

Impact and prospective of fungal pre-treatment of lignocellulosic biomass for enzymatic hydrolysis

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Abstract: The presence of lignin in lignocellulosic biomass leads to a protective barrier which prevents enzymes from being accessible to cellulose and hemicellulose for hydrolysis. As a result, pre-treatment is a 'must' step for subsequent enzymatic hydrolysis. Bio pre-treatment is normally conducted at low temperatures and low pressures without using expensive equipment, chemical agents, reactors, and additional energy for lignin removal and biomass structure destruction. Therefore, it is a green, safe, and inexpensive method. White-rot fungi (WRF), a group of fungi (more than 1500 different species) are successfully applied in bioconversion processes such as sewage treatment, biopulping, conversion of forest and agricultural residues to animal feeds, and the production of edible or medicinal mushrooms. In the bio pre-treatment process, WRF are mostly used for secreting ligninolytic enzymes, a variety of donor substrates and selective degradation of lignin. Current research related to WRF bio pre-treatment is mainly focusing on the following four aspects: (i) selection of candidate strains for certain biomass materials; (ii) optimization of cultivation methods; (iii) characterization of fungal treated materials; and (iv) evaluation of combining bio pre-treatment with chemical or physicochemical approaches. Future prospects and recommended research work on applying WRF in bio pre-treatment are also briefly introduced and summarized in this review. These include (i) integrated methods (i.e. co-treatment with organic solvents, diluted acids, supercritical CO₂ and ionic liquids) to resolve problems existing in fungal pre-treatment applications; (ii) mutation breeding and crossbreeding of fungal mycelia to obtain engineering strains; and (iii) integration of fungal pre-treatment with simultaneous saccharification and fermentation to produce biofuels and value-added products. © 2012 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: bio pre-treatment; white-rot fungi; ligninolytic enzymes; bio-organosolve process; organic electrolyte solution; green technology

Introduction

World energy demand, particularly the demand in emerging countries such as China, is increasing rapidly. At present, petroleum is still the major raw material for the production of transportation fuels and industrial chemicals. However, fossil oil resource is limited to provide sustainable raw material. Therefore, abundant and low-cost renewable cellulosic biomass is an ideal alternative for the production of biofuels (e.g. ethanol and methane) and chemicals through fermentation and chemical processes. Since the 1970s, billions of dollars have been invested globally in process technology development (biorefining) to convert cellulose into bioethanol. Many companies in North America have taken the lead and developed process technologies to convert lignocellulosic biomass into bioethanol; some are at advanced pilot-plant levels. For enzymatic hydrolysis, pre-treatment (e.g. physical, chemical, physical-chemical, and biological methods) is required to break down lignin that binds cellulose and to destroy the crystalline structure of cellulose and increase its surface area so that fragments become accessible to enzyme active sites. Pre-treatment is the first-step in the conversion of lignocellulosic biomass to biofuels because it significantly improves processing and reduces operating costs of downstream units. In this work, fungal bio pre-treatment and its combination with other methods are introduced, reviewed, and discussed in detail. Future prospects and recommended studies for biorefinery are also given and discussed.

Bio pre-treatment in the biorefinery industry

For biorefining lignocellulosic materials into bioethanol and value-added chemicals, hydrolysis of cellulose and hemicellulose to sugars is a key step. Compared with traditional acid hydrolysis, the enzymatic hydrolysis approach (with cellulases and cellobiases) is recognized as one of the most promising and common methods.¹ It includes four steps: (i) pre-treatment of biomass for cellulose being accessible to enzymes; (ii) enzyme production; (iii) enzymatic saccharification of the pre-treated biomass to fermentable sugars; and (iv) subsequent microbial or chemical conversions of these sugars to desired end-products (e.g. fermentation to bioethanol).

Various studies were conducted with different cellulase systems and an array of cellulosic substrates.² The presence

of lignin, however, leads to a protective barrier that prevents cellulase enzymes from accessing cellulose and hemicellulose for their conversion to corresponding sugars.^{3,4} Some degree of pre-treatment is necessary to overcome this problem. Many different methods have been studied for biomass pre-treatment during the past few decades, and are loosely divided into four categories: physical (e.g. grinding, ball milling and irradiation), chemical (e.g. alkali, dilute acids, oxidizing agents, and organic solvents), physicochemical (e.g. steam-explosion, ammonia fiber explosion (AFEX), hydrothermolysis, and wet oxidation) and biological pre-treatments or a combination of these.¹

Compared with other approaches, the bio pre-treatment method does not require expensive equipment, chemical agents, and additional energy to remove lignin. It is considered as an environmentally friendly green method. By employing white-rot fungi (WRF), bio pre-treatment could remove 11.6–41.7% lignin from lignocellulosic materials in 5–120 days.^{4–14} Subsequent enzymatic hydrolysis yield was enhanced by 1.5–10 times than that of untreated materials.^{4–16} However, the biggest barrier for large-scale applications of bio pre-treatment is the slow processing of delignification. Additionally, a portion of carbohydrates in lignocelluloses is lost as micro-organisms will consume parts of hemicellulose and cellulose as an energy requirement for growth and metabolism.^{17,18} Nevertheless, bio pre-treatment technology has potential applications in biofuels that could be used alone or combined with other types of pre-treatment methods to increase the overall biorefinery efficiency.^{1,19}

White-rot fungi (WRF) and their applications

In the bio pre-treatment process, fungi (including white-, brown- and soft-rot), are mostly used to degrade lignin and hemicellulose in lignocellulosic biomass.²⁰ Among them, white-rot fungi (WRF; Fig. 1), with more than 1500 different species, are able to decompose lignin with little consumption of cellulose component. They are mostly used in the bio pre-treatment of biomass to produce biofuels including biogas.^{21–23} Besides bio pre-treatment, they can also be directly used in other bioconversion processes such as sewage purification,^{24–27} bio-pulping,^{28–32} bioconversion of forest and agricultural residues to animal feeds,^{33,34} as well as the production of edible or medicinal mushrooms.³⁵



Figure 1. Fruit bodies of some white-rot fungi. (A) *Armillariella mellea* (Vahl) P. Karst., (B) *Collybia velutipes* (Curtis) P. Kumm., (C) *Cryptoporus volvatus* (Peck) Shear, (D) *Ganoderma australe* (Fr.) Pat., (E) *Oudemansiella mucida* (Schrad.) Höhn., (F) *Ganoderma sinense* J.D. Zhao, L.W. Hseu & X.Q. Zhang, (G.) *Pleurotus rudis* (Fr.) Pilát, (H) *Schizophyllum commune* Fr., (I) *Laetiporus sulphureus* (Bull.) Murrill.

Mechanisms of WRF pre-treatment

The hyphae of WRF can secrete many ligninolytic enzymes which catalyze oxidative reactions during lignin depolymerization. These extracellular enzymes are divided into two families: polyphenol oxidases and heme-containing peroxidases (Table 1).³⁶ Under natural conditions, these ligninolytic enzymes only have limited reactions because their size impedes their penetration into plant cell wall to attack the inner lignin component.³⁷ Therefore, a variety of smaller-sized molecules are initially oxidized to donors for these enzymes. Afterward, the oxidized donors move onto the substrate and catalyze the removal of an electron from phenolic hydroxyl or aromatic amino groups. This process forms free phenoxy radicals and aromatic radicals, respectively. Following the initial cracking reaction caused by the radicals, the lignin polymers start to decompose and the radicals are finally depredated.³⁸⁻⁴² The molecules which act as donors include organic acids (e.g. oxalic and 3-hydroxyanthranilic acids), mediators (e.g. 3,4-dimethoxybenzyl alcohol (synonyms: veratryl alcohol); 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (synonyms: ABTS); [4- [[4- (dimethylamino) phenyl]

[4- (ethylamino)-1- naphthyl] methylene] cyclohexa-2,5-dien-1-ylidene] dimethylammonium chloride (synonyms: basic Blue 11 or Victoria Blue R)) and accessory enzymes (e.g. cellobiose-quinone oxidoreductase and cellobiase dehydrogenase).^{38,39,43-45} Furthermore, fungal mycelia can also cause some non-enzymatic catalytic reactions, such as quinone redox cycling and fenton reactions, to produce hydroxyl radical. The hydroxyl radical attacks lignin monomer bonds by oxidative reactions to decompose lignin. Dashtban *et al.*³⁶ summarized the detailed lignin decomposition pathways with WRF as shown in Fig. 2. Based on the synergy of oxidative systems led by the mycelia, WRF can destroy lignin polymer structure spontaneously to monomers, then to the final products of CO₂ and H₂O in the natural environment.

Owing to their strong ability to degrade lignin, aqueous solutions of ligninolytic enzymes are used directly (*in vitro*) to pre-treat lignocellulosic biomass. Unfortunately, their performance is not as good as that of solid-state cultivation (SSC) of WRF.^{46,47} It is found that WRF have additional positive effects than enzyme mixtures on enhancing enzymatic sugar-releasing ability. These effects include (i) increasing hydrophilicity of lignin by the modification of lignin

Table 1. Features of the fungal ligninolytic enzymes.^a

Type of enzymes	Reaction	Cofactor	Metals or ions	Mediators	Subunits & molecular mass (kDa)
Phenol oxidase (Laccases)	$4 \text{ benzenediol} + \text{O}_2 = 4 \text{ enosemiquinone} + 2 \text{ H}_2\text{O}$	N/A	Ca^{2+} , Cd^{2+} , Cu^{2+} , H_2O_2 , imidazole, K^+ , K_2SO_4 , Mn^{2+} , Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$	Phenols, aniline, 3-HAA, NHA, syringaldehyde, hydroxybenzotriazole and ABTS	Monomeric (43-100), dimeric, trimeric & oligomeric
Peroxidases:					
1. Lignin peroxidase (LiP)	$1,2\text{-bis}(3,4\text{-dimethoxyphenyl})\text{propane-1,3-diol} + \text{H}_2\text{O}_2 = 3,4\text{-dimethoxybenzaldehyde} + 1\text{-(3,4-dimethoxyphenyl)ethane-1,2-diol} + \text{H}_2\text{O}$	Heme	Iron	Veratryl alcohol	Monomeric (37-50)
2. Manganese peroxidase (MnP)	$2\text{Mn(II)} + 2\text{H}^+ + \text{H}_2\text{O}_2 = 2\text{Mn(III)} + 2\text{H}_2\text{O}$	Heme	Ca^{2+} , Cd^{2+} , Mn^{2+} , Sm^{3+}	Organic acid as chelators, thiols, unsaturated fatty acids	Monomeric (32-62.5)
3. Versatile peroxidase (VP)	$\text{donor} + \text{H}_2\text{O}_2 = \text{oxidized donor} + 2\text{H}_2\text{O}$	Heme	Mn^{2+} , Ca^{2+} , Cu^{2+} , Iron	Veratryl alcohol, Compounds similar to LiP and MnP mediators	Monomeric

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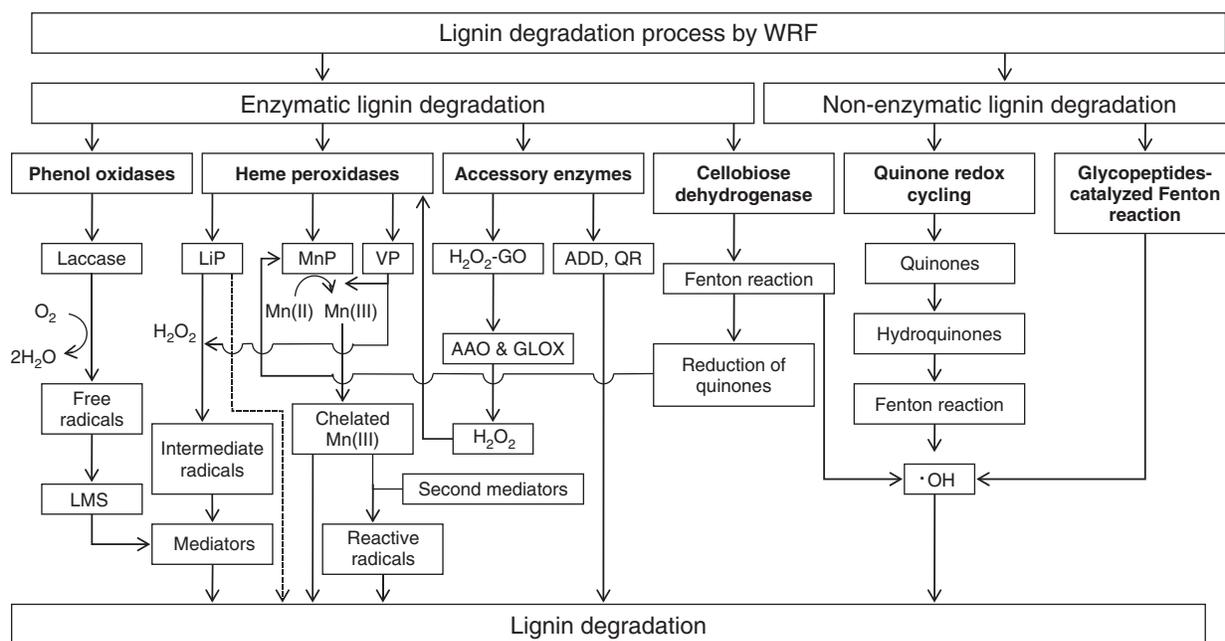


Figure 2. Schematic diagram of lignin degradation by white-rot fungi. Reproduced with the permission.³⁶ Copyright © 2010, e-Century Publishing Corporation. LMS: laccase-mediator system, H_2O_2 -GO: H_2O_2 -generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, ADD: aryl-alcohol dehydrogenases, QR: quinone reductases.

molecules; (ii) expanding the surface of forming pore structures on fiber walls via hyphal penetration; and (iii) reducing the concentration of lipophilic and hydrophilic inhibitors

caused by mycelia metabolism.^{6,47} Moreover, compared with commercial enzyme preparation, WRF pre-treatment is less expensive and much easier to conduct.⁴⁸

Table 2. Techniques of reported biopretreatment using white-rot fungi.

Species	Substrate(s)	Methods/ Moisture (%)	Temp. (°C)	Days	Additives	Ref.
<i>Ceriporia lacerate</i> N. Maek., Suhara & R. Kondo	Soft wood ^a	SSC/62 ^g	30	56	–	[8]
<i>Ceriporiopsis subvermispora</i> (Pilát) Gilb. & Ryvarden	Corn stover	SSC/75 ^g	28	18	–	[4]
<i>Coriolus versicolor</i> (L.) Quél.	Bamboo residues ^b	SSC/60 ^g	–	–	mixture I ⁱ	[10]
<i>Cyathus stercoreus</i> (Schwein.) De Toni	Corn stover	SSC/88 ^g	27	29	–	[15]
<i>E. taxodii</i>	Bamboo culms ^b	SSC/66 ^g	25	120	–	[9]
<i>E. taxodii</i>	China-fir ^c	SSC/75 ^g	25	90	–	[6]
<i>E. taxodii</i>	Chinese willow ^d	SSC/71.4 ^g	25	90	–	[6]
<i>Grifola frondosa</i> (Dicks.) Gray	Sawdust matrix ^e	SSC/65 ^g	28	88	–	[11]
<i>Ischnoderma benzoinum</i> (Wahlenb.) P. Karst.	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>T. versicolor</i>	Bamboo culms ^b	SSC/66 ^g	25	120	–	[9]
<i>T. versicolor</i>	China fir ^c	SSC/71.4 ^g	25	90	–	[14]
<i>T. versicolor</i>	Chinese willow ^d	SSC/75 ^g	25	90	–	[14]
<i>Ph. chrysosporium</i>	Corn stover	SSC/88 ^g	27	29	–	[15]
<i>Ph. chrysosporium</i>	Cotton stalks	SSC/75 ^g	39	14	–	[5, 12]
<i>Ph. chrysosporium</i>	Cotton stalks	SMC/5 ^h	39	14	–	[5, 57]
<i>Ph. chrysosporium</i>	Rice straw	SMC/98 ^h	29	15	mixture III ^k	[13]
<i>Ph. sordid</i>	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>Phlebia radiata</i> Fr.	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>Pl. ostreatus</i>	Rice Straw	SSC/60 ^g	25	60	–	[7]
<i>Pl. ostreatus</i>	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>Polyporus brumalis</i> (Pers.) Fr.	Soft wood ^a	SSC/62 ^g	30	56	–	[8]
<i>Polyporus versicolor</i> (L.) Fr.	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>Py. cinnabarinus</i>	Straw ^f	SSC/90 ^g	28	35	mixture II ^j	[16]
<i>Stereum hirsutum</i> (Willd.) Pers.	Soft wood ^a	SSC/62 ^g	30	56	–	[8]

^a *Pinus densiflora* Sieb. et Zucc., ^b *Phyllostachys pubescens* Mazel ex J.Houz., ^c *Cunninghamia lanceolata* (Lamb.) Hook., ^d *Salix babylonica* L., ^e contains wood chips (corn:bran = 9:1, w/w), ^f *Triticum aestivum* L., ^g Solid state cultivation, ^h Submerged cultivation, ⁱ Contains 0.1g wheat bran per gram of bamboo, ^j Contains KH₂PO₄, MgSO₄, CaCl₂, NH₄NO₃, ZnSO₄, MnSO₄, CoCl₂, and CuSO₄, ^k Contains yeast extract, KH₂PO₄, and CaCl₂.

In the following sections, status, trends and future prospects of bio pre-treatment with WRF are briefly reviewed. Subsequently, bio pre-treatment and its combination with other techniques, as well as their evaluation methods are introduced. Finally, further studies in bio pre-treatment for practical applications are proposed.

Status and trends of bio pre-treatment with WRF

Techniques to apply and evaluate fungi in lignocellulosic bio pre-treatment are introduced and discussed in this section.

Table 2 summarizes the representative techniques of WRF pre-treatment for different types of biomass, and Table 3 gives their corresponding evaluations for the component changes and hydrolysis yields of the pre-treated samples in the past decades.

Selection of strains for different types of biomass materials

Investigators have applied different WRF strains to evaluate their efficiency for bio pre-treatment.^{4,7-15,49,50} Thirteen types of lignocellulosic biomass including wheat straws, corn stover, cotton stalks and wood residuals were treated

Table 3. Effects of biopretreatment by white-rot fungi on lignocellulosic materials.

Species	Substrate(s)	Component changes of substrate (wt%)				Hydrolysis conditions				Yields of hydrolysis (wt%) (untreated/pretreated)			Ref.
		Lignin reduction	Cellulose recovery	Hemi-cellulose recovery	Solid recovery	Enzyme loading ^j	Solid to liquid ratio (w/v, %)	Time (h)	Glucose	Hemi-cellulose sugars	Reducing sugars		
<i>Ce. lacerata</i>	Soft wood ^a	13.10 ± 0.4	92 ± 0.5 ^g	-	90.50 ± 0.5	-	80/72 ^k	1.25	72	8.83/10.78	-/-	14.6/15.03	[8]
<i>Cer. sub-vernisporea</i>	Corn stover	29.54 ± 0.33	95.39 ± 0.24	81.73 ± 0.42	85.03 ± 0.42	6.40	10/-	2.5 ^m	72	21/57.67	13/38.21	-/-	[4]
<i>C. versicolor</i>	Bamboo residues ^b	48.60	98.4	45.6	-	30.38	20/-	2	48	-/-	-/-	9.55/22.32	[10]
<i>Cy. stercoreus</i>	Corn stover	-	-	-	91.70 ± 1.1	-	60/-	1	136	16.1/35.7	-/-	-/-	[15]
<i>E. taxodii</i>	Bamboo culms ^b	29.14	98.32	71.54	73.69	14.8 ⁱ	20/-	2.5	120	-/-	-/-	3.8/37 ^o	[9]
<i>E. taxodii</i>	China-fir ^c	38.80 ± 1.3	89.4 ± 2.7	75.1 ± 2.5	76.60 ± 0.8	3.7	20/-	2.5	120	-/-	-/-	2/15	[6]
<i>E. taxodii</i>	Chinese willow ^d	41.70 ± 4.9	79.1 ± 0.3	55.2 ± 0.6	69.30 ± 0.7	2.0	20/-	2.5	120	-/-	-/-	5/26	[6]
<i>Gr. frondosa</i>	Sawdust matrix ^e	21.05	80.5	-	-	1.08	-/-	3.3	48	5/88	-/-	-/-	[11]
<i>I. benzoinum</i>	Straw ^f	-	-	-	79.8	-	10/-	5	72	5.1 ± 0.9/23.9	12.2 ± 1.3/35.9	-/-	[16]
<i>T. versicolor</i>	Bamboo culms ^b	12	90.65	81.11	59.98	2.61 ⁱ	20/-	2.5	120	-/-	-/-	3.8/23.8 ^o	[9]
<i>T. versicolor</i>	China fir ^c	12.80	-	-	-	-	20/-	2.5	120	-/-	-/-	3/11	[14]
<i>T. versicolor</i>	Chinese willow ^d	15.70	-	-	-	-	20/-	2.5	120	-/-	-/-	8/37.5	[14]
<i>Ph. chrysosporium</i>	Corn stover	-	-	-	80 ± 1.8	-	60/-	1 ⁿ	136	16.1/12	-/-	-/-	[15]
<i>Ph. chrysosporium</i>	Cotton stalks	35.53	56.74	39.95	59.18	0.82	40/-	5	72	18 ± 1/3 ± 0.5	33 ± 2/24 ± 3	-/-	[5, 12]
<i>Ph. chrysosporium</i>	Cotton stalks	19.38	72.25	64.05	74.87	0.70	40/-	5	72	18 ± 1/11 ± 0.5	33 ± 2/26 ± 3	-/-	[5, 57]
<i>Ph. chrysosporium</i>	Rice straw	21	81.2	-	-	3.13	60/30	-	120	20/64.9	-/-	-/-	[13]
<i>Ph. sordida</i>	Straw ^f	-	-	-	57.7	-	10/-	5	72	5.1 ± 0.9/9.8	12.2 ± 1.3/20.3	-/-	[16]
<i>Ph. radiata</i>	Straw ^f	-	-	-	54.8	-	10/-	5	72	5.1 ± 0.9/13.2	12.2 ± 1.3/17.5	-/-	[16]
<i>Pl. ostreatus</i>	Rice Straw	41	83	52	75	2.41	10/ ^j	1	48	12.9/31.7	-/-	13.6/32.7	[7]
<i>Pl. ostreatus</i>	Straw ^f	-	-	-	76.4	-	10/-	5	72	5.1 ± 0.9/18	12.2 ± 1.3/32.6	-/-	[16]
<i>Po. brunnalis</i>	Soft wood ^a	11.60 ± 0.3	89.4 ± 0.3 ^g	-	90.10 ± 0.4	-	80/72 ^k	-	72	8.83/9.68	-/-	14.6/14.91	[8]
<i>Po. versicolor</i>	Straw ^f	-	-	-	69.2	-	10/-	5	72	5.1 ± 0.9/8.5	12.2 ± 1.3/14.8	-/-	[16]
<i>Py. cinibarinus</i>	Straw ^f	-	-	-	64.4	-	10/-	5	72	5.1 ± 0.9/26.4	12.2 ± 1.3/32	-/-	[16]
<i>S. hirsutum</i>	Soft wood ^a	14.5 ± 0.4	92.2 ± 0.3 ^g	-	89.30 ± 0.7	-	80/72 ^k	1.25	72	8.83/13.56	-/-	14.6/21.01	[8]

^a *P. densiflora*, ^b *Phy. pubescens*, ^c *Cun. lanceolata*, ^d *Sa. babingtonia*, ^e contains wood chips (corn:bran=9:1, w/w), ^f *Tr. aestivum*, ^g Holocellulose (combination of hemicellulose and cellulose), ^h Abbreviation of lignin decomposing selectivity, defined as the ratio of lignin degradation over cellulose loss, ⁱ In 4 weeks, ^j Cellulase loading (FPU/g substrate) / Cellulose Loading (IU/g substrate), ^k unit of Cellulase loading is EGU/g substrate, ^l 10mg cellulase powder/g substrate, ^m w/w, ⁿ Ratio of cellulose amount in substrate to liquid, ^o Fermentable sugars, ^p not available.

with *Ph. chrysosporium*, *Echinodontium taxodii* (Lentz & H.H. McKay) H.L. Gross, *T. versicolor*, and 13 other different species of WRF (Table 2). These experiments were carried out in two ways: (i) by treating different types of biomass with a specific WRF strain; and (ii) by treating a specific type of biomass with different WRF strains. These works revealed large variations in digestion of lignin and carbohydrates in lignocellulosic biomass after WRF pre-treatment (Table 3).^{49,50}

Optimization in cultivation of WRF for bio pre-treatment

Solid-state cultivation (SSC) as main fungal pre-treatment pattern

Both SSC and submerged cultivation (SMC) techniques are adopted in bio pre-treatment, but SSC performed better because it has five unique biotechnological advantages: (i) **easy attachment of the enzymes secreted along mycelia to substrates**, the hyphae of WRF attach biomass stably under natural conditions;⁴⁶ (ii) **rapid diffusion of oxygen**, it is favorable for WRF growth and oxidic depolymerization of lignin with higher oxygen diffusion rate and concentration;³⁸⁻⁴⁰ (iii) **adaptable to either continuous or batch processes**, SSC bioreactors are not as complex as those used in submerged fermentation; (iv) **lower cost**, it requires less stirring and aeration in operation as well as less heating steam in sterilizing feedstocks; and (v) **smaller reaction volumes relative to pre-treated biomass**, it is due to the absence of excess water in reactors.⁵¹

Optimum temperature and moisture in solid-state pre-treatment

In a typical WRF pre-treatment procedure, incubated temperature ranges from 25 to 35°C, which are mild and optimum for WRF growth, but far below the temperature applied in chemical and physiochemical pre-treatments.

Fungal primary growth and secondary metabolism require appropriate moisture in biomass.^{12,52} In previous work,⁵³ 60–90 wt% moisture usually served as the inchoative humidity. It was revealed that insufficient humidity in biomass may even cause fungal death, whereas excess moisture inhibits fungal growth, especially in the deep layers with little air and mycelia.⁵³ Heat removal and respiration of mycelia may lead to an uneven distribution of water in the system as a result of water evaporation.⁵¹ Adding some additional

water and inverting substrates during pre-treatment process could resolve the problems but it is rarely reported in literatures.

Effect of additives

For most wood and soils, the most likely limiting nutrient for fungal growth is nitrogen.^{7,54} Therefore, lignin degradation generally occurs under conditions of nutrient starvation and usually with high C/N ratios.⁵⁵ However, Shi *et al.*¹² found that addition of salts (4‰ NaNO₃, 1% KCl, 1.4% MgSO₄·7H₂O, 0.14‰ FeSO₄·7H₂O, 4% KH₂PO₄, 0.02‰ thiamine and 1.8% MnSO₄·H₂O, *w/w*) to the cotton stalks pre-treated by *Ph. chrysosporium*, only resulted in a slightly higher solid recovery of substrates and available carbohydrates. This may be attributed to the type of strains or the substrate material. However, effects of additives are not reported in most of the previous works (Table 2).

Effect of oxygen

WRF growth, enzyme production, and lignin oxidation depend on sufficient supply of oxygen.⁵ Concentrated oxygen can reduce treatment time with some WRF, such as *Ph. chrysosporium*, *Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvardeen and *Pycnoporus cinnabarinus* (Jacq.) P. Karst. There is also a positive correlation between oxygen concentration and delignification rate. Most studies were conducted in an atmosphere of air due to its lower cost and convenient applications even in a large-scale.^{16,56,57}

Evaluation of pre-treated biomass

Pre-treated biomass is often characterized by many physical and chemical techniques, as well as enzymatic hydrolysis tests to determine: (i) lignin reduction and biomass structure alteration; (ii) selectivity and cellulose recovery; and (iii) enzymatic hydrolysis yield and reaction rate.

Lignin reduction and biomass structure alteration

The enhancement of enzymatic hydrolysis after WRF pre-treatment is usually attributed to (i) biodegradation of lignin component that hinders the penetration and irreversible adsorption of cellulases, and (ii) biomodification of the physical properties of substrates.

Structural and component changes of pre-treated biomass are commonly characterized via the following techniques:

(i) the analyses of cellulose, hemicelluloses and lignin according to US National Renewable Energy Laboratory (NREL) standard biomass analytical procedures and some developed gravimetric methods;^{4,5,6,12,13} (ii) the variations in chemical bonds of biopolymers by Fourier transform-infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA), and differential thermal analysis (DTA);^{9,58} (iii) The examination of surface structures by scanning electron microscope (SEM);^{7,28} (iv) the reduction of degree of crystallization by X-ray diffraction (XRD) and FTIR;^{6,8,13} and (v) the measurement of pore size distribution by nitrogen gas adsorption.⁸

Cellulose recovery and lignin decomposing selectivity
Although lignin reduction is a critical factor for evaluating the effectiveness of bio pre-treatment, total availability of glucose in the hydrolysis stage is definitively determined by solid and cellulose recovery after pre-treatment.¹² Lignin decomposing selectivity is defined as the ratio of lignin degradation to inevitable cellulose consumption after pre-treatment. It was employed to recognize the *fitness* of a certain WRF strain for processing.¹⁵ WRF bio pre-treatment with a higher selectivity for lignin degradation would lead to a more economic pre-treatment process. As summarized in Table 3, the cellulose recovery rate and selectivity of lignin decomposition are 56.74–98.4% and 0.7–30.38, respectively after bio pre-treatment with different species of WRF for various types of biomass under different conditions.^{5–12}

Enzymatic hydrolysis yield and reaction rate

Though the various methods mentioned play an important role in pre-treatment evaluation, subsequent hydrolysis is necessary to estimate the possible usefulness of fungal bio pre-treatment for lignocelluloses.²⁶ In the published works related to fungal pre-treatment (Table 2), enzymatic hydrolysis was necessarily performed to confirm if the fungal treatment was efficient on a certain series of biomass (Table 3). How fast (reaction rate) and how far (conversion yield) hydrolysis reaction can proceed reveals the accessibility of pre-treated lignocelluloses to an enzyme system.² Most of the fungal pre-treatment gave positive effects on the subsequent enzymatic hydrolysis. The glucose and reducing sugar yields of biomass pre-treated by WRF were 1.22 to 17.6 and 1.03 to 7.5 times as those of untreated materials,

respectively.^{4–16} Enzymatic hydrolysis, however, are affected by other three factors: (i) the origin of cellulases and cellobioases; (ii) enzyme activity and biomass loading; and (iii) reaction temperature and time. It is hard to compare all of the experimental data since different methods are used for quantitative sugar analyses without restriction to a unique hydrolytic condition. Therefore, it is needed to authenticate a standard analytical approach for fungal pre-treatment evaluation. Procedures (NREL/TP-510-42629) published by NREL are considered as one of the possible analytical standards.⁵⁹

Techniques for determining the inhibitors (e.g. phenolic compounds) in hydrolyzed solutions were seldom involved in some bio pre-treatment works.^{6,9,18} But, some researchers measured the density of fermentative microbes and their productivity to determine the fermentability of solutions hydrolyzed from bio pre-treated biomass.^{13,60} It is a simple way to evaluate the effects of these inhibitors produced from the bio pre-treatment stage.

Combination of fungal method with other techniques

Compared with the well-developed physical, chemical and physicochemical approaches, fungal delignification is time-consuming and has not yet used in practice.¹ Moreover, its subsequent hydrolysis yields and rates are relatively low.³ Therefore, novel techniques have been explored to integrate fungal delignification with physical, chemical, and physicochemical methods to (i) reduce the severity and time in the combined pre-treatment; and (ii) enhance the hydrolysis yields and rates. Lower severity does not only mean lower chemical agent and energy consumption, but also leads to less biomass degradation and consequently lower inhibitor levels.¹⁵ These integrated techniques include bio-alkali, bio-acid, bio-AFEX, bio-organosolve, ultrasonic-bio, and H₂O₂-bio processes.

Bio-alkali process

Hatakka¹⁶ applied an alkali treatment (2% (*w/v*) sodium hydroxide) for the wheat straw at 115 °C for 10 min. The wheat straw had been previously pre-treated separately with three strains of WRF (*Ischnoderma benzoinum* 108, *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* 115) for 14 days. Results showed that combination of fungal method with alkali treatment did not prove to be more effective than either of the treatments. A possible explanation is that

the strong alkali treatment most likely masked the effect of fungal treatment. Treatment with mild alkali might be an option for further studies.

Bio-acid process

Pre-treatment of water-hyacinth with *Echinodontium taxodii* combined with mild acid hydrolysis was also evaluated.⁶¹ Reducing sugar yield increased by 1.1–2.1 times as compared with that from a single-acid-treatment procedure. The experiments suggested that the promising combination improved enzymatic hydrolysis and subsequent ethanol production. Kuhar *et al.*⁶² employed four WRF strains (*Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, and two unidentified fungal isolates RCK-1 and RCK-3) to treat wheat straw and algarroba (*Prosopis juliflora* (Sw.) DC) under SSC conditions for 10 days. Results indicated that structural polysaccharides were decayed by both fungi and acid, fermentable sugars were increased, while inhibitors were reduced. In this way, ethanol yield and volumetric productivity were eventually increased.

Bio-AFEX process and bio-steam explosion

Balan *et al.*¹⁹ treated rice straw with *Pl. ostreatus* and AFEX. It gave significant higher glucan and xylan conversions under less-severe conditions than those with direct AFEX. Sawada *et al.*⁶³ found that consecutive treatments by *Ph. chrysosporium* and steam explosion were more effective for the conversion of wood-meal into sugars. The highest efficiency was achieved after 28 days of fungal treatment coupled with steam explosion at 215 °C for 6.5 min. Their positive results were mainly due to the pre-reduction of structural strength and the growth of pores on biomass surfaces caused by WRF.

Bio-organosolve process

In bio-organosolve process, *Ceriporiopsis subvermispota* (Pilát) Gilb. & Ryvarden, *Dichomitus squalens* (P. Karst) D. Reid, *Pl. ostreatus*, and *C. versicolor* were used to pre-treat beech wood chips for 2–8 weeks. Considerable efficient delignification and hydrolysis of hemicellulose were achieved by using *C. subvermispota* followed by ethanolysis at 140–200 °C for 2 h. In subsequent SSF, ethanol yield obtained was 1.6 times higher than that without fungal treatment. In addition, fungal pre-treatment saved 15% of electricity needed for ethanolysis.^{60,64} On the other hand, the remained

lignin can be easily removed in a relative shorter time after fungal pre-treatment, and less energy is consumed in the subsequent organosolve pulping process.⁶⁵

Ultrasonic-bio and H₂O₂-bio processes

A two-step method consisting of mild ultrasonic or H₂O₂ treatment step followed by treatment with *Pl. ostreatus* was proposed for the enzymatic hydrolysis of rice hulls.¹⁸ These combinations led to a significant increase in lignin degradation as compared with that from any single pre-treatment. At the same time, yields of total soluble sugar and glucose were 3.3–5.8 and 4.2–6.5 times higher than those of sole fungal pre-treatment, respectively. In ultrasonic-bio or H₂O₂-bio process, the efficient breakdown of lignin and cellulose polymers arises from the synergy of enzymatic fungal and physicochemical effects on the biomass.

Future prospects and recommended studies

Despite recent advances in WRF bio pre-treatment, several major challenges (e.g. low delignification rate and lose of carbohydrates) limit its uses. Perspective and recommended studies in basic and applied research are discussed to overcome the drawbacks and challenges in WRF bio pre-treatment for its practical applications.

Basic research

Screening and engineering of fungi

Four directions to select fine strains for pre-treatment via screening and breeding techniques are proposed below:

1. There are more than 1500 different species of WRF, but, less than 20 species are applied in bio pre-treatment (Tables 2 and 3). Unknown WRF strains with high delignification capability could be found in nature and in the culture collections all over the world. Furthermore, the effects of delignification differ in various fungi-substrate combinations.^{34,65,66} Therefore, further studies are needed to select fast-growing WRF and other ligninolytic microbe (e.g., bacteria, actinomycetes, and brown-rot fungi) species that rapidly colonize on specific types of biomass (e.g. sawdust, corn straw, grass, and bagasse) with efficient preference for degrading lignin. A relatively easy and fast method using Simons' stain can be

introduced from biomechanical pulping to evaluate the efficacy of fungal pre-treatment on biomass.⁶⁵ It can help to estimate the change in cellulose crystallinity, which is an important factor in enzymatic hydrolysis.⁶⁷

2. Strains that have a fast-growing rate and high selectivity for lignin removal are essential to reduce pre-treatment time and cost in scaled-up processing.⁶⁸ Through mutation breeding and cross-breeding technologies, genetically engineered WRF strains were proposed that consume hemicelluloses only as an energy source for growth (i.e. cellulase-less) and subsequent lignin degradation. These proposals were suggested many years ago, but never succeeded. With the development of fungal genome research, they would be realized in the near future. Recombinant ligninolytic enzymes, however, have been produced in large quantities from engineered *Pichia pastoris* (Guillerm.) Phaff. Nowadays, they are successfully incorporated into the delignification and bleaching processes.^{69,70}
3. Higher temperatures can accelerate delignification reaction rate. But the optimal temperature range of fungal primary growth is relatively low (25–35 °C). Selection of extremophilic fungi (thermophiles), or genetic engineering of fungi that can survive and degrade lignin at higher temperatures could reduce processing time. Additionally, when employing a scale-up reactor system, fungal growth produces a significant amount of heat that should be removed. Using extremophilic fungi can reduce the cost in controlling temperature and ventilation.^{65,71}
4. Selection of the fungi that produce fewer aerial hyphae on biomass surfaces will reduce the requirement for ventilation.⁶⁵

Optimization of cultivation

1. Optimization of symbiotic fungal pre-treatment with two or more fungi applied simultaneously or in subsequent phases.¹⁵ Physical characteristics and compositions of biomass vary at different processing stages. A single strain of fungus is not always effective because of the limited integration of enzymes. The application of two or more fungi can complement their different enzyme systems. It can be accomplished either by adding all of the strains simultaneously at the beginning of the process or stepwise adding them in different periods.
2. Regulation of temperature at two different stages (lower temperature for growing and higher temperature for delignification) may accelerate the pre-treatment efficiency by employing of the mesophilic fungi.
3. It is perfect to inoculate substrates by spores. However, for most fungi that do not produce spores under cultivating conditions, using liquid-mycelia inocula is a good choice. Compared with solid-mycelia inocula, liquid-mycelia ones can help fungi colonize on biomass more easily and rapidly. Addition of corn steep liquor⁶⁵ or tree barks in liquid-mycelia inoculation can be facilitated in a large-scale.

Integrated methods

Combination of WRF with other pre-treatment methods can reduce the severity of the whole process. Furthermore, integrated strategies may also overcome some problems, such as slow colonization on biomass (e.g. sawdust), hemicellulose loss, and low enzymatic hydrolysis rate and yield caused by hydrolysis-inhibitory molecules from degraded lignin polymer. The integrated methods are introduced and proposed:

1. Croan³⁵ pointed that WRF do not always readily colonize on conifer wood (e.g. *Pinus taeda* L., *Pinus ponderosa* Dougl. ex Laws.) due to the nature of resins produced by the plants. Therefore, for softwood, a 'sandwich' method was designed to improve hydrolysis yield and reduce pre-treatment time. In Fig. 3, organic solvents are applied first, then fungi are used for pre-treatment. After inhibitors (e.g. resins) are removed by the organic solvents, fungi can be colonized more easily on the wood. After delignification by the fungi, small molecular materials are dissolved and taken away by the next organic solvent extraction step.⁷² The solvents are recycled by distillation.
2. Hemicellulose is easily hydrolyzed and consumed by fungi. A combined approach is proposed in Fig. 4. Hemicellulose is hydrolyzed initially by dilute acid under mild conditions before WRF treatment. The removal of most of hemicellulose leads to fewer carbohydrates to be consumed subsequently by fungi. In addition, WRF growth and delignification could possibly be promoted by the structure disruption of biomass at the chemical pre-treatment step.¹⁸ Additionally, since hemicellulose is selectively soluble in hydrothermal water, the integration

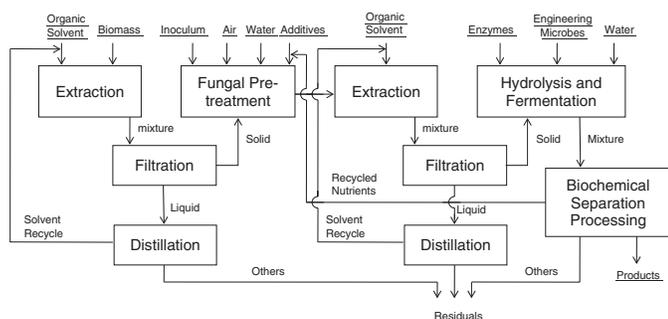


Figure 3. Block flow diagram of (organic solvents-bio-organic solvents) technique.

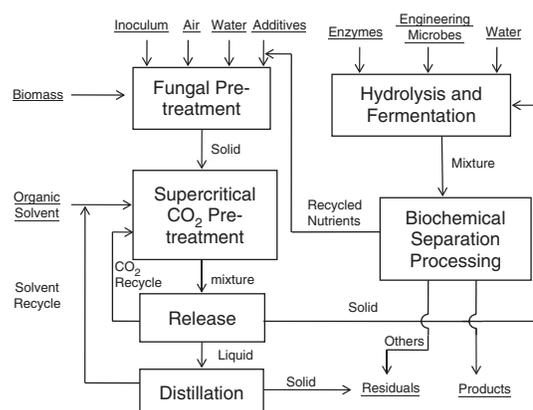


Figure 5. Block flow diagram of (bio-organic solvents-supercritical CO₂) technique.

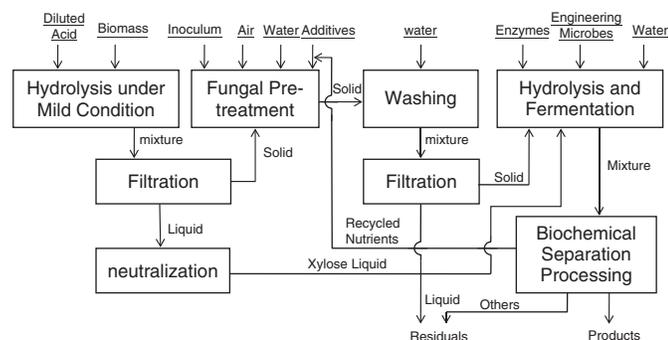


Figure 4. Block flow diagram of (diluted acid-bio) technique.

of hydrothermal and fungal methods has a great potential application for pre-treatment.^{73,74}

- Fungal pre-treatment can reduce the viscosity of materials.¹⁵ This result will reduce energy requirement (e.g. milling and stirring) or the severity of physical pre-treatment (e.g. steam explosion and supercritical CO₂ (ScCO₂) explosion). Integrating organic solvents with ScCO₂ explosion to process WRF-treated lignocellulosic materials could be a feasible way (Fig. 5). This combined treatment enhances enzymatic hydrolysis through the following principles: Small hydrolysis-inhibitory molecules generated by lignin decomposition will be rapidly dissolved in organic solvents and removed by ScCO₂; Moreover, rapid release of ScCO₂ will further destroy the structure of biomass previously degraded by fungi.
- Shi *et al.*⁷⁵ inferred that after fungal pre-treatment, enzymatic hydrolysis yield could obviously decrease by the presence of fungal mycelia that hinder catalytic binding of cellulolytic enzymes to the substrate. We propose a method in Fig. 6 to overcome this problem: The bio pre-treated

biomass sample with mycelia is firstly put into an organic electrolyte solution (OES) composed of a small fraction of ionic liquid (e.g. 1-allyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium chloride) and polar organic solvent [e.g., dimethylsulfoxide and acetone] since biomass is soluble in the OES.⁷⁶ After the biomass with mycelia dissolves in the OES, it is regenerated by precipitation by adding anti-solvent (e.g. water and ethanol). In this way, the structures of fungal mycelia and microcrystalline cellulose are destroyed and these mycelia may also be separated. So, hydrolysis yield and reaction rate will be increased.

Applied research

Many engineering challenges are involved in redesigning laboratory procedures of fungal pre-treatment to be practical on a large-scale. These include several key areas: (i) reactor design; (ii) decontamination of biomass materials; (iii) production of inocula; (iv) control of temperature and ventilation; (v) cost evaluation of industrial process; and (vi) cost decrease by integrating different processes.

Hereby, achievements of these challenges are briefly discussed. Conceptions are also proposed that may be helpful for the commercial applications of fungal pre-treatment.

Reactor design

For large-scale applications of aerobic fungal pre-treatment, a constant-humidity incubator that can maintain constant humidity and provide good mixing, controlled temperature, and proper airflow is necessary.¹⁵ Since most reported

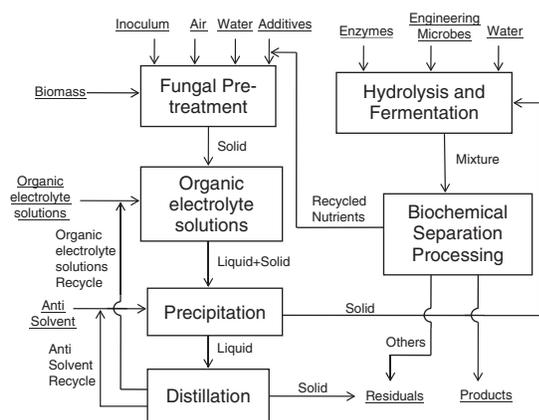


Figure 6. Block flow diagram of (bio-organic electrolyte solution) technique.

works were at flask- or fomentor-level, some reactor systems need to be developed for collecting the kinetic data necessary for pilot-scale process and industrial implementations. Scott *et al.*⁷¹ have developed two scaling-up types of reactor systems for the study of WRF pre-treatment of wood: (i) cylindrical reactors containing 6–140 kg of dry chips for collecting the engineering data, and (ii) chip piles containing 40–380 kg of dry chips for analogizing industrial implementation. Based on engineering and economic analyses, the developed chip-pile was technologically feasible and economically attractive.⁷¹ It is a good demonstration of reactor system for fungal pre-treatment. Alternatively, the ready-to-use fermentation tunnel techniques, which are commercially adopted in mushroom composting industry, are suitable, available and easily applied for industrial fungal pre-treatment of biomass.⁷⁷

Decontamination of biomass materials

WRF are not aggressive enough to compete with indigenous micro-organisms in unsterilized wood chips.⁷¹ Decontamination provides several challenges as transferring laboratory scale to larger reactors. The energy requirements and time costs for autoclaving and chemical treatment do not make them practical sterilization methods on a large scale.⁷¹ Although steaming is a predominant method, the optimized conditions for continuous process, such as steam temperature, dosage, retention time, and the control of biomass temperature for inoculation during active cooling should be interested. Encouragingly, Scott *et al.*⁷¹ discovered

that sterilization can be accomplished by steaming wood chips for a very short time because only the surfaces of wood chips need to be decontaminated. It gives us a new insight to develop a low-cost and high-efficiency approach to sterilize biomass by steaming.

Production of inocula

The quality of inocula has a major impact on all microbial processes. The inocula for a large-scale bio pre-treatment should be stable, pure, vigorous, specific (adapted to grow on desired biomass) and high-quality ones for the culture of certain WRF or their mixtures.⁷⁸ For the commercial production of inocula, liquid fermentation is the first choice for its higher efficiency in the production of fungal mycelia than SSF. Moreover, it is easier to be processed to pure mycelia powders or concentrated to liquid inocula that are easy to be stored and transported for pre-treatment mills.⁷⁸

Control of temperature and ventilation

In large reactors or piles, a significant amount of heat produced by respiration of fungi will cause biomass overheating that inhibits the growth of fungi and delignification. As a result, the heat must be removed efficiently according to its characteristic generation pattern. Forced ventilation with filtered and humidified air might be one appropriate method to remove the metabolic heat from a pile.⁷¹ At the same time, the ventilation process can benefit the gas exchange between colonized biomass and environment. To apply this technique in different reactor systems, ventilation capacity should be further optimized based on the loading, density, and granularity of biomass, as well as the time variations and species of inoculated fungi.⁷¹ Additionally, a combination of pressure drop and forced ventilation techniques can be more efficient for heat removal.⁷¹ It has a potential use in tunnel reactors.

Cost evaluation of industrial process

In industrial applications, the cost of bio pre-treatment process must be evaluated appropriately according to the engineering data at a specific production scale.⁶⁵ The economic benefits with fungal pre-treatment result from the following advantages: energy savings, chemical agent savings, lower equipment investment, higher throughput, and environmental benefits (reduction of gas, water and solid wastes). Akhtar *et al.*⁶⁵ indicated that the economics

of fungal pre-treatment for pulping were attractive. By the analysis, people will be convinced of a bright future of industrial-scale applications of fungal pre-treatment.

Cost decrease by integrating different processes

Fungal pre-treatment can be integrated with others and used according to the following patterns, which are proposed to be more economical.

1. Integrating decontamination with inoculation processes. Operation based on a continuous basis and employing existing equipment is one of the trends.⁷¹ One conceptual operation is the continuous transportation formulated by Scott *et al.*^{65,71} Two conveyors and a surge bin are included in the system. The chipped and screened biomass is briefly steamed on one working screw conveyor and then transferred into the surge bin to release the steam. While the sterilized biomass chips fall on another conveyor, and are cooled by forced air. As arriving at the terminal of the conveyor, the chips are inoculated with liquid inocula. This continuous transportation system fits well to woodyard operation.⁶⁵ It can be easily realized and has a high processing capability.
2. It is feasible to apply fungal pre-treatment concurrently with on-farm wet storage or long-distance shipping transport.⁴ Rather than controllable incubators, it is complex to control changeable temperature and humidity caused by day-night and seasonal effects in the storehouses or containers. Thus, the combination of film mulching, water re-circulating, and ventilation techniques, and modeling of kinetics of fungal growth and delignification during treatment processing should be developed.
3. By taking the benefits from biocompatibility, fungal pre-treatment can be coupled with SSF and SSC to produce biofuels, crude enzymes, fungal oils, organic acids and biogas.^{50,79-83} The combined process has the advantages of fewer substrate inhibitors produced and less biomass consumed, as well as lower energy and investment required.⁸⁴

Compared with conventional methods, fungal bio pre-treatment of biomass is cost-efficient and environmentally friendly. Based on previous results and suggestions, some challenges (e.g. slow process of delignification and loss of carbohydrates) for biochemical process research and

commercial applications of WRF pre-treatment still need to be examined. However, further development and incorporation of multiple key techniques will make fungal pre-treatment a bright future in large-scale applications. By integrating WRF with other types of treated methods, efficient pre-treatment of lignocellulosic biomass can be achieved for subsequent hydrolysis for biorefinery industry.

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