AIMS Energy, 6 (5): 866–879.
DOI: 10.3934/energy.2018.5.866
Received: 30 June 2018
Accepted: 10 October 2018
Published: 18 October 2018

http://www.aimspress.com/journal/energy

Research article

Lignocellulose bioconversion to ethanol by a fungal single-step consolidated method tested with waste substrates and co-culture experiments

Hans Mattila, Dina Kačar, Tuulia Mali and Taina Lundell*

Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Helsinki, Finland

* **Correspondence:** Email: taina.lundell@helsinki.fi.

Abstract: The *Polyporales* phlebioid white rot fungus *Phlebia radiata* is efficient in decomposing the wood main components, and in producing ethanol from lignocelluloses and waste materials. Based to these qualifications, the fungus was adopted for design of a consolidated bioprocess method to convert wood waste materials into ethanol without pretreatments. Higher ethanol yield was aimed by introducing collaborative fungal cultivations including isolates of *Saccharomyces cerevisiae*, other yeasts, and a brown rot fungus. Various waste lignocellulose materials such as wheat and barley straw, recycled wood-fiber based core board, recycled construction waste wood, spruce saw dust, and birch wood were applied to represent wood and non-wood waste lignocellulose of different origin, chemical content and structure. In solid-state single cultivations with the white rot fungus *P. radiata*, both core board and barley straw turned out as suitable substrates for the consolidated bioprocess. Up to 32.4 ± 4.5 g/L of ethanol accumulated in the solid-state core board cultivation in 30 days whereas with barley straw, 7.0 ± 0.01 g/L of ethanol was obtained. Similar concentrations of ethanol were produced in increased-volume and higher gravity bioreactor cultivations without chemical, physical or enzymatic pretreatment. In all, our consolidated method adopting a white rot fungus is a promising and economic alternative for second generation bioethanol production from waste and residual lignocelluloses.

Keywords: *Phlebia radiata* 79; consolidated bioprocess; bioethanol; white rot fungi; waste lignocellulose; bioconversion; solid state cultivation; bioreactor

Abbreviations: CBP: Consolidated bioprocess; FBCC: Department of Microbiology, University of Helsinki, fungal biotechnology culture collection; HPLC: High-performance liquid chromatography; ITS-PCR: Internal transcribed spacer-Polymerase chain reaction; YE: Yeast extract.

1. Introduction

From currently available options for renewable transportation fuels, bioethanol is the cheapest choice from the consumer's perspective, as well as the most straightforward alternative for fossil fuels to be used in vehicles equipped with combustion engines [1,2]. Global production of ethanol is currently over 41×10^6 gallons, and the increasing production trend can lead to decreasing consumption of crude oil [1]. In contrast to the environmentally acceptable but yet expensive electric cars, bioethanol utilizing flexible combustion engine is an accessible option for hundreds of millions of car users, especially in developing countries and newly industrialized economies. Bioethanol is currently predominantly produced by the first generation bioethanol processing methods that rely on starch and sucrose containing feedstocks, i.e. from maize, sugar cane, sugar beet and cereal grains [2].

However, first generation bioethanol production conflicts with current global challenges in the food supply chain, reduction of farming land area, climate change, and environmental conservation aspects and policies [2]. Substrate cost is also one of the major expenses in the first generation bioethanol production industry [1]. For these reasons, wood-based and non-wood lignocellulose waste fractions from industry, communities and agriculture would make a low cost, more sustainable and ethically acceptable choice for bioethanol production.

Our consolidated process for bioethanol production uses *Polyporales* phlebioid white rot fungi [3], which have proven to be particularly adoptable among wood-decay fungi for wood and wood-based lignocellulose bioconversions [4,5] and bioethanol production from cellulosic substrates [3,6]. Especially the species *Phlebia radiata* isolate 79 expresses a versatile array of carbohydrate-active enzymes and oxidoreductases that enzymatically decompose the main components of wood (cellulose, hemicellulose and lignin) [5]. This fungal isolate is capable for simultaneous or subsequent ethanol fermentation from the released sugars [3]. Due to the efficient wood and solid substrate colonization ability, and decomposition characteristics of the wood-decay fungus, enzyme additions and commonly used pretreatments are not required in this process [3,7].

Traditional biological pretreatment applications on lignocellulosic feedstocks utilize white rot fungi only for modification and saccharification of the solid substrates into fermentable sugars [8]. The released fermentable sugars are then subsequently transformed into ethanol by a strong yeast isolate during consecutive cultivation and bioprocess [9]. In the single-species, single-step consolidated bioprocess concept, however, fermentation step to ethanol is carried out by the same lignocellulose-decaying and sugar-releasing fungus and thereby, the second (yeast fermentation) phase may be avoided [3,6,9].

This study explores the potential of using *P. radiata* for production of bioethanol on various waste lignocellulose substrates, either in single-species or in combination cultivations with yeast or another wood-decaying fungus. The aim of the co-cultivations including white rot fungus and yeast were to increase the degree of bioconversion of the solid substrate by advancing cellular intake rate of the released sugars and thereby, to enhance total ethanol yield in the process.

Our goal was also to explore the capacity of *P. radiata* in utilizing various heterogenic lignocellulose residuals and waste substrates, in order to convert them into ethanol without generally

adopted physical, chemical or enzyme-supplementation pretreatment steps. Upscaling from laboratory scale into industrial scale has been one of the major obstacles in commercializing lignocellulose-based second-generation bioethanol production. Taking this into account, the first trials of larger volume-scale bioconversions and bioreactor cultivations on the lignocellulosic waste substrates are presented.

2. Materials and methods

2.1. Substrate materials

Lignocellulose waste substrates of different origins and recycling properties were assessed in order to develop the solid state bioethanol production method described previously [3]. Both wood-based substrate materials (core board from tissue paper rolls, recycled construction and municipal wood waste, spruce wood sawdust, birch wood sticks) and non-wood agricultural wastes (wheat and barley straw) were adopted due to their good availability and lack of established further recycling possibilities. Cereal straw acts as an example of agricultural wastes, recycled wood waste and core board in turn represent the community wastes, and spruce sawdust and shavings are side-stream sources of forest-based industry.

2.2. Fungal isolates

Fungal isolates of this study are stored at the HAMBI-FBCC fungal culture collection of the Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki [http://www.helsinki.fi/hambi/HAMBI_eng/mainpage_eng/index.html]. Cultivation of the isolates was first conducted on 2% malt extract agar plates for one week at +25 °C. Identification of the filamentous fungal and yeast isolates was verified by ITS-PCR and sequencing [10,11].

2.3. Selection of the yeast isolate and co-cultivations

To select the most suitable yeast isolate for co-cultivations, all 12 yeast isolates (Table 1) were first screened for their sugar consumption capability, ethanol production and growth (OD_{600}) properties in liquid medium. All isolates were first cultivated overnight at 28 °C, 120 rpm, in a 0.5% (w/vol) yeast extract −2% (w/vol) glucose medium, pH 6.0, glucose as the carbon source supporting yeast growth. Yeast growth was assessed after 24 h by measuring optical density (OD_{600}) of the liquid cultures. Ethanol production rate and glucose consumption rate were assessed by first diluting the overnight cultivations into 0.5 OD_{600}. Equal cell densities of all the 12 yeast isolates were then incubated at 25 °C under agitation of 50 rpm for 24 h in the basal medium for ethanol production supplemented with 2% (w/vol) glucose and under semi-aerobic conditions [3].

Table 1. Fungal isolates used in this study.

	Isolate identifier	Species name
Wood decay fungi	FBCC 0004	*Phlebia acerina*
	FBCC 0043	*Phlebia radiata*
	FBCC 1181	*Fomitopsis pinicola*
Yeasts	FBCC 2577	*Saccharomyces cerevisiae*
	FBCC 2471	*Saccharomyces cerevisiae*
	FBCC 2525	*Saccharomyces cerevisiae*
	FBCC 2576	*Saccharomyces cerevisiae*
	FBCC 2539	*Saccharomyces cerevisiae*
	FBCC 2526	*Saccharomyces cerevisiae*
	FBCC 2527	*Candida humilis*
	FBCC 2573	*Kluyveromyces lactis*
	FBCC 2494	*Pichia anomala*
	FBCC 2458	*Candida albicans*
	FBCC 2574	*Zygosaccharomyces rouxii*
	FBCC 2575	*Rhodosporidium toruloides*

Second screening for the suitable yeast isolates assessed the tolerance towards oxalic acid, which is generally produced by wood decay *Polyporales* brown and white rot fungi [4,11,12], or tolerance against the culture fluids of phlebioid fungi. Fresh cultivation liquid medium with glucose [3,6] was supplemented with oxalic acid (Sigma) by adding either 0.0 g L^{-1}, 0.009 g L^{-1}, 0.09 g L^{-1} or 0.9 g L^{-1} to get final concentrations of 0.0 mM, 0.1 mM, 1.0 mM or 10.0 mM of oxalic acid in the medium, respectively. Yeast isolates were cultivated under aerobic conditions in glass test tubes at 25 °C with agitation at 160 rpm. Samples were taken after 4 h, 6 h, 18 h and 24 h of inoculation.

Effect of the phlebioid secretome was tested by using a culture filtrate liquid from an 18 day-cultivation of the white rot phlebioid fungus *Phlebia acerina* (Table 1) as the basal growth medium for the yeasts. Glucose (2% w/vol) was added to the phlebioid culture filtrate and the solution was autoclaved (115 °C, 1 atm, 15 min). The yeast isolates were all cultivated overnight (25 °C, 160 rpm) in the phlebioid filtrate—glucose medium, and yeast growth (OD_{600}) was assessed after 18 h.

Three fungal species co-cultivations were carried out with 100 ml glass Erlenmeyer flasks under anaerobic conditions adopting rubber plugs as culture flask seals [3]. Core board (1.0 g dry weight) was used as the solid substrate for the two filamentous fungi (*P. radiata* and *P. acerina*, Table 1) and thereby, as the original carbon source also for the selected *S. cerevisiae* yeast isolate (FBCC 2525, Table 1), which was inoculated in the liquid phase of the culture 7 days after initiating the solid-state cultivation with the filamentous fungi.

2.4. Solid-state cultivations on lignocelluloses for ethanol production

The solid-state cultivations on different waste lignocelluloses were performed with three biological replicate cultures in 100 ml glass Erlenmeyer flasks containing 20 ml of 1% (w/vol) yeast extract (YE) solution, or additionally, 20 ml of Milli-Q ultrapure water including 1 g (dry weight) of

brewery spent barley mash. Larger scale bioreactor cultivations (2.0–20 L scale) were performed without replicates. Barley mash represented an inexpensive waste source for organic nitrogen and vitamins for the fungi.

Dry matter load varied between 2.0 and 4.0 g depending on the cultivation type and substrate. Wheat and barley straw were cut into 2–3 cm long pieces, the air-dried Norway spruce shavings and sawdust were sieved before use with a 2–5 mm mesh size metal sieve, and the milled waste core board and recycled wood waste were sieved with an 8 mm mesh size metal sieve before autoclaving. Bioreactor cultivations were carried in tightly sealed glass vials of 2.0 L and 20 L with 46 g (dry weight) of cut wheat straw, 50 g (dry weight) of core board, and 800 g (dry weight) of spruce sawdust as the solid-state lignocellulose waste material substrates. Solid state bioreactor cultivations were conducted without any agitation mechanism and overpressure formed during the process was automatically released through a fermentation airlock. Inlets of the lid were sealed with rubber plugs. *P. radiata* was used as the single fungus in the flask scale cultivations. In bioreactors containing straw and core board, co-cultivation with *P. radiata* and *F. pinicola* was carried out. All solid substrates were dry-heated (110 °C, 10 min) prior to mixing with the autoclaved (121 °C, 1 atm, 15 min) liquids, and initiation of the cultivations.

2.5. Solid substrate optimization and bioconversion yield calculations

All cultivations were followed until the formation of overpressure gas ceased, indicating the end of ethanol fermentation. Concentration of ethanol and other soluble extracellular metabolites were measured during and at the end of the cultivations. Cultivations for optimization of the dry weight ratio of the substrate lignocelluloses were conducted by adding 2.0, 3.0 or 4.0 g of dry core board, or dried and cut barley straw, into 100 ml Erlenmeyer glass vials containing 20 ml of 1% YE solution, pH 3. Carbohydrate content of the core board (arabinose 5.8 mg, galactose 5.8 mg, glucose 577.5 mg, xylose 100.1 mg and mannose 41.7 mg, or 1.0 g of dry core board) was previously analyzed [13]. The carbohydrate content of barley straw varies depending on the plant variety, growth location and land, seasonal conditions, crop batch and harvest time [14]. An estimation based on literature values (glucan content 357 mg and xylan content 233 mg/g of dry barley straw) was used for barley straw [15]. Theoretical yields were calculated as described in [16]:

$$Max\ yield = (substrate\ hexose\ and\ pentose\ content\ (g) * 0.51) \quad (1)$$

or

$$Max\ yield = (cellulose\ or\ glucan\ (g) * 0.5679) + (xylan\ (g) * 0.5808) \quad (2)$$

2.6. Ethanol formation and other culture compound analyses

Optical density of the yeast cultivations in liquid media, and concentrations of reducing sugars in the culture fluids were determined by 96-well plate analyses in 150 µl volume using Infinite M200 multiwell reader spectrophotometer and Magellan software (Tecan, Austria). Sterile culture medium was used as blank reference in the measurements. Quantity of the reducing sugars in the yeast cultivations was determined by using the dinitrosalicylic acid based method adopted previously [17].

Statistical computational analyses (Student's T-test) were conducted using the IBM-SPSS statistics 24 software.

Ethanol concentrations were measured as previously [3] and by adopting an HPLC method, in which detection of dissolved ethanol, and other fungal-secreted extracellular metabolites was conducted with Waters Alliance separation module e2695 HPLC coupled with two consecutive detectors, Waters 996 photodiode array detector and Hewlett Packard HP1047A RI (refractive index) detector. Analyte separation was conducted with Agilent Hi-Plex H 300 × 6.5 mm column together with Agilent PL-Hi-Plex H Guard Column (50 × 7.7 mm). Sample injection of 20 µl was used and the analytes were eluted with isocratic 5 mM H_2SO_4 at 0.6 ml/min flow rate. Column temperature was kept at 65 °C. Commercially available analytical grade or higher quality reference compounds of ethanol, methanol, glycerol, glucose, xylose, cellobiose and acetic acid were used to quantify and identify the extracellular metabolites and lignocellulose carbohydrate degradation products.

To measure the dry weight of the core board substrate and the fungal biomass as formation of mycelia on the substrate, the fungal hyphae were peeled off from the core board at the end of the cultivations. The separated substrate (used core board) and mycelium were dried at 105 °C for 24 h for dry mass determination.

3. Results

3.1. Selection of the ethanol producing yeast isolate

Yeast isolates (Table 1) were screened by adopting several tests to establish the best adoptable and tolerable yeast for ethanol production in co-cultures with the phlebioid wood-decay fungi. At first, the capability of the yeast cultures to consume glucose, to yield ethanol, and to grow on 20 g/L of glucose as the carbon source was tested.

All of the *S. cerevisiae* isolates and the *Candida humilis* isolate (FBCC 2527) produced ethanol from glucose (Figure 1). Five *S. cerevisiae* isolates (FBCC 2577, 2471, 2525, 2539 and 2526) were able to almost completely utilize the available glucose resulting with production of a reasonable quantity of ethanol. Thus, based on these features, these five yeast strains were selected for further experiments.

The ability of the selected five yeasts to tolerate oxalic acid and phlebioid fungal culture fluid was also tested (Figure 2). The white-rot fungal culture fluid adopted in the experiments was derived from a liquid culture of *P. acerina* (Table 1). To conclude, growth of two *S. cerevisiae* yeast isolates (FBCC 2526 and 2577) was slightly affected by addition of oxalic acid up to 10 mM concentrations (Figure 2A) whereas two *S. cerevisiae* isolates (FBCC 2539 and 2471) were significantly (T-test $p < 0.05$) affected by the phlebioid culture fluid (Figure 2B). Therefore, the most tolerable isolate of *S. cerevisiae* (FBCC 2525) was selected for the co-cultivation together with *P. radiata* and *P. acerina*.

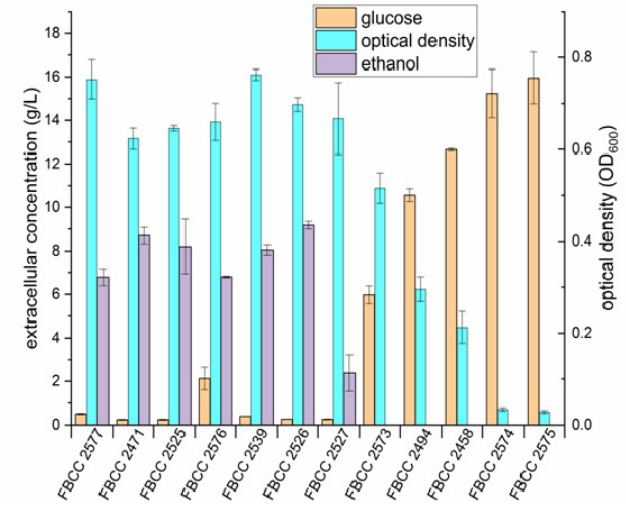

Figure 1. Growth, ethanol yield and glucose consumption of twelve yeast isolates (for species and isolate identity, see Table 1).

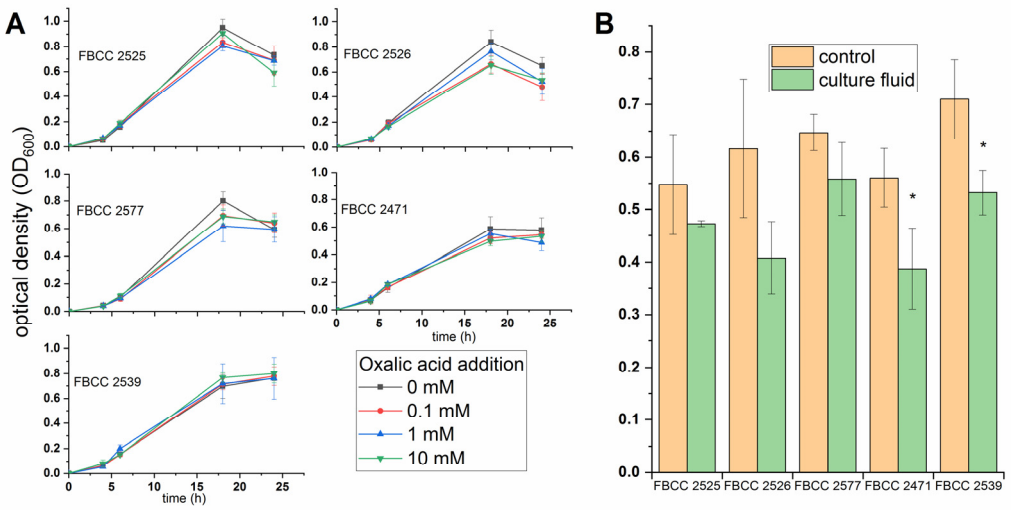

Figure 2. Growth (OD_{600}) of five *S. cerevisiae* isolates on (A) different concentrations of oxalic acid, and (B) growth on without (control) and treated with phlebioid culture fluid. * Statistically significant difference between the growth of the yeasts without (control) and in the presence of fungal culture fluid.

3.2. Ethanol yield in the fungal co-cultures

Inoculation of the selected *S. cerevisiae* isolate FBCC 2525 into the co-cultivations, either on day 3 or 7, resulted with no evident increase in final concentration of ethanol on cultivation day 9 (Figure 3A). However, addition of the yeast isolate caused a decrease in the quantity of available sugars in the growth liquid (Figure 3A), which is an indication of effective sugar consumption by the yeast. In all, presence of the yeast isolate caused a slight increase in decomposition of the core board lignocellulose substrate, and resulted in an increase of biomass (mycelia formation) by the wood decay fungi (Figure 3B).

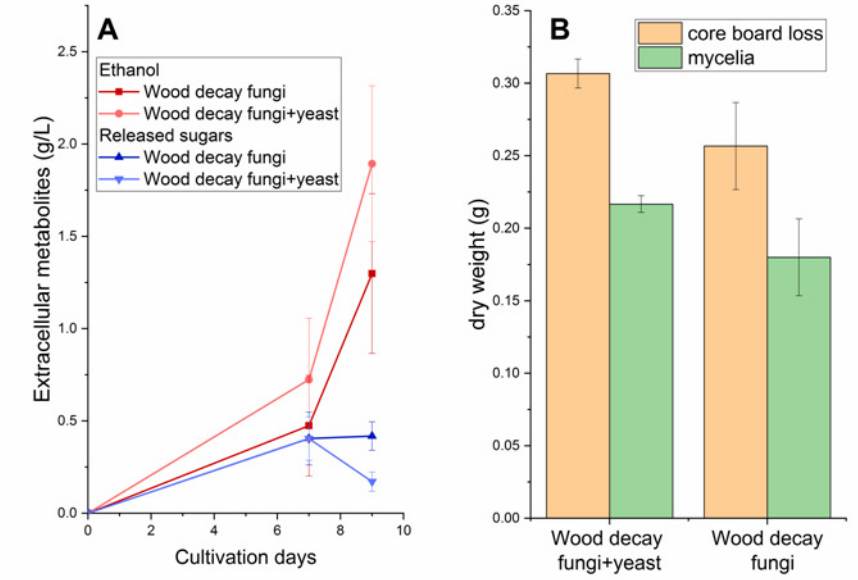

Figure 3. Effect of addition of *S. cerevisiae* FBCC 2525 yeast isolate into co-cultivations on core board with two phlebioid wood-decay fungi. (A) Ethanol production (g/L) and concentration of released sugars (g/l) in the cultures. (B) Mass loss of the core board substrate and generation of mycelium (biomass dry weight).

On the other hand, co-cultivation of a wood-decaying white rot fungus together with a brown rot fungus (*P. radiata* and *Fomitopsis pinicola*) revealed no additional advance to the outcome of ethanol fermentation and lignocellulose substrate conversion efficiency under the cultivation conditions adopted (anaerobic solid-state cultivation). Mixture of ground core board (1/5) and spruce wood sawdust (4/5, in dry mass relation) yielded 8.1 ± 0.2 g/L of ethanol after 14 days regardless of the presence of *F. pinicola*.

3.3. Ethanol production from various lignocellulose substrates

Since co-cultures with the *S. cerevisiae* yeast isolate or with the brown rot fungus *F. pinicola* resulted in no increase in the accumulation of ethanol on the core board cultivations, these steps were omitted from the subsequent experiments. The ability of *P. radiata* FBCC 0043 to produce ethanol in the single-step, single-organism process [3] was furthermore assessed with lignocellulose wastes used as solid substrates, together with addition of YE solution or spent barley mash as sources for organic nitrogen and trace nutrients for the wood-decay fungus.

Our results point to that *P. radiata* can convert core board into ethanol reaching concentrations over 30 g/L in the smaller flask cultivations (Figure 4). Noteworthy is that this high level of ethanol concentration can as well be achieved in a higher volume and mass scale bioreactor containing core board or wheat straw. Ethanol concentration of 25.6 g/L and interestingly, also 3.7 g/L of methanol was obtained with the 2 L bioreactor after prolonging the cultivation up to 146 days on core board.

Barley and wheat straw demonstrated potentiality as solid substrates although the ethanol yields in the single-step, single organism cultivations remained at the level of approximately 10 g/L (Figure 4). *P. radiata* converted wheat straw into ethanol (up to 10.7 g/L) only in the presence of

supplementation of barley mash whereas barley straw was adoptable as growth substrate for the fungus as such. In other words, barley straw was converted into ethanol irrespective of the additional source for nutritional nitrogen (Figure 4). In accordance to wheat straw as the lignocellulose substrate, also recycled waste wood as well as birch and spruce wood required addition of spent barley mash in the cultivations to initiate production of ethanol. Apparently, barley spent brewery mash acted as the most adoptable nutritional nitrogen source for the white rot fungus under these conditions.

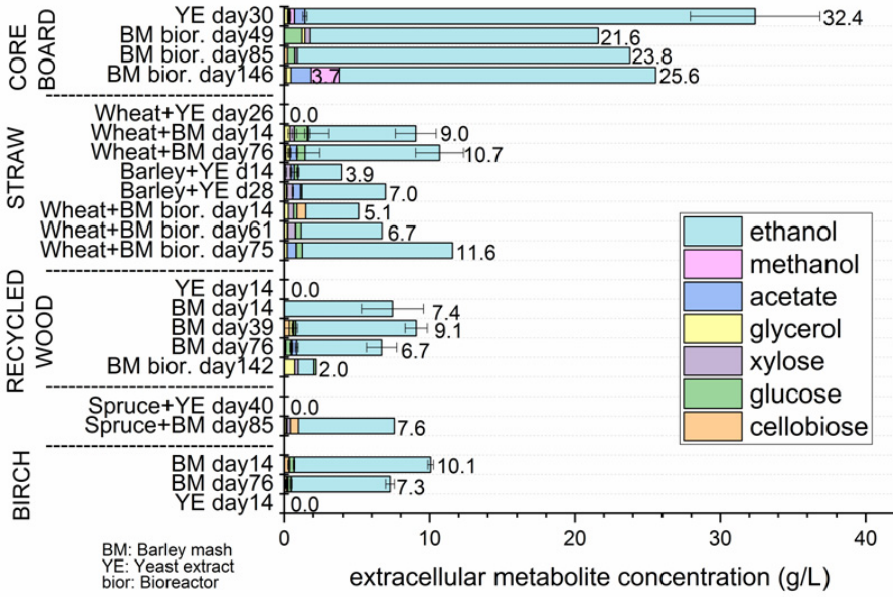

Figure 4. Concentrations of extracellular metabolites and released sugars in the solid-state cultivations of *P. radiata* on lignocellulosic waste and residual substrates (BM = barley mash,YE = yeast extract), bior = bioreactor.

3.4. Efficiency in conversion of core board and barley straw into ethanol

Different substrate load ratios were assessed in order to find the optimal dry weight percentage of the substrate for consolidated bioconversion of core board and barley straw to ethanol by *P. radiata*. With core board as the solid-state lignocellulose substrate, the highest efficiency for ethanol production was obtained with 10% dry mass of the substrate (Figure 5A). Up to 89% of the total fermentable carbohydrates (calculated as theoretically available released sugars from the amount of fermentable polysaccharides present in the substrate) of the core board matrix were converted into ethanol (Figure 5A).

With 10% dry mass substrate load of barley straw, however, only 17% of the total fermentable carbohydrates were converted into ethanol (Figure 5B). Nearly the same percentage (16%) was obtained with 15% barley straw substrate dry mass ratio in the cultivation. With both of these adoptable solid-state substrates, core board and barley straw, a clear drop in the efficiency in ethanol production emerged between 15% and 20% of lignocellulose dry mass. This was also observed as slower generation of overpressure (gas production) during the cultivations.

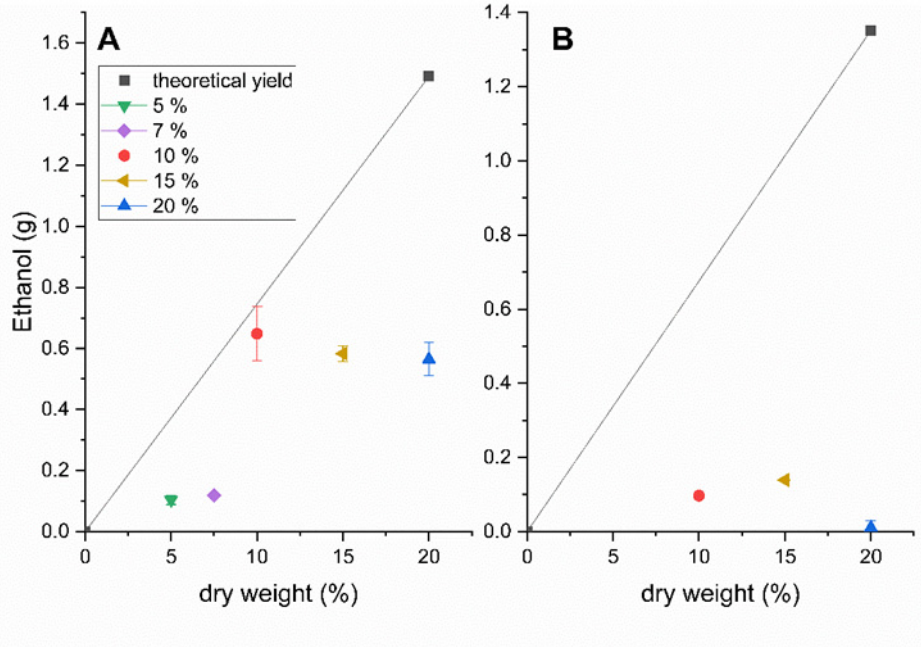

Figure 5. Ethanol yield obtained with different dry mass weight ratios of the solid lignocellulose substrates adopted in the solid-state cultivations. (A) Core board, (B) barley straw as substrates. Black line represents the calculated theoretical yield of ethanol production from the substrate.

4. Discussion

S. cerevisiae and other yeast isolates were assessed to find the most suitable isolate for consolidated co-cultivations with filamentous wood-decay fungi on solid lignocellulose substrates. As was expected, most of the *S. cerevisiae* isolates demonstrated fast conversion of glucose to ethanol. However, the adopted cultivation conditions may have affected ethanol production and fermentation efficiency of the *S. cerevisiae* isolates. For instance, the semi-aerobic culture conditions [3] may influence yeast metabolism due to potential limitations in dissolved oxygen content and accumulation of fermentation gases in the cultures, factors that may have affected production of ethanol and its subsequent oxidation and loss as fermentation product. The concentration (20 g/L) of glucose adopted in the initial screening tests may have been a selective feature since the most robust, industrially applicable yeast strains may tolerate even ten times higher sugar concentrations [18,19].

It has been proposed that co-cultivation of a white rot fungus with a brown rot fungus could lead to more rapid decomposition of wood, partially due to the combative situation encountered by the interacting organisms [11]. This type of fungal interaction situation may as well have occurred in our study in the co-cultivations of the two white rot fungi with the selected yeast isolate. In part, it may be suggested that competition of the released sugars from the solid lignocellulose substrate with yeast cells could stimulate the wood decay fungus to higher degradation and bioconversion efficiency. However, our results indicate that co-cultivation with yeast or a brown rot fungus was not such a promotive effect for the white rot fungus. At the best cases, the initial production of ethanol by the white rot fungus was only slightly increased. This indicates that our organism of choice for the

lignocellulose conversion and ethanol production, *P. radiata* (isolate 79, FBCC 0043) [3,5,10,13] is alone powerful when it comes to ethanol fermentation from solid lignocellulose substrates.

Oxalic acid or culture fluid of a phlebioid fungus caused no direct effect on the growth of the selected yeast isolate *S. cerevisiae* FBCC 2525. This finding opposes with previous observations obtained with another isolate of *P. radiata* that is isolate CBS 184.83 which was previously applied as pretreatment organism for wheat straw [20]. However, simultaneous co-cultivation with phlebioid fungi may have affected the growth and ethanol fermentation capability of the yeast isolate. Further studies are required to evaluate the applicability of the selected *S. cerevisiae* isolate in fungal co-cultivations on diverse lignocelluloses.

Overall, core board and barley straw demonstrated great potential as applicable solid-state substrates for ethanol production in our consolidated bioprocess. In this study, we found *P. radiata* capable of converting especially the core board lignocellulose carbohydrates into ethanol with a remarkable efficiency. Interestingly, *P. radiata* produced also methanol from core board. One possibility for the origin of methanol could be enzymatic de-esterification of the core board pectins during lignocellulose degradation [21]. Core board and barley straw both served as fungal growth supporting substrates with formation of ethanol irrespective of the organic nitrogen source adopted (yeast extract or spent brewery barley mash). All the other waste and residual lignocellulose substrates tested produced ethanol only in combination with the spent barley mash, which apparently provided additional carbohydrates for fungal growth together with acting as nitrogen source.

In comparison to conventional solid-state or high gravity cultivations, our presented method offers an alternative in the context of consolidated bioprocessing aiming at ethanol production. Reaching sufficient agitation and heat transfer are one of the major problems in solid-state cultivations [9]. Since our method requires no agitation or additional heating, these obstructions can be avoided. Untreated lignocellulose substrates are insoluble in water, and contain fermentable sugars up to 50% of their mass, depending on plant tissue type. In order to become economically feasible and technically potential to yield satisfactory levels of ethanol, the dry mass ratio (weight percentage) in the solid-state cultivation must be high. Our single-step, single-organism version of consolidated bioprocess method is applicable with at least 10% of substrate dry mass, as is shown in this study with the wood-based lignocellulose (core board) and with the non-wood residual lignocellulose (barley straw).

Upscaling the volume and size of the solid-state cultivations to 2 L bioreactor scale adopting larger glass vials led to production of ethanol from the lignocellulose substrates by the white rot fungus. Increase in substrate load of core board or wheat straw by more than twelve- or fifteen-fold, respectively, resulted with minor changes in ethanol production and acetate concentrations. In the solid-state cultivations on core board, ethanol yield was already reaching concentration levels which are promising for bioprocessing scale. Our intention is to put more effort to optimization of cultivation conditions for a suitable bioreactor type of larger size, with trials on higher substrate loads to facilitate even better yields of ethanol production.

Noteworthy portions of the wood-based wastes processed and transported from communities, as well as residuals and side streams from forest sector industry and agriculture have low recycling potential. These heterogenic lignocelluloses are often utilized for generation of bioenergy and heat power by burning, especially in bioenergy combustion plants. However, by combustion to provide heat and electricity, substantial portion of the lignocellulose carbon as CO_2 is released into the atmosphere thereby causing more increase in accumulation of greenhouse gases. The attractions in a

single-step, consolidated bioprocess from lignocellulose wastes and residuals for bioethanol production are on the estimated low production costs and on low infrastructure requirements, both features supportive for design of a decentralized, at the site operating ethanol production process [22]. Decentralized bioprocess may better fit to smaller scale production of bioethanol thereby broadening the range of substrates to lignocelluloses currently not suitable for industrial scale due to their weak or seasonal availability [23]. Our trials indicated that the presented consolidated method may be extended from the laboratory flask scale to bioreactor cultivations. Notable also is that the same cultivation conditions may apply for a range of different lignocelluloses.

5. Conclusions

Although our consolidated bioprocess method is promising and functional with using only one organism for lignocellulose bioconversion and ethanol production, upscaling the method towards commercial scale still requires further research and efforts. For instance, comparative experiments with other sources of lignocellulose waste and residual substrates are required, in order to allow process flexibility and high productivity of ethanol fermentation. Our consolidated method, however, opens possibilities for a new array of substrates applicable to second-generation bioethanol production. Moreover, our alternative bioprocessing approach, further advanced in this study, aims to exploit the biology of a wood-decaying and ethanol fermenting fungus in a single-step consolidated method, and is based on utilization of waste lignocellulose substrates without a need for pretreatments such as steam explosion or addition of enzymes. Thereby, our consolidate bioprocess method on waste and residual materials supports bio-based and more sustainable circular economy, and may thus allow development of an inexpensive method for second generation bioethanol production.

Author's contribution

HM, TL, designed the study; HM, DK, performed the experiments; HM, DK, TM, TL, analyzed and interpreted the results; HM, TL, wrote the manuscript. All authors read and accepted the final manuscript.

Acknowledgements

The study was funded by the Academy of Finland project no. 285676 (Fungco) and grants from the "Marjatta ja Eino Kollin säätiö" and Niemi Foundation. EU Erasmus+ grant for DK is gratefully acknowledged. Miikka Olin is thanked for the advice concerning design of the HPLC method.

Conflict of interest

The authors confirm no conflict of interest.

References

1. Niphadkar S, Bagade P, Ahmed S (2018) Bioethanol production: Insight into past, present and future perspectives. *Biofuels* 9: 229–238.

2. Markandya A, Dhavala K, Palma A (2018) The role of flexible biofuel policies in meeting biofuel mandates. *AIMS Energy* 6: 530–550.
3. Mattila H, Kuuskeri J, Lundell T (2017) Single-step, single-organism bioethanol production and bioconversion of lignocellulose waste materials by phlebioid fungal species. *Bioresour Technol* 225: 254–261.
4. Lundell TK, Mäkelä MR, de Vries RP, et al. (2014) Genomics, lifestyles and future prospects of wood-decay and litter-decomposing Basidiomycota. *Adv Bot Res* 70: 329–370.
5. Kuuskeri J, Häkkinen M, Laine P, et al. (2016) Time-scale dynamics of proteome and transcriptome of the white-rot fungus *Phlebia radiata*: growth on spruce wood and decay effect on lignocellulose. *Biotechnol Biofuels* 9: 192.
6. Kamei I, Hirota Y, Mori T, et al. (2012) Direct ethanol production from cellulosic materials by the hypersaline-tolerant white-rot fungus *Phlebia* sp. MG-60. *Bioresour Technol* 112: 137–142.
7. Mood SH, Golfeshan AH, Tabatabaei M, et al. (2013) Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renew Sust Energ Rev* 27: 77–93.
8. Saha BC, Kennedy GJ, Qureshi N, et al. (2017) Biological pretreatment of corn stover with *Phlebia brevispora* NRRL-13108 for enhanced enzymatic hydrolysis and efficient ethanol production. *Biotechnol Progr* 33: 365–374.
9. Zabed H, Sahu JN, Suely A, et al. (2017) Bioethanol production from renewable sources: Current perspectives and technological progress. *Renew Sust Energ Rev* 71: 475–501.
10. Kuuskeri J, Mäkelä MR, Isotalo J, et al. (2015) Lignocellulose-converting enzyme activity profiles correlate with molecular systematics and phylogeny grouping in the incoherent genus *Phlebia* (Polyporales, Basidiomycota). *BMC Microbiol* 15: 217.
11. Mali T, Kuuskeri J, Shah F, et al. (2017) Interactions affect hyphal growth and enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes. *PLoS One* 12: 1–21.
12. Shah F, Mali T, Lundell TK (2018) Polyporales brown rot species *Fomitopsis pinicola*: Enzyme activity profiles, oxalic acid production, and Fe^{3+}-reducing metabolite secretion. *Appl Environ Microbiol* 84: e02662-17.
13. Mäkinen MA, Risulainen N, Mattila H, et al. (2018) Transcription of lignocellulose-decomposition associated genes, enzyme activities and production of ethanol upon bioconversion of waste substrate by *Phlebia radiata*. *Appl Microbiol Biotechnol* 102: 5657–5672.
14. Pahkala K, Kontturi M, Kallioinen A, et al. (2007) Production of bio-ethanol from barley straw and reed canary grass: A raw material, In *15th European Biomass Conference Exhibition, Berlin, Germany*, 7–11.
15. Sáez F, Ballesteros M, Ballesteros I, et al. (2013) Enzymatic hydrolysis from carbohydrates of barley straw pretreated by ionic liquids. *J Chem Technol Biot* 88: 937–941.
16. Dien BS (2010) Mass balances and analytical methods for biomass pretreatment experiments. *Biomass to Biofuels: Strategies for Global Industries* 2010: 213–231.
17. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31: 426–428.
18. Kasavi C, Finore I, Lama L, et al. (2012) Evaluation of industrial *Saccharomyces cerevisiae* strains for ethanol production from biomass. *Biomass Bioenerg* 45: 230–238.
19. Mohd SA, Abdulla R, Jambo SA, et al. (2017) Yeasts in sustainable bioethanol production: A review. *Biochem Biophys Rep* 10: 52–61.

20. Salvachúa D, Prieto A, López-Abelairas M, et al. (2011) Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. *Bioresource Technol* 102: 7500–7506.
21. Rytioja J, Hildén K, Yuzon J, et al. (2014) Plant-polysaccharide-degrading enzymes from basidiomycetes. *Microbiol Mol Biol Rev* 78: 614–49.
22. Rastogi M, Shrivastava S (2017) Recent advances in second generation bioethanol production: an insight to pretreatment, saccharification and fermentation processes. *Renew Sust Energ Rev* 80: 330–340.
23. Arevalo-Gallegos A, Ahmad Z, Asgher M, et al. (2017) Lignocellulose: a sustainable material to produce value-added products with a zero waste approach—A review. *Int J Biol Macromol* 99: 308–318.

AIMS Press