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Isolation of cellulolytic fungi and their application for production of organic fertilizer from water hyacinth (*Eichhornia crassipes*)

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Abstract

The purpose of this study is to isolate the cellulolytic filamentous fungi involved in the decomposition of water hyacinth to produce organic fertilizer. In this study, 13 different fungi were isolated and screened. Among them, isolate N.S8 showed the highest cellulase activity with the diameter of the clear zone of 35 mm. The isolate N.S8 was identified by sequencing the D1/D2 region of 28S rRNA coding gene. BLASTN analysis of sequenced 28S rRNA segment revealed that the isolate N.S8 is *Aspergillus oryzae* with identity value and E-value of 100% and 0.0, respectively. Additionally, culturing the isolate N.S8 in rice bran medium (pH 6.5) for 144 hours is the optimal method of improving cellulase activity. Moreover, the use of this isolate for composting water hyacinth created an organic fertilizer with nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) contents of 3.35%, 0.43% and 0.74%, respectively after 45 days. Because of the high contents of nutrients, this organic fertilizer could solve the problems of fertilizer for crops with an efficiency being equivalent to that of chemical fertilizers which are known as one of the causes of soil degradation, environmental pollution and the negative effect on the quality of agricultural products.

Introduction

Fungi play an important role in the ecosystem by involving in nutrient cycling and energy flows (Buee et al., 2009). Fungi such as saprophytes convert organic matters in the soil into inorganic nutrients which plants could absorb (Morris et al., 2005). Many fungi which could successfully grow on various types of substrates and help to decompose organic matters to beneficial inorganic nutrients have been investigated (Fuchs et al., 2010).

Cellulolytic fungi are considered as the key factors to create high quality compost (Hubbe et al., 2010). Previous research indicated that the application of cellulolytic fungi increased the ability of holding water in fungal inoculated samples and balanced the C:N ratios of cellulose waste (Hart et al., 2002; Sivaramanan et al., 2014). Additionally, the addition of microorganisms such as fungi, bacteria and other microbes was demonstrated to shorten the composting duration (Mishra et al., 2013).

Water hyacinth (*Eichhornia crassipes*) is a free-floating freshwater plant of the family Pontederiaceae that has a cosmopolitan distribution but are mostly found in sub-tropical and tropical countries (Agunbiade et al., 2009; Jafari et al., 2010). Water hyacinth has been proven to be a highly problematic invasive species due to its detrimental impacts of preventing sunlight and lowering disolved oxygen concentration. Controlling this plant is difficult because of its fast growth and large biomass (Akter et al., 2009). Therefore, there is a great attention of treating water hyacinth biomass.

Water hyacinth can be processed into compost (Zimmels et al., 2006). Previous studies demonstrated that compost produced from water hyacinth is good for the yields and growths of many plants (Osoro et al., 2014). Additionally, the supplement of fungi such as *Trichoderma* for composting water hyacinth was proved to be useful for improving nutrition quality (Ghosh et al., 2010).

The purpose of the present research is to introduce some results about the selection of cellulolytic fungi and its application for the production of organic fertilizer from water hyacinth to create a high-quality organic fertilizer source that could increase the porosity, soil fertility and alleviate environmental pollution.

Materials and Methods

Sample collection

Soil samples (30 samples) were collected from several areas including gardens and fields in Thua Thien Hue, Vietnam. Sterile spatula and plastic bags were used for sample collection. Before being used for fungi isolation process, the samples were stored at 4 ^{o}C for 12 hours (Singh et al., 2013).

Water hyacinth (*Eichhornia crassipes*) was obtained from river and lakes in Thua Thien Hue Hue, Vietnam. After being collected, waterhyacinth would be dried for 5-7 days and used as raw material for the process of organic fertilizer production.

Isolation of cellulolytic fungi

The potato glucose agar (PGA) medium was used for the isolation of filamentous fungi. To isolate the fungi, 1 g of soil sample was suspended in 9 ml of sterile distilled water and vortexed thoroughly. From this 10 ml stock solution, serial dilutions were performed to 10^{-6}. 100 µl from the dilutions of 10^{-5} was plated in triplicates on the culture medium. Cultures were then incubated at 30 ^{o}C. The growths of fungi were daily checked. Pure isolates were maintained at 4 ^{o}C in a refrigerator for further analyses (Akharaiyi et al., 2016).

Determination of cellulase activity

25 ml of medium, given 2% agar and 1% carboxymethylcellulose, was poured into each petri dish. Next, fungus isolates were subcultured on the medium. Cultures were then incubated at 30 ^{o}C in the dark. After 4 days of incubation, 5 ml of Lugol's reagent was plated on each petri dish to detect clear zones. Finally, the diameters of clear zones were obtained (Coronado-Ruiz et al., 2018).

Effect of pH and temperature on cellulase activity

The activity of cellulase was measured by changing the pH with 0.05 M sodium phosphate buffer. The pH was varied between 5.0-7.5, after which cellulase activity was determined. The effect of temperature on cellulase activity was investigated at different temperatures of range 30-70 ^{o}C with pH being adjusted based on cellulase activity determined during pH studies (Talekar et al., 2011).

Effect of culture duration

To determine the effect of culture duration on cellulase activity, the filamentous fungi was grown on rice bran medium supplemented with carboxymethylcellulose substrate and incubated at 30 ^{o}C. After 24-168 hours of incubation, the enzyme activity would be determined based on the diameters of clear zone (Bansal et al., 2012).

Classification of filamentous fungi

Isolates were firstly pre-classified by observing morphological features of colonies on agar plates and reproductive organs on glass slide at 40X magnification (Klich et al., 2002).

The D1/D2 region of 28S rRNA coding gene was amplified using the primer pair U1/U2 (U1FGC (5 '- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA - 3'; Sigma) and U2R (5 '- GAC TCC TTG GTC CGT GTT - 3'; Sigma)). 260 bp amplicons were then sequenced by Nam Khoa Trade Production & Service Company Limited, Vietnam. After that, the isolate N.S8 was classified using BLASTN, with the sequenced segment as query sequence (Sandhu et al., 1995).

Compost preparation

The filamentous fungi with high cellulase activity was cultured in 250 ml Erlenmeyer flasks containing 1% of carboxymethylcellulose and rice bran. During the culture process, the humidity would be maintained appropriately (Tolan et al., 1999). The experiment of composting water hyacinth consisted of two treatments which are presented in Table 1.

Table 1. Treatments for composting water hyacinth

Treatment	Water hyacinth (kg)	Cellulolytic microorganism	Concentration (%)
Experiment	100	Strain with the highest capacity of degrading cellulose	5
Control	100	-	-

100 kg of dried water hyacinth and 5 kg of fungus were well mixed. The mixture was then compacted, covered by polyethylene sheets and incubated for 45 days. Control experiment was carried out in the same way as above-mentioned without the addition of fungus.

After 45 days of incubation, the total nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O) of the compost were determined by ISO 11261:1995, wet digestion spectrophotometer (420 nm) and flame photometer respectively (Barton, 1948; Hesse & Hesse, 1971).

Data analysis

The data were analyzed by ANOVA using IBM® SPSS® Statistic software (version 20). Means were compared by Duncan's test with the significance level of

0.05. All the graphs were constructed by Microsoft® Office Excel software (2013). Each experiment was done in triplicates.

Results and Discussion

Isolation and screening of cellulolytic fungi

Thirteen filamentous fungi were isolated from different soil sources. The isolates were screened and compared for their ability of degrading cellulose. Isolates' mean clear zone diameters, obtained after 4 days of incubation, are presented in Table 2.

Table 2. Growth and enzyme activity of isolates

Isolate's code	Colony diameter (mm)	Clear zone diameter (mm)
N.S1	17(0.421)^{f*}	17(0.234)^{g}
N.S2	28(0.528)^{d}	28(0.442)^{d}
N.S3	19(0.526)^{e}	19(0.521)^{f}
N.S4	16(0.415)^{f}	16(0.218)^{g}
N.S5	17(0.432)^{f}	19(0.322)^{f}
N.S6	42(0.846)^{b}	42(0.865)^{c}
N.S7	13(0.408)^{g}	17(0.831)^{g}
N.S8	**45(1.52)^{a}**	**60(0.963)^{a}**
N.S9	35(0.835)^{c}	45(0.958)^{b}
N.S10	9(0.405)^{h}	16(0.524)^{h}
N.S11	15(0.524)^{g}	15(0.519)^{h}
N.S12	14(0.423)^{g}	22(0.633)^{e}
N.S13	15(0.432)^{g}	23(0.631)^{e}

*Within a column, means having a letter in common are not significantly different at the 5% level. The values are presented as mean (standard deviation).

The insignificant differences between colonies and clear zones' diameters were observed in 6 isolates namely N.S1, N.S2, N.S3, N.S4, N.S6 and N.S11. In contrast, isolates including N.S5, N.S7, N.S8, N.S9, N.S10 N.S12 and N.S13 obviously showed high cellulase activity, with the largest clear zones' diameters, 60 mm on average, created by N.S8. Thus, among 13 isolated fungi, 7 isolates were identified as cellulase producers. Additionally, because of the highest cellulase activity, isolate N.S8 was selected to use for further studies.

Morphological characterization of the isolate N.S8

Morphological examination showed that N.S8's colonies were initially white and then turned to the yellow color of areca flower as cultures aged. The surfaces of colonies were velvety, with clearly observed hyphae. From the microscopic observation, it is clear that the hyphae, containing septa, are olive green and branched. The conidiophore is not branched. Conidia are round and rough (Figure 1).

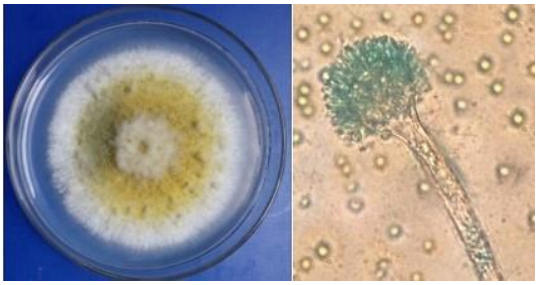

Figure 1. Features of isolate N.S8.

Identification on the basis of phylogenetic analyses

The results show that the sequenced D1/D2 region of NS.8 is highly similar to the one of *A. oryzae* (Accession number: AP007172.1), with the identity value and E-value of 100% and 0.0 respectively. Thus, it can be concluded that NS.8 belongs to *A. oryzae* species (Table 3 and Table 4).

Table 3. Evaluating homologous rate of 28S RNA gene sequence

Species name	Strain name	Accession number	Homologous rate (%)
Aspergillus oryzae	RIB40	AP007172.1	100

Table 4. The sequencing of the 28S rRNA gene of the isolated strain

Query	1	CACGGGCGCGGACACCCCATCCCAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAG	60
Sbjct	3119	CACGGGCGCGGACACCCCATCCCAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAG	3060
Query	61	GGCACTTAGACAGGGGCCGCACCCGAAGCATCCTCTGCAAATTACAATGCGGACCCCGAA	120
Sbjct	3059	GGCACTTAGACAGGGGCCGCACCCGAAGCATCCTCTGCAAATTACAATGCGGACCCCGAA	3000
Query	121	GGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATCCCG	180
Sbjct	2999	GGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATCCCG	2940
Query	181	GTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGAT	240
Sbjct	2939	GTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGAT	2880
Query	241	CCGAGGTCAACCTGGAAAAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGA	300
Sbjct	2879	CCGAGGTCAACCTGGAAAAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGA	2820
Query	301	GCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGG	360
Sbjct	2819	GCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGG	2760
Query	361	CCCGTccccccGGAGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCA	420
Sbjct	2759	CCCGTCCCCCCCGGAGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCA	2700
Query	421	ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC	480
Sbjct	2699	ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC	2640
Query	481	TCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCAT	540
Sbjct	2639	TCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCAT	2580
Query	541	CGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACT	600
Sbjct	2579	CGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACT	2520
Query	601	CAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTG	660
Sbjct	2519	CAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTG	2460
Query	661	AGAGCCCCCGGCGGCCATGAATGGCGGGCCCGCCGAAGCAACTAAGGTACAGTAAACACG	720

Determination of the suitable conditions for cellulase synthesis

Effect of culture duration

It is illustrated in Figure 2 that when the culture duration was increased, the cellulase activity of *A. oryzae* rose. However, when the optimal culture duration was exceeded, the enzyme activity began to drop. The maximum cellulase activity was attained after 144 h of incubation with a clear zone diameter of 38.67 mm on average.

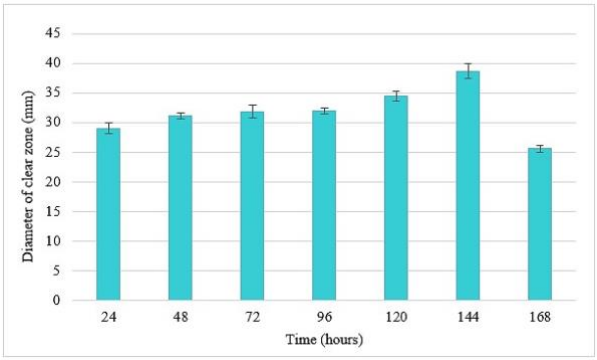

Figure 2. Effect of time on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

Compared to the previous research, differences were observed in the results of our present study. Specifically, according to Acharya et al. (2008), the highest cellulase activity was obtained after 96 hours of fermentation using *A. niger* (Acharya et al., 2008). Additionally, another research on cellulase production by *A. niger* from coastal mangrove debris conducted by Devanathan et al. (2007) also indicated that the optimal fermentation duration is 96 hours (Devanathan et al., 2007). Thus, all the above-mentioned results proved that the culture duration can affect the ability of producing cellulase of the filamentous fungi. Moreover, the optimal culture duration and enzyme amount are different among fungus strains (Nochure et al., 1993).

Effect of pH

As it is highlighted in Figure 3, the highest cellulase activity was obtained at pH 6.5 with the average clear zone diameter of 37.55 mm. According to Anita et al. (2009), the optimal pH for cellulase activity produced by *A. heteromorphus* is 6.0 (Anita et al. 2009). Furthermore, in a study conducted by Akiba et al. (1995), the authors concluded that the optimal pH for cellulase activity from *A. niger* is 6.0-7.0, and the stable pH range is 5.0-10.0 (Akiba et al., 1995). These different results might be caused by the genetic differences within the same genus (Acharya et al., 2008).

Effect of temperature

The effect of temperature on cellulase activity was determined in the range of 30-70 °C. As illustrated in Figure 4, the optimum temperature was 40 °C. Beyond 40 °C, thermostability decreased, possibly due to thermal denaturation of enzyme. The results obtained from this study are similar to the findings of Ali et al. (1991) who concluded that the optimal temperature for cellulase activity from *A. niger* "Z10" and *A. terreus* was at 40 °C. Loss of cellulase activity was observed beyond 40 °C and 50 °C (Ali et al., 1991).

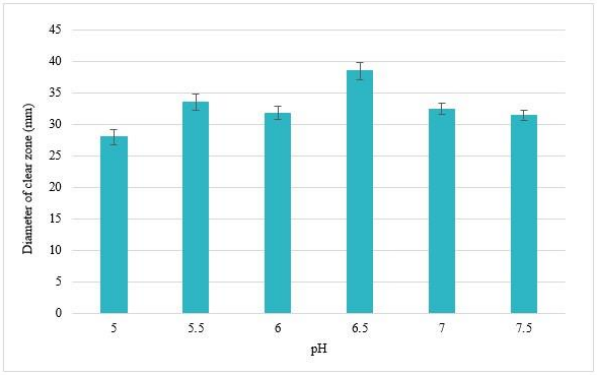

Figure 3. Effect of pH on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

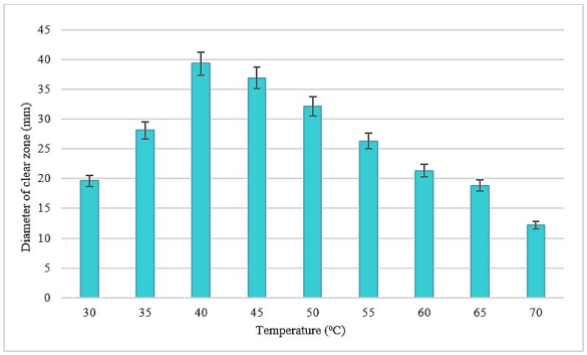

Figure 4. Effect of temperature on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

Creating organic fertilizer from water hyacinth and *A. oryzae* "N.S8"

After 6 days of culturing the fungus in the optimized condition, 100 kg of dried water hyacinth and 5 kg *A. oryzae* were mixed. After 45 days of incubation, chemical parameters of the organic fertilizer were shown in Table 3.

The provided results in Table 5 indicated that after being incubated, the total N, P, and K contents in all treatments increased. However, the contents of N, P, K in the treatment containing microorganisms was higher than those of the control with 1.53%, 0.19% and 0.38%, respectively.

The nutrient compositions of organic fertilizer from water hyacinth in the present research are higher compared to that in the previous research of Thanaporn et al. (2019) who concluded that the ranges of chemical parameters in the liquid organic fertilizer produced from agricultural residues and industrial wastes are 0.14-0.33% (total N), 0.002-0.017% (total P_2O_5) and 0.81-11.8% (total K_2O) (Thanaporn et al., 2019).

Table 5. Analysis results of total N, P, K contents in organic fertilizer sample

Content (%)	Before incubation		After incubation	
	Control	Experimental treatment (Supplemented with 5% *A. oryzae* "N.S8")	Control	Experimental treatment (Supplemented with 5% *A. oryzae* "N.S8")
Total N	1.42	1.42	1.53	3.35
Total P	0.16	0.16	0.19	0.43
Total K	0.36	0.36	0.38	0.74

Conclusion

Thirteen filamentous fungi with the ability of degrading cellulose were isolated from different soil sources in which the isolate N.S8 showed the highest cellulase activity with the average diameter of clear zone of 35 mm. The results of classification indicated that this isolate is *A. oryzae*.

The optimal culture duration and pH for growth and development of *A. oryzae* "N.S8" in rice bran medium are 144 hours and pH 6.5.

The use of *A. oryzae* "N.S8" for composting water hyacinth created an organic fertilizer with nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) contents of 3.35%, 0.43% and 0.74%, respectively after 45 days.

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