilution from Megazyme kit, 210.0 μL of Megazyme ADH and 840.0 μL of Milli-Q water was gently mixed with a pipette in an Eppendorf tube. To prepare Megazyme aldehyde dehydrogenase (Al-DH) dilution from Megazyme kit, 500.0 μL of Al-DH and 700.0 μL of Milli-Q water was mixed together, as described for the ADH. Next step was preparing a series of dilutions from the samples. Dilution ratio depended on the ethanol concentration expected to be found in the sample. Method was optimised for samples that contain ethanol concentrations in range 0.1-0.2 μg μL^{-1}. Firstly, 185.0 μL of Milli-Q water was pipetted to all wells. Then 10.0 μL of diluted sample or Standard solution 3, 2, 1 or 0 was added in well. Volume of 2.0 mL of Megazyme buffer, 2.0 mL of Megazyme NAD^+ solution and 1.2 mL of diluted Al-DH were mixed together in order to prepare enzyme-precursor mixture. Volume 52.0 μL of prepared mixture of NAD^+ and Al-DH was pipetted to each well. Then, the plate was inserted to Tecan reader 811001737 (GmbH, Grödig Austria) and executed integrated pre-prepared programe „Megazyme EtOH“. Now, first value was acquired (Absorbance 1). Afterwards, as plate moved out automatically,

10.0 µL of diluted ADH was quickly added into each well and the program continued. So. The second value was acquired (Absorbance 2).

The subtraction of Absorbance 1 from the Absorbance 2 gave absorbance value which was related to ethanol concentration that was present in tested samples (Equation [2]).

$$Abs_{ethanol} = Abs_2 - Abs_1 \quad [2]$$

The ethanol concentration was calculated from the standard curve (Equation [3]) and multiplied by the dilution factor. Each set of ethanol determination was done with new curve and corresponding equation. One of equations is presented below.

$$y = 5.6292x + 0.1014 \qquad R^2 = 0.99 \quad [3]$$

y= determined absorbance at λ of 340 nm

x= concentration of ethanol in g L^{-1}

3.2.8. Fermentative ability of 12 yeast strains

Fermentation ability test was made by using 12 yeast species from HAMBI (see Table 1. in Materials) in order to select best performing yeast (see Chapter 3.1.1.). Main criteria for the selection were: efficiency in glucose (initial concentration of 20.0 g L^{-1}) consumption and ethanol production over period of 24.0 h under defined conditions (temperature of 25°C, shaking at 50 rpm, and relatively low amount of dissolved oxygen). An overnight pregrown culture of 12 yeast species was prepared as described previously (see Chapter 3.2.4.). Volume of 500.0 µL of each overnight pregrown culture was pipetted into 5.5 mL of modified Okamoto medium 1 (see Chapter 3.2.1.) in a sterile test tube under aseptic conditions and incubated in incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C and shaking at 160 rpm for 6 h. After incubation OD_{600} of suspensions was determined (see Chapter 3.2.5.). Then, suspensions were diluted by using basic Okamoto medium to obtain OD_{600} of 0.05. Prepared diluted suspension was used as inoculum (1.0 mL) of 50.0 mL of modified Okamoto medium 1 in 100.0 mL sterile Erlenmeyer flask. Then, flasks with inoculated medium were closed with sterile rubber plugs with a hole in the middle (VWR, Radnor, Pennsylvania, SAD) carrying filter-tip in order to ensure microaerophilic conditions

and placed in the incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C and shaking at 50 rpm. Samples were aseptically taken 24.0 h after the inoculation. Withdrawn sample (200.0 μL) was pipetted in Eppendorf tube and fermentation test was conducted in three biological replicates. As control modified Okamoto medium 1 without inoculum was used.

3.2.9. Oxalic acid sensitivity test

During lignocellulose conversion filamentous fungi produce different organic acids, mainly oxalic acid. Therefore, six yeast species were subjected to cultivation in modified Okamoto medium 1 with different concentrations of added oxalic acid. Selected yeast species were pre-grown as overnight cultures (see Chapter 3.2.4.) and OD_{600} was determined, as described previously (see Chapter 3.2.5.). In order to adjust initial OD_{600} of 0.01, different volumes of pregrown yeast suspensions were added in 5.0 mL of modified Okamoto medium 1 supplied with oxalic acid (Sigma-Aldrich, St.Luis, MO, USA) in concentrations of 0.0 mM (0.0 g L^{-1}), 0.1 mM (0.009 g L^{-1}), 1.0 mM (0.09 g L^{-1}) and 10.0 mM (0.9 g L^{-1}). Experiment was performed in sterile test tubes and incubation was performed in the incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C and shaking at 160 rpm. Samples were aseptically taken after 4.0 h, 6.0 h, 18.0 h and 24.0 h of inoculation. Volume of 150.0 μL of sample was pipetted in 96-well plate (producer) and OD_{600} was determined (see Chapter 3.2.5.). Each experiment was conducted in three biological replicates and as control modified Okamoto medium 1 was used.

3.2.10. Yeast growth in monoculture FBCC4 filtrate with glucose

Production of yeast growth inhibitors might occur during lignocellulose conversion by filamentous fungi. In order to test inhibitory effect of fungal products present in liquid phase of broth, filtrate of pre-grown monoculture *Phlebia acerina* FBCC4 was prepared and used as medium for yeast growth. Monoculture FBCC4 was cultivated in modified Okamoto medium 2 over 18 days (see Chapter 3.2.3.) at 25°C and without shaking. Filtrate was obtained, as described later (see Chapter 3.2.20.) and the pH value of the filtrate (pH 4.89) was determined using pH-meter Orion SA720 (Merck, Darmstadt, Germany). Volume of 100.0 mL of the filtrate was supplied with 2% D-(+)-glucose (Sigma-Aldrich, Lyon, France) and autoclaved (115°C, 1 atm,15 min).

An OD_{600} value (see Chapter 3.2.5.) of overnight pre-grown yeast suspension (see Chapter 3.2.4.) was determined and adjusted to be 0.01 in all samples by pipetting suspensions to sterile 5.0 mL of fungal filtrate with 2% D-(+)-glucose (Sigma-Aldrich, Lyon, France) (FBCC4 FG) in test tube. Inoculated FBCC4 FG was incubated in the incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C and shaking at 160 rpm. After 18.0 h sample (150.0 μL) was withdrawn and pipetted in 96-well plate (producer) and then OD_{600} was determined (see Chapter 3.2.5.). Three biological replicates were performed and control was FBCC FG.

3.2.11. Inoculation of pre-grown yeast suspension in modified Okamoto medium 2 in Erlenmeyer flasks with pre-grown filamentous fungi FBCC4 and FBCC43

An overnight pre-grown suspension of yeast HAMBI1164 and HAMBI1459 (see Chapter 3.2.4.) were used as inocula for modified Okamoto medium 2 in Erlenmeyer flasks with pre-grown filamentous fungi FBCC4 and FBCC43 after 7 days of FBCC4 and FBCC43 cultivation. First, OD_{600} value of yeast suspension was determined (see Chapter 3.2.5.) and certain volume of pre-grown suspension of yeast was pipetted to a sterile Eppendorf tube and then centrifuged (3500 rpm, 5 min) using centrifuge ScanSpeed Mini (LaboGene, Lynge, Denmark). The volume was calculated from obtained OD_{600} value in order to get initial OD_{600} of 0.01 in 20.0 mL of total volume of modified Okamoto medium 2 used for cultivation of FBCC4 and FBCC43 over first 7 days. The supernatant was carefully removed by pipette and the cells were re-suspended in basic Okamoto medium (see Chapter 3.2.1.) by mixing it gently with a pipette. Whole volume was added to Erlenmeyer flask in which the bioprocess started 7 days before. Flasks were carefully rotated manually to spread the yeast cells over the FBCC mycelia. Afterwards, prepared co-cultures were incubated in the incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C and constantly shaking at 50 rpm.

3.2.12. Lignocellulose conversion and ethanol production in three-species co-culture FBCC4 + FBCC43 + HAMBI1164 or HAMBI1459

Co-culture consisted of *Phlebia acerina* FBCC4, *Phlebia radiata* FBCC43 and *Saccharomyces cerevisiae* HAMBI1164 or HAMBI1459 was cultivated in modified Okamoto medium 2 over 15 days. The cultivation started (day 0) by inoculation of FBCC4 and

FBCC43 in modified Okamoto medium 2 (see Chapter 3.2.3.) at 25°C and without shaking. After 7.0 days, inoculum of HAMBI1164 or HAMBI1459 was added to pre-grown FBCC4 + FBCC43, as described above (see Chapter 3.2.11.), and cultivation was continued at 25°C and shaking at 50 rpm. From 7^{th} to 14^{th} day of described bioprocess samples were aseptically taken from the liquid phase of broth by Pasteur pipette (≈ 300 μL) and transferred to Eppendorf tube (sample taken on the 7^{th} day of bioprocess was taken right before yeast inoculation), stored at -20°C, and analysed as required. Concentration of reducing sugars and ethanol were determined as described previously (Chapters 3.2.6. and 3.2.7., respectively) while procedure for determination of colony forming units (CFU) is described in Chapter 3.2.13.

Sample taken at the end of bioprocess (14^{th} day) consisted of core board, mycelia and liquid phase was used for determination of core board weight loss and weight of dry mycelial biomass (see Chapter 3.2.20.). Before determination core board, mycelia and liquid phase were separated. The experiment was conducted in three biological replicates and with three controls. One control was modified Okamoto medium 2. Another control was modified Okamoto medium 2 inoculated with filamentous fungi FBCC4 and FBCC43, and the third control was modified Okamoto medium 2 inoculated with yeast HAMBI1164 or HAMBI1459.

3.2.13. Determination of yeasts colony forming units (CFU)

On the 7^{th} day of the bioprocess that was carried out by FBCC4 + FBCC43 + HAMBI 1164 or HAMBI1459, liquid phase (≈ 300 μL) was aseptically taken by Pasteur pipette as sample for determination of yeasts CFU. Sample was transferred into a sterile Eppendorf tube and 100.0 μL of the sample was pipetted into another sterile Eppendorf tube containing 900.0 μL of Ringer salt solution and marked as 10^{-1} dilution. The tube was vortexed and 100.0 μL of diluted sample (10^{-1}) was pipetted in another sterile Eppendorf tube also containing 900.0 μL of Ringer salt solution and marked as 10^{-2} dilution. The procedure was repeated until the dilution 10^{-4} was prepared. Volume of 100.0 μL of each of previously mixed dilutions10^{-3} and 10^{-4} was pipetted onto MEA as a droplet and spread equally using sterile glass spreader, previously rinsed with 96.1% ethanol (Altia, Helsinki, Finland) and flamed. The plates were marked and two replicates of each dilution were made. Inoculated MEA were incubated in the incubator Innova 4230 (New Brunswick Scientific, Edison, New Jersey, USA) at 25°C. After

48 h grown colonies were counted manually and CFU mL^{-1} was calculated according to following equation:

$$N = \frac{\text{sum of all colonies}}{\text{sum of all volumes}} = \frac{\sum_{i=1}^{n} N}{\sum_{i=1}^{n} V} \quad [\frac{CFU}{mL}] \qquad [4]$$

The procedure was repeated every 24.0 h over next three days.

3.2.14. Polymerase chain reaction (PCR) of the Internal Transcribed Spacer (ITS) of rDNA sequence

Phire Plant Direct PCR Kit (Thermo Scientific, Vantaa, Finland) was used for the PCR amplification of the internal transcribed sequence (ITS) from the rDNA of the 12 yeast species using ARKTIK Thermal Cycler TCA4848 (Thermo Scientific, Vantaa, Finland). The amplified region consisted of, as follows: a part of the conserved 18S, variable ITS1, conserved 5.8S, variable ITS2 and a part of the conserved 28S region of rRNA genes. First, cell lysis was done by pipetting 20.0 µL of Phire Plant Direct PCR dilution buffer into a sterile Eppendorf tube. Yeast colonies were scratched from the MEA plates by inoculation microbiological loop and transferred in an Eppendorf tube containing a dilution buffer. The samples were vortexed vigorously for 10 seconds and put on the ice. Before putting it on the ice, the samples and the dilution buffer were kept on a room temperature. The primers used for PCR amplification were ITS1 and ITS4 (see Chapter 3.1.2.3.). The 30.0 µL of PCR reaction mixture was prepared by mixing 12.40 µL of nuclease-free water, 15.00 µL of Phire-Plant buffer, 0.75 µL of ITS1-primer (10.0 µM), 0.75 µL of ITS4-primer (10.0 µM), 0.60 µL of Phire Hot Start II- DNA polymerase and 0.50 µL of DNA sample. The PCR amplification protocol is described in Table 4. All PCR reagents, enzyme and primers were stored at -20°C or kept on the ice right before usage.

Table 4. PCR-amplification program set in ARKTIK Thermal Cycler TCA4848 (Thermo Scientific, Vantaa, Finland)

PCR-amplification steps	Temperature (°C)	Time (s)	
Initial denaturation	98	300	
Denaturation	98	5	
Primer annealing	58	5	x30
Extension phase	72	20	
Continued extension phase	72	60	
Storage temperature	4	∞	

3.2.15. Sequencing and processing of the ITS-sequences

All PCR products were examined by running an agarose gel electrophoresis at room temperature and 120 V for 40 minutes. The formed DNA bands were imaged under UV-illumination using gel imaging system BioRad SN:720BR/01908 (BioRad,Hercules, CA, USA). The resulting PCR products were sent for DNA-sequencing to the Laboratory for genome and DNA sequencing in the Institute of Biotechnology at the University of Helsinki. PCR products were sequenced directly from the PCR reaction mixture using the same primers as in the ITS-PCR (see Chapter 3.2.14.). The sequences obtained and their electropherograms were analysed with Molecular Evolutionary Genetics Analysis software version 6.0 (MEGA6) (Tamura et al. 2013). Forward and reverse sequences were modified and a single trimmed sequence was created. ITS sequences comprising the regions ITS1, 5.8S and ITS2 were aligned using ClustalW and contig sequence was created. Sequences obtained were compared to sequence information database of National Center for Biotechnology Information (NCBI, Rockville Pike, Bethesda MD, USA) using Basic Local Alignment Search Tool (BLAST) and blast algorithm for species identification.

3.2.16. Construction of the phylogenetic tree

Evolutionary analyses were conducted and the phylogenetic tree was constructed using MEGA6 software (Tamura et al. 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join (Saitou and Nei, 1987) and BioNeighbor-Join (Gascuel, 1997) algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 24 nucleotide sequences. HAMBI sequences comprising rDNA regions ITS1, 5.8S and ITS2 were obtained by PCR amplification (see Chapter 3.2.14.) and sequenced (see Chapter 3.2.15.). Processed ITS-sequences were compared to corresponding reference sequences obtained by BLAST. Bootstrap values (100 replications) higher than 50% were indicated for the nodes. Scale bar represented 0.05 nucleotide substitutions per position.

3.2.17. Reverse-phase microscopy of yeast cells

Overnight cultures of 12 yeast strains were analysed by reverse-phase microscopy using optical microscope Leica DM 750 (Leica, Wetzlar, Germany) and the images of each yeast species (data not shown) were obtained by digital camera Leica EC 3 (Leica, Wetzlar, Germany). Microscope scale in µm was photographed separately and the respective scale was constructed using Dia Diagram Editor version 0.97.2. The scale was attached to photograph of the selected yeast strain *Saccharomyces cerevisiae* 1164, HAMBI (see Fig. 15) in order to be able to estimate the size of cells.

3.2.18. Determination of the termination of the lignocellulose conversion and ethanol production process in three-species co-culture FBCC4 + FBCC43 + HAMBI1164

In order to determine the optimal duration of the lignocellulose conversion and ethanol production process, the sampling was done on the 9^{th} and the 10^{th} day of the process. The aim was to check if the ethanol concentrations remain in the same range after 9^{th} day. The duration of the process was 10 days in total. It started by cultivating only filamentous fungi in modified Okamoto medium 2 (see Chapter 3.2.3.). On the 7^{th} day of the process, *Saccharomyces cerevisiae* HAMBI1164 was aseptically inoculated (see Chapter 3.2.11.). There were two sets of parallel experiments. From one set, samples were taken on the 7^{th}, 9^{th} and 10^{th} day and

from the other set, samples were taken on the 7th and 10th day. Samples were aseptically taken from the liquid phase by Pasteur pipette (≈ 300 μL), passed to a clean Eppendorf tube and stored at -20°C. Core board, mycelia and fungal liquid were separated on the 10th day of the process and the masses of dry core board and dry mycelium were determined (see Chapter 3.2.20.). The experiment was conducted in three biological replicates and there were two controls. One control was modified Okamoto medium 2 inoculated with filamentous fungi FBCC4 and FBCC43 and another was modified Okamoto medium 2. Almost anaerobic conditions were ensured by using rubber plugs without holes (VWR, Radnor, Pennsylvania, USA).

3.2.19. Lignocellulose conversion and ethanol production using three-species co-culture FBCC4 + FBCC43 + HAMBI1164

After selection of the best yeast candidate for the ethanol production in three-species co-culture with filamentous fungi and after determination of the optimal time-frame of the process, lignocellulose conversion and ethanol production was performed simultaneously during nine days in total. The process procedure was the same as previously described (see Chapter 3.2.18.) with single change. The last sample was taken on the 9th day, as well as the core board, mycelia and fungal liquid separation (see Chapter 3.2.20.).

3.2.20. Core board, mycelia and fungal liquid separation procedure and the measurement of their weight

At the end of the bioprocess of lignocellulose conversion into ethanol, vacuum filtration was performed to separate liquid from the rest of solid core board and biomass. Created semi-solid layer of mycelium and core board in the Erlenmeyer flask was gently pushed out using forceps, taking care that mycelium does not break while pouring the content in the vacuum filtration system. The bottom of flask was used to press and squeeze out the remaining liquid while the mycelium with core board remained glued to the bottom of the flask due to the slimy mycelia texture. Then the solid complex was gently removed by hand and placed face-down on the flat area on the table. Unused core board was carefully scratched with the forceps away from the mycelia. Both mycelia and core board were dried in a drying oven 40050-IP-20 (Memmert, Schwabach, Germany) at 105°C for 24 h and weighed using lab balance Precisa junior 3100C (Precisa Gravimetrics AG, Dietikon, Switzerland). As both

mycelia and the core board were dried in an aluminium foil, the mass of the folium was subtracted from the total weight to get the mass of each sample.

3.2.21. Ethanol and mycelia yield calculation

The ethanol yield ($Y_{P/S}$) was calculated as quotient of mass of ethanol (g) obtained at the end of the process (9th day) and dry mass of consumed or total core board (g):

$$Y_{P/S} = \frac{\gamma V}{m} \ [g\ g^{-1}] \qquad [5]$$

γ = concentration of ethanol [g L^{-1}]
V = total volume [L]
m = dry mass of consumed or total core board [g]

The mycelia yield ($Y_{X/S}$) was calculated as quotient of dry mass of produced mycelia (g) at the end of the process (9th day) and dry mass of consumed core board (g):

$$Y_{X/S} = \frac{m_X}{m_S} \ [g\ g^{-1}] \qquad [6]$$

m_X = dry mass of mycelia [g]
m_S = dry mass of consumed core board [g]

The consumed core board (CB) at the end of the bioprocess was calculated as a percentage of the control sample:

$$CB(consumed) = \left(1 - \frac{m(CB)}{m(control)}\right) x100 \ [\%] \qquad [7]$$

m(CB) = dry mass of core board left at the end of the bioprocess [g]
m(CB) = dry mass of core board from the control sample without working organisms [g]

Dry mass of consumed core board used in calculations for ethanol and mycelia yield was calculated as the percentage of consumed core board of the total (1.0 g) core board. Ethanol and mycelia mass was obtained as the average of two independent experiments.

4. RESULTS AND DISCUSSION

Aim of this Thesis was improvement of bioprocess for ethanol production from untreated lignocellulosic raw material. Filamentous fungi *Phlebia acerina* FBCC4 and *Phlebia radiata* FBCC43 were employed as biocatalysts which possess hydrolytic and fermentative activity and are able to convert lignocellulose to fermentable sugars and ferment sugars therefrom to ethanol. In order to improve direct conversion of lignocellulose to ethanol, two-species co-culture of *Phlebia acerina* FBCC4 and *Phlebia radiata* FBCC43 (two-species co-culture FBCC4 + FBCC43), instead of monoculture of either species, was used as biocatalyst. In order to get more efficient bioprocess for ethanol production 12 yeast species from HAMBI were tested and two yeast strains, *Saccharomyces cerevisiae* HAMBI1459 and *Saccharomyces cerevisiae* HAMBI1164, were selected as the most appropriate candidates for bioprocess conducted by three-species co-culture of *P. acerina* FBCC4, *P. radiata* FBCC43 and *S. cerevisiae* HAMBI1459 (three-species co-culture FBCC4 + FBCC43 + HAMBI1459) and *P. acerina* FBCC4, *P. radiata* FBCC43 and *S. cerevisiae* HAMBI1164 (three-species co-culture FBCC4 + FBCC43 + HAMBI1164).

Two different modified Okamoto media were used: modified Okamoto medium in which glucose (20.0 g L^{-1}) was added as carbon source (modified Okamoto medium 1) and modified Okamoto medium in which core board (50.0 g L^{-1}) was added as carbon source (modified Okamoto medium 2).

Experiments were performed in test tubes with working volume of 5.0 mL or in Erlenmeyer flasks with working volume of 20.0 or 50.0 mL, as indicated in every figure caption. Cultivation of *Phlebia* sp. was performed without shaking while experiments with yeasts were performed with shaking (50 or 160 rpm). All experiments were done in triplicates and data in this Thesis represent the average of three biological replicates. Values are means ±SD for samples.

4.1. Bioconversion of lignocellulose to fermentable sugars by monocultures and two-species co-cultures of *P. acerina* FBCC4 and *P. radiata* FBCC43

Degradation of lignocellulose to fermentable sugars was followed in modified Okamoto medium 2. Monoculture FBCC4, monoculture FBCC43 and two-species co-culture FBCC4 + FBCC43 were used as biocatalyst (Figs. 4-6). Concentration of fermentable sugars was determined by DNS assay, as described in Chapter 3.2.6. in Experimental.

Different portions of inoculum were used: single disc of pre-grown *Phlebia* sp. mycelium was used in experiment presented in Fig. 4 while two discs of pre-grown *Phlebia* sp. mycelium were used in experiment presented in Figs. 5 and 6.

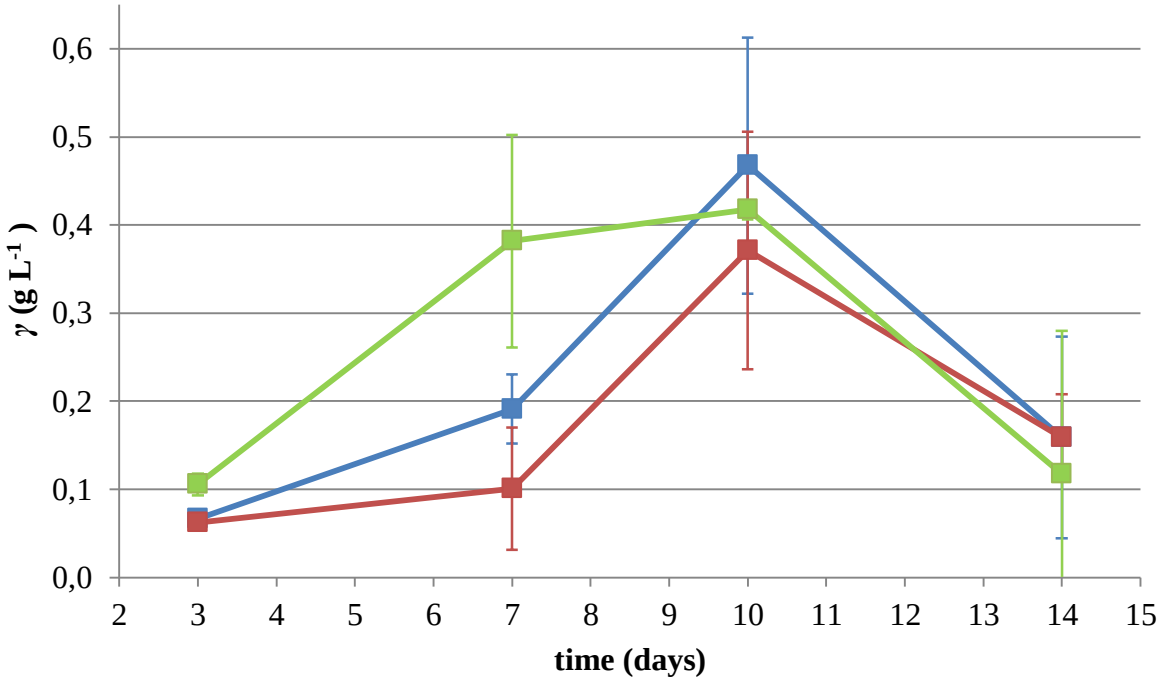

Figure 4. Changes in concentration of reducing sugars during cultivation of monoculture FBCC4 (*Phlebia acerina* FBCC4; ■) and monoculture FBCC43 (*Phlebia radiata* FBCC43; ■) and the two-species co-culture FBCC4 + FBCC43 (■) in modified Okamoto medium 2 in Erlenmeyer flask (working volume of 20.0 mL) at 25°C without shaking. For inoculation procedures see Chapter 3.2.3. in Experimental.

Concentration of reducing sugars presented in Fig. 4 is a result of two processes - lignocellulose hydrolysis and reducing sugars uptake *via* fermentation and mycelial biomass synthesis. In all three cultivations the concentration was in range from 0.06 to 0.47 g L^{-1} and the highest reducing sugars concentration was determined on the 10^{th} day. The first two days of the bioprocess the concentration was not followed because it was rather low.

During bioprocess catalysed by monocultures of two *Phlebia* sp. similar trend of reducing sugars concentration was observed from the 3^{rd} to 14^{th} day. Reducing sugars concentration increased until 10^{th} day of bioprocess when started to decrease until the end of the bioprocess. It might be assumed that rate of lignocellulose hydrolysis and rate of reducing sugars uptake were not balanced.

When grown in two-species co-culture FBCC4 + FBCC43 biocatalysts produced almost 2-fold higher concentration of reducing sugars over first seven days of the bioprocess. That indicated the possibility to shorten the bioprocess in total if using both species in two-species co-cultivation rather than monoculture. In that case, yeast might be inoculated on the 7^{th} day of bioprocess when the concentration of reducing sugars is high enough for growth and fermentative activity of the yeast. Between the 7^{th} and the 10^{th} day of the bioprocess reducing sugars concentration was rather constant. It might be due to balanced rate of lignocellulose hydrolysis and reducing sugars uptake. After the 10^{th} day the concentration decreased in similar trend as during the bioprocesses catalysed by the monocultures.

In order to investigate effect of mycelial inoculum portion on reducing sugars concentration during bioprocesses catalysed by two monocultures (FBCC4 and FBCC43) instead of one disc (see Chapter 3.2.3.) two discs were used for inoculation (Fig. 5).

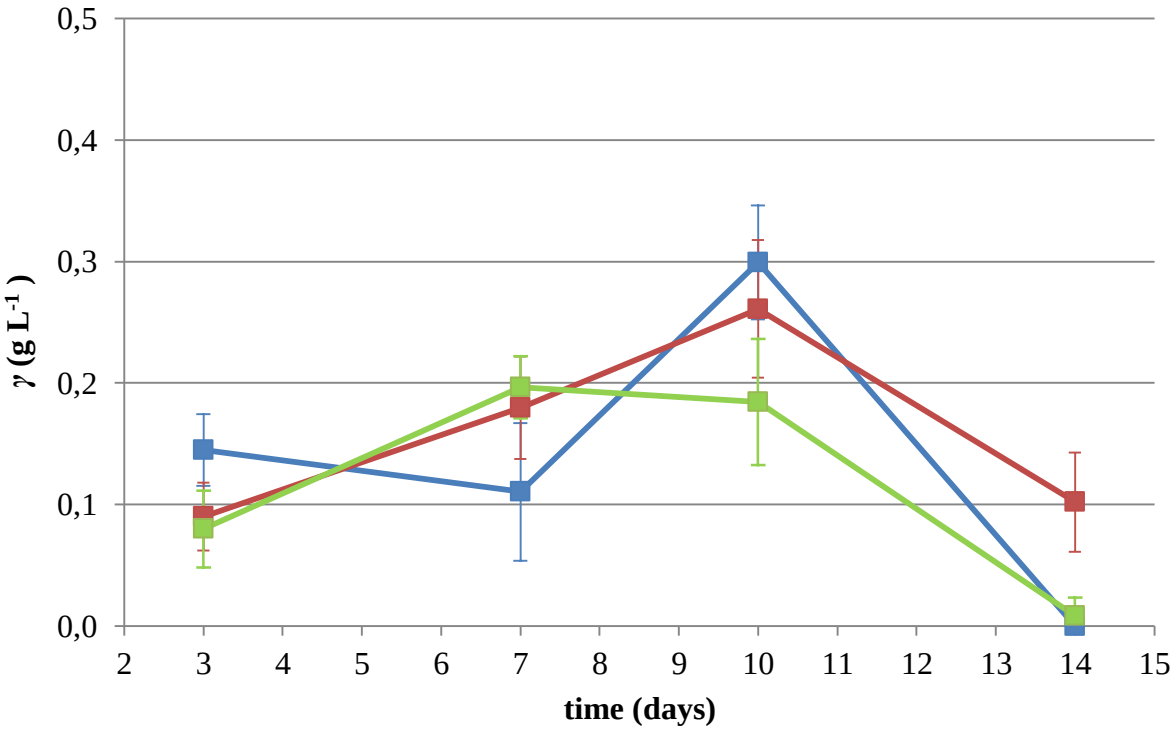

Figure 5. Changes in concentration of reducing sugars during cultivation of monoculture FBCC4 (two discs of inoculum) (■), monoculture FBCC43 (two discs of inoculum) (■) and the two-species co-culture FBCC4 + FBCC43 (■) in modified Okamoto medium 2 in Erlenmeyer flask (working volume of 20.0 mL) at 25°C without shaking. For inoculation procedures see Chapter 3.2.3. in Experimental.

In this set of experiments reducing sugars concentration was in range from 0.0 to 0.3 g L^{-1}. However, similar trend like in previous set of experiments (Fig. 4) was observed with the highest concentration of reducing sugars on the 10^{th} day. Therefore, it was concluded that in all next sets of experiments one disc of pre-grown mycelia of each monoculture and two-species co-culture FBCC4 + FBCC43 will be used.

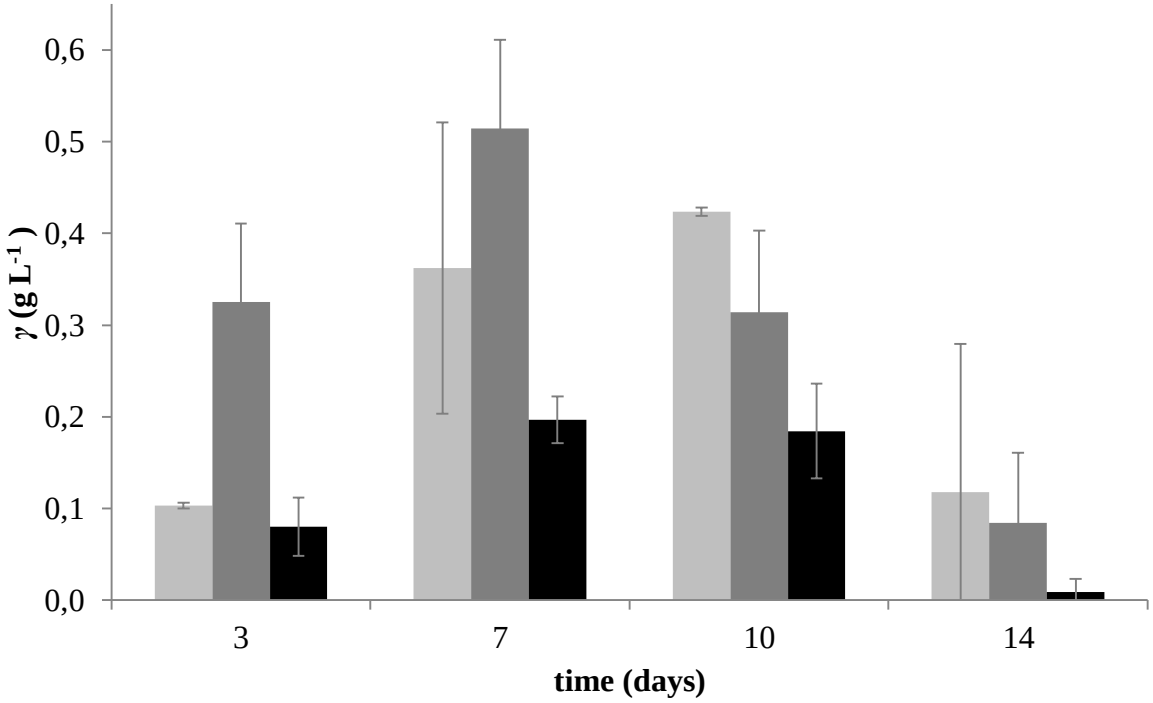

Figure 6. Changes in reducing sugars concentrations during three independent cultivations (A; ■, B; ■ and C; ■) of two-species co-culture FBCC4 + FBCC43 in modified Okamoto medium 2 in Erlenmeyer flask (working volume of 20.0 mL) at 25°C without shaking. For inoculation procedure see Chapter 3.2.3. in Experimental.

Although there are high standard deviations between biological replicates, which are rather characteristic for semi-solid media and mycelial growth of fungi, data presented in Fig. 6 show similar trend as observed within three independently performed experiments, with the highest reducing sugars concentrations determined on the 7th and 10th day of the bioprocess.

4.2. Screening studies for the selection of yeast species for ethanol production

In order to improve reducing sugars uptake *via* fermentation, yeast candidate for three-species co-culture was selected. Fermentative ability of 12 yeast species from HAMBI (see Table 1. in Experimental) was investigated (Chapter 4.2.1.) and six of them were cultivated in modified Okamoto medium 1 in which oxalic acid, usual by-product of *Phlebia* sp. metabolism, was added. Results are presented in Chapter 4.2.2. Five out of those six yeast species were further selected based on their growth in filtrate of monoculture FBCC4 (FBCC4

FG, see Chapter 4.2.3.) obtained after cultivation of the fungal strain in modified Okamoto medium 2 at 25°C after 18 days. Two yeast candidates were selected for three-species co-cultivation (Fig. 9 in Chapter 4.2.3.).

4.2.1. Fermentative ability of 12 yeast species

Ethanol producing yeast species available in HAMBI were used in this set of screen study. Six *S. cervisiae* strains (HAMBI10, 785, 1164, 2108, 1459 and 1165), two species from genus *Candida* (HAMBI1169 and 261) and one from genera *Kluyveromyces* (HAMBI2238), *Wickerhamomyces* (HAMBI811), *Rhodosporidium* (HAMBI2246) and *Zygosaccharomyces* (HAMBI2239) were candidates for fermentation in modified Okamoto medium 1. The results are presented in Fig. 7.

Glucose consumption was monitored by DNS assay (Chapter 3.2.6. in Experimental), optical density of yeast suspensions was followed spectrophotometrically (Chapter 3.2.5. in Experimental) and ethanol production by enzymatic method (Chapter 3.2.7. in Experimental).

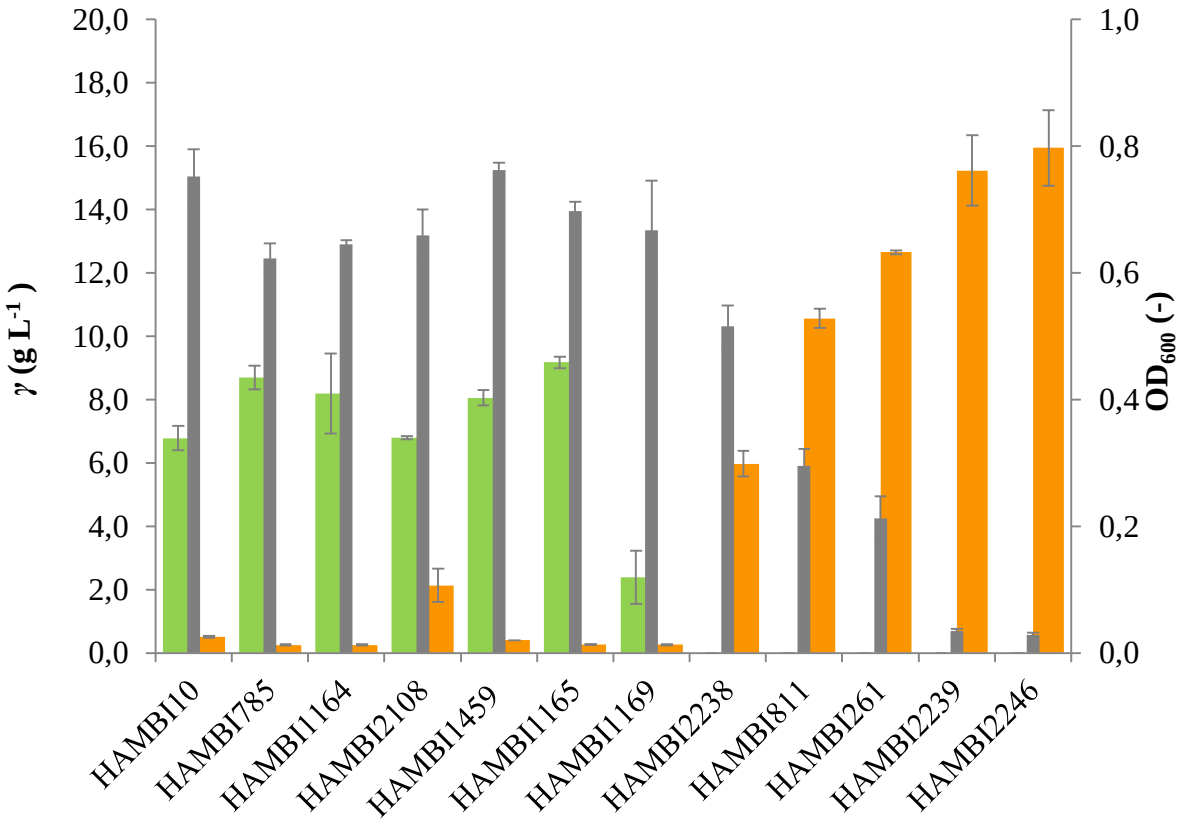

Figure 7. Optical density (OD_{600}, ■; right y-axis) of 12 yeast species (see Table 1. in Experimental), concentration of remaining glucose (■) and produced ethanol (■) after 24.0 h of cultivation of monocultures of yeast species in modified Okamoto medium 1 in Erlenemeyer flask (working volume of 50.0 mL) at 25°C and shaking at 50 rpm (see Chapter 3.2.8. in Experimental).

All species were able to grow under chosen conditions. Optical density had similar values in all suspensions of *S. cerevisiae* strains as well as *C. humilis* HAMBI1169. Five yeast species: HAMBI2238, 811, 261, 2239 and 2246 showed very modest growth which was in agreement with low sugar uptake.

The results presented in Fig. 7 are showing the ability of 12 different yeast strains to ferment glucose. Only seven of them were capable of producing ethanol (HAMBI10, 785, 1164, 2108, 1459, 1165 and 1169). Regarding concentration of produced ethanol, the best producers were *S. cerevisiae* strains (HAMBI10, 785, 1164, 2108, 1459 and 1165). Nevertheless, six species (HAMBI10, 785, 1164, 1459, 1165 and 1169) which spent almost all glucose were considered for further investigation.

4.2.2. Effect of different concentration of oxalic acid on growth of yeast species

Oxalic acid production is widespread phenomena among fungal species. One of its hypothetical functions is inhibition of the growth of other microorganisms in different habitats, while it is also involved in hydrolysis of lignocellulose. Therefore, screen study on sensitivity of yeast species on the oxalic acid was performed to select the appropriate candidate for a three-species co-cultivation.

Growth rate of yeasts was followed by measuring optical density as described in Chapter 3.2.5. in Experimental after 2.0, 4.0, 18.0 and 24.0 h after inoculation of six yeast species (HAMBI10, 785, 1164, 1459, 1165 and 1169) into modified Okamoto medium 1 and modified Okamoto medium 1 containing different oxalic acid concentrations, as indicated in Figure 8, and cultivation of listed yeast species as monocultures at 25°C and shaking (160 rpm).

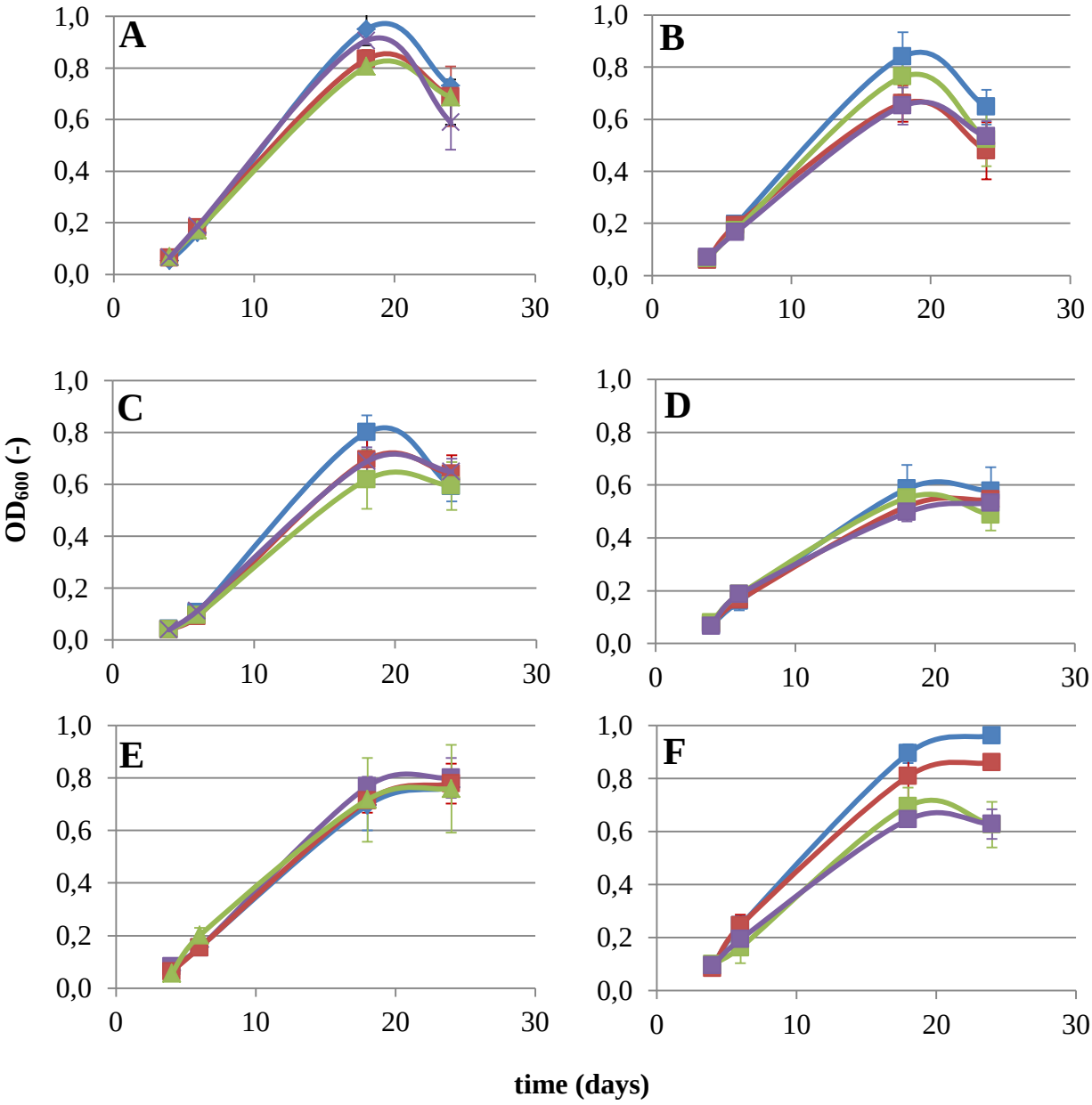

Figure 8. Optical density (OD_{600}) of six yeast species cultivated in modified Okamoto medium 1 and different concentrations of oxalic acid, as follows: 0.0 mM (0.0 g L^{-1}; ■), 0,10 mM (0.009 g L^{-1}; ■), 1.0 mM (0.09 g L^{-1}; ■) and 10.0 mM (0.9 g L^{-1}; ■). Yeast species tested were: HAMBI1164 (A), HAMBI1165 (B), HAMBI10 (C), HAMBI785 (D), HAMBI1459 (E) and HAMBI1169 (F). Experiments were performed in test tubes (working volume 5.0 mL) at 25°C and shaking at 160 rpm (see Chapter 3.2.9. in Experimental).

Obtained values of optical density during the cultivation of monocultures of all tested *Saccharomyces* strains (HAMBI10, 785, 1164, 1459 and 1165) showed very little inhibitory effect of oxalic acid on their growth. The biggest inhibitory effect proportional to increasing oxalic acid concentrations was observed with *Candida humilis* HAMBI1169 (Fig. 8F) and, therefore, it was excluded from further experiments. On the other hand, *S. cerevisiae* HAMBI1459 (Fig. 8E) showed almost no inhibitory effect which made this yeast species a good candidate for three-species co-cultures. Yeast species HAMBI1164 (Fig. 8A), HAMBI1165 (Fig. 8B), HAMBI10 (Fig. 8C) showed decreasing values of OD_{600} at 20.0 h after inoculation compared to OD_{600} values determined at 18.0 h after inoculation, while OD_{600} values of yeast species HAMBI785 (Fig. 8D), HAMBI1459 (Fig. 8E) and HAMBI1169 (Fig. 8F) determined at 20.0 h after inoculation remained similar as at 18.0 h after inoculation in the modified Okamoto medium 1 without oxalic acid.

4.2.3. Effect of FBCC4 filtrate with glucose on growth of selected yeast species

Monoculture FBCC4 was cultivated in modified Okamoto medium 2 for 18 days and mycelium and core board was separated by filtration from liquid phase of cultivation broth, as described in Chapter 3.2.20 in Experimental. To obtained filtrate, containing all metabolites of FBCC4, 2.0% of glucose was added. Sterile FBCC4 filtrate with glucose (FBCC4 FG) was used as a medium for cultivation of five *S. cerevisiae* strains (HAMBI10, 785, 1164, 1459 and 1165). Growth of five yeast monocultures in FBCC4 FG was compared to the growth of five yeast monocultures in modified Okamoto medium 1, as showed in Figure 9.

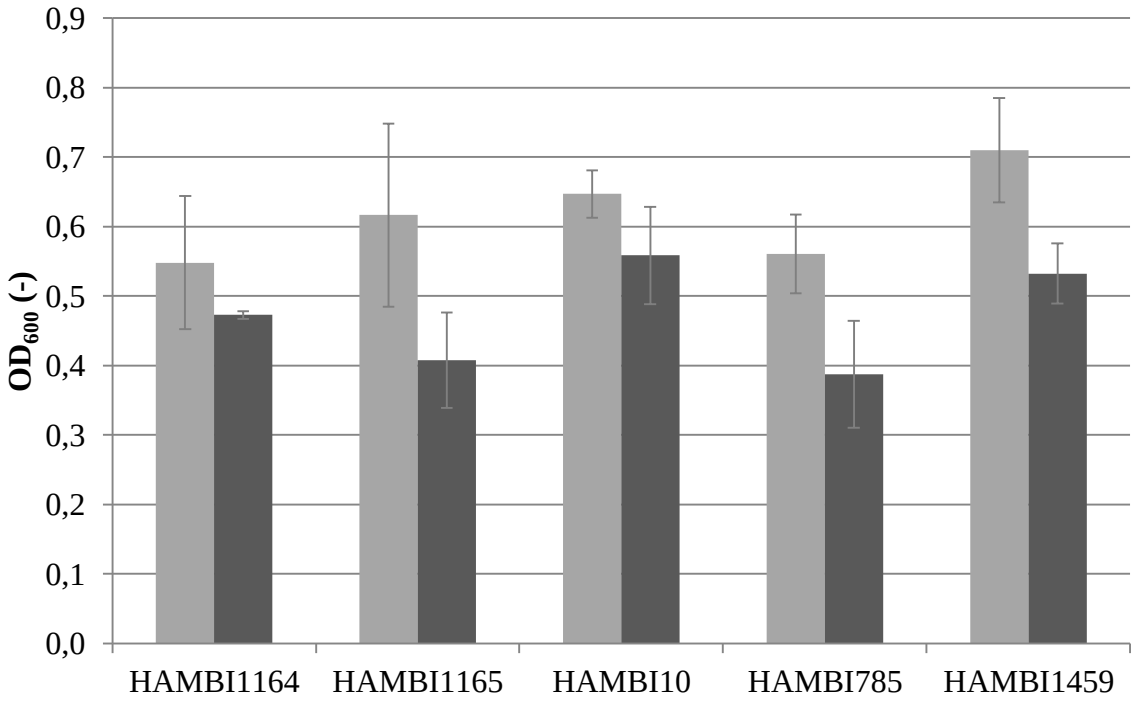

Figure 9. Optical density (OD_{600}) of five yeast species (HAMBI1164, HAMBI1165, HAMBI10, HAMBI785, and HAMBI1459) cultivated in modified Okamoto medium 1 (■) and in FBCC4 FG (■). Optical density was determined after 18.0 h of cultivation in modified Okamoto medium 1 or FBCC4 FG (both with working volume of 5.0 mL) in test tubes at 25°C with shaking (160 rpm). Cultivation procedure is described in Chapter 3.2.10. in Experimental.

All yeast strains presented in the Figure 9 were able to grow in FBCC4 FG, although less successfully when compared to their growth in modified Okamoto medium 1. According to similarities in growth curves obtained in experiments with modified Okamoto medium 1 without addition of oxalic acid (0.0 mM or 0.0 g L^{-1}; Fig. 8), *S. cereviasiae* strains were grouped in two groups: group A (HAMBI1164, 1165, 10) and group B (HAMBI785, 1459). Namely, *S. cerevisiae* strains from group A have similar growth curves with the highest value at 18 h and with decreasing values determined afterwards while *S. cerevisiae* strains from group B also have the highest value at 18 h but afterwards OD_{600} value remains almost constant (as in stationary growth phase).

Based on results from Fig. 8 and 9, one yeast strain from each group, A and B, was chosen for experiments performed with three-species co-cultivation: HAMBI1459, because of the greatest tolerance to oxalic acid (Fig. 8), and HAMBI1164, due to the lowest growth inhibition in monoculture FBCC4 FG when compared to the growth of the strain in modified Okamoto medium 1 (Fig. 9). In addition, both yeast strains were able to ferment glucose to ethanol under conditions used for *Phlebia* sp. cultivation (see Fig. 7).

4.3. Selection of yeast strain for ethanol production from lignocellulose by three-species co-culture

In this set of experiments ethanol production from lignocellulose catalysed by three-species co-cultures FBCC4 + FBCC43 + HAMBI1164 or HAMBI1459 was investigated. Changes in reducing sugars concentrations and ethanol production during the bioprocess catalysed by three-species co-cultures in modified Okamoto medium 2 are presented in Chapter 4.3.1. and growth of *S. cerevisiae* strains HAMBI1164 and HAMBI1459 during the bioprocess under described conditions is shown in Chapter 4.3.2.

Based on experimental results, strain HAMBI1164 was chosen as a good candidate for bioprocess conducted by three-species co-cultures FBCC4 + FBCC43 + HAMBI1164 (see Chapter 4.4.). Before further experiments phylogenetic analysis of all 12 yeast species used in this Thesis was done, as shown in Chapter 4.3.3.

4.3.1. Changes in reducing sugars concentrations and ethanol production during bioprocess by three-species co-cultures

Previously experimentally selected *S. cerevisiae* strains HAMBI1164 and HAMBI1459 were used in three-species co-cultivation with *Phlebia* species FBCC4 and FBCC43 in modified Okamoto medium 2. Reducing sugars concentrations and ethanol concentrations determined during 15 days long bioprocess in three-species co-cultivations FBCC4 + FBCC43 + HAMBI1164 or HAMBI1459 were compared to two-species cultivation FBCC4 + FBCC43. Results are shown in Figs. 10 and 11, respectively.

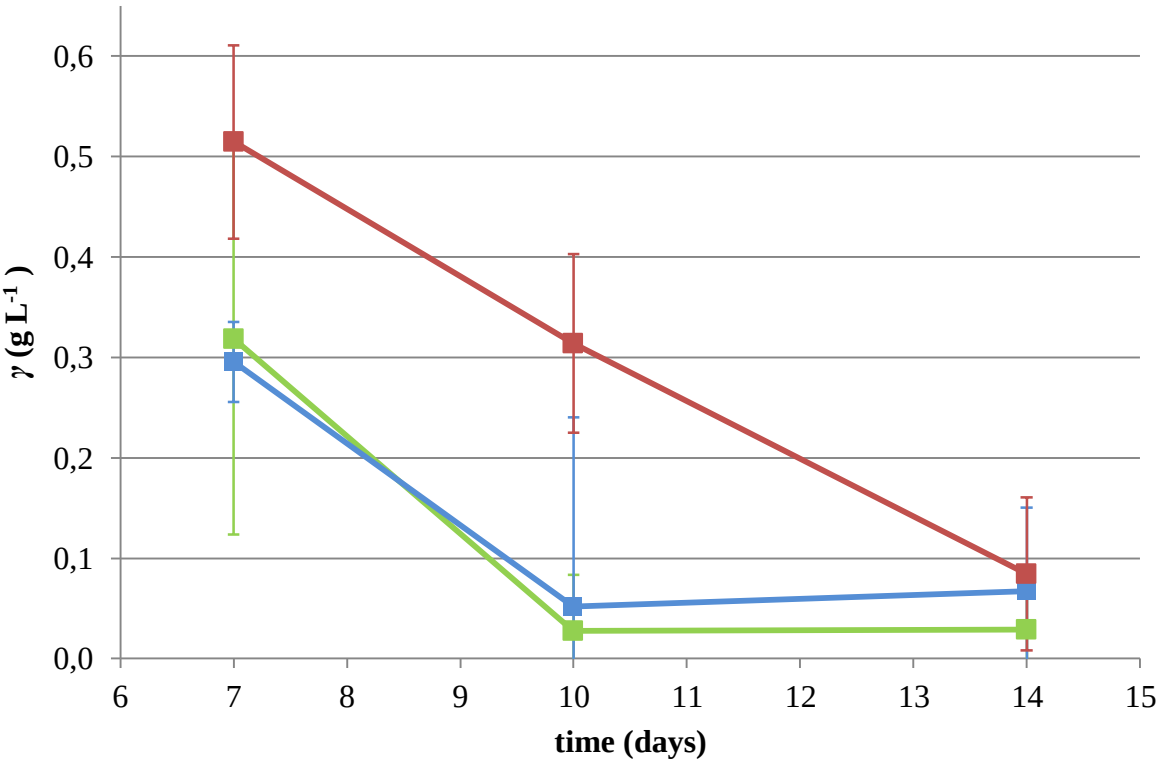

Figure 10. Changes in concentration of reducing sugars during cultivation of three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (■), three-species co-culture FBCC4 + FBCC43 + HAMBI1459 (■) and two-species co-culture FBCC4 + FBCC43 (■). The cultivations were carried out in modified Okamoto medium 2 in Erlenmeyer flasks (working volume 20.0 mL) at 25°C. Shaking (50 rpm) started after inoculation of yeast strains (HAMBI1164 and HAMBI1459) on the 7th day of bioprocess (see Chapter 3.2.11. in Experimental).

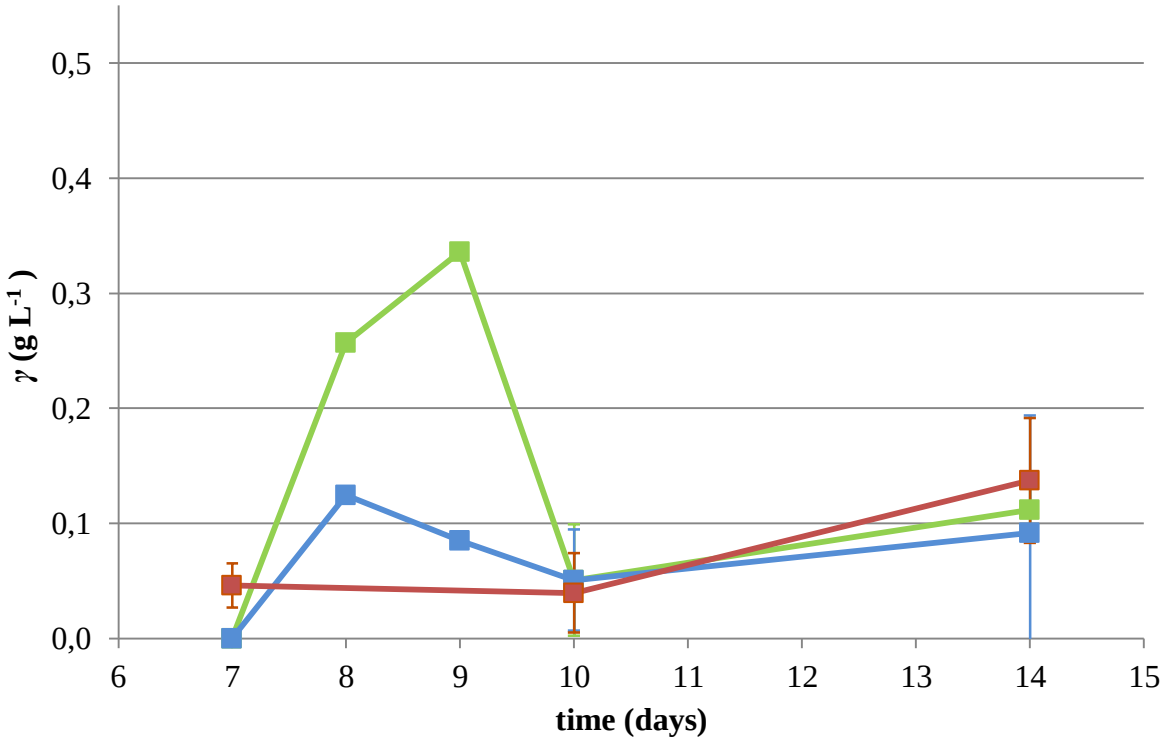

Figure 11. Ethanol production by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (■), three-species co-culture FBCC4 + FBCC43 + HAMBI1459 (■) and two-species co-culture FBCC4 + FBCC43 (■). The cultivations were carried out in modified Okamoto medium 2 in Erlenmeyer flasks (working volume 20.0 mL) at 25°C. Shaking (50 rpm) started after inoculation of yeast (HAMBI1164 and HAMBI1459) on the 7th day of bioprocess (see Chapter 3.2.11. in Experimental).

Changes in reducing sugars concentrations and ethanol production were followed after inoculation of *S. cerevisiae* HAMBI1164 or HAMBI1459 on the 7th day of bioprocess. Reducing sugars concentrations were in the range from 0.02 to 0.50 g L^{-1} (Fig. 10) and ethanol concentrations in range from 0.00 to 0.34 g L^{-1} (Fig. 11).

During first two days of three-species co-cultivation FBCC4 + FBCC43 + HAMBI1164 the production of ethanol increased rapidly. After the 9th day, reducing sugars concentration was below 0.1 g L^{-1} (see Fig. 10) and concentration of ethanol was rapidly decreased. Since fermentable (reducing) sugars were almost depleted (< 0.1 g L^{-1}) and ethanol was the only remaining simple carbon source (lignocellulose from core board was still present in the flask, see Figs. 19 and 20), it seems that the three-species co-culture FBCC4 + FBCC43 + HAMBI1459 under microaerophilic conditions used ethanol as a main carbon source.

On the 14th day of the bioprocess ethanol concentration slightly increased when compared to 10th day (Fig. 11), while the reducing sugars concentration remained the same as on the 10th day (Fig. 10).

Similar trend in reducing sugars consumption and ethanol production was observed in three-species co-culture FBCC4 + FBCC43 + HAMBI1459 with main difference in ethanol production between 7th and 10th day of bioprocess. *S. cerevisiae* strain HAMBI1164 is better ethanol producer under these conditions than *S. cerevisiae* strain HAMBI1459 as indicated in Fig. 11.

In two-species co-culture FBCC4 + FBCC43 reducing sugars concentration was constantly decreasing from 7th to 14th day of bioprocess, as indicated in Figure 10. Ethanol concentration measured on the 7th day of bioprocess was the same as measured on the 10th day, but slightly increased on the 14th day (Fig. 11). Compared to ethanol concentrations in three-species co-cultures on the 14th day of bioprocess, there was slightly more ethanol produced in two-species co-culture. However, the highest ethanol concentration (0.34 g L^{-1}) was detected on the 9th day of bioprocess in three-species co-culture FBCC4 + FBCC43 + HAMBI1164, therefore *S. cerevisiae* strain HAMBI1164 was selected for lignocellulose conversion to ethanol in three-species co-culture.

4.3.2. Growth of *S. cerevisiae* strains HAMBI1164 and HAMBI1459 during bioprocess in modified Okamoto medium 2

Growth of *S. cerevisiae* strains HAMBI1164 and HAMBI1459 (expressed as CFU mL^{-1}) in three-species co-culture FBCC4 + FBCC43 + HAMBI1164 and FBCC4 + FBCC43 + HAMBI1459 in modified Okamoto medium 2 was followed every 24.0 h from 7th to 10th day of bioprocess (see Chapter 3.2.13. in Experimental) in order to investigate if yeast strains were able to grow and be active in three-species co-culture. Results are compared to the growth of monoculture HAMBI1164 and monoculture HAMBI1459 also cultivated under the same conditions, as presented in Figure 12.

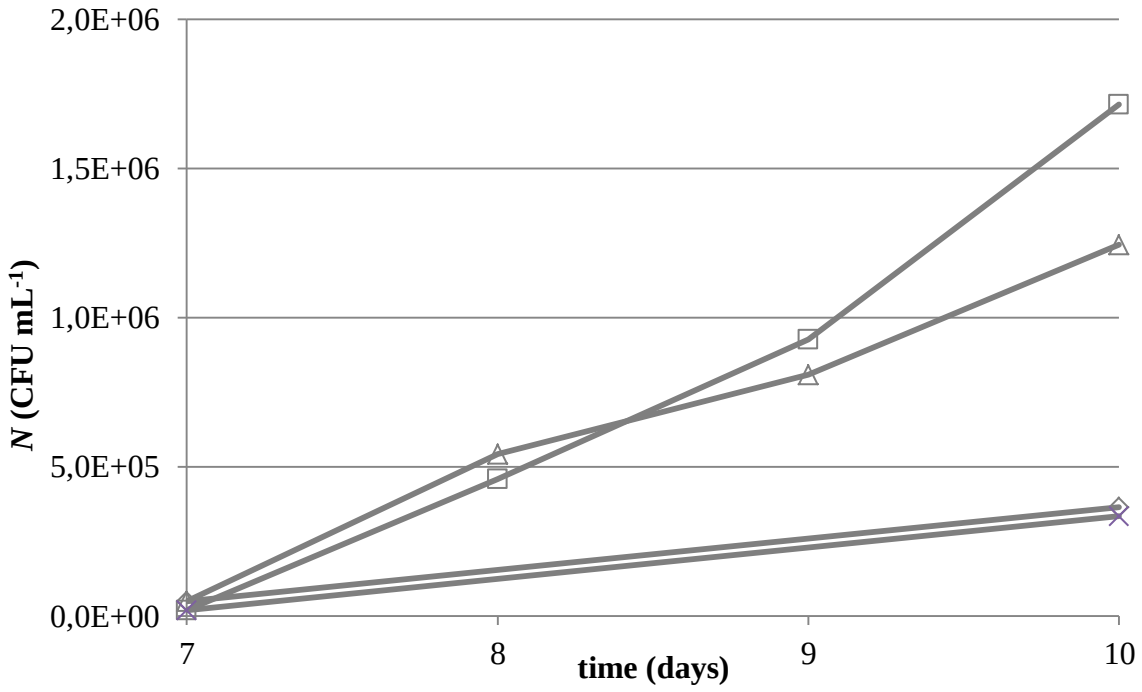

Figure 12. Growth of *S. cerevisiae* HAMBI1164 during bioprocess catalysed by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (Δ) and *S. cerevisiae* HAMBI1459 during bioprocess catalysed by three-species co-culture FBCC4 + FBCC43 + HAMBI1459 (□). Monocultures of HAMBI1164 (◊) and HAMBI1459 (×) were also cultivated as monocultures in modified Okamoto medium 2 in Erlenmeyer flasks (working volume 20.0 mL) at 25°C and shaking (50 rpm). Samples were withdrawn, as indicated, and inoculated on malt extract agar and incubated at 25°C over 48.0 h for determination of CFU, as described in Chapter 3.2.13. in Experimental.

Number of cells of both tested yeast strains HAMBI1164 and HAMBI1459 increased from the 7th to the 10th day of bioprocess catalysed by three-species co-cultures and this results showed that yeasts were able to grow and retain their metabolic activity during co-cultivation with filamentous fungi. When compared to control samples (monoculture HAMBI1164 and monoculture HAMBI1459 cultivated under the same conditions), number of cells of two yeast strains in three-species co-cultures were 10-fold higher. In addition, core board mass loss at the end of the bioprocess conducted by monoculture HAMBI1164 or monoculture HAMBI1459 was not detected (data not shown) while core board mass loss

during the bioprocess catalysed by three-species co-culture was observed, as shown in Figs. 17 and 20.

Based on these results it may be assumed that yeast strains HAMBI1164 and HAMBI1459 cannot hydrolyse hemicellulose from core board in medium in which core board was the only carbon source. As a consequence, the yeast strains do not grow, as confirmed experimentally. All in all, it might be concluded that three-species co-cultures create favourable conditions for growth and activity of two yeast strains.

4.3.3. Phylogenetic analysis of 12 yeast species

Phylogenetic tree comprised 12 yeast species (see Table 1. In Experimental) is constructed as described in Chapter 3.2.16. in Experimental and presented in Fig. 13. Reverse-phase microscopy was used to create image of selected *S. cerevisiae* HAMBI1164, as shown in Fig. 14.

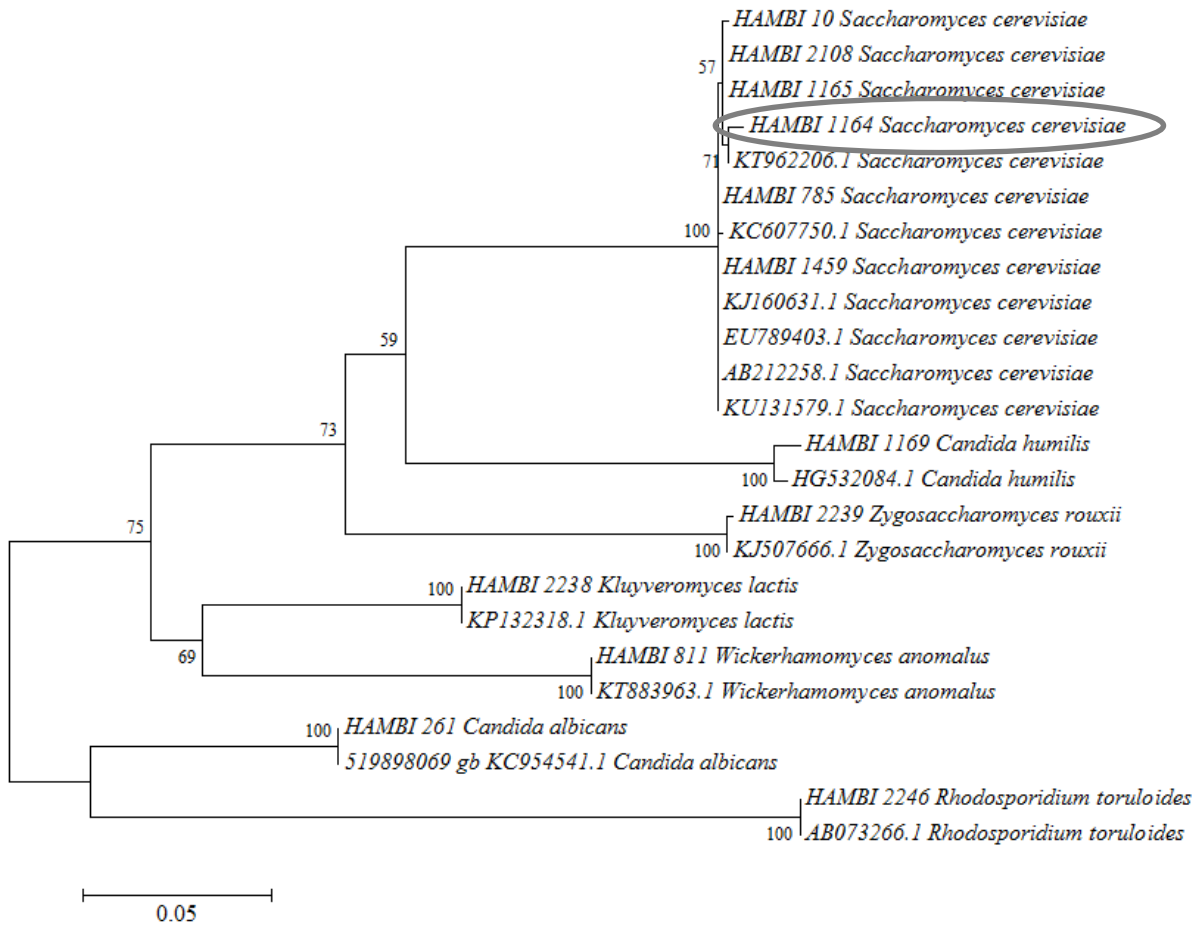

Figure 13. Molecular phylogenetic analysis of 12 yeast species from HAMBI collection (see Table 1. in Experimental) and 12 reference sequences from NCBI, based on ITS1-5.8S-ITS2 sequences, created by Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Bootstrap values (100 replications) higher than 50% are indicated for nodes. Scale bar represents nucleotide substitutions per position. The yeast species *Rhodosporidium toruloides* was chosen as an outgroup sequence and used as a tree root. Yeast strain *Saccharomyces cerevisiae* HAMBI1164, marked with grey circle, was chosen for bioprocess for ethanol production from lignocellulosic material.

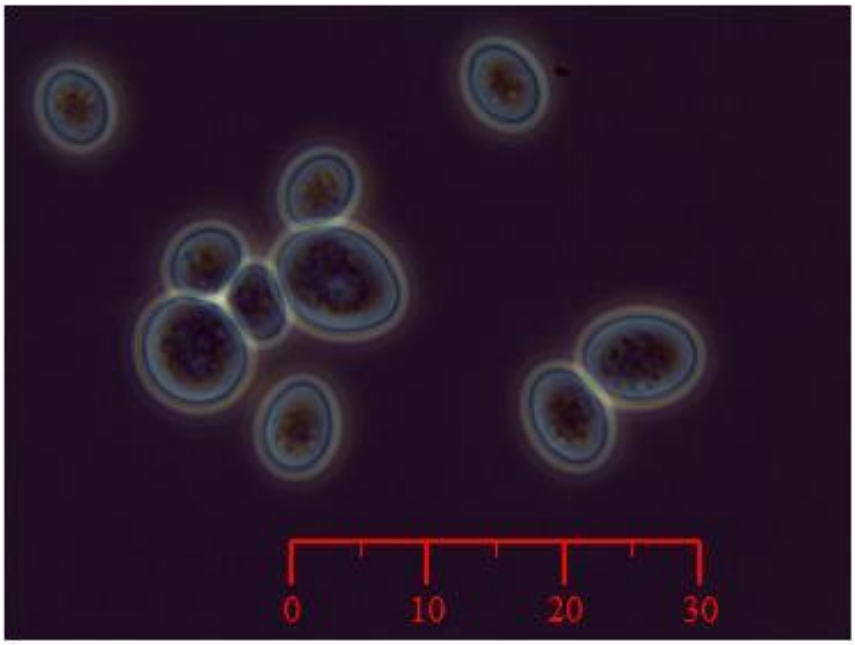

Figure 14. Image of *S. cerevisiae* HAMBI1164 cells obtained by reverse-phase microscopy (Leica E3 camera; Leica, Wetzlar, Germany) with respective size-scale in µm created by using Dia Diagram Editor version 0.97.2 (see Chapter 3.2.14.).

4.4. Ethanol production from lignocellulose by three-species co-culture FBCC4 + FBCC43 + HAMBI1164

After selecting the yeast strain for the ethanol production by three-species co-culture, the optimal duration of the bioprocess was determined, as described in Chapter 4.4.1. Ethanol production by lignocellulose conversion using three-species fungal co-cultures is discussed in Chapter 4.4.2.

4.4.1. Determination of the bioprocess duration

Direct ethanol production from lignocellulose catalysed by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 in modified Okamoto medium 2 was performed in two sets of experiments, as described in Chapter 3.2.18. in Experimental. Ethanol and reducing sugars concentrations during bioprocess 1 and 2 are shown in Figure 15.

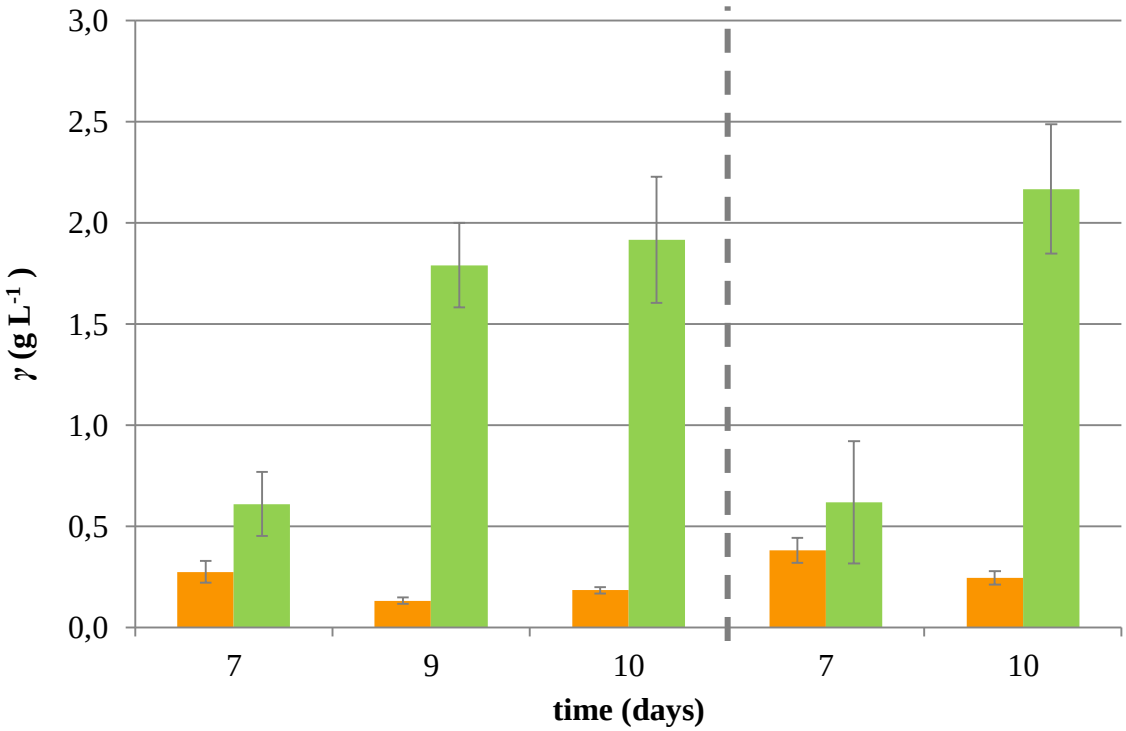

Figure 15. Ethanol (■) and reducing sugars concentration (■) during bioprocess 1 (on the left side of the Fig.) and bioprocess 2 (on the right side of the Fig.) (separated by dashed grey line), both catalysed by three-species co-culture FBCC4 + FBCC43 + HAMBI1164. Both bioprocesses were carried out in modified Okamoto medium 2 in Erlenmeyer flasks (working volume of 20.0 mL) at 25°C and shaking at 50 rpm. (see Chapter 3.2.18. in Experimental).

In both bioprocesses reducing sugars concentration from 7th to 10th day was below 0.5 g L^{-1}. In the bioprocess 1 reducing sugars concentration is decreasing from the 7th until the 9th day when it started to increase to the 10th day of the bioprocess. In bioprocess 2 reducing sugars concentration determined on the 10th day was lower than reducing sugars concentration determined on the 7th day. The highest ethanol concentration (2.17 g L^{-1}) was reached on the 10th day of the bioprocess 2. In the bioprocess 1 and the bioprocess 2 ethanol concentrations were increasing from 7th to 10th day of bioprocess. Since the ethanol concentrations were not significantly higher on the 10th day compared to 9th day of bioprocess, it was reasonable to reduce duration of the bioprocess to 9 days.

4.4.2. Ethanol production by lignocellulose conversion using three-species fungal co-cultures

Ethanol production from lignocellulose using three-species co-culture FBCC4 + FBCC43 + HAMBI1164 was compared to ethanol production from lignocellulose using two-species co-culture FBCC4 + FBCC43 and the results are presented in Figure 16. Both fungal co-cultivations, three- and two-species, were performed in modified Okamoto medium 2 under the same conditions. In addition, core board mass loss, mycelia weight and ethanol yield are discussed below.

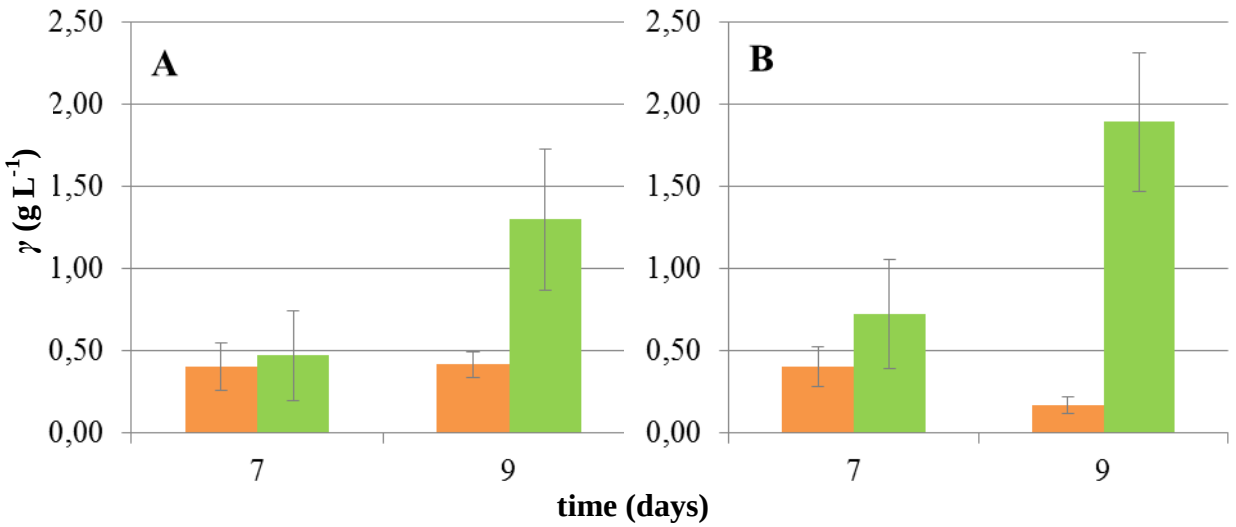

Figure 16. Ethanol (■) and reducing sugars concentration (■) during bioprocess catalysed by two-species co-culture FBCC4 + FBCC43 (A) and bioprocess catalysed by three-species co-culture FBCC4 + FBCC43 + FBCC1164 (B). Both bioprocesses were carried out in modified Okamoto medium 2 in Erlenmeyer flasks (working volume 20.0 mL) at 25°C and shaking at 50 rpm (see Chapter 3.2.19. in Experimental).

Reducing sugars concentration in experiments performed by two-species co-culture (Fig. 16A) remained almost constant over two days of the bioprocess (from the 7th to 9th day) while in experiments performed by three-species co-culture (Fig. 16B) reducing sugars concentration decreased on the 9th day of the bioprocess. Results clearly show that the yeast strain contributed to reducing sugars consumption. This conclusion was further confirmed by data obtained for ethanol concentration which were higher at the end of the bioprocess catalysed by three-species co-culture (1.89 g L^{-1}) than in the bioprocess catalysed by two-species co-culture (1.30 g L^{-1}).

Ethanol yield was calculated according to equations [2] and [3] in Chapter 3.2.21. in Experimental. The bioprocess procedure created in this Thesis, catalysed by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 over 9 days in total, resulted in 139.5 mg and 37.8 mg ethanol produced per gram of consumed and total core board, respectively. In comparison, in the bioprocess conducted by two-species co-culture FBCC4 + FBCC43 over 9 days 122.1 mg and 26.0 mg of ethanol per g of consumed and total core board was produced, respectively.

Further, the highest ethanol yield (37.8 mg g^{-1}) obtained by simultaneous saccharification and fermentation of untreated core board by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 over 9 days can be compared to ethanol production from sugarcane bagasse where ethanol yield of 64.2 mg g^{-1} bagasse was achieved after four weeks of delignification followed by 20 days of fermentation catalysed by *Phlebia* sp. (Kondo et al.; 2014). Results from this Thesis might be also compared to oak wood utilization in ethanol production although the carbon sources composition differs. When the oak wood was used as only carbon source, 359.7 mg of ethanol per g of wood was produced after 56 days of delignification and 20 days of fermentation (Kamei et al. 2012b).

Results obtained in this Thesis seem to be promising for further investigation on lignocellulose conversion into ethanol by using tested co-cultures. On the other hand, more promising results have been already published. In bioprocess with unbleached hardwood kraft pulp as substrate strain *Phlebia* sp. MG-60 produced 420 mg of ethanol per g of unbleached hardwood kraft pulp over 7 days (Kamei et al. 2012a). More efficient bioprocess might be result of utilization of different *Phlebia* strains as biocatalysts, more favourable carbohydrate types in the substrate and inocula preparation.

4.4.2.1. Mycelial biomass growth and core board weight loss

Core board and mycelia weight were determined as described in Chapter 3.2.20. in Experimental. Calculations for core board weight loss and mycelia weight were done according to equations presented in Chapter 3.2.21. in Experimental. Comparison of core board weight loss and mycelial synthesis between 9th and 15th day of bioprocess is shown in Figs. 17 and 18, respectively. Changes in consistency of modified Okamoto medium 2 and mycelial growth of tested co-cultures is shown in Fig. 19. Separated mycelial biomass and core board, which remained after the bioprocesses, were shown in Fig. 20.

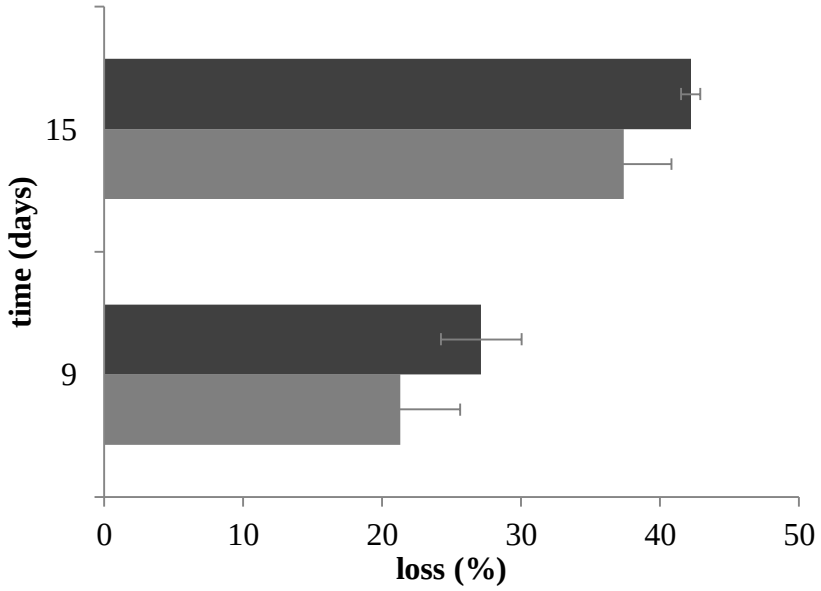

Figure 17. Core board weight loss (%) after 9 and 15 days of bioprocess catalysed by two-species co-culture FBCC4 + FBCC43 (■) and three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (■).

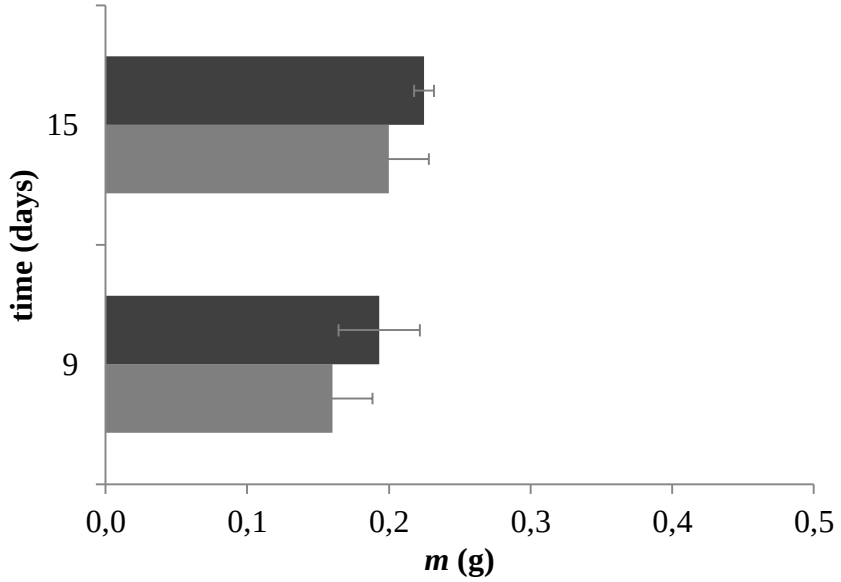

Figure 18. Weight of dry mycelial biomass (*m*) as determined after 9 and 15 days of bioprocess catalysed by two-species co-culture FBCC4 + FBCC43 (■) and three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (■).

The mycelial biomass produced in three-species co-culture was 0.70 g of dry mycelial biomass per g of consumed core board and in two-species co-culture 0.75 g dry mycelial biomass per g of consumed core board was produced.

The core board mass loss was higher in three-species co-culture (27.1%) than in two-species co-culture (21.3%). It might be assumed that reducing sugars concentration during the bioprocess catalysed by two-species co-culture (γ = 0.40-0.42 g L^{-1}; Fig. 16A) affects the hydrolysis of lignocellulose while lower and decreasing concentration of reducing sugars during the bioprocess conducted by three-species co-culture (γ = 0.40-0.17 g L^{-1}; Fig. 16B) has reduced effect on the lignocellulose hydrolysis. So, addition of yeast strain HAMBI1164 to the co-culture (FBCC4 + FBCC43 + HAMBI1164) decreased concentration of reducing sugars and increased core board mass loss.

It was previously reported that presence of relatively low concentration of glucose (0.05% w/v) decreased the cellulose breakdown by *Phlebia* sp. (Cho et al.; 2009). Also, bagasse weight loss during the bioprocess conducted by *Phlebia* sp. MG-60 after 4 weeks was 16.4% (Kondo et al. 2014).

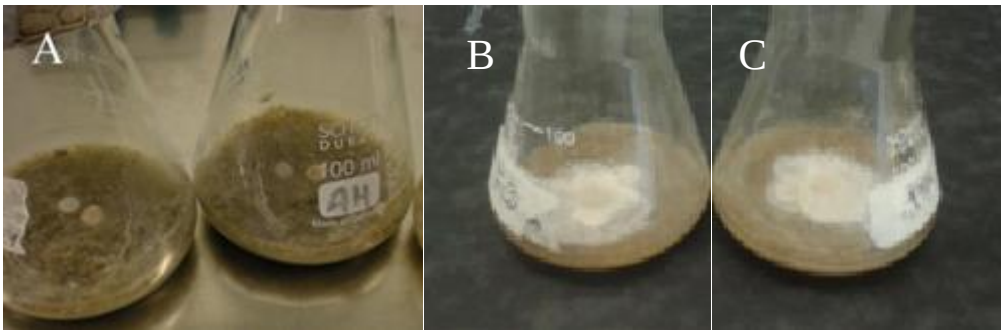

Figure 19. Consistency of modified Okamoto medium 2 inoculated by two-species co-culture FBCC4 + FBCC43 at the beginning of the bioprocess (A) and after 9 days of bioprocess carried out by two-species co-culture FBCC4 + FBCC43 at 25°C without shaking (B), and three-species co-culture FBCC4 + FBCC43 + HAMBI1164 after 9 days of bioprocess carried out at 25°C with shaking at 50 rpm (C).

After 9 days the consistency of modified Okamoto medium 2 utilised by two-species co-culture FBCC4 + FBCC43 (Fig. 19B) was visually the same as the consistency of modified Okamoto medium 2 utilised by three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (Fig. 19C). It might be assumed that the addition of yeast HAMBI1164 in modified Okamoto medium 2 containing two-species co-culture FBCC4 + FBCC43 at the 7^{th} day of the bioprocess did not affect the mycelial growth of the white-rot fungi FBCC4 and FBCC43.

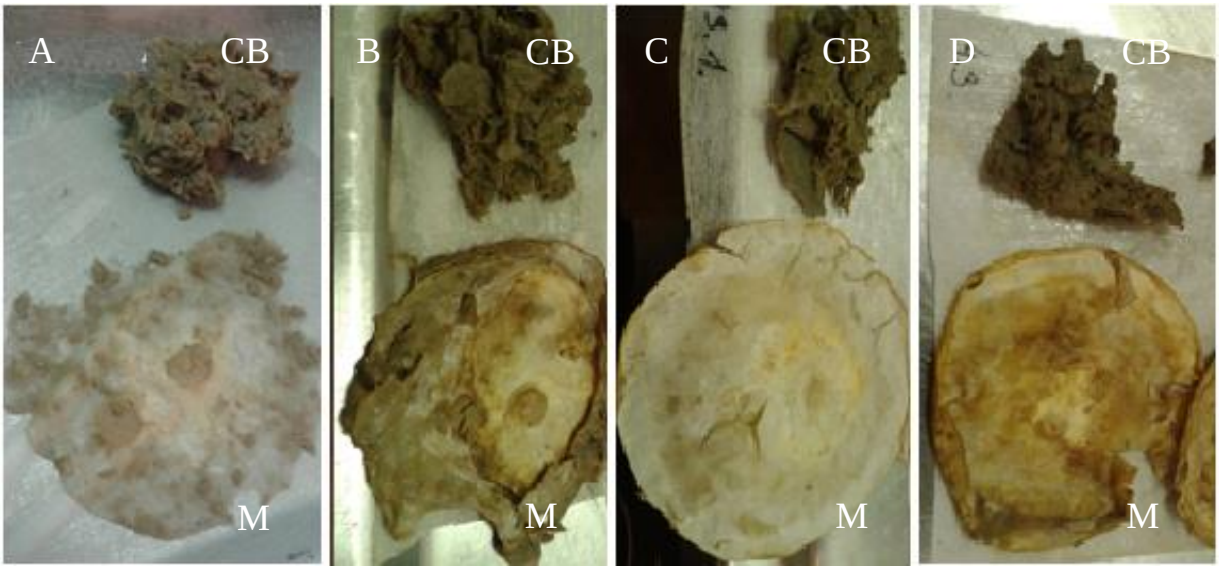

Figure 20. Mycelia (M) and remaining core board (CB) withdrawn from the bioprocesses carried out in modified Okamoto medium 2 in Erlenmeyer flasks at 25°C after 9 or 18 days by: three-species co-culture FBCC4 + FBCC43 + HAMBI1164 (after 9 days; A), two-species co-culture FBCC4 + FBCC43 (after 18 days; B), monoculture FBCC4 (after 18 days; C), and monoculture FBCC43 (after 18 days; D).

5. CONCLUSIONS

Based on the results presented in this Thesis following conclusions can be made:

1. Bioprocess for ethanol production from lignocellulosic raw material was investigated. Filamentous fungi *Phlebia acerina* FBCC4 and *Phlebia radiata* FBCC43 were employed as biocatalysts in direct conversion of lignocellulose to ethanol and the bioprocess was improved by employing three-species co-culture of *Phlebia acerina* FBCC4, *Phlebia radiata* FBCC43 and yeast *Saccharomyces cerevisiae* HAMBI1164 (three-species co-culture FBCC4 + FBCC43 + HAMBI1164). Experiments were performed in modified Okamoto medium 2 in which core board (50.0 g L^{-1}) lignocellulose was main carbon and energy source.
2. Two-species co-culture FBCC4 + FBCC43 hydrolysed core board lignocellulose more efficiently than the monoculture FBCC4 and monoculture FBCC43 and produced reducing sugars in range from 0.11 g L^{-1} to 0.42 g L^{-1}. The highest reducing sugars concentrations were observed between 7^{th} and 10^{th} day of the bioprocess. Increasing portion of inocula of monoculture FBCC4 and monoculture FBCC43 which was added to the medium did not increased concentration of reducing sugars.
3. Between 12 tested yeast species *S. cerevisiae* HAMBI1164 was selected as the best candidate for three-species co-culture FBCC4 + FBCC43 + HAMBI1164. Monoculture HAMBI1164 fermented glucose from modified Okamoto medium 1 and very efficiently produced ethanol. In addition, growth of the monoculture was almost not affected by oxalic acid nor ingredients of monoculture FBCC4 filtrate with glucose. Furthermore, HAMBI1164 efficiently grew as a member of three-species co-culture FBCC4 + FBCC43 + HAMBI1164 in modified Okamoto medium 2.
4. Three-species co-cultures FBCC4 + FBCC43 + HAMBI1164 produced the highest concentration of ethanol (0.34 g L^{-1}) on the 9^{th} day of the bioprocess carried out in modified Okamoto medium 2 under microaerophilic conditions. When almost anaerobic conditions were created, higher concentration of ethanol (1.89 g L^{-1}) was produced on 9^{th} day of the bioprocess. During this period weight of core board from modified Okamoto medium 2 was reduced for 27.1 % of the initial weight while weight of dry mycelial biomass increased to 0.19 g in one Erlenmeyer flask.

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