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ORIGINAL ARTICLE

Novel fungal consortium pretreatment of waste oat straw to enhance economical and efficient biohydrogen production

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Abstract *- Bio-pretreatment using a fungal consortium to enhance the efficiency of lignocellulosic biohydrogen production was explored. A fungal consortium comprised of T. viride and P. chrysosporium as microbial inoculum was compared with untreated and single-species-inoculated samples. Fungal bio-pretreatment was carried out at atmospheric conditions with limited external energy input. The effectiveness of the pretreatment is evaluated according to its lignin removal and digestibility. Enhancement of biohydrogen production is observed through scanning electron microscopy (SEM) analysis. Fungal consortium pretreatment effectively degraded oat straw lignin (by >47% in 7 days) leading to decomposition of cell-wall structure as revealed in SEM images, increasing biohydrogen yield. The hydrogen produced from the fungal consortium pretreated straw increased by 165% 6 days later, and was more than produced from either a single fungi species of T. viride or P. chrysosponium pretreated straw (94% and 106%, respectively). No inhibitory effect on hydrogen production was observed.*

Keywords *- bio-hydrogen; fungal consortium; pretreatment; oat straw; lignin degradation.*

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1. Introduction

Reducing the reliance on fossil fuels, by converting abundant lignocellulosic biomass to biofuels presents a viable option (Chang et al., 2011). Straw is one of the major lignocellulosic wastes produced during agricultural cultivation of cereals in temperate climates. Its hemicellulose typically contains high fractions of pentoses and has a dried-lignin content of 16–25% (Rio et al., 2012). It could be an excellent source for bio-energy production. Oat straw is one of the major crops in Russia, North America and Europe containing around 66.3% carbohydrates according to the USDA Database (USDA, 2013). Converting agricultural wastes such as oat straw to fuel achieves provides useful resources and potentially reduces the negative effect of fossil fuels on climate change.

Hydrogen is a clean energy carrier which can be produced from waste materials, such as organic waste crop straw (Pakarinen et al., 2008). Some believe that hydrogen will replace fossil fuels in the next generation (Verhelst, 2014). Considerable research in recent years has been focused on the conversion of biomass reproducible resources to hydrogen (Pakarinen et al., 2008; Kim et al., 2012; Wu et al., 2013; Zhou and Li, 2016). Biological hydrogen production is recognized as one of the most promising technologies in the successful evolution of fuels (Das, 2009).

Despite considerable research on biohydrogen production from lignocellulosic wastes, a major challenge remains due to its low conversion efficiency. The primary reason is that the cellulose fraction of straw is recalcitrant to enzymatic breakdown owing to the complex structure of cellulose and the rigid shielding of hemicellulose by lignin, resulting in stable biomass (Balat, 2011; Monlau et al., 2013). Accordingly, pretreatment of the straw is necessary to soften the lignin shield, exposing the cellulose and hemicellulose for microbial consumption, resulting in hydrogen production. A variety of pretreatment techniques have been developed to improve decomposition of cellulosic fibers: physical (Bak et al., 2009; Wu et al., 2013), chemical (Ballesteros et al., 2008; Zhao et al., 2009), and physio-chemical (Chen et al., 2008). However, chemical methods, whether acid or alkaline, leave strong acidic, basic, or toxic residues in the treated biomass, causing significant environmental risks (Wu et al., 2013), whereas physical pretreatment without the use of harmful chemicals, such as thermal, microwave and freezing, require high energy input which is not sustainable. In addition, most of those processes produce inhibiting compounds, such as weak acids, furan derivatives and phenolic compounds which negatively

affect the subsequent fermentation to produce hydrogen (Palmqvist and Hahn-Hagerdal, 2000).

Biological pretreatment is an attractive pretreatment method since it does not involve hazardous processed chemicals and metabolite repression problems (Kausar et al., 2010), and has remarkable advantages of simplicity, low cost and low requirements for equipment (Mshandete et al., 2008). Recent research has found that some fungi consortia are able to degrade the cell walls of straw through composting. These include *Trichoderma viride* and *Aspergillus niger* (Kausar et al., 2010, 2011, 2013) and *T. viride* and *Phanerochaete chrysosporium* (Lin et al., 2011) used in composting at around 40-50°C. To the authors' knowledge, there is no reported study on fungal consortium pretreatment of oat straw for hydrogen production. In this study, we explore a low-cost and environmentally sound alternative using fungal consortium to enhance the efficiency of lignocellulosic biohydrogen production. Two fungi species (*T. viride* and *P. chrysosporium*) were selected to pretreat the oat straw facilitating subsequent hydrogen fermentation. *P. chrysosponium* has been proven to be highly reactive for lignin degradation (Michael and Margaret, 1993; Zeng et al., 2014). *T. viride* has shown a significant celluloytic power to rice straw leading to a decrease in the straw cellulose and hemicellulose contents, although lignin is recalcitrant to many other degradation methods (Wu et al. 2013). This fungal bio-pretreatment was carried out at atmospheric condition with limited external energy input. In this study, the effectiveness of the pretreatment is evaluated according to its lignin removal and digestibility. Enhancement of biohydrogen production is observed through scanning electron microscopy (SEM) analysis.

2. Materials and methods

2.1. Raw material

The straw was obtained from a farm in Richmond, a suburb of Vancouver, Canada. The oat straw waste was cut to pieces ~1-2 cm long, then milled and sieved through a 2.0 mm screen, and dried at 70 ± 1°C for 4 hours. The dried straw was then ground to a powder and stored in a sealed plastic bag at room temperature.

Two fungi species for the pretreatment, *T. viride* and *P. chrysosporium*, were isolated by the College of Architecture & Environment in Sichuan University, Chengdu, China. Each single colony of these two species and their combination were enriched at 28 ± 1°C by shaking at 150 rpm for 3 days in Mandels medium (Mandels and Weber, 1969), prepared by blending and dissolving 22 g Ammonium tartrate; 20 g glucose; 20 g KH_2PO4; 8.7 g $MgSO_4$; 1.0 g $CaCl_2$; 0.6 g NaCl; 0.35 g $MnSO_4$; 60 mg $FeSO_4$; 110 mg $CoCl_2$; 60 mg $ZnSO_4$; 95 mg $CuSO_4$; 6 mg H_3BO_3; 6 mg Na_2MoO_4 and 100 mg VB1 in 1L of deionized water.

Cow manure, used as the digesting microflora for hydrogen production, was obtained from a farm in a suburb of Vancouver, Canada. Cow manure was heat-treated by boiling for 30 min to inactivate H_2-consuming bacteria and to enrich spore-forming H_2 producers (Chang et al., 2011). The nutrient stock solution for hydrogen fermentation contained 4 g of yeast extract, 12.4 g of KH_2PO_4; 0.1 g of $MgSO_4.7H_2O$; 0.01 g of NaCl; 0.01 g of $Na_2MoO_4.2H_2O$; 0.01 g of $CaCl_2.2H_2O$; 0.015 g of $MnSO_4.7H_2O$ and 0.0278 g of $FeCl_2$ per liter, modified from Lay et al. (1999).

The physical and chemical characteristics of raw waste oat straw and cow dung are given in Table. 1.

Table 1. Characteristics of raw materials

	TS[a] (%)	VSb[b] (%)	pH	TCOD[c] (g/l)	SCOD[d] (g/l)
Cow dung	42.05	20.69	8.28	7.53	5.02
Oat straw	99.00	88.43	NA	NA	NA

[a] TS represents total solids content
[b] VS represents volatile solids
[c] TCOD represents total chemical oxygen demand
[d] SCOD represents soluble COD
NA – Not applicable

Main elemental composition (% weight of total) of raw oat straw is as follows: **C**: 38.48, **N**: 0.00**, P**: 0.19, **K**: 0.12, **H**: 5.79, **S**: 0.15, **Fe**: 1.68, **Na**: 0.74, **Ca**: 1.82.

2.2. Fungi pretreatment of oat straw

For bio pretreatment, a fungal consortium comprised of *T. viride* and *P. chrysosporium* was chosen as the microbial inoculum, and each member was used for comparison. The substrate was inoculated with 1 ml of inoculum at a concentration of 10^6 cell ml^{-1} in 250 ml Erlenmeyer flasks containing 100 ml of Mandels medium as described in section 2.1. The Mandels and Weber (1969) method was modified, with the glucose replaced by 6 g of dried waste oat straw. The culture was incubated at 28 ± 1 °C for 14 days with shaking at 150 rpm. This pretreated oat straw was then washed with deionized water three times to remove any by-products, and finally dried at 105 ± 1°C for 24 h for degradation analysis by SEM and for biohydrogen production by fermentation.

2.3. Degrading effects of pretreatment

The residue of the oat straw after fungi pretreatment was collected to determine the cellulose, hemicellulose and lignin contents according to Goering and Van (1970). The reducing sugar content of the pretreated samples produced for each incubation time was analyzed by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Scanning electron microscopy (SEM) investigations of the effects of fungi pretreatment on straw cell wall disruption, composition, ultrastructure and surface properties were carried out in order to better understand the increased susceptibility to digestion in hydrogen

fermentation. The SEM was carried out in a JEOL microscope model JSM-5900. Prior to analysis the samples were coated with gold to a thickness of ~7 nm using a Polaron SC7620 sputter coater. Microscopy was performed at 10 and 12 kV acceleration voltage and with magnification from 50 to 1,000.

2.4. Anaerobic fermentation of hydrogen production

The anaerobic experiments were performed with 150 ml serum vials as batch reactors containing the mixture of 6 g of the pretreated oat straw, 6 g cow dung and 60 ml of nutrient stock solution. Untreated oat straw was used as a control. These vials were infused with nitrogen to remove oxygen from the headspace of the reactors to maintain anaerobic conditions. The bottles were incubated in an orbital shaker at 65°C, with a rotation speed of 90 rpm to provide better contact among substrates. The volume of biogas was determined using glass syringes of 5 to 50 ml.

The gas composition (H_2, CH_4 and CO_2) was analyzed with a gas chromatograph (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and a 6-foot stainless column packed with Porapak Q (80/100 mesh). The operating temperatures of the injection port, oven and detector were 100°C, 80°C and 150°C, respectively. Nitrogen was the carrier gas at a flow rate of 20 ml/min.

All experiments were performed in triplicate (n=3), with average values reported, and the maximum and minimum values identified by means of error bars.

2.5. Data analysis

The hydrogen yield (ml/g straw) was obtained by dividing the cumulative hydrogen production (ml) by the dry weight of straw used for the fermentation. Analysis of variance (ANOVA) was used to estimate the statistical parameters. Two additional confirmation experiments were conducted later to confirm the validity of the statistical procedures.

3. Results and discussion

3.1. *Effects of fungi consortium on lignin degradation*

The effects of two individual fungi species and their combination on lignin degradation are shown in Fig. 1.

The consortium of *T. viride* and *P. chrysosponium* demonstrates better degradation effects on oat straw lignin than any single species. It also indicates that the delignification increased with time. After 3 days of fungal consortium digestion, the lignin content decreased from 22.6% to 17.4% (% of dry wt.). When the pretreatment time was extended to 5 days, the lignin content decreased to 14.4%. In the 7th day of treatment, the lignin content further decreased to 11.9%, representing 47.35% of the total lignin removal. However, increasing the processing time from 7 to 14 days did not significantly increase lignin degradation. Hence, 7 days processing time was selected for the applied effective *T. viride* and *P. chrysosponium* consortium bio-pretreatment for the subsequent experiments.

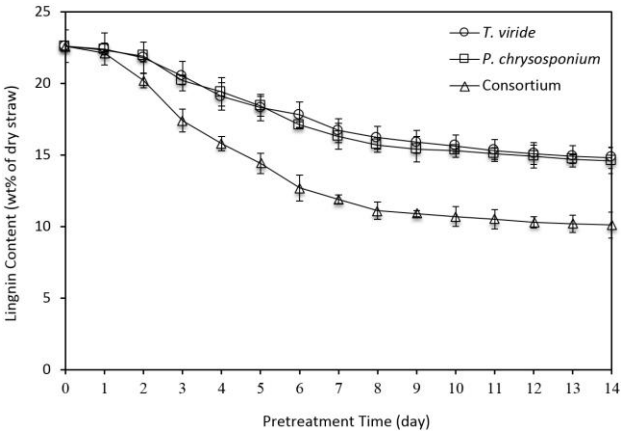

Fig. 1. Effect of fungi pretreatment on lignin content of the oat straw. The results are expressed as mean ± standard error of duplicate samples.

In this study, we demonstrated that the fungal consortium using *T. viride* and *P. chrysosponium* displayed synergistic effects, degrading the oat straw lignin more effectively than a single fungi species, indicating that fungal consortium fermentation could potentially be an excellent pretreatment option to enhance biohydrogen production from lignocellulosic biomass.

3.2. Effects on the straw cell-wall matrix

SEM images show that the surface of oat straw particles changed significantly with fungi pretreatment (Fig. 2). Images of the untreated oat straw particles revealed the elongated nature of the cells in the inner parts of the straw, and displayed the smooth, palisadic surfaces of the epidermal layers of oat straw, which shield and could resist fungal enzymatic attack (Figure 2A-C). They also show that the tubing making up the porous elongated particle structures remained relatively intact, whereas fungi-treated substrate images in Fig. 2D-L appear to have altered the surface structure causing rupture of the parenchyma cells.

Images of the straw particles with one fungi strain treatment revealed partial presence of the initial structures of the oat straw (Fig. 2 D-I). After *T. viride* treatment, mainly granular structures remained, but ordered, physiological structures, presumably rudiments of the epidermis and xylem, could also be seen (Fig. 2 D-F), whilst after *P. chrysosponium* treatment, the cell structures were more loosely connected (Fig. 2 G-I). The cell structures seemed more loosely connected and more exposed cellulose chains or cellulose (micro) fibrils after *T. viride* and *P. chrysosponium* consortium pretreatment (Fig. 2J-L). The epidermal surface layer appeared more torn with irregular edges, and the particles had few intact epidermal structures left, although some areas of palisadic surfaces were still visible (Fig. 2L). In general,

compared to the fungal consortium treated sample, the samples treated by either *T. viride* or *P. chrysosporium* presented fewer signs of rupture of the internal parenchyma, phloem and xylem structures, but crevices and holes appeared after those pretreatments.

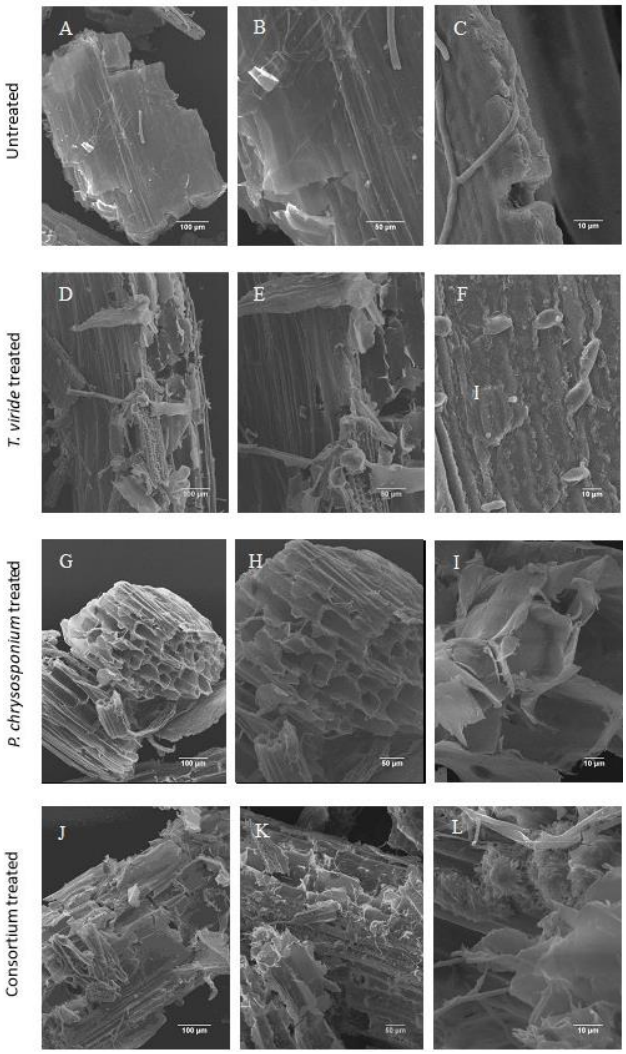

Fig. 2. SEM images of untreated (A-C), *T. viride*-treated (D-F), *P. chrysosponium*-treated (G-I) and consortium of *T. viride*- and *P. chrysosponium*-treated (J-L) oat straw.

These SEM images thus clearly indicated that significant changes of the straw structures resulted from the fungal consortium, apparently resulting in improved substrate–enzyme interactions.

3.3. Effects on biohydrogen production

Given that fungi can degrade lignin, it is logical to expect that fungi pretreatment could enhance biohydrogen production. To confirm the enhancement of lignin degradation in biohydrogen production, an anaerobic fermentation experiment was conducted to determine the overall effect of fungal consortium pretreatment on biohydrogen production from the oat straw.

3.3.1. Effects of fungi *pretreatment on biohydrogen production*

A batch fermentation experiment was conducted to determine the effect of fungi pretreatment on biohydrogen production. This experiment was performed over 8 days with samples pretreated by *T. viride*, *P. chrysosponium* and their consortium, as well as an untreated (control) sample. Both untreated and bio pretreated samples produced hydrogen (Fig. 3). No methane was detected, indicating the absence of methanogens. As shown in Fig. 3, fungal consortium pretreatment significantly increased biohydrogen yield above that of the single strains and the control. After 6 days of fermentation, the sample pretreated by fungal consortium produced 82 ml/g straw hydrogen, while *T. viride* treated, *P. chrysosponium* treated and untreated samples produced 60, 64 and 31ml/g straw, respectively. When the fungi pretreated samples are compared with the control (untreated oat straw), hydrogen produced by the fungal consortium treated straw increased by 165%, while the *T. viride* and *P. chrysosponium* treated samples increased by 94% and 106% respectively. This is consistent with the observations through SEM in Fig. 2. This hydrogen yield is compatible to previous studies on hydrogen production by the physical, chemical and thermal pretreatment options (Chang et al., 2011; Kim et al., 2012; Wu et al., 2013). It can be seen that the lag stage of the control was much longer than for the fungi pretreated samples. This suggests that the aerobic digestion of the oak straw by fungi pretreatment broke lignin barriers in the cell walls (Fig. 2), improving the substrate conversion and reducing the time required for anaerobic fermentation.

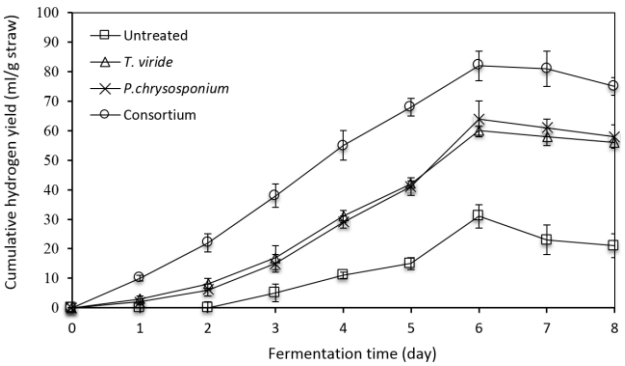

Fig. 3. Cumulative hydrogen produced by the untreated fungi-treated samples. The results are expressed as mean ± standard error of duplicate samples.

Changes in accumulative hydrogen and carbon dioxide yield during the conversion of the fungi pre-digested oat straw wastes to biohydrogen by cow dung compost are shown in Fig. 4. The results also provide evidence that regardless of pretreatment or no treatment, all groups produced a similar ratio of H_2 to CO_2 (4.9–5.3).

The results of the present fermentation experiment unequivocally demonstrate that under our experimental conditions, the fungal consortium pretreatment enhanced overall biohydrogen production. In our work, possible

inhibitors were removed by washing the substrate before hydrogen fermentation, so that there limited inhibition was observed with the samples pretreated by the fungi group. This is an improvement of the fermentation processes as most existing pretreatment processes form undesirable byproducts which inhibit fermentation processes (Palmqvist and Hahn-Hagerdal, 2000). Quéméneur et al. (2012) concluded that all the putative inhibitory compounds have a significant negative impact on H_2 production performance.

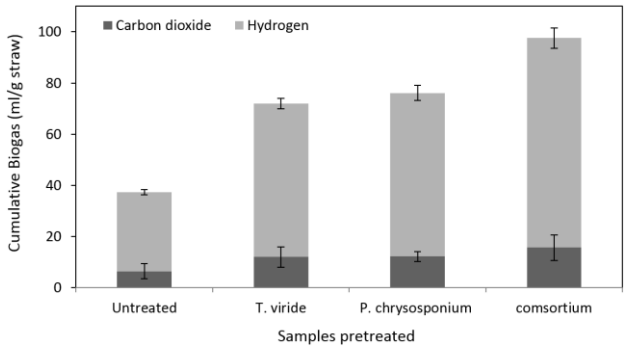

Fig. 4. Cumulative biogas produced after 7 days of fermentation by the untreated, *T. viride*, *P. chrysosponium* and a combination of these two fungi species. The results are expressed as mean ± standard error of duplicate samples.

3.3.2. Straw degradation versus hydrogen production

Naturally, when lignin is being reduced by pretreatment, the reducing sugar content yield increased relatively. Fig. 5 marks the relationship between sugar yield and lignin removal in the samples pretreated by the consortium of *T. viride* and *P. chrysosponium*. This clearly proves that the increasing pattern of reducing sugar has a similar trend as the change in lignin removal, in agreement with results reported (García-Cubero et al., 2009; Schultz-Jensen et al., 2011).

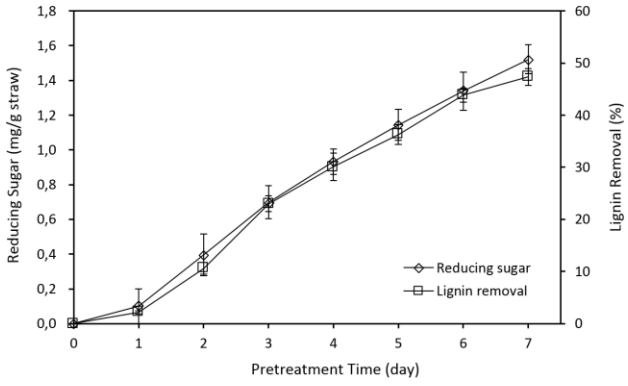

Fig. 5. Reducing sugar yield and lignin removal of fungi-group-treated sample after 7 days of incubation. The results are expressed as mean ± standard error of duplicate samples.

It is assumed that fungi digestion of oat straw decreased insoluble lignin content, increasing considerably the digestibility in the hydrogen production stage by increasing the accessibility in comparison with the non-pretreated raw materials (Singh et al., 2011). Lignin degradation versus hydrogen production is plotted in Fig. 6, showed that hydrogen production is positively related to the degradation of lignin in the straw. To achieve efficient biohydrogen production, a pretreatment targeting lignin removal is usually adopted (Ren et al., 2009; Cheng et al., 2011; Quéméneur et al., 2012) so that cellulose and hemicellulose can be released, exposed to microbes and subjected to enzymatic attack. Results of this study presented in Fig. 6 clearly demonstrate that, under the given experimental conditions, fungal consortium reduced lignin contents substantially, thereby enhancing biohydrogen production.

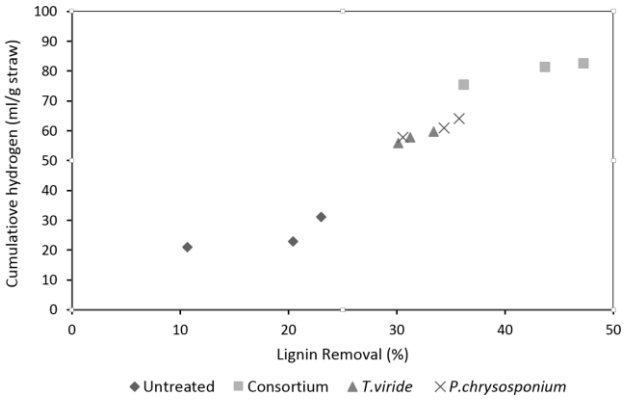

Fig. 6. Cumulative hydrogen production versus lignin removal in straw samples with different pretreatments.

4. Conclusions

Fungal consortium pretreatment effectively degraded oat straw lignin (by 47.35% in 7 days) leading to decomposition of cell-wall structure, thereby increasing biohydrogen yield. The hydrogen produced from the fungal consortium pretreated straw increased by 165% 6 days later, which was more than produced from either a single fungi species of *T. viride* or *P. chrysosponium* pretreated straw (94% and 106%, respectively). No inhibitory effect on hydrogen production was observed. Overall, this fungal consortium gave higher hydrogen yield, indicating that production of biohydrogen from oat straw can be significantly enhanced by *T. viride* and *P. chrysosponium* consortium pretreatment.

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