SZENT ISTVÁN UNIVERSITY
DOCTORAL SCHOOL OF FOOD SCIENCE

UTILISATION OF WHEAT BRAN AND WASTEPAPERS FOR PRODUCTION OF ETHANOL

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1 BACKGROUND AND OBJECTIVES

Recently, lignocellulosic materials i.e. crop residues, wastes from food processing, forestry residues etc., receive more attention as abundant and raw materials for production of renewable energy. Plant-based bioenergy (biogas, biohydrogen, biobuthanol, bioethanol etc.) has the potential to partially replace fossil fuels and secure the energy supply as well as reduce the environmental pollution. In recent years, some pilot-plants are developed and adapted for production of second generation ethanol, but industrial application is still unrealised. The technological challenges and limitations are mainly caused by the complex chemical structure of cellulose, hemicellulose, lignin, that are the main compound in the second generation, lignocellulose-based biomass. It is widely believed that the structure of lignocellulose is resistant to degradation due to its compositional heterogeneity, which depends on its species, variety, growth conditions and maturity. It follows, that pretreatment is needed and it is a critical step, which alters the physical and chemical properties of the biomass and makes cellulose and hemicellulose more available to hydrolytic enzymes generated simple sugars to fermentation process.

Biological pretreatment of lignocellulosic biomass is considered as an efficient, eco-friendly and cheap alternative method. It has potential advantages over physical, chemical and combined processes, such as lower energy requirement, lower pollution generation and reduced inhibitor production under mild reaction conditions. The drawbacks of biological pretreatment are a longer duration, lower degradation level of carbohydrates and the risk of sugar loss. Paper-based waste materials often contain (besides cellulose) additional barrier components such as waxes, fats, rubbers, synthetic polymers and resins, and other substances that prevent the enzymatic hydrolysis and ethanol fermentation of paper fibers. In the past few years, researchers have developed several methods in addition to traditional ones, such as microwave irradiation and sonication or even biosorption with natural biopolymers.

In my PhD researches, development of new strategies for second generation ethanol production based on two different plant-based waste products wheat bran and waste papers was focused. The main goal was to make the crucial process steps of the pretreatment methods sustainable and more efficient by increasing the ethanol and sugar yields. These waste products were chosen for modelling the second generation bioethanol production due to their high cellulose and low lignin content.

The objectives of the research

Development of new microbial pretreatment method for wheat bran

- Collect and maintain mesophilic microorganisms (fungal and yeast strains) isolated from decomposing plant residues.
- Determine the degradation ability of the applied strains on wheat bran under solid state conditions. Screening of the strains.
- Analyse the interactions in the 18 different microbial consortia, containing two or three strains.
- Determine the degradation efficiency of the selected microbial consortium on various conditions: water content, pH, ratio of inoculated fungal conidium and inoculation methods.

Development of a new deinking method for waste papers

- Determine the deinking ability of different physical, and physico-chemical methods: washing and soaking, microwave irradiation and sonication, adhesion-based processes, such as flotation and chitosan-based biosorbents.

Enzymatic hydrolysis and fermentation of the pretreated biomass

- Develop new enzymatic saccharification strategies for pre-treated wheat bran and wastepaper materials.
- Determine the ethanol productivity of the applied yeast and bacteria strains in mono- and co-cultures. Analyse the ethanol fermentation profiles at different fermentation processes: separated and simultaneous hydrolysis and fermentation.

2 MATERIALS AND METHODS

Wheat bran (*Triticum aestivum* L.) was purchased from Alnatura GmbH (Austria) and waste papers - office papers and printed papers - were collected locally.

Microbial biodegradation (pretreatment) of wheat bran was investigated using a total of 35 decomposing strains from different genera of *Aspergillus* (11 strains), *Penicillium* (7 strains), *Mucor* (1 strain), *Phanerochaete* (2 strains), *Rhizopus* (3 strains), *Trichoderma* (5 strains), *Candida* (3 strains) and *Pichia* (3 strains). The microorganisms were either kindly provided by National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest, Hungary) or purchased from the International Mycological Institute (IMI, Engham, Surrey, United Kingdom). Biological pretreatment was carried out with single strain cultures and microbial consortium (strains were selected by their degradation ability) at reduced moisture content on 30°C for 7-9 days. Effect of the pH (between pH 3-7), the moisture content (citrate-phosphate and wheat bran ratio between 4:1 and 7:1), the ratio of the strains in the consortium and the inoculation techniques were also studied.

Physical and physico-chemical deinking methods were used with and without chemicals, on roughly shredded samples and fiber suspensions. The flotation experiments were conducted in a laboratory scale with air flotation equipment at 1 (w/v) % paper samples, 10-50 (v/v) % ethanol, 30 °C and 50 °C for 5-30 min. The irradiation methods were tested in 1 (w/v) % paper mash containing 15 (v/v) % ethanol at 30°C for 5-30 min. The microwave treatment was operated at 750 W, while the sonication method was at 50-60 Hz. Furthermore, chitosan-based bioadsorbents, produced from chitin powder by deacetylation step, such as chitosan, cross-linked chitosan with glutaraldehyde and chitosan coated Ca-alginate beads were prepared. Different sorbent doses (2.5-20 % w/v), times (5-30 min), temperatures (30-55°C) and pHs (3.5-6.5) were investigated for determination of maximal adsorption capacity at 220 rpm shaken rate using 100 ppm model solutions of Carbon black ink pigments. Printed papers were also deinked by selected biosorbent at optimal conditions in batch and continuous modes.

Commercial enzyme preparations (Novozymes A/S, Denmark), such as amyloglucosidase (AMG 300L), cellulase (Celluclast®1.5L), cellobiase (Novozyme 188), xylanase from *Aspergillus niger*, *Trichoderma reesei* and *Thermomyces lanuginosus*, respectively, were used individually and in combination to hydrolyse pre-treated materials into fermentable sugars.

Ethanol fermentation was performed with yeast strains of *Lanchancea thermotolerans* and *Kluyveromyces marxianus* (NCAIM, Budapest, Hungary), active dry yeast Levuline Fb

type *Saccharomyces cerevisiae* (from Danstar Ferment A.G. Ltd.) as well as a bacterial strain of *Zymomonas mobilis* (from the NCAIM, Budapest). Fermentation profiles of different fermentation modes of mono- and co-culture (in a ratio of 1:1), separated and simultaneously hydrolysis and fermentation were studied at 30 °C for 7 days.

3 RESULTS AND DISCUSSION

Microbial pretreatment of wheat bran and ethanol production

Total of 29 filamentous fungal strains and 6 yeast strains were screened for their ability to degrade highly resistant and recalcitrant lignocellulosic structure under solid-state conditions. The quantity and the composition of the water-soluble products (mono-, di- and polysaccharides) were different in each microbial pretreatments due to the different cellulase and other hydrolytic enzyme activities as well as the presence or absence of oxidative enzymes. The strains of *Penicillium* exhibited the best results with more than 25 % of the theoretical sugar yield was achieved. The pretreatment abilities were ranked in order of strains of *Aspergillus*, *Trichoderma*, *Mucor*, *Rhizopus*, *Phanerochaeta*, *Candida* and *Pichia*. Based on the screening results, *A. brasiliensis* F.00892, *A. niger* F.00632, *A. wentii* F.00167, *P. chrysogenum* F.00814, *P. granulatum* F.00913 and *T. viride* F.00795 strains were selected for further studies. Total of 18 fungal consortia comprising of two- and three strains were developed and investigated. All tested combination increased the soluble carbohydrate content in the mashes due to their synergistic effects. With the simultaneous use of *A. niger* F.00632, *P. chrysogenum* F.00814 and *T. viride* F.00795 in equal ratios a total amount of 41.6 g soluble carbohydrate from 100 g substrate were achieved, which is about 50 % of theoretical yield. Optimal ratio of liquid to solid, pH and temperature as well as inoculum ratio were determined to be 5:1, pH 5.0, 30°C and 65:25:15 % in total 10^7 spores (of *A. niger, P. chrysogenum* and *T. viride*) per gram dry substrate, respectively. The application of fungal multi-culture resulted in about 58.8 g soluble carbohydrate (about 10 g glucose) from 100 g substrate (67 % of theoretical yield) after 3 days of bio-treatment.

Enzymatic saccharification profiles of microbially pretreated wheat bran showed high glucose concentration (454.5 mg/g), which is equal to 53 % of the theoretical glucose yield, when the mixture of commercial enzyme preparations cellulase (2100 U/g), cellobiase (780 U/g) and xylanase (7000 U/g) was applied at 50 °C, pH 5.5, agitation speed of 220 rpm for 24 hours.

Ethanol-producing microbes were tested for their ability to convert fermentable sugars to ethanol at various sugar concentration (up to 20 %) in fermentation media containing treated and untreated (as control) wheat bran. *Saccharomyces cerevisiae* (Levuline Fb type), *Kluyveromyces marxianus* Y.00959 and *Zymomonas mobilis* subsp. *mobilis* B.01327^T showed similar fermentation profiles, ethanol yield was about 2.4 % (v/v) and 2.8 % (v/v) in untreated

fermentation medium (initial reducing sugar content was about 5 - 7 %, w/v) at 30 °C. The bacteria strain showed a greater glucose and ethanol tolerance during the fermentation of enzymatically treated samples. The ethanol concentration reached 4.6 % and 5.1 (v/v) % at 15-20 % (w/v) initial glucose concentration. A further increase in ethanol production was found with the mixed culture of two yeast strains (*Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) in equal ratios. In searching for the best fermentation process, simultaneous application of two yeast strains with an additional inoculation step of the bacteria at 48 hours showed 7.6 % (v/v) of ethanol after 7 days of fermentation at 30 °C.

Deinking waste papers and ethanol production

According to the results obtained from chemical and physico-chemical treatments, application of ethyl alcohol in a concentration of 25 % (v/v) as an organic solvent in laboratory-scale flotation turned out to be more effective at deinking than washing with a number of other chemicals and irradiation techniques. The best parameters at alcoholic flotation were the following: 1 % (w/v) mixed waste papers in 25 % (v/v) ethyl alcohol solution, 50 °C and 30 min.

As natural biosorbents, chitosan and its two derivates: cross-linked chitosan with glutaraldehyde and chitosan-coated Ca-alginate beads showed adsorption properties in the model solution containing 100 ppm of carbon black from HP laser jet ink cartridges. However, adsorption capacities and interactions between biosorbents and ink pigments significantly changed during their application. Chitosan-coated Ca-alginate beads were the most effective adsorbent with more than 80 % of ink removal using 1.5 g of adsorbent at 30-35 °C and pH 6-6.5 with constant shaking at 220 rpm for 30 minutes. Adapting these adsorption techniques for the pretreatment of mixed paper waste treatment with chitosan-coated Ca-alginate beads at the same operational parameters, except with a dosage equal to 10 %, showed similar ink removal than with the model solutions.

Saccharification with industrial enzyme preparation of deinked paper samples was carried out and sugars released were converted into ethanol. The conversion rate was about 21 % and 56 % (with 16 % and 33 % glucose yield) at flotated paper samples, while bioadsorbents led to 65 % soluble carbohydrate yield (glucose yield was 23 %) performed with a mixture of commercial cellulase (2100 U/g) and glucoamylase (320 U/g) enzyme preparation at 50 °C, pH 5.5 and 220 rpm of agitation for 24 hours.

Saccharomyces cerevisiae (Levuline Fb type) produced more ethanol on treated paper-based materials (mixed paper waste and office paper) than other yeast strains from *Lachancea* genera produced. Comparison of two fermentation processes - ShF and SSF- using *Saccharomyces cerevisiae* showed that the ethanol content observed was not above 5 % (v/v) at both fermentation modes, thus ethanol fermentation still was not economically accepted. As a result of co-fermentation processes sugar consumption partially increased, but the ethanol yield only slightly increased (1.2-1.3 % v/v) with the combination of *Saccharomyces cerevisiae* with *Lanchancea thermotolerans* Y.00775 and *Zymomonas mobilis* subsp. *mobilis* B.01327^{T} (in equal ratio) on treated mixed paper waste, and a higher ethanol concentration (2.2-2.7 % v/v) was observed in treated office papers at 30 °C after 7 days of fermentation.

As a conclusion of the results described here, these new methodologies are very promising and can serve as a basis for further experiments to develop more economical lignocellulosic ethanol production from plant-based wastes

4 NEW SCIENTIFIC RESULTS

1. A new microbial consortium formed from mesophilic, decomposing fungal strains *Aspergillus niger* F.00632, *Penicillium chrysogenum* F.00814 and *Trichoderma viride* F.00795 was successfully developed that was able to degradation wheat bran to release high amount of soluble sugars, thus it was suitable for biological pretreatment of lignocellulosic biomass.

2. Different parameters of biological pretreatment of wheat bran with microbial consortium were optimised for maximum degradation capacity. The optimal biological pretreatment parameters are the following: 30 °C, citrate-phosphate buffer and wheat bran ratio of 5:1, pH 5, inoculation conidium number is 107 per gram dried wheat bran (fungal strains ratio: 60 % of *Aspergillus niger* F.00632, 25 % of *Penicillium chrysogenum* F.00814 and 15 % of *Trichoderma viride* F.00795). About 63 % of theoretical carbohydrate yield can be achieved after 3 days of pretreatment at optimised condition.

3. Saccharification process of biologically pretreated wheat bran with enzyme cocktail of commercial enzyme preparations cellulase (T. reesei, 2100 U/g), cellobiase (*A. niger*, 780 U/g) and xylanase (*T. lanuginosus*, 7000 U/g) at 55 ° C and pH 5.5, 220 rpm for 24 hours was developed and tested. More than four-fold increase in glucose yield (fermentable sugar) was observed in wheat bran mash by this technology.

4. Chitosan coated Ca-alginate beads is suitable for removal of ink dyes from the printed papers. Maximum adsorption capacity of biosorbent was determined to be 80-85 ppm carbon black ink/1.5 g sorbent at pH 6-6.5 and 30-35 °C. With this modified chitosan biopolymer, ink removal achieved 80-85 % effectiveness.

5. A new saccharification technique was developed for the printed papers deinked with chitosan coated Ca-alginate beads. In this technology, cocktail of commercial enzyme preparations cellulase (*T. reesei*, 2100 U/g) and amyloglucosidase (*A. niger*, 320 U/g) was applied at 50 °C and pH 5.5 for 24 hours , and it resulted about 65 % of theoretical carbohydrate yield.

6. New co-fermentation mode on wheat bran hydrolysates was developed and introduced. Ethanol fermentation was initiated with the inoculation of *Saccharomyces cerevisiae* (Levuline Fb) and *Kluyveromyces marxianus* Y.00959 in a ratio 1:1 (10 % w/v), and *Zymomonas mobilis* subsp. *mobilis* B.01327T (10 % w/v) was inoculated on the day 3. Application of this technique resulted 7.6 (v/v) % ethanol content in the wheat bran mash with 20 % (w/v) initial sugar content after 7 days of fermentation at 30 °C.

5 CONCLUCIONS AND SUGGESTIONS

Biorefinery technologies based on lignocellulose biomass has not yet reached the required level in the field of environmental interests. For researchers and manufacturers, the main drawback of the biofuel and bioproduct production (second generation) is the efficient and economical pretreatment methods. It can account for up to 40% cost of the whole recovery technology. Ethanol research has also confirmed that pretreatment can yield up to 90 % of sugar while its absence can result in a yield of less than 20 % of the total available sugars, thus pretreatment has also a major impact in the subsequent fermentation step.

Doctoral research was focused on modelling the second generation ethanol production based on wheat bran and paper-based wastes, and development the essential technological steps: pretreatment, enzymatic saccharification and ethanol fermentation in laboratory-scale experiments. I have successfully developed new pretreatment techniques for both plant-based residues. Biological degradation of wheat bran provides further research opportunities such as isolation/selection mesophilic and thermophilic (thermotolerant) decomposing strains, examination the interactions between strains and with microbial consortium as well as mapping the pretreatment of other lignocellulosic materials: wheat straw, corn cobs, etc. at laboratory-scale. In the case of paper samples, the main problem was not primarily the exploration of complex and resistant structure of carbohydrates, rather it was the removal of dyes and other additives from paper fibers. Among deinking methods, adsorption-based methods: alcohol flotation chitosan biosorbents, mainly chitosan coated Ca-alginate gel beads were successfully applied. However, the changes in environmental parameters easily caused desorption, which still needs improvement.

One-step enzymatic saccharification resulted an increase in carbohydrates conversion in case of both pretreated materials, but the amount of polymers and oligomers in the hydrolyzates remained at very high level. For this reason, multi-hydrolytic enzymes or enzyme mixtures should be tested. Presumably, the presence of lignin may have reduced the active linkage of the enzymes to the carbohydrates, which could be solved by the lignin digestion (with white-rot fungal strains) or lignin-degrading oxidative enzyme preparations. Ethanol fermentation, depends on the composition of hydrolysates and fermentation modes, resulted different ethanol yields.

Based on wheat bran hydrolysates, economical ethanol concentration was reached, however the significant amount of carbohydrates remained in the fermentation broths. At paper-based ethanol production, high carbohydrate content was also observed after fermentation. It

can be explained by the inhibition effect of ink pigments and other additives, which reduced the productivity of ethanologenic strains. It would be worthwhile separating the pre-treated paper samples or developing further pretreatment techniques.

6 PUBLICATIONS RELATED TO THE THESIS

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