Chiang Mai J. Sci. 2018; 45(2) : 645-652
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

# Cooperative Decomposition of Rice Straw by Co-cultivation of Cellulolytic Fungi

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Received: 2 August 2016
Accepted: 9 November 2016

## ABSTRACT

Cooperative action of cellulolytic microbes provides the basis for lignocellulose decomposition in nature and is considered as a potent approach for developing efficient microbial agents for biotechnological applications, such as degradation of agricultural wastes. In this study, the co-cultivated cellulolytic fungal cultures on effective decomposition of rice straw was explored. Single-strain culture of cellulolytic fungi led to 2-14% weight loss of rice straw after incubation at 30°C for 14 d under static conditions in a simplified medium containing 0.1% (w/v) urea. Co-cultivation of *Penicillium oxalicum* BCC4504 and *Cheatomium globusum* BCC5776; *P. oxalicum* BCC4504 and *Trichoderma reesei* BCC62305; and *Aspergillus niger* BCC5772 and *C. globusum* BCC5776 could increase rice straw decomposition as high as 20%, compared to a single-strain culture. This work shows potential of the fungal co-cultures as active biological agents in order to diminish rice straw for more sustainable farming.

**Keywords:** fungal cultivation, fungal interaction, lignocellulosic biomass, cellulase

## 1. INTRODUCTION

Rice is among the world's most important staple food with the annual production of 730 million tons, and most of its plantation is in Asia [1]. Thailand is recognized as one of the top world rice producers during the past decades. In 2014, Thai farmers grew rice on 12 million hectares of land, producing 40 million tons of rice and massive amount of straw waste. Most of the rice straw is commonly eliminated by open-air burning, which decrease soil fertility and causes subsequent environmental problems and pollution [2]. Decomposition of rice straw by potential microorganisms is

considered as a potent alternative method for paddy field preparation after harvesting with minimal environmental problems. This results in incorporation of degraded straw into soil which would help to enrich soil organic matter and reducing fertilizer utilization in the long term [3].

Rice straw is basically a lignocellulosic biomass which is a complex structure comprising highly organized cellulose microfibers linked to the network of hemicelluloses and shielded by lignin. This structure makes it recalcitrant to external physical, chemical, and biological attacks. The bulk of straw in the field is generally decomposed slowly by indigenous microbes. Attempts to increase decomposition rate of straw have been done by augmenting cellulolytic microorganisms into the field. Fungal genera such as *Aspergillus*, *Chaetomium*, and *Trichoderma* have been shown to enhance straw decomposition in the field [4-5]. However, the decomposition efficiency in open environment is limited probably due to unsuitable environmental conditions and incompatibility of the supplemented cellulolytic microbes with indigenous microorganisms [6].

Cooperative action of microorganisms producing a wide array of cellulolytic, hemicellulolytic, and lignolytic enzymes provides the basis for efficient lignocellulose decomposition of the complex structure of plant biomass in nature. A highly active enriched microbial consortia comprising a variety of cellulolytic bacteria and fungi has been reported with high capability of degradation of rice straw in paddy fields [7]. However, the use of enriched mixed culture is limited by long-term stability of the consortia which result in difficulties for up-scaling production of the microbial agents. Direct application of exogenous cellulase in soil to promote straw decomposition has also been reported elsewhere [8]; however, its practical use is limited due to the additive cost of enzyme production which makes this method not economically attractive.

Co-cultivation of biomass-degrading microbes has been reported as a potent approach to increase decomposition efficiency of various agricultural residues. These included coffee pulp waste, forest waste, and wheat straw [7-10]. In order to obtain the best co-cultivation of rice straw-degrading fungi, strains of cellulolytic and hemicellulolytic fungi (*Aspergillus* sp., *Chaetomium* sp., *Penicillium* sp., and *Trichoderma* sp.) isolated from active lignocellulose-degrading environment have been systematically studied for their cooperative interactions on rice straw decomposition. The works provide a basis for improving the efficiency of microbial agents used in straw decomposition.

## 2. MATERIALS AND METHODS

### 2.1 Materials and Microbial Strains

Rice straw was collected from a local field in Pathum Thani province, Thailand. The biomass was physically processed using a cutting mill (Retsch SM 2000, Hann, Germany) in the size range of 3 cm and used without any further pretreatment. Six selected fungi, *A. niger* BCC5772, *A. acculeatus* BCC17849, *C. globusum* BCC5776, *P. oxalicum* BCC4504, *T. asperellum* BCC62292, *T. reesei* BCC62305, were obtained from the BIOTEC Culture Collection, (BCC), (National Center for Genetic Engineering and Biotechnology, Thailand BIOTEC). These fungal strains were isolated from active lignocellulose-degrading environments and maintained on PDA (potato dextrose agar). A commercial product (Microbial Activator "Super LDD.2"), provided by the Land Development Department, Ministry of Agriculture and Cooperatives of Thailand (http://

www.ldd.go.th/ldd_en/en-US/microbial-activator-super-ldd-2/) was used as a benchmark for comparison of rice straw decomposition efficiency.

**2.2 Fungal Strain Cultivation and Enzyme Activity Assay**

The fungi were grown aerobically in submerged liquid culture in 250 ml conical flasks. The culture was prepared from the fungi grown on PDA by using a number 2 cork borer to obtain 4 agar pieces covered with profuse mycelium that was then placed into 50 ml of wheat brand and soy bean (WS) medium [2% (w/v) wheat bran and 3% (w/v) soy bean] and incubated at 25°C for 5 d with rotary shaking at 200 rpm. The mycelia were separated from the culture supernatant by filtration with sterile gauze and the supernatant was used as a source of the crude enzymes for activity assay. The cellulase (FPase and CMCase) and hemicellulase (endo-xylanase) activities were determined based on the amount of reducing sugar released by 3,5-dinitrosalicylic acid (DNS) method [11]. Reaction mixture with 1 ml total volume contained an appropriate dilution of enzyme in 100 mM sodium acetate buffer, pH 5.0 with 0.01 mg Whatman filter paper, 1% (w/v) carboxymethyl cellulose sodium salt, or beechwood xylan as a substrate and incubated at 50°C for 10 min. The amount of reducing sugars was determined the absorbance at 540 nm using a VICTOR3 V microplate reader (Perkin Elmer, Waltham, MA, USA) compared to the standard curve of glucose, and xylose, respectively. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars from a substrate in 1 min under the assay condition. The β-glucosidase and β-xylosidase activities were determined spectrophotometrically at 540 nm for the detection p-nitrophenolate product released from hydrolysis of p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-xylopyranoside substrates, respectively, after incubation at 50°C for 10 min in 100 mM sodium acetate buffer, pH 5.0. Protein concentration was quantified by Bradford assay [12]. Bovine albumin was used as a standard. All analysis were performed in triplicates.

**2.3 Evaluation of Decomposition Performance of Rice Straw by Fungal Single-strain Culture**

The fungal cultures were prepared by inoculating 125 mg (wet weight) of fungal mycelia grown in PDA for 5 d into 50 ml of sterile rice straw-urea medium (RUM; 5% w/v rice straw in 0.1% w/v urea in water) in 250 ml-Erlenmeyer flasks. Rice straw decomposition was determined after 15 d of incubation at 30°C under static condition by measuring dry weight loss of the rice straw. Control experiments with no fungal inoculation were included to determine the degradation in the absence of external microbes. A benchmark experiment using Super LCC2 cultivated under identical conditions was included for comparison. All experiments were done in triplicate. Means and standard deviation were calculated and Duncan's new multiple range test was used to compare the decomposition efficiency of the rice straw.

**2.4 *In vitro* Compatibility Evaluation of Co-fungal Strains**

The fungi were evaluated for their compatibility before co-cultivation. The fungal interactions were studied by dual direct opposition mycelia cultures [13]. Two discs of different strains were inoculated 4 cm apart from each other in the same PDA plate. A single culture was included as the

control. The co-inoculated plates were incubated at room temperature for 14 d. Fungal interaction were determined by using the observation key of Molla et al. [13]: (1) mutual intermingling interaction (MII); (2) partial mutual intermingling interaction (PII); and (3) inhibition interaction (IHI). The fungi with either MII or PII compatibilities were subjected for co-cultivation and determination of rice straw decomposition potential.

### 2.5 Evaluation of Decomposition Performance of Rice Straw by Co-fungal Cultivation

The fungal co-cultures were prepared by inoculating 125 mg (wet weight) of mycelia of each strain grown in PDA for 5 d into 50 ml of sterile rice straw-urea medium (RUM; 5% w/v rice straw in 0.1% w/v urea in water) in a 250 ml-Erlenmeyer flasks. The co-fungal cultures were incubated at 30°C for 15 d under the static conditions. Weight loss of rice straw in a flask without fungal inoculation was used as a control. "Super LDD.2" was used as a benchmark to compare the decomposition efficiency. The rice straw weight loss was analysed as following the technique mentioned in section 2.3.

## 3. RESULTS AND DISCUSSION

### 3.1 Determination of Lignocellulolytic Enzyme Activities

Six fungal stains were selected from (BCC) based on their ability to produce significant levels of the endo-acting and exo-acting hydrolytic enzymes. The six fungal strains showed varying levels of cellulolytic and hemicellulolytic enzymes (Table 1). *Penicillum oxalicum* BCC4504 showed the highest specific activities for most cellulases and hemicellulases among the selected fungal strains, while relatively low activities were observed for *C. globusum* BCC5776, *T. asperellum* BCC62292, and *T. reesei* BCC62305. *A. niger* BCC5772 was found to be the highest xylanase producer (299.38±16.55 U/mg of protein). Relatively high FPase (approximately 1) and CMCase (approximately 50) activities were detectable from *A. niger* BCC5772 and *A. aculeatus* BCC17849. These strains were used further for investigation of rice straw hydrolysis.

**Table 1**. Enzyme activities of selected fungal strains.

| Strains | Specific activity (U/mg of protein) | | | | |
|---|---|---|---|---|---|
| | FPase | CMCase | β-glucosidase | Xylanase | β-xylosidase |
| BCC4504 | 4.66±0.38$^{a}$ | 248.61±4.51$^{a}$ | 6.36±0.61$^{a}$ | 104.59±6.34$^{b}$ | 20.05±1.77$^{a}$ |
| BCC5772 | 1.09±0.01$^{b}$ | 50.20±2.99$^{b}$ | 6.29±0.10$^{a}$ | 299.38±16.55$^{a}$ | 4.78±0.03$^{b}$ |
| BCC5776 | 0.13±0.01$^{c}$ | 6.46±1.08$^{d}$ | 5.12±0.24$^{a}$ | 6.06±0.64$^{d}$ | - |
| BCC17849 | 1.57±0.01$^{b}$ | 45.20±7.40$^{b}$ | 4.28±1.07$^{a}$ | 26.29±0.01$^{c}$ | 2.28±0.10$^{cd}$ |
| BCC62292 | 0.13±0.04$^{c}$ | 6.51±1.44$^{d}$ | 1.60±0.24$^{b}$ | 20.19±8.63$^{c}$ | 0.70±0.15$^{d}$ |
| BCC62305 | 0.29±0.04$^{c}$ | 18.73±1.17$^{c}$ | 1.09±0.67$^{b}$ | 109.70±14.79$^{b}$ | 1.01±0.02$^{d}$ |

- indicates undetectable activity.

Duncan's new multiple range test was used to identify the significant level among each activity. Different letters indicate the significant difference of activity between strains ($p < 0.05$).

### 3.2 Rice Straw Decomposition by Individual Fungal Strains

The rice straw decomposition capability of the individual fungi was determined in the simplified medium containing rice straw as the sole carbon source and urea as a nitrogen source (Figure 1). Small reduction in the straw weight (10%) was observed in the control experiment with no fungal inoculation after 15 d of incubation. The highest straw degrading activity was found for *P. oxalicum* BCC4504, resulting in 14.89 ± 0.53% higher weight loss compared to the control. Relatively lower degradation rates in the range of 9.86-10.91% was observed for *A. niger* BCC5772 and *A. aculeatus* BCC17849, which was comparable to that observed for Super LDD.2 (10.00 ± 1.32%). Lower degradation efficiency (2.27-4.15%) was obtained for the other fungal strains.

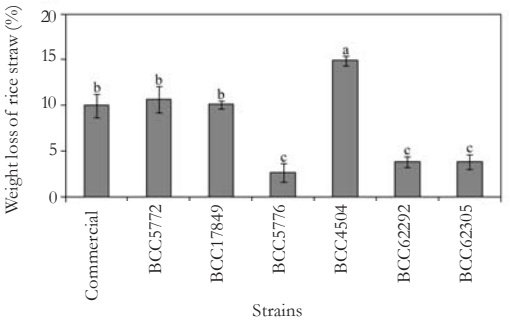


**Figure 1**. Rice straw decomposition experiment of 6 selected fungal isolates. The differences between the treatments were evaluated by a Duncan's new multiple range test. Different letters indicate the significant difference between treatments ($p < 0.05$).

### 3.3 Compatibility Test of Fungal Strains

Compatibility of the multiple fungi was studied *in vitro* using the dual direct opposition mycelia culture method. BCC4504, which showed the greatest rice straw decomposition efficiency, was used as a main culture in the compatibility test. The compatibility result showed that most of the fungi could mutually grow on PDA (Table 2). Combinations of the six fungi showed that *P. oxalicum* BCC4504, *C. globusum* BCC5776, *A. aculeatus* BCC17849, and *T. reesei* BCC62305 were compatible (mutual intermingling interaction; MII) to each other. Only one combination, *P. oxalicum* BCC4504 and *T. asperellum* BCC62292 showed partial intermingling interaction (PII) as their mycelium growth patterns were rendered at their touching area. These results suggested that all selected fungi could be further used in co-cultivation study.

**Table 2**. Compatibility of selected fungi.

| Fungal combinations | | Fungal interactions |
|---|---|---|
| BCC4504 | BCC5772 | MII |
| | BCC5776 | MII |
| | BCC17849 | MII |
| | BCC62292 | PII |
| | BCC62305 | MII |
| BCC5772 | BCC5776 | MII |
| | BCC17849 | MII |
| | BCC62292 | MII |
| | BCC62305 | MII |
| BCC5776 | BCC17849 | MII |
| | BCC62292 | MII |
| | BCC62305 | MII |
| BCC17849 | BCC62292 | MII |
| | BCC62305 | MII |
| BCC62292 | BCC62305 | MII |

MII: mutual intermingling interaction.
PII: partial intermingling interaction.

### 3.4 Rice Straw Decomposition by Co-cultivated Fungi

The (MII) or (PII) were subjected to co-cultivation study to observe their cooperative action on rice straw decomposition (Figure 2). The maximal decomposition efficiency was achieved using co-culture of *P. oxalicum* BCC4504

combined with *C. globusum* BCC5776 or *T. reesei* BCC62305, which was higher than that obtained using BCC4504 alone and Super LCC2, which was used as a benchmark. The degradation efficiency on native rice straw by the developed co-cultures was also remarkably higher than that obtained using single fungal strain in most previous study, although this could not be directly compared due to the differences in the characteristics of lignocellulosic substrates and the cultivation conditions. The higher decomposition rate is most likely related to the complemented enzymatic activities between cellulases (FPase, CMCase, and β-glucosidase) of *P. oxalicum* BCC4504 to xylanse of *T. reesei* BCC62305. This should result in enzyme synergism among various cellulases and hemicellulases which resulted in the overall increased efficiency on decomposition of the target biomass [14]. Furthermore, successful decomposition of biomass required not only cellulases (cellobiohydrolases, endoglucanase, β-glucosidase) and hemicellulases (xylanses, β-xylosidase, α-arabinofuranosidase and α-glucuronidase) but also some auxiliary proteins such as expansin [15]. Therefore, synergistic interaction of BCC5776, the low-enzyme producer, to BCC4504 and BCC5772 could come from the other undetermined hemicellulases and auxiliary proteins.

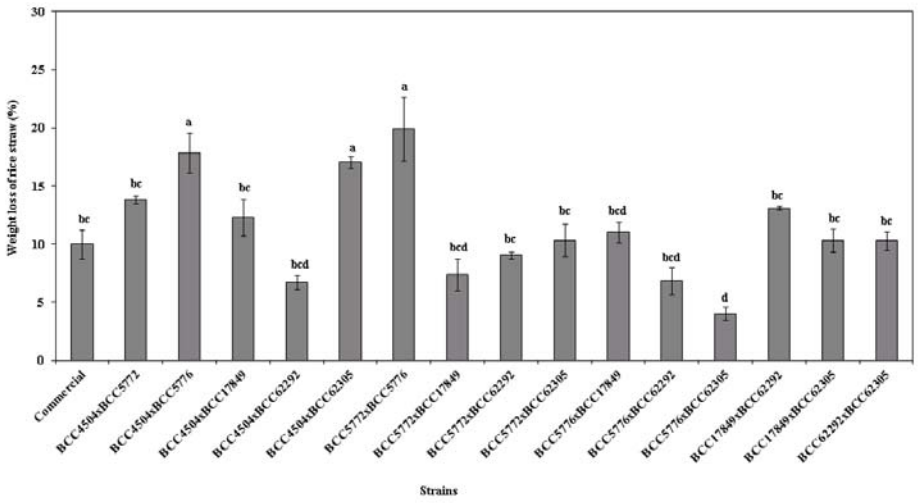


**Figure 2**. Rice straw decomposition experiment of co-cultivated cultures. The differences between the groups were evaluated by a Duncan's new multiple range test. Different letters indicate the significant difference between groups ($p < 0.05$).

The effectiveness of using microbial consortia containing 3 or more microorganisms can also be further investigated (Table 3). To date, the highest biomass decomposition capability was obtained from a complex microbial consortium reported by Pei-pei et al. [7]. The consortium, containing at least 20 bacterial strains and 7 fungal species, could reduce rice straw dry weight by 63.80% within 15 d of incubation at 30°C in the enriched culture medium. However, maintaining a complex microbial consortium on a larger scale could face difficulties in stability and production [16]. Compared to the complex microcosms, the co-culture developed in

this study provides a promising way for practical application in term of stability and could be grown in a simplified medium containing only rice straw and urea.

**Table 3**. Biomass decomposition comparison by different microorganisms.

| Microorganisms | Biomass | Duration | Culture | Weight loss (%) | Reference |
|---|---|---|---|---|---|
| Fungi | | | | | |
| *Aspergillus turreus* | RS | 3 weeks | 28°C | 21.00 | [17] |
| *A. fumigatus* | SGL | 10 weeks | 26°C | 11.66 | [18] |
| *Chaetomium bostrychodes* | HPL | 10 weeks | 26°C | 7.69 | [18] |
| *P. citrinum* | JBL | 8 weeks | 20°C | 2.40 | [19] |
| *Penicillium* sp. 2 | SGL | 10 weeks | 26°C | 11.03 | [18] |
| *P. oxalicum* | RS | 15 days | 30°C | 14.89 | This study |
| *T. harzianum* | JBL | 8 weeks | 20°C | 4.60 | [19] |
| *T. reesei* | RS | 3 weeks | 28°C | 22.00 | [17] |
| *Trichoderma* sp. 2 | SGL | 10 weeks | 26°C | 10.05 | [18] |
| Microbial consortia | | | | | |
| BCC5772xBCC5776 | RS | 15 days | 30°C | 19.95 | This study |
| ADS-3 | WHS | 15 days | 30°C | 63.80 | [7] |

RS: rice straw.
SGL: sweet gum litter.
HPL: horsetail pine litter.
JBL: Japanese beech litter.
WHS: wheat straw.

## 4. CONCLUSION

Fungi are key lignocellulose degraders under aerobic conditions with an important role in biogeochemical cycling of organic carbon. A range of fungi are capable of producing a variety of hydrolytic and non-hydrolytic enzymes with different specificities involving the degradation of the cellulose, hemicellulose, and lignin fractions in plant biomass. Attempts to decompose agricultural residues with fungi have been reported with the use of single strains, dual compatible strains, and complex microcosms comprising multiple strains of fungi and bacteria. The use of single fungal strains usually resulted in relatively low decomposition rate. To increase decomposition capability of the fungi, rational co-cultivation of selected fungi was employed. The co-cultivation approach increased the rice straw decomposition to 17.84±1.72% higher weight loss than control in BCC4504xBCC5776. The highest rice straw decomposition was achieved from BCC5772xBCC5776 (19.92±2.74% higher weight loss than control). These results indicated that rational co-cultivation of fungi with cellulolytic/hemicellulolytic activities could efficiently use to increase decomposition rate of rice straw. Improvement on biomass decomposition could be achieved by more combination of fungal strains including the ones active on degradation of pectin and lignin. These show the high potential for using co-culture of selected fungi for effective straight-forward and inexpensive degradation of rice straw in an open environment.

**ACKNOWLEDGMENTS**

The authors thank the National Center for Genetic Engineering and Biotechnology for financial support (grant no. P-13-50020). Manuscript proofreading by Dr. Piyanun Harpichanchai is appreciated.

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