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Application of quadratic regression orthogonal design to develop a composite inoculum for promoting lignocellulose degradation during green waste composting

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ABSTRACT

The aims of this study are to determine the feasibility of applying QROD (quadratic regression orthogonal design) to optimize a combination of microorganisms and to develop a composite inoculum for promoting lignocellulose degradation during GWC (green waste composting). This feasibility was studied in a laboratory scale experiment, using three lignocellulolytic microorganisms, isolated from the mature phase of GWC by the dilution plating method. After the feasibility was confirmed, a composite inoculum was developed through the results of the optimization, whose effect was evaluated by comparing it with *Phanerochaete chrysosporium* and EM (Effective Microorganisms) in a pilot scale experiment of GWC. The use of QROD to finish this optimization was proven feasible, because the p value of the regression equation was less than 0.05 (0.0108), meaning that the quadratic regression model is suitable for describing the relationship between the combination of the three microorganisms and their ability to degrade lignocellulose. Additional proof of this feasibility is that the composite inoculum in the quadratic regression orthogonal experiment demonstrated lignocellulose degradation ability similar to the GWC experiment. Although the lignin degradation ability of the composite inoculum did not surpass *Phanerochaete chrysosporium*, it was stronger than EM. Meanwhile, cellulose degradation ability and humus synthesis ability of the composite inoculum were stronger than for *Phanerochaete chrysosporium* and were close to EM. It is hard to tell which inoculum is the best since each inoculum had advantages in different aspects, while the composite inoculum still showed a considerable effect of lignocellulose degradation during GWC.

1. Introduction

Composting is replacing incineration and landfill use, becoming the main strategy of green waste disposal in urban China. However, the long cycle of GWC (green waste composting) caused by high lignocellulose content is still under investigation (Gong et al., 2017; Zhang and Sun, 2014; Zhang and Sun, 2015, 2017). Microbial inocula showing positive effects in lignocellulose degradation have been widely investigated in GWC and are regarded as the key strategy for accelerating the composting process (Gazi et al., 2007; Gong et al., 2017; Liu et al., 2018). As the inocula with single function microorganisms cannot meet multiple requirements, composite inocula with various microorganisms attract attention. Such inocula can comprise multiple functions into one single inoculum and may acquire extra functional improvement due to interactions among different microorganisms (Choudhury and Kabi, 2004). Obviously, the most important factors that determine the effect of a composite inoculum are the microbial species and their quantities; however, the latter are rarely emphasized. In 2018, the most widely used strategy for developing a composite inoculum is to directly use material containing various microorganisms such as cattle dung, mature compost, slurry, digestate, etc. as composite inocula (Alkoaik and Ghaly, 2005; Bolta et al., 2003; Di Maria et al., 2016; Kalemelawa et al., 2012; Lins et al., 2012). Other

Abbreviations: QROD, quadratic regression orthogonal design; GWC, green waste composting; EM, Effective Microorganisms; cfu, colony forming unit; TOC, Total Organic Carbon; TN, Total Nitrogen; rpm, revolutions per minute; PDA, Potato Dextrose Agar; Mnp, Manganese peroxidase; Lip, lignin peroxidase; Lac, laccase; *B1*, *Bacillus* sp. 1; *B2*, *Bacillus* sp. 2; *A*, *Aspergillus* sp.; dw, dry weight; CK, the treatment adding equal volume of water instead of inoculuminocula; GI, Germination Index; ANOVA, One Way Analysis of Variance; LSD, Fisher's Protected Least Significant Difference Test.

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strategies such as obtaining a composite inoculum from cultured material containing various microorganisms (Ming et al., 2008; Shen et al., 2015), or developed by cocultiviation of different microorganisms (Wei et al., 2007; Zhou et al., 2015) have also been reported. The aforementioned strategies did not discuss quantities of component microorganisms, which may lead to the problem that the composite inoculum cannot be reproduced (as an example, using the strategy in which mature compost is directly used as composite inoculum, microbial species and quantities of mature compost can be influenced by the composition of raw material, composting management, time, etc. (Liu et al., 2018). Thus, it is difficult to guarantee that the composite inoculum developed using this strategy is the same in different batches). Some strategies referring to microbial quantities are also available, though in these strategies, different microbial suspensions were equally or proportionally composed together, without discussing the combination optimization of the component microorganisms (Lu et al., 2004; Rashad et al., 2010; Singh and Sharma, 2003; Zhao et al., 2017). However, this optimization may be an opportunity to improve the function of the composite inoculum.

In the current study, QROD (quadratic regression orthogonal design) was carried out to finish this optimization and to develop a composite inoculum for promoting lignocellulose degradation during GWC. Additionally, the quantity (cfu, colony forming unit) of each component microorganism was clarified. QROD is a statistical method that can give a clear understanding of the relationship between multiple factors and response value through a regression equation, it can also provide an extremum of response value with a relevant value of each factor (Cao et al., 2017). This method has been widely used in engineering, but has not yet been used to optimize a combination of microorganisms (Cao, 2016; Cao et al., 2017; Subramani and Panda, 2014). Thus, the feasibility of this method needs to be confirmed and was therefore studied in a laboratory scale experiment using three lignocellulolytic microorganisms isolated from the mature phase of GWC. If the p value of the regression equation is less than 0.05 (Cao, 2016), indicating that the quadratic regression model is suitable to describe the relationship between the combination of the chosen microorganisms and the chosen response value (total degradation rate of lignocellulose), it can be stated that QROD is feasible for the development of composite inocula. Once the feasibility was confirmed, a composite inoculum was prepared according to the results of the optimization and was applied in a pilot scale experiment of GWC for the effect evaluation. If the composite inoculum demonstrated similar lignocellulose degradation ability in the quadratic regression orthogonal experiment of laboratory scale and GWC experiment of pilot scale, this would be further proof of the feasibility of the approach. Meanwhile, the strongest lignin degrading microorganism, *Phanerochaete chrysosporium*, and the most widely used composite inoculum, EM (Effective Microorganisms, which mainly consists of *Lactobacillus sp*., *Saccharomyces sp*., and *Rhodopseudomonas sp*.) (Khaliq et al., 2006) were also applied as comparisons for the effect evaluation.

Objectives of the current study were: 1. To determine the feasibility of applying QROD to optimize the combination of microorganisms and to develop a composite inoculum. 2. To evaluate the effect of the composite inoculum in GWC.

2. Materials and methods

2.1. Methods of composting and microorganism screening

2.1.1. Method of composting

The composting was performed to obtain compost at a mature phase for microorganism screening. The experiment was conducted at the composting plant of the Beijing botanical garden and began on 15 September 2016. The raw material was composed mainly of branches with the leaves, which was obtained lately before the experiment from the Beijing botanical garden. These were smashed into less than 20 mm chips and piled on the ground. Raw material for the experiment was collected from the surface of this pile and then piled into a 1 m^3 conical heap (basal area was 3 m^2, height was 1 m). Subsamples were taken from the top, middle, and bottom of this heap and mixed into one composite sample for chemical analysis (TOC (Total Organic Carbon): 47.8% (dry basis), TN (Total Nitrogen): 1.4% (dry basis), C/N: 34.14). The C/N was then adjusted to 25 by urea (11.15 g urea/1 kg dry raw material, the urea was dissolved to 5 L water and sprayed onto the heap, the heap was then stirred). The moisture content was adjusted to 60–70% by watering and was kept in this range (Gong et al., 2017). One hundred and fifty grams rhamnolipid and 2 ml bamboo vinegar were dissolved in 1 L and 2 L water, respectively, and then added to per 100 kg dry raw material at the beginning of composting and then once every 6 days (Zhang and Sun, 2014). Addition of rhamnolipid was to promote water distribution around compost particles, which can optimize the air-water condition for the microbial reaction. Addition of bamboo vinegar (mainly consisting of acetic acid) was to lower the pH of the compost, which can reduce nitrogen loss as ammonia during composting (Zhang and Sun, 2014, 2015). After composting started, the temperature in the center of the heap was determined by a thermometer once every 3 days (the temperature measurement was always conducted before watering and turning, in order to eliminate the effect of watering and turning on temperature variation). Once the temperature exceeded 60 °C, the heap was turned. If the temperature no longer exceeded 60 °C, the heap was turned once every 6 days. When the temperature was equal to ambient level and the equilibrium lasted for two determinations, samples were taken by the aforementioned method for microorganism screening. The reason for the timing in sampling is that the microorganisms were supposed to be composed to a composite inoculum added at the beginning of mature phase. Because the mature phase is the longest and most thermally stable period of a composting, adding the microorganisms to their source during a thermally stable period may preserve their optimal adaptations and maintain their effects as long as possible (Tran et al., 2015; Zhou et al., 2015). Futhermore, the sampling temperature was recorded to determine the culturing temperature, which was intended to simulate the temperature of the mature phase.

2.1.2. Method of microorganism screening

Microorganisms were screened by the dilution plating method (Sen and Chandra, 2009). Ten grams of fresh sample was added to 90 ml of sterile water (1/9, w/w) in a plastic bottle, and the bottle was shaken at 200 rpm (revolutions per minute) for 30 min and left to stand for 15 min. This was to separate the microoganisms from the compost into the supernate. The supernate was diluted between 10 and 10^5 times; each 0.1 ml aliquot of liquid was respectively plated on beef extract peptone agar medium and rose bengal agar medium for the colonization of bacteria and fungi, respectively (Atlas, 2010). All the media were cultured at the sampling temperature (28 °C). After clear colonies appeared on the media, each colony with a different appearance was streak cultured on a new batch of the same medium until single colonies appeared. Then, one of the single colonies was transferred to a liquid medium (bacteria to beef extract peptone medium, fungi to potato dextrose medium (Atlas, 2010)) and cultured at 28 °C, and 200 rpm. After the liquid media turned turbid, each suspension of 10 μl was respectively inoculated onto filter paper (d = 6 mm) on selective medium A (CMC-Na: 10.0 g, K_2HPO_4: 1.0 g, $MgSO_4 \cdot 7H_2O$: 0.1 g, $FeSO_4 \cdot 7H_2O$: 0.1 g, $MnSO_4$: 5×10^{-4} g, peptone: