HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

DEVELOPMENT OF MICROBIAL CONSORTIUM FOR BIOLOGICAL PRETREATMENT OF LIGNOCELLULOSIC RAW MATERIALS

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1. INTRODUCTION AND OBJECTIVES

Nowadays, the world has witnessed a rapid increase in population as well as economic growth, resulting in the depletion of fossil fuel and an increase in energy-related carbon dioxide emissions which account for negative environmental impact. Many governments have been stimulating the utilization of renewable energies and resources, which are able to overcome the environmental problem and energy scarcity. Different types of renewable energy are currently being extensively researched, namely solar, wind, geothermal, hydrothermal and biofuel. Among renewable energy sources, bioenergy (energy from bio-based sources) is the largest renewable energy present in the liquid fuels such as biofuel, diesel, or gasoline.

Biomass sources may be obtained from many sources such as forestry or agriculture waste streams. There are three classifications of biofuel, listed as first-, second-, and third-generation biofuels. Edible biomass was employed as feedstock in first-generation biofuel while non-edible feed stocks were used as the substrate sources for second-generation biofuel and third-generation biofuels use algal biomass and gases. Agricultural residues such as wheat straw, rice straw, rice husk, switchgrass, etc. have been intensively researched for second-generation biofuel production. However, the recalcitrant structure of lignocellulose by the strong association of lignin, cellulose and hemicellulose cause technological challenges and limitations in the lignocellulose conversion into valuable products. Therefore, a pretreatment step is acquired to break down the harsh structure of lignocellulosic biomass, making it accessible for the enzymatic attack on cellulose and hemicellulose chains to produce mono-carbohydrates. There are many pretreatment routes, including physical, chemical, physicochemical, biological, and combined pretreatment (**Figure 1**).

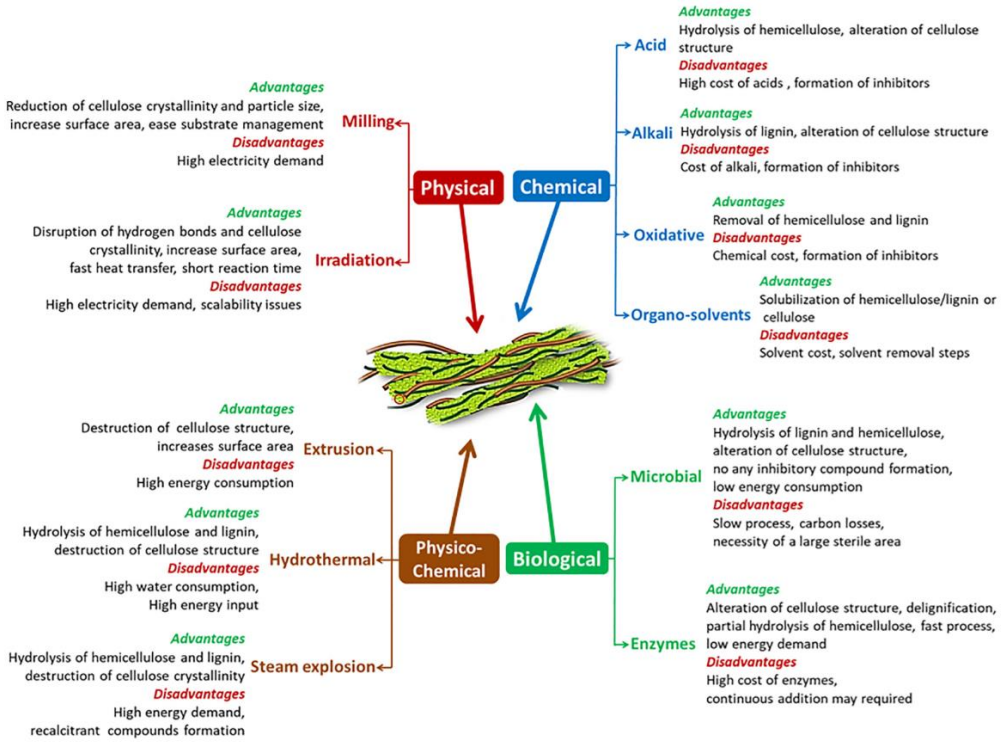

Figure 1. Different lignocellulose pretreatment approaches (Abraham et al., 2020)

Among these routes, biological pretreatment is considered a safe and environmentally friendly method, because it has many advantages over others with cost efficiency, lower energy requirement, mild working condition and free of toxic chemicals. However, biological

pretreatment still faces some drawbacks such as low degradation efficiency, taking long time, and the risks of carbohydrate loss. The promising approach to enhance the effectiveness of biological processes is utilizing microbial consortia, taking advantage of co-cultures synergistic actions. The appropriate pretreatment is presented through high adaptability, increasing degrading enzyme activities, control of pH and substrate utilization. Therefore, a great challenge should be the organization of consortium members for specific tasks to maximize the degradation rate and efficacy of the bioprocess.

In the last decade, the application of microorganisms in every aspect of life has become promising due to their great advantages over the conventional process. The utilization of microbial communities comprised of various types of microbial species has become a new promising avenue to enhance the efficiency of bio-based processes. Connecting to this field, my PhD research focuses on the construction and tailoring of efficient microbial consortia for biological pretreatment of lignocellulosic biomass.

The objective of the research

Effects of different individual strains and their consortia in degradation of lignocellulosic biomass

- Bacteria strains and their consortia
- Yeast strains and their consortia
- Fungi strains and their consortia

Optimization of operating conditions for microbial pretreatment with different microbial consortia

- Design and construction of microbial consortia
- Construction effective microbial comprised various microorganisms for biological pretreatment of lignocellulosic biomass
- Evaluation of efficacy of the complex microbial consortia on the pretreatment of various types of lignocellulosic biomasses

Application potential of newly developed microbial consortia – cases study

- Investigation of new saccharification method in combination of microbial pretreatment with exogenous enzyme preparations
- Ethanol fermentation of pretreated biomass

2. MATERIALS AND METHODS

2.1. Lignocellulose substrate and microorganisms

Wheat (*Triticum aestivum*) bran was purchased from Denes-Natural Kft. (Pecs, Hungary). The label chemical components of wheat bran were about 3.4% (w/v) fat (0.8% (w/v) saturated fatty acids), 56.2% (w/v) carbohydrates (5.0% (w/v) sugar), 16.3% (w/w) protein, 0.03% (w/w) salt. Wheat (*Triticum aestivum*) straw was collected from Jászberény village in Hungary. Wheat straw was dried and then cut into small pieces, then grounded and passed through an 80-mesh sieve. Wheat bran and wheat straw were kept separately in plastic bags in the desiccator for at least 24 hrs until used.

Microbial pretreatment was investigated using a total of 29 strains from different genera of *Bacillus* (8 strains), *Rhodococcus* (6 strains), *Pseudomonas* (3 strains), three species of fungi (*Aspergillus niger*, *Penicillium chrysogenum*, *Trichoderma viride)*, *Yarrowia* (5 strains), and *Pichia* (4 strains). These strains were kindly provided by National Collection of Agricultural and Industrial microorganisms (NCAIM, Institute of Food Science and Technology, MATE, Hungary).

Cellulolytic and ligninolytic bacteria were refreshed for 24 hrs in nutrient medium; fungal strains were grown for 5 days on yeast extract peptone dextrose (YEPD) agar slants except *P. chrysogenum* F.00814, which was grown on malt agar slants before being used. Yeast species were refreshed for 24 hrs in yeast extract peptone dextrose (YEPD) agar slants until used.

2.2. Effect of bacteria, yeast and their consortia on the pretreatment of lignocellulose

The monoculture and microbial consortium constructed from the effective strains were cultivated in a basal medium containing wheat bran 2% (w/v). The pH was adjusted to pH 6.5 using 1M NaOH solution before autoclaving. After cooling down the flasks, the equivalent inoculum was added to 250 ml flask containing 150 mL of medium to obtain 105 CFU/mL. The biological pretreatment was conducted at 30 ± 2oC for 7 days, 140 rpm agitation speed. Samples were taken at 24 hrs intervals, then centrifuged at 17.968 x g centrifugal force for 10 min at room temperature to remove cells and supernatant. All samples were kept at -20^{o}C for further analysis.

2.3. Fungal biological pretreatment

Five-day old fungal strains were transferred into sterilized glass tubes containing 5 ml Triton-X solution and dispersed with glass beads to separate fungal cells from the agar slants completely. Bucker chamber with Olympus Plan 40x/0.65 Ph2 objective was used to determine the number of fungal conidia. 10g of wheat bran was added to 250 ml Erlenmeyer flasks with liquid to solid ratio of 9:1. The pH value of the medium solution was adjusted to pH 6.5 by 1M NaOH solution. The flasks were sterilized at 121^{o}C for 30 min and cooled down at room temperature before cultivating microbes. Monoculture or mixed-cultures were added to flasks, then incubated at 28-30^{o}C and 140 rpm agitation speed for 7 days. The solid samples were periodically taken at every 24 hrs, and were mixed with 0.1M acetate buffer solution pH 4.5, centrifuged and filtered before analysis.

2.4. Optimization of microbial pretreatment

Effect of culture medium and pH

The effect of culture medium and pH was evaluated using complex microbial consortium including filamentous fungi and ligninolytic bacteria. Culture media including 0,15M citrate buffer solution supplemented with mineral compounds (g/L) such as $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 1.025; KCl, 0.5 and basal medium were studied. The pHs were adjusted to pH 4.5 and 6.5 with 1M NaOH or 1M HCl solution. The suspended pretreatment with liquid:solid ratio 9:1 and the initial inoculum ratio of fungi and bacteria 1:1 was applied.

Effect of liquid:solid ratio

Various liquid:solid ratios were tested to study the effects of moisture content on the pretreatment of lignocellulose using complex microbial consortium.

Effect of cultivation method

Co-culture of filamentous fungi *A. niger* F.00632 (FA) and lignocellulolytic bacterial co-culture *B. subtilis* B.01162 (A) and *P. putida* B.01522 (K*) were cultivated under suspended pretreatment (liquid:solid ratio of 9:1) or submerged pretreatment. 90 ml of basal medium was added in 250 ml Erlenmeyer flask containing 10 g of dry wheat bran in suspended pretreatment, while in submerged condition, microbes were added in basal medium containing 2% (w/v) wheat bran.

Different routes in which fungi or bacteria co-culture were cultivated 24 hrs before the addition of others or simultaneously were also investigated.

2.5. Construction of complex microbial consortia

In order to select the consortium members, the bottom-up strategies are usually used. The microbial species were firstly screened and evaluated individually, then incorporated into community. Various microbial communities were constructed by combination of different strains of bacteria, fungi and yeast. The bacterial and yeast strains were freshly incubated for 24 hrs in a suitable culture medium, while the 5 day-old fungal spores were separated from agar slants by mixing with Triton X solution. Two-member, three-member and complex microbial consortia were constructed by adding 10^5 cells/gds of each strain into testing flasks at the ratios of 1:1, 1:1:1 and equal ratios of each member in communities, respectively. The microbial pretreatment was carried out similarly as procedure used in the case of individual species.

2.6. Effect of lignocellulosic biomass quality

Lignocellulosic substrates composed of wheat bran and wheat straw at different ratios were evaluated to study the effect of substrates quality on the pretreatment efficiency. The substrates were pretreated by microbial consortia constructed artificially.

2.7. Saccharification and fermentation of pretreated biomass: cases study

The effects of substrate loading and enzyme dosage during the saccharification of pretreated substrates were evaluated in preliminary trial tests. Biologically pretreated wheat bran and soluble carbohydrates in the extract were enzymatically hydrolyzed in 250 mL Erlenmeyer flasks containing 120 mL slurry with suitable substrate loadings at 50^{o}C and pH 5.0 for 4 hrs in a shaker with an agitation speed of 140 rpm. A commercial cellulase enzyme (Celluclast 1.5L) extracted from *Trichoderma reesei* was used in efficient enzyme dosages. The hydrolysis was terminated by

autoclaving the hydrolysates at 121^{o}C for 15 min. Then, pH of the mash was adjusted to pH 4.0 by sterilized 1M NaOH solution. The mash was supplemented with 5 mL nutrient solution. Commercial yeast preparation *Saccharomyces cerevisiae* Danstill A was firstly activated in warm distilled water containing 2% glucose at 32^{o}C for 20 min and then cultivated in YEPD medium. One day grown-yeast inoculum with cell concentration of 10^8 cell/gds was added to the substrate. The fermentation was carried out in static incubation and anaerobic conditions throughout the cultivation period of 7 days at 30^{o}C.

Analytical methods

Different degradation parameters were analyzed. The degradation rate was quantitatively determined by gravimetric analysis. The reducing sugar was determined using Somogyi-Nelson. Regarding enzyme activities, they were monitored by measuring changes in concentration of correspondent substrates during the reaction. The total phenolic content was optically measured according Folin-Ciocalteau method, in which gallic acid was used as standard solution. Amino acid content was qualitatively evaluated by observing the appearance of a yellow precipitate of xanthoprotetic acid. HLPC was utilized for mono-sugar and ethanol content evaluation. The bioconversion rate was calculated based on the difference between actual amount of ethanol in fermented mash and theoretical ethanol which is equivalent to 51% of fermented sugar concentration.

2.8. Statistical analysis

All experiments were run in triplicates. The data were processed in Microsoft Excel spreadsheet an expressed as the mean ± SE of different independent replicates. One-way analysis of variables (One-way ANOVA) followed by TUKEY post hoc multiple comparison tests was conducted using SPSS software (version 20.0) to test the differences between the variances. Data were considered significant at $p < 0.05$ and reported as the mean ± SD (standard deviation). Mean values with different letters above the bars differ according to Tukey's test at $p < 0.05$.

The strength of a linear association between reducing sugar and weight loss was interpreted based on the covariance method, called Pearson's Correlation analysis.

Multivariate methods as cluster analysis with Euclidean shortest distance were applied to describe diversity patterns of hydrolysis capacity between strains and consortia.

The Principal Component Analysis (PCA) method was used for multi-variables. The correlation matrix between variables is calculated to transform orthogonal, creating new axes (eigenvectors) installed as the original variables' linear combination. The percentage variations of two principal components in investigated variable were obtained in the PCA diagram using SPSS 20.0. The contribution rates of each variable to PC1, PC2 and their interrelations were also performed.

3. RESULTS AND DISCUSSION

3.1. Bacterial pretreatment of wheat bran

Cellulolytic bacilli

A total of 8 *Bacillus* strains and 11 bacilli co-cultures was examined for their hydrolytic ability on lignocellulosic biomass. *B. subtilis*, *B. cereus*, *B. coagulans* species were considered the best degraders with relatively high weight loss ranging from 54% to 60% after 7 days of submerged pretreatment. The correlation between weight loss and reducing sugars was introduced in pretreatment by single *Bacillus* strains but did not apply to their mixed culture. Among these species, *B. cereus* and *B. subtilis* released the highest yield of reducing sugars ranging from 240-252 mg/gds after 72 hrs of biological pretreatment, and 72 hrs was also the optimal time for most efficient *Bacillus* strains to achieve the peak of reducing sugar yields. *B. subtilis* B.01162, *B. coagulans* B.01123 and *B. cereus* B.00076 showed the highest enzyme production and their collaboration in 2-member consortia showed the synergistic relationship with the great enhancement of cellulolytic enzyme activities, specifically FPase and xylanase, which were almost 2-fold higher and over 3-fold higher than that of pure cultures. However, the role of β-glucosidase in degradation by the consortium was negligible.

Ligninolytic bacteria

In order to select lignin-degrading strains, various species of *Rhodococcus* and *Pseudomonas* were screened. Among them, *R. erythropolis* B.01914, *R. opacus* B.01915 and *P. putida* B.01522 have the greatest effect on lignocellulose degradation, performed by high weight loss of solid residues ($p<05$) and xylanase with high activities of 1.716, 1.644 and 2.011 IU/mL, respectively. Reducing sugar performed a moderately positive linear relationship with FPase activities ($r=0.571$, $p<0.05$) but a strong negative association with laccase enzymes ($r=-0.898$, $p<0.05$). The co-culture of ligninolytic strains, however, caused the reduction of reducing sugar yields in general. It was found that *P. putida* B.01522 and its co-culture with *R. opacus* B.01915 could release relatively high degrading enzyme activities than other bacterial mixtures. On the other aspects, the co-culture of ligninolytic strains could break down the biopolymer structure and release a higher concentration of fermented sugars which were able to be converted into valuable products.

Construction the mixed culture of cellulolytic and ligninolytic strains

Selected cellulolytic and ligninolytic strains were constructed in various bacterial communities, which significantly affected the degradation of lignocellulose substrates. In general, ligninolytic consortia accounted for a higher solid loss than cellulolytic consortia, a high percentage of weight loss, and an increase of total phenolic compounds in some microbial mixtures in the presence of *R. opacus* B.01915-*P. putida* B.01522 (C*K*) and *R. fascians* B.01608-*P. putida* B.01522 (D*K*). In addition, these consortia showed a more effective effect on breaking down the lignin structure, facilitating the enzymatic attack on cellulose and hemicellulose structure while the others' degradation effects were dominated by laccase. The highest total cellulase enzyme activity was found in cellulolytic consortia AB and mixed culture of *P. putida* B.01522 (K*) and 2 *Bacillus* species (K*-AC), values of 0.213 and 0.206 IU/mL. On contrary, glucose yield was highly released in the extracted hydrolysate of species with lower degrading enzyme activities.

3.2. Fungal pretreatment of lignocellulosic biomass

Three species of filamentous fungi including *A. niger* F.00632, *P. chrysogenum* F.00814 and *T. viride* F.00795 were investigated in suspended pretreatment, using the basal medium with the ratio of liquid:solid as 9:1, acidic pH of 4.5. Among them, *A. niger* F.00632 could need a short processing time of 48 hrs to achieve the best sugar yield while others acquired more time. Also, this fungus could release degrading enzymes with as high activities as those secreted from fungal co-cultures. The most effective fungal co-culture is *A. niger* F.00632-*T. viride* F.00795 with outstanding hydrolysis capacity, in which enzyme activities of total cellulase, endo-glucanase and xylanase were 0.411, 1.827 and 12.990 IU/gds, respectively. Additionally, these consortia could release a high amount of glucose which 4-7 times higher than in other case studies.

3.3. Utilization of yeast as supplements

Role of yeast in the bioprocess could be highly evaluated when applying their co-culture in the pretreatment of lignocellulosic biomass. It was found that co-cultures of *Y. divulgata* and *P. stipitis* (Y.02062-Y.00888 and Y.02062-Y.01047) gave xylanase activity with enzyme titers 0.48 IU/gds and 0.33 IU/gds, respectively. They also released a larger amount of amino acids than single cells using quantitative analysis and produced a significantly high amount of fermented sugar. The maximum glucose concentration of 67.36 mg/gds was measured in pretreated lignocellulose substrates by co-culture of *Y. divulgata* Y.02062 and *P. stipitis* Y.00888.

3.4. Optimization of operating parameters

Factors such as culture medium, pH value, moisture and cultivation methods were studied to figure out the best operating parameters for biological pretreatment. In submerged pretreatment, basal medium stimulated the production of more reducing sugar and higher hydrolytic enzyme activities under fungal or bacterial pretreatment than utilization of citrate buffer.

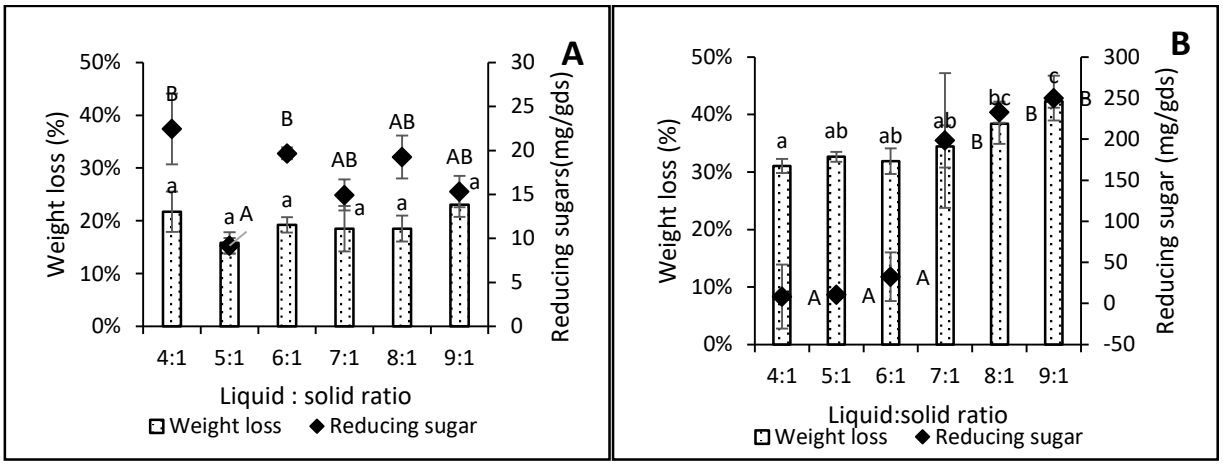

Figure 2. Effect of moisture in degradation efficiencies in biological pretreatment using bacteria (A) and fungi (B)

It was found that degradation efficiency was not effected by pH values. Suspended pretreatment was more favorable for filamentous cultivation than utilization of bacteria. It was observed that the increase of moisture content under fungal cultivation caused greater solid loss after 72 hrs of pretreatment and at liquid:solid ratio of 7:1, 8:1, 9:1, reducing sugar yield was recorded around 200 mg/gds while at moisture content less than 75%, the highest yield was only 32.53 mg/gds

(Figure 2). The complex culture of fungi and bacteria was applied to investigate the effects of incubation order, in which strains were cultivated simultaneously or at different times. Two opposite degradation profiles under suspended and submerged pretreatment were found when each species have its favor habitat conditions. Under solid state pretreatment, microbial co-culture could produce reducing sugar yield with 10 times higher than under the submerged pretreatment. The cultivation route in which fungi were cultivated 24 hrs before the addition of bacteria accounted for the higher weight loss but no significant differences in reducing sugar yields or enzymatic activities. Therefore, simultaneous incubation of microbes was selected to construct complex microbial consortia in suspended pretreatment.

3.5. Development of the effective microbial consortia

The construction of complex microbial communities including fungi, bacteria and yeast is a new and promising avenue to reduce the metabolic burden and execute multiple tasks simultaneously. They perform unique characteristics, associated with the interactions between members in the same habitat. A greater weight loss of lignocellulose treated with mixed cultures than with simple culture was obtained, revealing its strong correlation with reducing sugar yield produced during the biological pretreatment. Bacteria itself showed less impact on the lignocellulose degradation, however, its combination with fungi or yeast was capable of boosting pretreatment efficiency. The role of yeast in microbial communities is to improve the hydrolytic enzyme activities and sugar conversion. It was found that the co-culture of yeast with fungi or bacteria generated a tremendous concentration of glucose and xylose, around 102-103 mg/gds and 47-66 mg/gds, respectively. The highest glucose concentration of 235.91 mg/gds was determined in the hydrolysate after 72 hrs pretreatment by a consortium comprised of *B. subtilis* B.01162, *P. putida* B.01522, *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795, *Y divulgata* Y.02062 (Table 1).

The effect of substrates on biological pretreatment by complex consortia was investigated. As a consequence, BFY4 and BFY5 showed a similar degradation rate to each investigated substrate. Mixtures of wheat bran and wheat straw substrate at the ratio of 25:75 and 50:50 obtained the highest accumulation of sugars on the 4^{th} day and the 3^{rd} day of the pretreatment. After 72 hours, highest FPase (0.213 IU/gds) and xylanase (7.588 IU/gds) activities were detected in the wheat straw, meanwhile CMCase activity peaked highest (0.928 IU/gds) on wheat bran substrate. The amount of released glucose increased during the treatment process, when the two substrates were used in the same ratio. Consortium BFY4 could stimulate the bioprocess by producing the maximum yield of glucose of 156.47 mg/gds, and 89.02 mg/gds after 72 hrs from substrates containing only wheat bran and the mixture of wheat bran and wheat straw at an equal ratio.

Table 1. Degradation parameters of wheat bran substrate in the cultivation of mono and mixed culture in the pretreatment

Cluster	Microbes	Codes	Weight loss (%)	Reducing sugar accumulation	pH
Cluster A	*A. niger* F.00632	**F1**	42.32 ± 1.13	3.78 ± 0.41	4 ± 0.17
	A. niger F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795	**F2**	44.45 ± 0.54	10 ± 1.63	4.23 ± 0.67
Cluster B	*B. subtilis* B.01162 + *P. putida* B.01522	**B1**	23.07 ± 2.3	0.23 ± 0.03	5.89 ± 0.02
	R. opacus B.01915 + *P. putida* B.01522	**B2**	33.93 ± 1.63	0.31 ± 0	8.81 ± 0.02
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522	**B3**	32.82 ± 0.67	0.32 ± 0.01	8.83 ± 0.04
	Y. divulgata Y.02062	**Y1**	23.5 ± 2.99	0.31 ± 0.14	5.88 ± 0.23
	Y. divulgata Y.02062 + *P. stipitis* Y.00888	**Y2**	29.64 ± 1.58	0.67 ± 0.08	6.28 ± 0.39
	A. niger F.00632 + *Y. divulgata* Y.02062	**FY**	35.14 ± 3.72	1.05 ± 0.17	4.81 ± 0.11
Cluster C	*B. subtilis* B.01162 + *P. putida* B.01522 + *A. niger* F.00632	**BF**	26.83 ± 0.39	3.21 ± 0.52	5.56 ± 0.03
	B. subtilis B.01162 + *P. putida* B.01522 + *Y. divulgata* Y.02062	**BY**	35.45 ± 2.65	3.5 ± 0.26	5.99 ± 0.01
	B. subtilis B.01162 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062	**BFY1**	36.15 ± 3.65	1.72 ± 0.36	5.65 ± 0.03
	B. subtilis B.01162 + *P. putida* B.01522 + *A. niger* F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795 + *Y. divulgata* Y.02062	**BFY2**	28.47 ± 1.3	1.62 ± 0.94	5.89 ± 0.01
	R. opacus B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY3**	39.11 ± 2.93	2.19 ± 0.47	5.91 ± 0.34
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY4**	36.23 ± 2.74	1.48 ± 0.36	5.62 ± 0.04
	R. opacus B.01915+*P. putida* B.01522+*A. niger* F.00632 + *P. chrysogenum* F.00814+*T. viride* F.00795+*Y. divulgata* Y.02062+*P. stipitis* Y.00888	**BFY5**	33.85 ± 1.28	1.78 ± 0.24	5.95 ± 0.01
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY6**	36.39 ± 1.72	1.83 ± 0.16	5.71 ± 0.02

3.6. Application of newly developed microbial consortia

Saccharification of pretreated wheat bran

After pretreatment, 4 hrs of hydrolysis with commercial cellulase at 50^{o}C was conducted, followed by ethanol fermentation using *Saccharomyces cerevisiae*. The 3 levels of substrate loadings as 3, 5, and 7% (w/w) and 4 levels of enzyme dosage as 5, 10, 20 and 40 FU/gds were tested to select optimal criteria for saccharification. It was found that the maximum fermented sugar yield was obtained at substrate loading of 5% and they showed no significant difference in sugar yields at various enzyme dosages which ranged from 10-40 FPU/gds. Therefore, substrate loading of 5% (w/w) and enzyme dosage of 10 FPU/gds were used in the saccharification process. Among samples pretreated by different strains and consortia, the maximum reducing sugar accumulation ratio achieved by consortium C7 comprised of *A. niger* F.00632, *B. subtilis* B.01162, *P. putida* B.01522, *R. opacus* B.01915, *Y. divulgata* Y.02062, *P. stipitis* Y.00888.

Ethanol fermentation of biologically pretreated wheat bran

After hydrolysis, pretreated samples were anaerobically fermented in 7 days. Glucose and maltose yield dropped during fermentation due to ethanol conversion by yeast. A diversity of optimal periods to obtain the peak of bioconversion rate was found when utilizing different species in lignocellulose pretreatment. The highest ethanol concentration of 5.7% (v/v) with an 89.21% conversion rate was observed on the first fermentation day in the case of consortium C7 containing *A. niger* F.00632, *B. subtilis* B.01162, *P. putida* B.01522, *R. opacus* B.01915, *Y. divulgata* Y.02062, *P. stipitis* Y.00888. The fermentation efficiency was enhanced markedly and the conversion of recalcitrant lignocellulose was accelerated by the synergism relationship of suitable fungi, bacteria and yeast in the same habitats.

4. CONCLUSIONS AND RECOMMENDATIONS

In this study, we discover the synergistic relationship of microorganisms including fungi, bacteria and yeast, which play an important role to enhance the degradation efficiency in biological pretreatment. The suspended process was proven to stimulate the growth of species in the same habitats and contribute a remarkable bioconversion rate of lignocellulose into fermented sugars. However, we could not achieve a sufficient ethanol yield in the fermentation process.

The knowledge of this study could be used as primary background for scaling up and improving the bioprocesses. Some directions for further research can be proposed as follows:

- Thermophilic bacteria can release degrading enzymes with high activities at temperature ranging from 40-50^{o}C, therefore, the construction of microbial consortia with thermophilic species is promising to achieve a higher efficiency in biological pretreatment.
- Scaling up and optimization pretreatment under suspended conditions using effective microbial consortium are needed for the adaptation of these results in the production of commercialized bio-based products from lignocellulose biomass.
- Utilization of enzyme cocktail and optimization saccharification process are recommended to maximize production of fermented sugars.

5. NOVEL CONTRIBUTIONS

1. Seventeen strains of bacteria, three mold strains and nine yeast strains were obtained and screened to check the biological treatment of biomass, and three *Bacillus* strains (*Bacillus subtilis* B.01162, *Bacillus coagulans* B.01123 and *Bacillus cereus* B.00076), one *Pseudomonas* strain (*P. putida* B.01522) and one *Rhodococcus* strain (*R. opacus* B.01915) were shown to be the best degraders with high enzyme activities. These strains were selected for development of consortia for biological pretreatment of lignocellulosic raw materials.
2. The 3-member consortium comprised the *Bacillus subtilis* B.01162, *Bacillus coagulans* B.01123 and *Bacillus cereus* B.00076 bacterial strains was found to be very promising degraders with high cellulase activities and efficient digestibility of solid substrates. Significant increase in the production of both cellulolytic and ligninolytic enzymes compared to monocultures was observed in the case of pretreatment of wheat bran substrate by co-culture of *P. putida* B.01522 and *R. opacus* B.01915 in submerged medium. Co-culture of yeast comprised by *Y. divulgata* and *P. stipilis* resulted the higher xylanase activity as well as amino acids than monocultures. Although, the combination of *A. niger* with *T. viride* F.00795 exhibited the outstanding hydrolysis capacity of total cellulase, endo-glucanase and xylanase (0.411, 1.827 and 12.990 IU/gds, respectively) in the case of suspended pretreatment.
3. Different microbial communities with various quality and quantity microbes were constructed and degrading profiles were studies. Generally, higher solid loss was observed in the cases of ligninolytic consortia (mould) than in the cases of cellulolytic consortia (bacterial), along with the increase in total phenolic compounds. Consortia of *R. opacus* B.01915-*P. putida* B.01522, *R. fascians* B.01608-*P. putida* B.01522, *R. fascians* B.01608-*P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076 and *R. opacus* B.01915-*P. putida* B.01522-*B. coagulans* B.01123- *B. cereus* B.00076 performed the high degradation capacity in the case of lignocellulose. The highest total cellulase enzyme activity was found in the cases of consortia of *B. subtilis* B.01162-*B. coagulans* B.01123 and *P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076. These values were 0.213 IU/mL and 0.206 IU/mL, respectively.
4. The parameters for the pretreatment of lignocellulosic biomass using complex consortia were optimized and they are basal medium, pH 6.5, liquid:solid ratio 9: 1. suspended pretreatment was proved more effective than the submerged approach. Numerically, 10 times higher reducing sugar yield after 72 hrs of pretreatment were obtained in the case of suspended biological pretreatment than in the case of submerged one.
5. In the case of suspended pretreatment with the microbial consortium, meanwhile the fungi played the most important role in degrading lignocellulosic, whereas the bacteria itself showed less impact to the lignocellulose degradation, however, its combination with fungi or yeast was capable of boosting pretreatment efficiency. In addition, the presence of yeast in the microbial consortia could improve the enzyme production and sugar conversion. The highest glucose concentration of 235.91 mg/gds was determined in the hydrolysate after 72 hrs pretreatment by a consortium comprised of *B. subtilis* B.01162, *P. putida* B.01522, *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795, *Y divulgata* Y.02062.
6. In the case of saccharification, 5% (w/w) substrate load and 10 FPU/gds enzyme dosage were found to be optimal parameters to archive the maximum fermentable sugar yield. The highest ethanol concentration of 5.7% (v/v) with 89.21% conversion rate at the first day of

fermentation was obtained in the case of pretreatment of wheat bran with 7-member microbial consortium consisted of *Aspergillus niger* F.00632, *Bacillus subtilis* B.01162, *Pseudomonas putida* B.01522, *Rhodococcus opacus* B.01915, *Yarrowia divulgata* Y.02062 and *Pichia stipitis* Y.00888. The fermentation efficiency was enhanced remarkly and the conversion of recalcitrant lignocellulose was accelerated by the synergistic actions of suitable fungi, bacteria and yeast in the same habitats.

6. PUBLICATIONS

Journal articles with IF

1. Emese Pregi, Dávid Kun, **Vi Vu**, Béla Pukánszky. (2019). Structure evolution in poly (ethylene-co-vinyl alcohol)/lignin blends: Effect of interactions and composition. *European Polymer Journal*, 111:74-81. https://doi.org/10.1016/j.fuel.2020.119259.
2. Duy H. Truong, Mai S. Dam, Erika Bujna, Judit Rezessy-Szabo, Csilla Farkas, **Vu Ngoc Ha Vi**, Olivia Csernus, Vuong D. Nguyen, Nicholas Gathergood, László Friedrich, Mohamed Hafidi, Vijai Kumar Gupta, Quang D. Nguyen. (2021). In situ fabrication of electrically conducting bacterial cellulose-polyaniline-titanium-dioxide composites with the immobilization of *Shewanella xiamenensis* and its application as bioanode in microbial fuel cell, *Fuel*, 285:119259. https://doi.org/10.1016/j.fuel.2020.119259.
3. **Vi Vu**, Csilla Farkas, Ouahab Riyad, Erika Bujna, Akos Kilin, Gizella Sipiczki, Minaxi Sharma, Zeba Usmani, Vijai Kumar Gupta, Quang D. Nguyen (2022). Enhancement of the enzymatic hydrolysis efficiency of wheat bran using the Bacillus strains and their consortium. *Bioresource Technology*, 343: 126092. https://doi.org/10.1016/j.biortech.2021.126092.
4. **Vi Ngoc Ha Vu**, Csilla Kohari-Farkas, Róbert Filep, Gábor Lászlovszky, My Thi Ban, Erika Bujna, Vijai Kumar Gupta, Quang D. Nguyen (2023). Design and construction of artificial microbial consortia for enhancement of degradation of lignocellulosic biomass. *Biofuel Research Journal* (**under revision**)

Conferences

1. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Effect of supplements on the pretreatment by Aspergillus niger strain. (*The IV SZIEntific Meeting for Young Researchers conference*. Szent Istvan University, Hungary, 07/12/2020)
2. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Screening of lignin degrading strains and construction of consortia for the bio-pretreatment of wheat bran. (*Chemical Engineering Day'21.* University of Pannonia, Veszprem, Hungary, 21/04/2021)
3. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Enhancement of cellulolytic enzyme production of lignocellulose during pretreatment of wheat bran by consortium Bacillus bacteria. (*The 4th International Conference on Biosystems and Food Engineering*, Budapest, Hungary, 04/06/2021)
4. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Enhancement of production of soluble carbohydrates during biological pretreatment of wheat bran. (*The 6th Central European forum for Microbiology*, Kecskemet, Hungary, 13-15/10/2021)
5. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Improving the solid-state degradation of lignocellulose biomass in biological pretreatment using microbial communities. (*9th Bi-annual Sunrise symposium: Transition to Net Zero*, UK, 10-11/2/2022)
6. **Vi Vu,** Csilla Farkas, Vijai Kumar Gupta, Quang D. Nguyen. Developing efficient microbial consortium for the pretreatment of different agriculture residues. (*The 4th Foodconf – International Conference on Food Science and Technology*, Budapest, Hungary, 09-11/06/2022).