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Design and construction of artificial microbial consortia to enhance lignocellulosic biomass degradation

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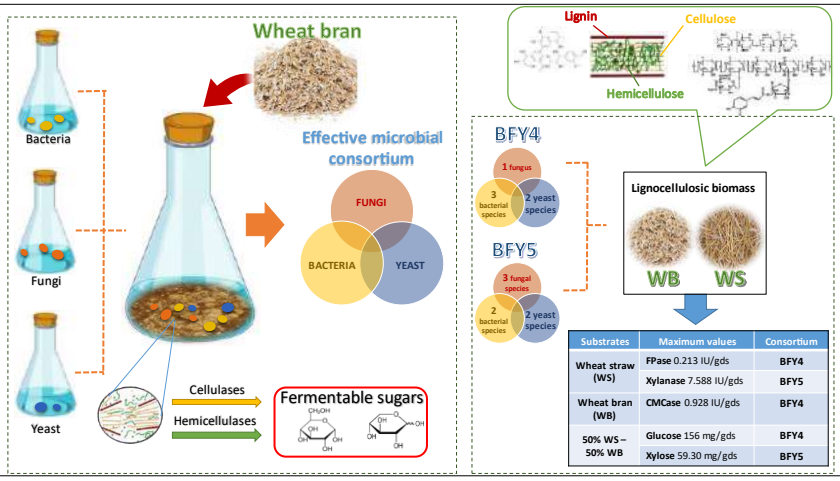
HIGHLIGHTS

- A new approach was introduced for designing and constructing microbial consortia for lignocellulosic pretreatments.
- Adding yeast into a mixed culture of fungi and bacteria improved lignocellulosic biodegradation.
- The developed artificial microbial consortia, BFY4 and BFY5, showed high lignocellulosic degradation capabilities.
- Pretreatment with BFY4 or BFY5 consortia resulted in the highest yield of reducing sugars.

GRAPHICAL ABSTRACT



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ABSTRACT

Cellulose-rich agricultural residues are promising renewable sources for producing various value-added products such as 2nd generation biofuels. However, the efficiency of the bioconversion process is not always satisfactory due to the slow and incomplete degradation of lignocellulosic biomass. An interesting approach would be using microbial communities with high lignocellulose-degrading ability for environmentally friendly pretreatment. This study focused on characterizing the degradation performance of bacteria, fungal, and yeast strains and designing and constructing different microbial consortia for solid-state treatment of wheat bran and wheat straw. The microbial consortia, namely BFY4 and BFY5, contained different bacteria, fungal, and yeast led to high ratios of sugar accumulation ranging from 3.21 to 3.5 with degradation rates over 33%, owing to more favorable hydrolytic enzyme activities and improved reducing sugar yield during the process. After 72 h, the highest FPase (0.213 IU/gds) and xylanase (7.588 IU/gds) activities were also detected in the wheat straw pretreated by BFY4 and BFY5, respectively, while CMCase activity peaked (0.928 IU/gds) when wheat bran was used as substrate. The amount of released glucose increased during the treatment process when the two substrates were used in the same ratio. Our results indicated that substrate composition also plays an important role in the degradation capacity of mixed cultures. These findings can be instrumental in advancing the primary knowledge required to apply such bioprocesses at the pilot scale.
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Contents

Abbreviations

ANOVA	Analysis of variance
CMCase	Carboxymethyl cellulase
FPase	Filter paper activity of cellulase
HPLC	High-performance liquid chromatography
IU	International units
gds	Gram of initial dry substrate
RI	Refractive index
SPSS	Statistical Package for the Social Sciences
YPD	Yeast peptone dextrose
YPX	Yeast peptone xylose

1. Introduction

A rapid population and economic growth increase has led to huge energy demands, 80% of which are met by fossil fuels. Such intensive use of these conventional energy carriers has contributed to the elevated atmospheric carbon dioxide concentration, causing major challenges for humanity, particularly climate change. In addition, fluctuating fossil fuel prices adversely affect livelihood and economic growth. In light of these, renewables have received more attention as a strategy to mitigate the vast reliance on fossil fuels.

Biomass energy (also known as bioenergy), mostly derived from plants and plant-based materials, makes up the largest part of the global renewable energy portfolio. In 2017, biomass energy accounted for 70% of renewable energy consumption (WBA, 2019). Biomass energy is present in solid, liquid, and gaseous fuel forms. Biomass fuels are classified into three groups based on the raw material used. Crops, mainly used for food purposes, serve as the basis of the 1st generation biofuels, while non-edible sources, such as residues rich in cellulose, are used for 2nd generation biofuels production. The 3rd generation biofuels are based on microalgae with high carbohydrate and lipid contents. Among lignocellulosic biomass, agricultural residues such as wheat straw, rice straw, rice husk, etc., have been intensively researched for 2nd generation biofuel production (Jain et al., 2017; Farkas et al., 2019). Lignocellulosic biomass consists of three major components, cellulose, hemicellulose, and lignin, linked together *via* covalent and non-covalent cross-linkages (Kumari and Singh, 2018). The lignocellulosic biomass conversion into biofuels includes three main steps, pretreatment to break down the recalcitrant structure of plant cell walls, enzymatic hydrolysis or saccharification to release fermentable sugars, and microbial yeast fermentation to produce ethanol (Himmel et al., 2007).

Pretreatment plays a key role in the lignocellulosic biomass conversion, where after the breakage of lignin-carbohydrate linkages and reduction of cellulose crystallinity, the accessibility of cellulose to enzymes significantly increases (Mosier et al., 2005; Baruah et al., 2018). Different pretreatment approaches exist, such as physical, chemical, physicochemical, biological, and combined. Among these routes, biological pretreatment has many advantages over other methods, with high-cost effectiveness, lower energy requirements, mild conditions, and being free from chemicals (Sharma et al., 2019). A wide taxonomic array of microorganisms is used in biological pretreatment. Fungi are advantageous biological elements due to producing a wide range of extracellular lignocellulose-degrading enzymes, especially species from Ascomycetes (*Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp.) and Basidiomycetes including white-rot fungi and brown-rot fungi (*Fomitopsis* sp., *Schizophyllum* sp., *Phanerochaete* sp.) (Dashtban et al., 2010; Paudel and Qin, 2015a).

Species belonging to Basidiomycetes have high selectivity in lignin degradation, with some capable of simultaneously degrading lignin, cellulose, and hemicelluloses. On the contrary, Ascomycota can degrade polysaccharides and slightly modify aromatic compounds of plant materials (Dicko et al., 2020; Ferrari et al., 2021). In nature, many bacteria can be found, including aerobic and anaerobic species, which have a potential for biodegradation. The Gram-positive *Bacillus* and *Rhodococcus* strains and Gram-negative *Pseudomonas* strains have the highest degrading efficiency on cellulose-rich materials (Paudel and Qin, 2015b). It has been confirmed that the synergistic interactions among enzymatic activities such as cellulases (endo-β-glucanase, cellobiohydrolases, β-glucosidase), xylanases (endo-β-xylanase, β-xylosidase) enhance the digestibility of the lignocellulosic biomass (Chang et al., 2014; Guo et al., 2018). Yeasts such as *Yarrowia lipolytica* also have the potential for cellulose, hemicelluloses, and lignin degradation (Gonçalves et al., 2014). Some yeasts, like *Candida* and *Pichia* strains, can also utilize and ferment pentose sugars (Ding et al., 2018). **Table 1** summarizes recent studies focusing on the potential capacity of microbial consortium for the pretreatment of lignocellulosic biomass.

Generally, biological pretreatment still has some drawbacks, such as low degradation efficiency, longer duration, and the risks of carbohydrate loss (Sindhu et al., 2016). The low degrading efficiency of individual strains towards the recalcitrant structure of natural lignocellulose and the high capacity of degrading enzyme production suggests an alternative approach for the efficient treatment of agricultural residues by taking advantage of the synergistic action of microorganisms. An appropriate biological pretreatment could be defined by its high adaptability, maximizing degrading enzyme activities, and control of environmental factors during the degradation of lignocellulosic biomass (Kalyani et al., 2013). Haruta et al. (2002) observed a very high degradation ability and stability of the bacterial communities isolated from compost samples under natural conditions, which resulted from the close and complex relationship between the strains. The interaction between the strains can be revealed by analyzing the composition of these bacteria and other mixed natural consortia, and more effective consortia can be developed for biological treatment (Kato et al., 2008). In our previous study, a consortium of two to three members of *Bacillus* species showed high synergism interaction, enhancing the cellulolytic activities during the pretreatment of wheat bran in submerged conditions, resulting in a significant increase in substrate degradation after 7 d of processing (Vu et al., 2022). Farkas et al. (2019) found that biological pretreatment of wheat bran using a three-member fungal consortium including strains of the genus *Aspergillus*, *Trichoderma*, and *Penicillium* could produce markedly more soluble carbohydrates compared to the individual strains. When used in consortia, some yeast strains have been shown to have other beneficial properties, including the production of metabolites (aromatics, flavonoids, etc.) from the released carbohydrates (glucose, xylose), serving as supplements for the other microorganisms in the consortia (Zha et al., 2021). Hence, although yeasts do not generally show substantial degradation activities in cellulose-rich materials, using yeasts in conjunction with the lignocellulose-degrading microorganisms opens a new avenue in the microbial processes. However, the big challenge to be considered during the development of microbial consortia with high degradation performance is the diverse metabolic functions of various microorganisms and their complex responses to each other in the communities.

In the present study, the characterization of the degradation performance of bacteria, fungal, and yeast strains, as well as their interactions, were focused. A new approach was also developed to design and construct different microbial consortia for the solid-state treatment of wheat bran and wheat straw. The biodegradation efficacy of selected consortia on the mixtures of the two cellulose-rich agro-industrial substrates was also tested on a laboratory scale.

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Table 1.
Comparison of the most abundant microorganisms present in artificial microbial consortia with their reported lignocellulose biomass conversion and the present study.

No	Strains	Substrates	Products	Achievements	Reference
1	*Micrococcus, Citrobacter, Exiguobacterium, Klebsiella, Lactococcus, Vanrija, Sugiyamaella*	Softwood sawdust	Methane	Enhancement of cumulative biogas yield up to 321 L/kg and methane yield up to 155.2 L/kg	Ali et al. (2017)
2	*Trichoderma reesei - Rhizopus delemar*	Microcrystal- line cellulose and alkaline pretreated corn stover	Fumaric acid	Achieved 6.87 g/L titer using consolidated bioprocessing	Scholz et al. (2018)
3	*Achromobacteri, Paenarthrobacter, Pseudaminobacteri, Paenibacillus, Candida, Rhodosporidium, Trichosporon*	Sugarcane bagasse	Not reported	Gene clusters related to aromatic degradation were detected by DNA sequence, and a recombinant pathway for vanillin production was developed	Moraes et al. (2018)
4	*Stenotrophomonas maltophilia - Paenibacillus sp.-Microbacterium* sp.- *Chryseobacterium taiwanense - Brevundimonas* sp.	Sugarcane bagasse, Sugarcane straw	Not reported	The degradation potential reached up to 96.5% of the degradation rates	Puentes-Téllez and Falcao Salles (2018)
5	*Aspergillus niger - Penicillium chrysogenum – Trichoderma viride* *Saccharomyces cerevisiae - Kluyveromyces marxianus - Zymomonas mobilis*	Wheat bran	Ethanol	Obtained 196.82 g/L of soluble carbohydrate content after 3 d of pretreatment; maximum ethanol content of 7.6% achieved after 7 d of fermentation	Farkas et al. (2019)
6	*Thermoanaerobacterium thermosaccharolyticum - Clostridium thermocellum*	Corn fiber	Ethanol	Improved the solubilization of corn fiber carbohydrate by over 90%; reduced inhibition of hemicellulose hydrolysis products	Beri et al. (2020)
7	*Trichoderma reesei - Clostridium tyrobutyricum - Veillonella criceti*	Beechwood	Butyric acid	Developed a stable cocultivation of spatial niches in a membrane reactor; obtained 196 kg of butyric acid per metric ton of feedstock	Shahab et al. (2020)
8	*Saccharomycetales, Shinella, Cupriavidus, Bosea*	Alkali lignin	Lignin degradation products	Improved lignin degradation enzyme activities; lignin degradation efficiency reached 54%	Zhang et al. (2021a)
9	*Citrobacter freundii - Sphingobacterium multivorum - Coniochaeta* sp.	Wheat straw		Discovered fungi played a key role in the degradation process and boosted the growth of bacteria	Wang et al. (2021)
10	*Bacillus subtilis - Bacillus coagulans - Bacillus cereus*	Wheat bran	Fermentable sugars	Achieved high enzymatic activities; improved digestibility of lignocellulosic biomass	Vu et al. (2022)
11	*Aspergillus niger - Penicillium chrysogenum - Trichoderma viride - Bacillus subtilis - Rhodococcus opacus - Pseudomonas putida - Yarrowia divulgata - Pichia stipitis*	Wheat bran, wheat straw	Fermentable sugars/Ethanol	Discovered the role of members in the consortium; obtained reducing sugar accumulation of 3.21-3.5 with a degradation rate of 33; highest FPase of 0.213 IU/gds and xylanase of 7.588 IU/gds from the degradation of wheat straw and maximum CMCase of 0.928 IU/gds from wheat bran	**Current Study**

2. Materials and Methods

2.1. Microorganisms and their cultivation

A total of 8 strains, including three bacterial strains: *Bacillus subtilis* B.01162, *Rhodococcus opacus* B.01915, and *Pseudomonas putida* B.01522, three filamentous fungal strains: *Aspergillus niger* F.00632, *Penicillium chrysogenum* F.00814, and *Trichoderma viride* F.00795, and two yeast strains: *Yarrowia divulgata* Y.02062 and *Pichia stipitis* Y.00888 were kindly provided by the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Hungary). The bacterial strains were refreshed in the nutrient medium (NCAIM 0025) containing 1 g/L yeast extract, 2 g/L meat extract, 5 g/L peptone, and 5 g/L NaCl for 24 h prior use. Fungal strains were pre-cultivated on yeast peptone dextrose (YPD) agar slants containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar for 5 d before incorporation into the consortium. *Yarrowia divulgata* Y.02062 strain was cultured on YPD agar slants, while *Pichia stipitis* Y.00888 strain in yeast peptone xylose (YPX) solution including 10 g/L xylose, 10 g/L yeast extract, and 10 g/L peptone for 24 h.

2.2. Substrates

For the laboratory-scale experiments, two cellulose-rich residues of the harvesting and processing of wheat (*Triticum aestivum* L.) were used. Wheat bran was purchased from Denes-Natural Kft. (Pécs, Hungary). Wheat straw was collected from a village field (Jászberény, Hungary). Wheat straw was dried and then ground with a laboratory grinder, and the coarse and fine fractions were separated through an 80-mesh sieve. The substrates were kept in the desiccator for at least 24 h until used.

2.3. Biological pretreatment

Wheat bran was used as the main substrate for mapping the performance of the individual strains. Fungal strains were cultured for 5 d and then transferred into sterilized glass tubes containing 5 mL 0.001% Triton X-100 solution. After that, the tubes were dispersed with sterilized glass beads to separate fungal conidia from the agar slants completely. The initial

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conidia number was determined by counting using a Burker chamber with Olympus Plan 40×/0.65 Ph2 objective. Erlenmeyer flasks (250 mL) containing 10 g substrate were autoclaved at 121ºC for 30 min. Then the flasks were cooled down at room temperature and moistened with adequate sterilized basal medium (pH 6.5) until the moisture content reached 80%. The basal medium contained (g/L): lactose 5, NH_4NO_3 5, KH_2PO_4 1, NaCl 1, $MgSO_4.7H_2O$ 0.6, $CaCl_2$ 0.1, and $FeCl_3$ 0.01. Single strains or mixed cultures were inoculated to the flasks, then incubated at 28-30ºC, 140 rpm agitation speed for 7 d. Liquid samples were taken daily and centrifugated at 17.968 ×g for 10 min at ambient temperature to separate solid residue from the supernatant. Then the liquid phase was filtered through a 45 µm PTFE membrane (Fisher Scientific, Leicestershire, UK). All the samples were kept at -20 ºC for further analysis.

The cultivation procedure of microbial consortium was similar to individual species. The mixed culture was constructed by combining single strains freshly grown separately. The initial conidia or cell number in the case of single strains was 10^6/g of the initial dry substrate (gds), and in the case of mixed cultures, it was 10^6/g per strain. In the case of the selected consortia, wheat bran was used in combination with wheat straw in various ratios. The biological treatment was conducted under solid-state conditions.

2.4. Degradation rate evaluation

After pretreatment for 7 d, the solid residues were suspended in distilled water to eliminate accompanying microorganisms. Then, they were filtered through Whatman filter papers (MN 612, Macherey-Nagel, Germany) and dried in an oven at 105 ºC for 24 h. The samples were stored in a desiccator for 2 d before weight loss determination. The weights of samples were measured by gravimetric methods and calculated by **Equation 1** (Qiu and Chen, 2012).

$$Weight\ loss\ ratio\ (\%) = \frac{W_o - W_p}{w_o} \times 100 \quad \text{Eq.1}$$

where W_o is the initial dry sample weight (g), and W_p is the dried weight (g) of the pretreated sample.

2.5. Determination of reducing sugars

Reducing sugar concentration was determined using the Somogyi-Nelson method with glucose as the standard solution (Rezessy-Szabo et al., 2003). The developed color was optically measured at 540 nm using a spectrophotometer (Helios Gamma, Unicam, United Kingdom). The reducing sugar accumulation was calculated by **Equation 2**.

$$Reducing\ sugar\ accumulation = \frac{mg\ sugar/gds\ of\ test\ sample}{mg\ sugar/gds\ of\ control\ sample} \quad \text{Eq. 2}$$

2.6. Enzymatic assays

The biological pretreatment process was investigated through the activity of the lignocellulose-degrading enzymes. Total cellulase (FPase), endo-β-glucanase (CMCase), and endo-β-xylanase were assayed as described in our previous study (Vu et al., 2022). Enzyme activity is expressed in International Units (IU) corresponding to the amount of enzyme releasing 1 µmol of corresponding sugar/min under room temperature.

2.7. HPLC analysis

High-Performance Liquid Chromatography (HPLC) analysis was conducted using a Surveyor HPLC system (Thermo Fisher Scientific Corporation, USA) equipped with a refractive index detector (RI). The analytical column utilized was a Hi-Plex Ca column (7.7 × 300 mm, 8 µm). The eluent was double-distilled water (ddH_2O) with a flow rate of 0.6 mL/min. The column temperature was maintained at 85 °C, and 10 µL of the sample solution was injected. Glucose, xylose, maltose, cellobiose, and maltotriose standards were prepared in ddH_2O at concentrations of 500 mg/mL for calibration. Additionally, internal standards were prepared using glucose and xylose and injected alongside the samples.

2.8. Statistical analysis

All experiments were prepared in three replicates; data are expressed as the mean and standard deviation. One-way analysis of variance (One-way ANOVA) and post-hoc Tukey multiple comparison tests were done using Statistical Package for the Social Sciences (SPSS) software (version 20.0); Pearson's correlation coefficient test was used to examine the correlation between different numerical data; only $p<0.05$ was accepted as the statistical significance level. The degradation profiles of monocultures and mixed cultures were evaluated by cluster analysis focusing on significant differences between groups or clusters. The enzyme activity, the released reducing sugar content, and weight loss were obtained in clustering results using the Euclidean shortest distance method.

3. Results and Discussion

3.1. Biodegradation performance of mono and mixed cultures

The results of our previous study confirmed that various combinations of strains *B. subtilis*, *B. licheniformis*, *B. cereus*, and *B. coagulans*, including a two-member consortium of *B. subtilis* B.01162 and *B. coagulans* B.01123 significantly enhanced the production of lignocellulose-degrading enzymes (Vu et al., 2022). Accordingly, in the present study, new combinations of previously investigated bacterial strains and some strains from *Pseudomonas* and *Rhodococcus* genera were designed and used for the biological treatment of wheat bran. A three-member consortium of *B. subtilis* B.01162, *Pseudomonas putida* B.01522, and *Rhodococcus opacus* B.01915 produced a higher amount of reducing sugars in comparison with the cocultures of *Bacillus* and *Pseudomonas* strains (data are not shown). Different artificial microbial communities were redesigned and used in the pretreatment of wheat bran substrate (**Table 2**). The consortium of bacteria and yeast (BY) resulted in significantly higher soluble sugar accumulation in the liquid phase than the respective mono-cultures (Y1) or co-cultures (B1, B2). The mixed culture of *Y. divulgata* Y.02062 and *P. stipitis* Y.00888 showed better bioconversion performance, effectively converting a two-fold higher amount of sugars than the mono-culture.

Biodegrading bacteria displayed the dominant role in enhancing the bioconversion of lignocellulosic biomass into valuable compounds, typically soluble sugars. After 72 h of cultivation, co-cultures of bacteria with fungi and co-cultures of bacteria with yeast led to better ratios of sugar accumulation, ranging from 3.2 to 3.5, respectively, while the mixture of fungi and yeast resulted in only 1.05. In addition, wheat bran pretreated with multiple cultures of the three types of microorganisms accumulated a higher yield of soluble sugars when compared with the co-culture of *A. niger* F.00632 and *Y. divulgata* Y.02062. Increases in substrate weight losses by complex microbes were observed, strengthening the high degradation effect when strains collaborated. Pearson's correlation coefficient was 0.596 ($p<0.05$), which showed a significantly high correlation between reducing sugar concentration and weight loss after 72 h of biological cultivation. The filamentous fungal strains such as *A. niger* F.00632, *P. chrysogenum* F.00814, and *T. viride* F.00795 showed high degrading capacity, leading to relatively high soluble sugar accumulations, especially when combined.

The co-cultures of fungal strains resulted in higher substrate weight loss than individual strains after 72 h of pretreatment. These values were reported at around 42.32 and 44.45% in the cases of the combinations of *A. niger* F.00632 strain with either *P. chrysogenum* F.00814 or *T. viride* F.00795, respectively. Generally, filamentous fungi have been known as effective degraders for decaying lignocellulosic residues because they can secrete various degrading enzymes, including cellulolytic, hemicellulolytic, and ligninolytic enzymes, while they can also interact synergistically during the biodegradation process (Nidetzky et al., 1994; Pérez et al., 2002). This phenomenon was also confirmed by Taha et al. (2015), who reported the improvement of straw saccharification efficiency through co-culturing degrading microorganisms, pointing out that the fungal insolates exhibited double-fold higher enzyme activities than the bacteria.

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, which cannot be achieved using mono-cultures. The selected members in the consortium could associate together and enhance the stability of the degradation process, thus improving the performance of the microbial system. One of

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Table 2.
Performance of different microbial communities in wheat bran degradation.

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

the most important environmental parameters is pH, which can indicate microbial growth. In the cases of the combinations B2 and B3 (**Table 2**), the pH values increased to the basic range (pH 8.8). This can be explained by the fact that the bacterial species can release ammonia gas converted into ammonium hydroxide in the liquid phase, increasing the pH value (Vu et al., 2022). On the contrary, a remarkable decline in pH values was observed

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when fungal strains were used during the treatment (**Table 2**). Additionally, during the treatment of wheat bran by the other microbial consortia, the pH values were around pH 6 or lower. This phenomenon could be explained by the nutrient cross-feeding interaction while multiple species compete for a single resource (Ali et al., 2020; Xu and Yu, 2021).

Microbial interaction is a typical characteristic of members in microbial communities. This was specifically observed in the present study leading to the reduction of reducing sugar yield caused by the metabolic consumption of microbes for their growth. Deng and Wang (2016) proved the higher metabolic pathway by synergistic co-cultures than pure culture, resulting in the competition of members for nutrients. Consequently, fermentable sugar yield was reduced during the treatment. Emphasizing the superior performance of microbial consortia, studies by Kato et al. (2004) and Levin et al. (2006) implied that pure culture isolates could only degrade substrates with simple structure and composition, such as artificial xylan and carboxymethyl cellulose, despite possessing high lignocellulolytic activities and could not degrade natural lignocellulosic biomass.

Activities of three extracellular hydrolytic enzymes (FPase, CMCase, and xylanase) were assayed at 72 h to elaborate the mechanism of carbohydrate degradation. It can be noticed that the fungal co-culture treatment had more elevated enzymatic activities than the fungal mono-culture pretreatment. Specifically, *A. niger* F.00632 and the three-member consortium of *A. niger* F.00632, *P. chrysogenum* F.00814, and *T. viride* F.00795 achieved the maximum values of FPase activities of 2.88 and 2.91 IU/gds, respectively. Additionally, CMCase and xylanase activities of fungal consortia were 14.6 and 95.15 IU/gds, while these activities for the single strain (F1 sample) stood at 11.2 and 45.4 IU/gds, respectively.

The FPase activities of complex cultures ranged from 1.00 to 1.69 IU/gds, which surpassed FPase secreted by bacteria and yeast in the pretreatment of lignocellulosic biomass in solid-state processing. Interestingly, the addition of yeast strain into the co-culture of bacteria or bacteria and fungi contributed to an increase in xylanase activity from 32.0 to 48.9 IU/gds, and it was responsible for higher weight loss of solid residues. The presence of the three filamentous fungi (BFY2) showed a slight increase in total cellulase and CMCase activities but led to a drop in xylanase activity. Likewise, ligninolytic species such as *R. opacus* B.01915 and *P. putida* B.01522 synergized with the fungal mixture to produce hydrolytic enzymes. The FPase, CMCase, and xylanase activities were 1.69, 7.28, and 51.1 IU/gds, respectively, in the case of a higher-member consortium (BFY5), including *R. opacus* B.01915, *P. putida* B.01522, *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795, *Y. divulgata* Y.02062, and *P. stipitis* Y.00888.

Microbial consortia composed of different types of species revealed unique characteristics associated with the interactions between them. The enzymatic assays revealed that the complex microbial consortia of bacteria, fungi, and yeast stably co-existed in the same habitat. Their co-existence was assumed to be important for the effective degradation by enhancing extracellular enzyme production. The enhancement of degrading enzyme activities of FPase and CMCase was found under the cultivation of filamentous fungi with other species. Xylanase, a key enzyme for xylan degradation, was also detected in the hydrolysates of mixed microbes, with activities fluctuating from 21.0 to 51.0 IU/gds. Overall, the presence of bacteria in a consortium with fungi or yeast resulted in a significant increase in degrading enzyme activities and reducing sugar yields (**Table 2**). Additionally, less weight loss of solid residues after 72 h of treatment was found in the samples cultured with *A. niger* F.00632, *B. subtilis* B.01162, and *P. putida* B.01522 compared to other co-cultures. These results suggest that communities with higher functional diversity increased the degradation of complex substrates.

The so-called division of labor caused by the diversity in the built consortia could be highlighted as a major driving force during lignocellulosic degradation. Filamentous fungi have been found to play an active role in the secretion of specific enzymes capable of degrading lignocellulose, while bacterial species can boost the production of hydrolytic enzymes and sugar compounds as nutrients, enhancing the ecological stability of the environment. Metabolic division of labor is a typical mutualistic interaction where distinct populations perform different but complementary tasks to diminish the metabolic burden of each population (Tsoi et al., 2018). This was referred to by Lee et al. (2021) in a constructed consortium of *Pseudomonas putida*, *Cellulomonas fimi*, and *Methylorubrum extorquens*, where *P. putida* acts as lignin degrader and *C. fimi* acts as cellulose degrader throughout the lignocellulose degradation. Another synergistic interaction, namely commensalism, was introduced by Minty et al. (2013). In a synthetic fungal-bacterial consortium, soluble saccharides produced by *Trichoderma reesei* as "public goods" was metabolized by *Escherichia coli* and converted into isobutanol. Together, these synergistic interactions enabled communities to accomplish tasks more metabolically intensive than pure culture could.

3.2. Sugar profile of the hydrolysates

The degradation effect during the pretreatment process resulted in the partial hydrolysis of polysaccharides (cellulose and hemicelluloses), thereby causing the accumulation of fermentable sugars in the liquid phase of the medium. After 72 h of biological pretreatment of wheat bran by different mono- and mixed cultures, the total amount of mono- (glucose, xylose) and disaccharides (maltose, cellobiose) varied from 4.4 to 398 mg/gds. Meanwhile, the use of consortia B1 and B2, as well as fungal mono-culture F1 in the biodegradation, led to a lower amount of simple sugars (<50 mg/gds), whereas the use of consortia BF, FY, BFY1, and BFY2 gave a quite high titer (>280 mg/gds). It is no doubt that bacteria and yeasts had less impact on degrading the structure of recalcitrant lignocellulose due to the deficient production of hydrolytic enzymes. Filamentous fungi also had inadequate sugar conversion even though they caused a large weight loss of lignocellulose substrate and a high reducing sugar accumulation. Nevertheless, incorporating these microbes together could improve the degree of degradation of lignocellulosic biomass. The consortia BY and BFY1 consisting of two bacterium strains, *B. subtilis* B.01162 and *P. putida* B.01522, and yeast strain *Y. divulgata* Y.02062 or the filamentous fungal strain *Aspergillus niger* F.00632 and yeast strain *Y. divulgata* Y.02062, respectively, exhibited good results in terms of the weight losses as well as in simple sugar spectrum.

Several previous studies have examined the roles of bacteria, in combination with fungi, in the degradation of lignocellulose. Suwannarangsee et al. (2012) found that the collaboration of hemicellulolytic enzyme secreted by *Aspergillus aculeatus* with non-catalytic protein produced by *B. subtilis* promoted rice straw degradation. It is worth noting that in the biological pretreatment by a microbial consortium, the correlation between the activities of hydrolases and sugar yield is not always strong and linear. In some cases, despite the enhancement of hydrolytic enzyme synthesis, lower content of fermentable sugars might be produced. Recent works by Kim et al. (2012 and 2018) reported that cellobiose could either be converted into other oligosaccharides or degraded into a monosaccharide (glucose) in the presence of *B. subtilis*, which could probably lead to the reduction of fermentable sugar yield.

In the present study, co-culturing yeast with fungal or bacterial strains generated a tremendous concentration of glucose and xylose, varying from 102 to 103 and 47 to 66 mg/gds, respectively. The highest glucose concentration of 236 mg/gds was determined in the hydrolysate after 72 h pretreatment by the BFY2 consortium. This can be explained by the fact that *Y. divulgata* can produce some metabolites for the growth promotion of other microorganisms (Zhang et al., 2021b), and *P. stipitis* can secrete xylanase, promoting the hemicellulose degradation process (Ding et al., 2018). Fermentable sugar yields released in the liquid phase varied based on the interactions among strains of the consortia. Nevertheless, the presence of yeast strains in the consortia was crucial to enhance the conversion rate and the amount of released fermentable sugars for further ethanol production or other applications.

3.3. Performance of microbial consortium on biological pretreatment

Cluster analysis using Ward's minimum variance divided the applied microorganisms into groups based on the biodegradation parameters, including extracellular lignocellulose-degrading activities (FPase, CMCase, and xylanase), released reducing sugars, and weight loss. Three clusters were formed from 16 variances and denoted as A, B, and C. Fungal strains and their consortia were grouped in *Cluster A*, which showed the highest enzyme activities and substrate weight loss. Fungal strains also performed a promising degradation capacity according to a large amount of reducing sugar released after 72 h of the pretreatment. *Cluster C*, including the mixed cultures of bacterial, fungal, and yeast strains, exhibited a

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moderate capacity for cellulolytic enzyme activities. The rest of the strains and co-cultures were classified into *Cluster B*, which indicated an insufficient effect on the bioprocessing of lignocellulosic biomass.

The solid-state process was applied with the solid-to-liquid ratio of 1:9, sufficient for the growth of diverse microorganisms. The fungal consortium of *A. niger* F.00632, *P. chrysogenum* F.00814, and *T. viride* F.00795 showed outstanding degradation performance in solid-state conditions. Similar results were obtained by several previous reports emphasizing the effect of rich enzyme systems on lignocellulosic residue degradation (Tsao et al., 2000; Kang et al., 2004). Our results also aligned with the high CMCase activity of isolated *A. niger* strains reported by Pirota et al. (2013). Rodríguez et al. (1996) also detected ligninolytic activities, especially laccase in the case of *P. chrysogenum* strains, capable of oxidizing aromatic compounds in cellulose-rich materials.

The superior hydrolytic enzymes and reducing sugar yields produced by fungal strains and their co-cultures were found in *Cluster A*, where the FPase activity varied very narrowly but was kept at a high level. The fungal lignocellulosic enzymes significantly affected biomass decomposition, as confirmed by the high reducing sugar yields. Meanwhile, microbial consortia belonging to *cluster B* showed moderate reducing sugar concentration and hydrolytic enzymes but lower polysaccharide loss than fungal consortia. The consortia in *Custer C* produced the lowest degrading enzymes and reducing sugar yields.

3.4. Effect of designed microbial communities on lignocellulosic biomass biodegradation

Among the tested consortia, BFY4 consortium including *B. subtilis* B.01162, *R. opacus* B.01915, *P. putida* B.01522, *A. niger* F.00632, *Y. divulgata* Y.02062, *P. stipitis* Y.00888, and BFY5 consortium including *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 were selected and inoculated into a solid-state system containing the mixtures of agro-industrial substrates, wheat bran and wheat straw, to investigate their degradation properties. Both consortia achieved similar biodegradation trends using different ratios of two substrates (**Fig. 1**). Increase in the percentage of wheat bran in the substrates increased weight loss. This can be explained by the lignin content that is significantly higher in the wheat straw (26.0 w/w% of the dry substrate) than in the wheat bran (5.5-6.0 w/w% of the dry substrate) (Guo et al., 2018; Kaprelyants et al., 2019). Therefore, the higher lignin content in substrates resulted in the lower degradation efficiency of biological pretreatment. Thus, to increase the digestibility of cellulose and hemicellulose, removing or breaking down recalcitrant lignin is crucial.

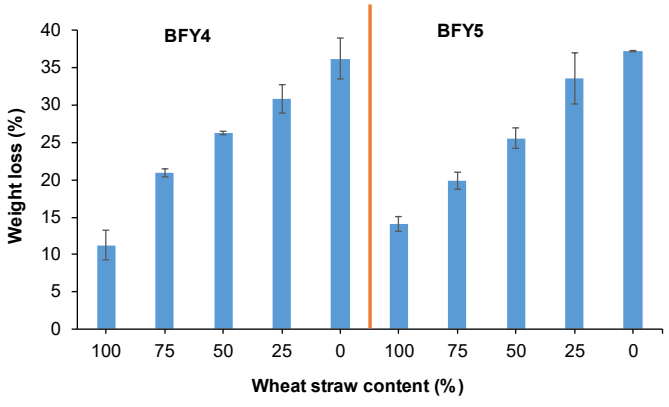

Fig. 1. Changes in weight loss of lignocellulosic substrates (containing wheat straw and wheat bran at different percentages) pretreated by microbial consortia BFY4 and BFY5. Values are reported as the mean ± SD (standard deviation) at p<0.05.

The influence of pretreatment time on the degradation performance of microbial consortia was investigated by monitoring the released reducing sugars daily up to 7 d of the pretreatment. In the case of wheat straw as a single substrate, the maximum sugar accumulation reached about 3.19 on day 2 and 2.09 on day 3 for the BFY4 and the BFY5 consortia, respectively (**Table 3**). In the cases of the mixtures of wheat bran and wheat straw, the highest accumulations of sugars were observed in the ratio of 75:25 on the 7th day of the pretreatment. These values were 7.16 and 5.67 for both consortia, the BFY4 and the BFY5, respectively. The low amount of lignin components in the substrate wheat bran might explain this observation. In the cases of the ratio of substrates 1:1, the maximum sugar accumulations were observed as 2.25 and 2.46 on the 3rd day of the pretreatment, respectively, for both investigated consortia and after that, a decline in sugar yields was detected.

Hydrolytic enzyme production is inducible and influenced by the nature of lignocellulosic substrates in the pretreatment; thus, selecting an inducer for the bioprocess is very important. Hydrolytic activities of enzymes including FPase, CMCase, and xylanase were evaluated after 72 h of the pretreatment process (**Fig. 2**).

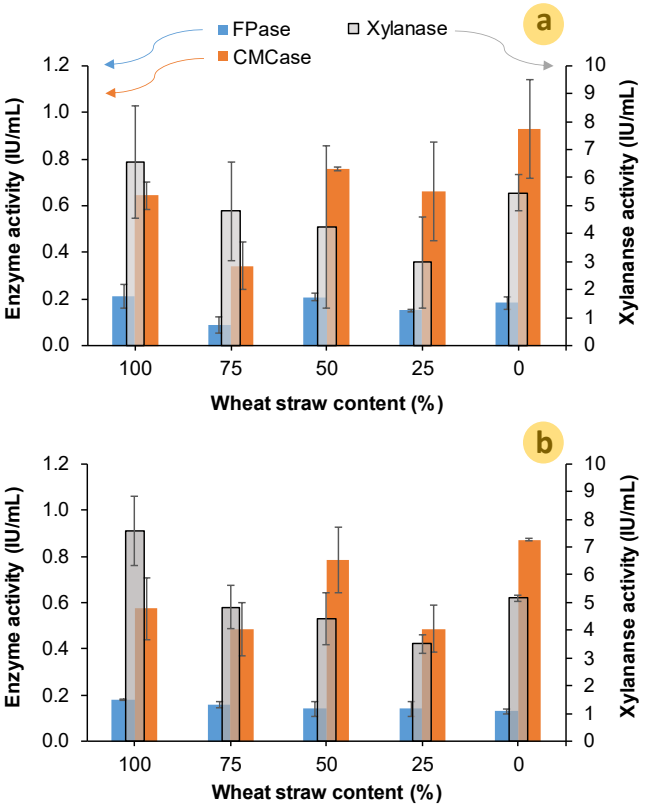

Fig. 2. Enzymatic activities (total cellulase - FPase, endo-β-glucanase - CMCase, and xylanase) for the pretreated lignocellulosic substrates containing wheat straw and wheat bran at different percentages under cultivation of two microbial consortia **(a)** BFY4 and **(b)** BFY5 at 72 h. Values are reported as the mean ± SD (standard deviation) at p<0.05.

Particularly, FPase and xylanase activities were markedly higher in the wheat straw alone than in the mixture of wheat straw and wheat bran. The highest FPase (0.21 IU/gds) and xylanase (6.56 IU/gds) activities were achieved with the BFY4 consortium for the wheat straw substrate. These values were about 2.4-fold and 1.3-fold higher, respectively, compared with values from the 75:25 wheat straw and wheat bran mixture. The BFY5 consortium also showed a promising degradation capacity when wheat straw was used alone, especially with outstanding xylanase activities (7.59 IU/gds) compared to others. Our results agree with the study of Kang et al. (2004), who claimed the maximum FPase and xylanase activities were obtained when using only rice straw treated with *A. niger* KK2. Besides, CMCase activities reached the maximum values of 0.93 IU/gds and 0.87 IU/gds when the medium contained wheat bran pretreated with BFY4 and BFY5 consortia, respectively. High activities of FPase and CMCase

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Table 3.
Sugar accumulation in response to the biological pretreatment of lignocellulosic biomasses by artificial microbial consortia.

Artificial microbial consortium	Substrates ratio (%)		Pretreatment time (d)						
	Wheat bran	Wheat straw	1	2	3	4	5	6	7
BFY4	0	100	1.58 ± 0.27	3.19 ± 0.65	2.12 ± 0.63	2.07 ± 0.21	1.31 ± 0.44	1.12 ± 0.28	1.36 ± 0.17
	25	75	1.57 ± 0.15	0.99 ± 0.10	0.89 ± 0.24	1.66 ± 0.29	1.63 ± 0.31	0.74 ± 0.21	0.58 ± 0.13
	50	50	0.96 ± 0.20	1.48 ± 0.43	2.25 ± 0.42	1.95 ± 0.13	1.58 ± 0.43	0.73 ± 0.28	0.78 ± 0.28
	75	25	1.00 ± 0.05	1.11 ± 0.13	2.37 ± 0.41	2.46 ± 0.31	3.75 ± 0.43	4.72 ± 1.38	7.16 ± 1.65
	100	0	0.62 ± 0.07	0.80 ± 0.21	1.48 ± 0.36	5.32 ± 0.29	3.09 ± 0.41	6.11 ± 0.56	8.21 ± 2.45
BFY5	0	100	1.63 ± 0.23	2.00 ± 0.49	2.09 ± 0.65	0.79 ± 0.17	0.77 ± 0.29	1.29 ± 0.18	1.57 ± 0.15
	25	75	1.15 ± 0.14	0.87 ± 0.22	1.19 ± 0.37	2.09 ± 0.33	0.90 ± 0.11	1.23 ± 0.41	0.60 ± 0.16
	50	50	1.56 ± 0.11	1.77 ± 0.13	2.46 ± 0.48	1.94 ± 0.19	1.00 ± 0.05	0.88 ± 0.22	1.02 ± 0.13
	75	25	1.35 ± 0.07	1.40 ± 0.50	1.95 ± 0.50	2.71 ± 1.02	3.15 ± 1.21	5.58 ± 1.71	5.67 ± 1.67
	100	0	0.66 ± 0.15	0.94 ± 0.19	1.69 ± 0.22	3.94 ± 0.89	5.75 ± 1.06	5.20 ± 1.51	5.79 ± 1.15

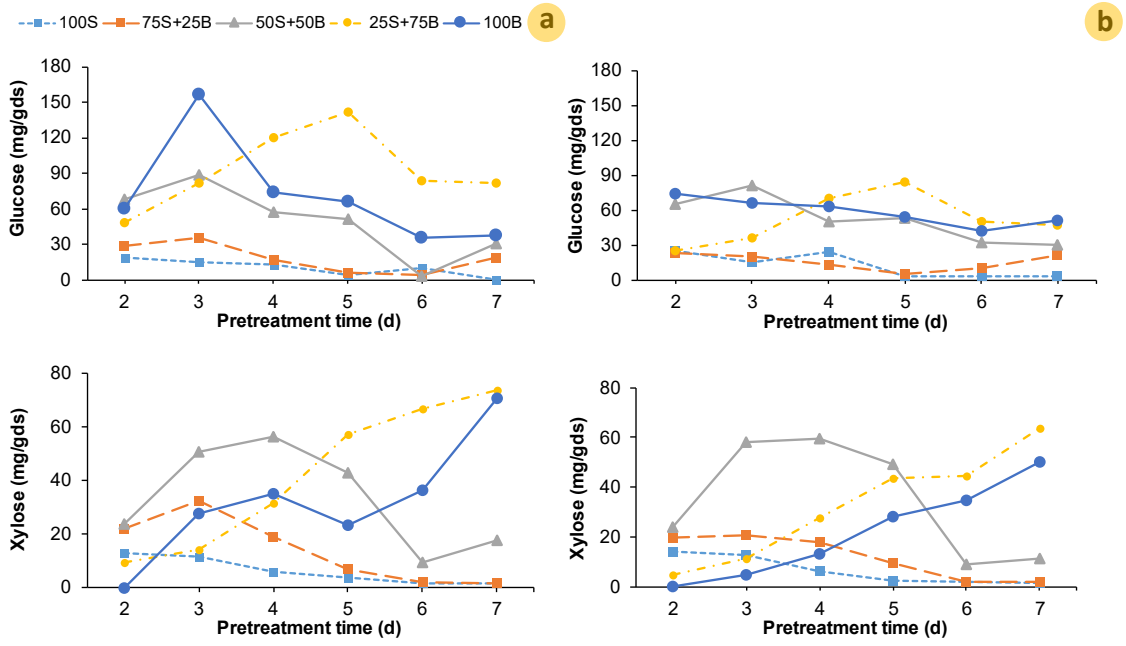

Fig. 3. Changes in glucose and xylose production during 7 d of biological pretreatment of different mixtures of substrates by the developed consortia **(a)** BFY4 and **(b)** BFY5. S: wheat straw and B: wheat bran.

were found in the case of substrates used in the same proportion (in the ratio of 50:50), contributing to the high accumulation of reducing sugars. Enzymatic activities reduced from 27 to 57% for FPase and from 13 to 53% for CMCase when the BFY4 consortium was inoculated to the medium containing 25% wheat straw and 75% wheat straw, respectively. A 38% decrease in CMCase activity was also observed when the BFY5 consortium was used in the same conditions. The BFY4 consortium released more glucose than the BFY5 consortium in the hydrolysate of pretreated mixed substrates containing over 50% wheat bran (Fig. 3).

The maximum glucose yield was 156.47 and 89.02 mg/gds 3 d after the pretreatment of wheat bran and a mixture of wheat bran and wheat straw at an equal ratio (50:50), respectively. Likewise, the highest glucose concentration was reached on the 5th day using a substrate composed of 25% wheat straw and 75% wheat bran, with the amount of 142.16 mg/gds (BFY4) and 84.73 mg/gds (BFY5). On the other hand, BFY4 and BFY5 consortia released xylose with a similar tendency. The amount of xylose reached the highest value at 3 d of the pretreatment, when the ratio of substrates was 1:1. Then, after 4 d, the xylose content dramatically reduced till the end of the process. However, substrates containing over 75% of wheat bran produced more xylose when increasing the time of treatment. This can be explained by the lower lignin content of wheat bran and by the increase in the secretion of cellulases with the process progressing. It is worth mentioning that in the ethanolic fermentation, the commonly used *Saccharomyces cerevisiae* yeast species cannot efficiently convert pentose sugars into ethanol, but other yeasts belonging to the genera *Kluyveromyces*, *Pichia* and *Candida* or bacterium *Zymomonas mobilis* can

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perform this process (Ding et al., 2018; Htet et al., 2018). Thus, the mixed cultural fermentation technique should be developed and adapted to utilize such pretreated biomass to produce ethanol (Rouhollah et al., 2007; Fu et al., 2009).

4. Conclusions and Prospects

Different microbial consortia were constructed and tested in solid-state systems to determine their degradation profiles and conversion efficiency. Fungi and mixed cultures of fungi, bacteria, and yeasts showed a relatively high degradation capacity under solid-state conditions due to their synergistic interactions in the microbial consortium. The source of substrates significantly impacted the synthesis of lignocellulose-degrading enzymes and the production of reducing sugars. The highest enzyme activities of FPase and endo-β-xylanase were found when wheat straw was used alone with CMCase was maximized when wheat bran was used under the cultivation of microbial consortium. Substrate mixtures containing over 50% wheat straw achieved the highest amount of reducing sugars within the 4 d of pretreatment. Our results open a new direction in the development of an artificial microbial consortium based on the role of members and natural interactions between members in the degradation of cheap lignocellulosic agro-wastes into value-added products.

The current study focused on the development of mesophilic aerobic microbial consortia, and it can serve as a model system for designing more complex artificial consortia in the future. Additionally, a combination of microbes in the consortium with thermophilic anaerobic ones screened and isolated from the environment would be an attractive and potential approach. Determination of population dynamics based on the analysis and differentiation of microbial DNA, such as sequencing and metagenomics, could also significantly help researchers predict metabolic fluxes and growth phenotypes of single strains or simple microbiota to understand the simultaneous involvement of several different microbial interactions in the same industrial process, and then to develop more effective control strategies.

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