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DEVELOPMENT OF A NEW COTTON WASTE COMPOSTING TECHNOLOGY FOR CULTIVATION OF OYSTER MUSHROOM (*Pleurotus ostreatus*)

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ABSTRACT

This applicable research has successful selected enzyme Ctec2 from Novozyme (Denmark) which gave the best cellulose degradation activity compared to the other enzymes. At optimum conditions of 50^{o}C, pH=5 and ratio between enzyme and substrate (E/S) being 0.40%, 0.45%, 0.50%, the time needed to substrate degrade was 60 hours. Based on this finding, a new cotton waste composting technology for cultivating oyster mushroom was developed. Mushroom productivities using the new technology had reached 62.3%, 65.1%, 59.8% respectively, over dry weight of the substrate. The application of this technology can help to reduce the substrate processing time from 06 days to 4.5 days and the fiber phase was finished 7-10 days earlier. The mushroom yield found 65.1% increase over the dry weight of the substrate and was 20% higher than using the natural fermentation method.

Keywords: cellulose, *Pleurotus ostreatus*, cotton waste, degradation, glucose activity.

1. INTRODUCTION

Textile industry of Vietnam is growing as fast as the development of the country and plays an important role in the national economy; however it also releases the high amount of cotton wastes as the result. In this context, the application of biotechnology to manage such post-harvest waste resources needs to be considered. Cotton waste is a potential material for edible mushroom production. Thus, utilization of such materials to produce valuable nutritious foods, to increase income as well as to protect environment is a special interest of scientific community in the country [3].

Currently, the science and technology, especially biotechnology, are developing tremendously, in which enzyme technology is one of the most attractive subjects for research and applications [5] [6] [8] [14]. However, the selection of a highly active enzyme having an efficient cotton degradation capable to provide substrate materials for production of high yield oyster mushroom cultivation in a short time cultivation with lower expenses compared to the other method, also to bring economical benefit as well as environment protection will be a target for practical use.

In recent years, edible mushrooms are growing widely in Vietnam and bring the great economic benefit for growers. The current cultivation technology has mainly based on two traditional methods named natural fermentation [4] and heat composting methods, which allow materials to converse into available substrate for mushroom to grow. However, a number of problems exit to negatively affect production processes. If using the first method, the productivity usually is low, a long substrate processing time is needed leading to the high possibility contaminated with other fungi, while the application of the second method requires ovens relatively expensive to build and causes air pollution due to emission CO, NO_x, CO_2, SO_2 during combustion, which directly affects grower health and environment in the long run [1]. Therefore, new technologies to shorten the substrate processing time and improving productivity are essential.

2. MATERIALS AND METHODS

2.1 Materials

Cotton waste was gotten from Viet-My Textile Manufacturing Company, Tuong Giang, Tien Son industrial zone, Bac Ninh, Vietnam. The substrate was digested into sugar by using enzyme Ctec2 purchased from Novozymes (Denmark) which is a combination of various activated cellulase, high performing β-glucosidases and hemicellulase in shape of brown liquid with the density approximately 1.2g/ml. *Pleurotus ostreatus* was obtained from the culture collection of edible mushrooms in the Biotechnology Center, Agriculture Genetic Institute.

2.2. METHODS

2.2.1. Enzyme Ctec2 activity determination

The determination of enzyme Ctec2 activity was carried as DNS method (Estimating Sugar by Dinitrosalicylic Acid Method) [12].

2.2.2. Isolate cellulose from cotton waste using Kurschner and Hanak method [9]

The method used to isolate and purify cellulose from draw cotton waste materials, named cleaning cellulose have to be done to remove impurities which may inhibit cellulase activity in hydrolyzing cellulose such as hemicellulose lignin, silic and inert substrate residues in starting materials [18]. Thus, separation of cellulose out of draw materials is also considered as cellulose cleaning and plays a very important role.

2.2.3. Pre-composting of cotton waste by biological method

Cotton waste is a kind of biomass originated from lignocelluloses. Many methods to pretreat lignocelluloses were reported such as physics, chemistry and physical chemistry [2] and biology [13]. Normally, the precomposting method used is dependent on selected substrates, which are different in their nature, characteristics and use purposes. Therefore, it is important to find appropriate methods to lower the cost and prevent environment pollution. Among said methods, biological precomposting using heat-resistant microorganisms [11] to hydrolyse lignin and hemicelluloses, which can facilitate the degradation capacity of cellulose, was a target of choices.

Cotton waste was soaked in lime water at pH=7 to have moisture content of 65% [19][20]. The mixture then was piled into 1.5 m long x 1.2 m wide x 1.0 m high block and blocks were naturally fermented for 2 days. The temperate of incubated material was recorded daily by placing the 0^oC-100^oC scale thermometer 25 cm deep inside the block for 2 minutes.

2.2.4. Determine optimum conditions for enzyme Ctec2 activation

In order to determine the specific optimal value range of each factor which may affect enzyme activity, it is necessary to keep non-target factors constant and the targeted one is changed in a particular range.

2.2.4.1. Determine optimum temperature

After precomposting, enzyme was added into substrates with the E/S ratio of 0.3%. The treated substrates was adjusted to pH=5 using 0.1M HCl and incubated in 5^oC difference from 30^oC to 60^oC range for 48 hours. Glucose content was measured at the end of degradation process. Each experiment repeats three times. Average value was taken for analysis and the highest one was selected.

2.2.4.2. Determine optimum pH

The experiment to determine the optimum pH was carried at condition described as above, but at the constant temperate of 50^oC. The pH was adjusted with 0.1M HCl to obtain pH value of 0.5 difference in the range of 3.5- 6.5.

2.2.4.3. Determine optimal E/S ratio

The experiment to determine optimal E/S ratio was conducted at conditions described as above, but at the constant pH=5 and temperature of 50^oC. E/S ratio (gram enzyme/gram pure cellulose) was changed from 0.3% to 0.6% with 0.05% difference.

2.2.4.4. Determine optimal degradation time

The optimal time for degradation was determined in the conditions described as above, but at the constant pH=5 and temperature of 50^oC. Degradation time was recorded every 12 hours during 36 to 84 hours.

2.2.5. Method to design tested mushroom cultivation models

Three testing models were designed using different E/S ratios in order to find the highest obtained sugar content. All were incubated in the same degradation time which was found optimum. Cotton waste pretreated by biological methods then was piled into 2.5m long, 0.8m wide, and 0.18m high block. Enzyme Ctec2 was soluble into water at the rate of 1/20 and then added to substrate materials in different concentrations. The substrate was incubated at temperature of 50°C, pH 5 for 60 hours. Degraded substrates then can be piled up in 25x35cm or 35x45cm bags for summer or winter use, respectively.

Before filling substrate and oyster mushroom spawn, it is necessary to make the bag bottom flat, so that they can stand up right. Bags were filled in layers of substrate and spawn. The oyster mushroom spawn was placed on each 5-7cm cotton layers and upper surface. The top of bags was covered by a paper pieces and then the bag mouth is closed tightly to prevent contamination.

The bags were than inoculated for spawn running under complete darkness. The completion of growth of mycelium on substrates, the time needed to hang and ditch bags and requirement cares were observed and analyzed. The data were also recorded for the yield of oyster mushroom in each model.

3. RESULTS

3.1. Enzyme Ctec2 activity

Determine glucose amount due to CMC degradation by enzyme using acid dinitro salicylic DNS method

Based the ability of dehydrogenation, Ctec2 can convert waste cotton substrate into glucose. When reacting with DNS reagent, glucose solution turned from transparency into red-brown colour [12]. The colour intensity of the reaction mixture is proportional to the concentration of glucose in a certain range. Therefore, glucose amount can be determined by using the optical density at 540 nm and standard curve produced from the reaction between pure glucose and DNS reagent.

Procedure

- Preparation standard curve for glucose analysis

In this experiment, a standard curve for the analysis of glucose was constructed using optical density (OD)of glucose at 0.5, 1.0, 1.33 and 2 mg/mL. The correlation between glucose concentration and absorbance density is shown in Figure-1.`

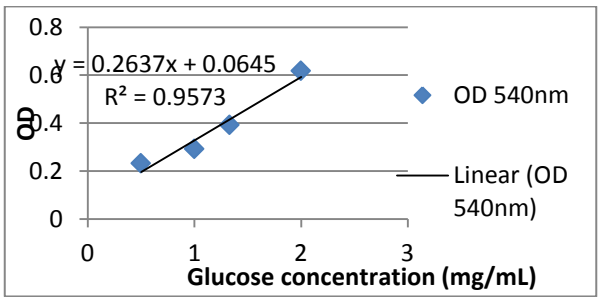


Figure-1. Standard curve for glucose.

- OD values of studied samples

The obtained solution was diluted 400 times and has an OD value equal to 1.759. From this value, glucose amount of tested sample was calculated by putting 1.759 numbers in the standard curve equation.

- Optical absorption and sugar concentration in tested samples

	Enzyme solution	Dilution coefficient
Optical absorption	1.759	x 400 times
Sugar concentration (mg/mL)	2578	

The above results indicate that enzyme Ctec2 of Novozymes has ability to convert CMC substrates into the high sugar concentration of 2578 mg/mL.

3.2. Cellulose purification from cotton waste

The purpose of separating cellulose from cotton waste is to obtain the exact amount of cellulose in used cotton waste. From that, the correct E/S ratio can be set up for mushroom cultivation models and mushroom yield can be evaluated.

Dried cotton was grounded into powder. The exact amount of 1.0 ± 0.1 g dry powder was prepared in two replicates and poured into 250mL flask. Addition of 35 mL alcohol: acid mixture, which is freshly prepared, into such flasks. They then were fitted with a converted condenser and heated in water bath for one hour. The temperature in water bath was increased gradually until boiling. The boiling water was keep at low level to avoid cotton powder moved to flask's surround or the above condenser. Take flask out of water bath and leave the mixture to be cool before filtered though porous filter funnel (or filter paper) having the known weight. Then freshly prepared ethanol-HNO3 mixture of about 5 mL was added into mixture. This step can be repeated several times (probably to 8-12 times or more depending on material types) until obtaining a white cellulose powder, which did not change into red colour when using tested agent. After the last composting, the remain powder was carefully transferred (as cellulose) into the filter funnel, and washed with 50mL freshly prepared ethanol-acid mix, then with distilled water several times until free from acid (using orange methyl). Filter funnels containing cellulose (or filter papers in weighting cup) were drying at 105 ± 3 ° C until obtaining constant weights.

Cellulose content (%) compared to absolute dry cotton was calculated as the following formula.

$$X=\frac{(m1-m)}{g}x100$$

Where:

m_1: weight of cellulose and filter funnel (filter paper + weighting cup) (g)
m: weight of funnel (filter paper + weight cup)(g)
g: absolute dry weight of cotton (g)

The difference between two parallel determinations should not exceed 0.05%.

Using Kurschner and Hanak method, 62% purified cellulose was recoved from cotton waste.

3.3. Precomposting by biological method

Biological precomposting period was carried in 02 days, corresponding to the temperature of incubated blocks reaching 63°C and 76°C due to the strong activities of microorganisms. At such high temperature, harmful microorganisms and pathogenic fungi were eliminated. In these experiments, the used appropriate inoculated blocks had stimulated the strong development of microorganisms [16] and helped them to break plant cell walls [7] making cellulose degradation easier. After biological precomposting step, cotton wastes colour was turned from the yellowish to dark brown and became very soft due to the formation of phenolic compounds, lignin hydrolysis products.

3.4. Effect of temperature into enzyme Ctec2 activity

Each enzyme has different optimal temperatures; however, the optimal temperature for each enzyme also is different depending on the substrate, pH and reaction time. Temperature making enzymes to complete lose their catalytic activity is called the critical temperature, usually around 70°C. At the critical temperature, the enzyme is denatured, less likely to recover its activity. Conversely, at temperatures below 0°C, the enzyme activity is reduced but may be recovered when the temperature was increased to normal [10]. After reaction, the products were measured their optical absorbance at 540 nm. Based on the standard curve of pure glucose with DNS reagent, sugar content in samples was determined.

OD values were determined based on standard sugar contents of 2mg/mL; 1.33mg/mL; 1mg/mL and 0.5mg/mL. From such value, a standard curve was set up and indicates the relationship between glucose concentration and optical density (Figure-2).

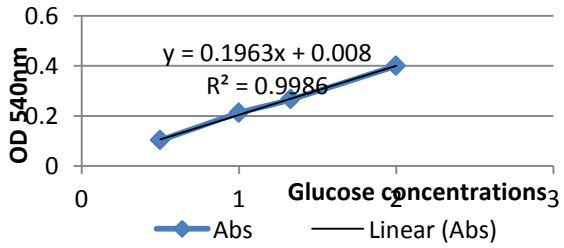


Figure-2. Standard curve of glucose using DNS method.

Based on the sugar contents obtained from tested models, in which cotton samples were incubated at different temperatures, a graph is built to show the changes of sugar content against temperature variation (Figure-3).

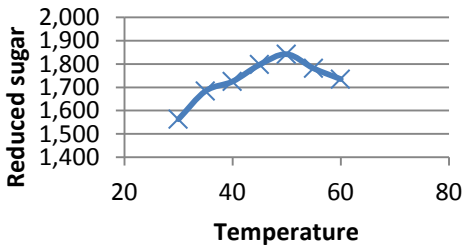


Figure-3. The graph showing sugar content variation according to temperature.

In the same condition of 48h degradation period and E/S ratio of 0.3%, the sugar amounts produced are different at different temperatures. They were increased as temperature up to 45-50°C. As a result, the optimal temperature for the substrate degradation of enzyme Ctec2 was found to be 50°C and the glucose content was reach as high as 1,842mg/mL. When the temperature deviates to either side of the optimal temperature, enzyme activity is decreased. As a consequence, the optimal temperature selected is 50°C.

3.5. Effect of pH on enzyme Ctec2 activity

Each enzyme has the highest activity at a certain optimal pH. Out of this pH, the activity of enzyme is lowered [15].

The sugar contents were calculated from using absorbance values at 540 nm of analyzed samples and the standard curve equation. From sugar content values (mg/mL) of cotton waste composting models incubated at different pH, a graph is built to show the changes of sugar content against pH variation (Figure-4).

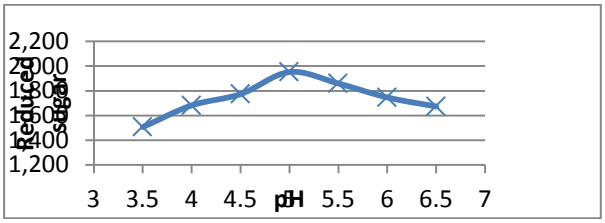


Figure-4. The graph showing sugar content variation (mg/mL) according to pH.

In the same conditions of 48h degradation period, temperature of 50°C and E/S ratio of 0.3%, the sugar amounts produced are different at different pH. Conversion activities of enzyme Ctec2 increased quickly from pH 4.0 to pH 5.0. At pH=5.0, the conversion efficient to produce sugar content is reached the maximal values as high as 1.952 mg/mL. Therefore, pH=5 is selected as the optimum pH.

3.6. Effects of E/S ratio to enzyme Ctec2 activity

Normally, substrate or enzyme is affected more or less depending on precomposting methods used [17]. Whenever E/S ratio is increased, reaction rate of enzyme also is increased due to a more interaction possibilities between enzyme and substrate to occur. When enzyme amount is high enough, enzyme is saturated by substrate. In that case, if bring enzyme concentration up, reaction rate only is increased slightly [14].

If substrates in culture media are available, the reaction rate is proportional to the amount of enzyme. The reaction rate is maximized when all enzymes incorporated into the substrate. When enzyme concentration exceeds the optimum level, reaction rate does not follow the rule and is shown as horizontal line in the graph [14].

Sugar concentrations are obtained when $OD_{540\ nm}$ of analyzed samples is located in the standard curve equation. Figure-5 performs the variation of sugar contents (mg/mL) among cotton waste samples incubated in different E/S ratios and recoded at different time points.

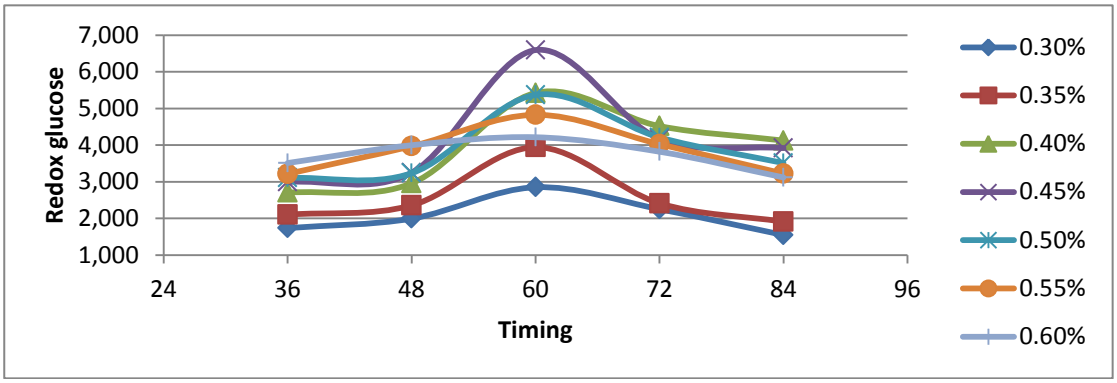


Figure-5. Graph showing the relationship between incubation time, enzyme concentration and sugar amount.

The results, which are shown in Figure-5, indicated that the sugar contents are increased when enzyme concentration is raised. It attained the highest level at E/S ratio of 0.45%, but decreased gradually when the higher E/S ratio of 0.50%, 0.55% and 0.60% used. The lower glucose inversion efficiency in the case of raised E/S ratios can be considered as an ineffective and wasted investment. In addition, the sugar content is increased during 36-60 hours substrate incubation and decreased in the longer period. The possible cause can be resulted from the stronger development of non-target microorganisms, and competitiveness with mushroom in using produced sugars and led to decrease in sugar contents.

Data analysis has revealed that the highest sugar content was achieved in 60-hour incubation of Ctec2 with E/S ratios of 0, 40%, 0, 45% and 0, 50%. Such optimal factors are selected for practical application.

The effects of enzyme concentrations on oyster mushroom yield with three replicated are shown in the Table below.

Time (hour)	E/S (%)	Average productivity (%) over dry weight of substrate
60	0.40	62.3
	0.45	65.1
	0.50	59.8

Effect of enzyme concentration to oyster mushroom productivity

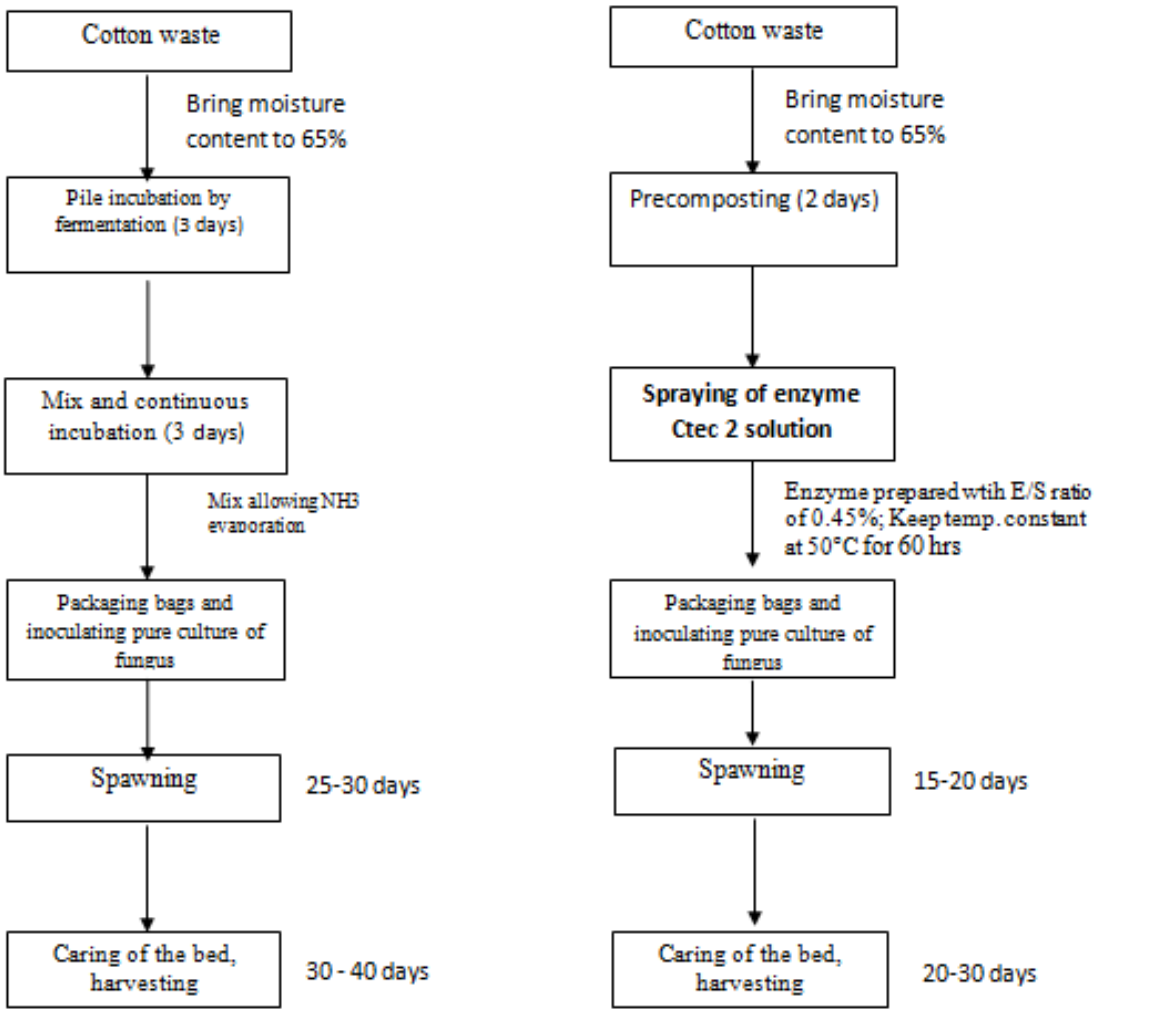
As shown in Tables, oyster mushroom can produce fruiting bodies on all three enzyme concentrations, but with different yields. At the low Ctec2 concentration of 0.40%, the enzyme is impossible to provide proper reactions to degrade a large amount of cotton waste substrates. Therefore, substrate materials are still hard and less modification compared to the original ones. Because of that, less available nutrition is produced for mushroom growth, leading to 23-25 day longer time needed for producing fruiting bodies and low yields although the appearance of white mycelia.

Enzyme Ctec2 reaches the optimal concentration at 0.45%. In this condition, the rate to degrade cellulose in incubated blocks is the strongest, making cotton properties deformed easily, providing enough nutrition for rapid developments of mycelia in quantities and qualities (thick and long), and creating the higher degradation efficiencies as well as the higher yields of mushroom.

At the relatively high Ctec2 concentration of 0.50%, substrate is degraded rapidly and turned into dark brown, which negatively affects the growth of oyster mushroom mycelium such as short, scattered and weak mycelium appearance and mushroom yield.

From different experiment results, enzyme Ctec2 concentration of 0.45% is chosen as the optimum and incorporated in the development of the new mushroom cultivation technology.

3.5. Development and yield assessment of new technological procedure for cultivation edible mushroom using cotton waste



Natural fermentation procedure

Mushroom yield:: 43.6%

New technological procedure using enzyme product

Mushroom yield: 65.1%

Commentation

The new technological procedure helps to reduce the substrate processing time from 06 days to 4.5 days and fiber phase was finished 7-10 days earlier. The mushroom yield attained 65.1% over the dry weight of the substrate and was 20% higher than that of the natural fermentation method.

CONCLUSIONS

- In production procedure of edible mushroom from lignocelluloses, the material precomposting process plays a very important role. The results from conducted experiments reveal that materials pretreated by biological methods are essential with many advantages such as reducing the amount of enzyme needed, having the high production efficiency, lowering the costs and reducing the pollution risk to environment.

- Cellulose content in cotton waste was identified as 62%.

- The optimal conditions for enzyme Ctec2 activity were determined, including temperature of 50^{o}C, pH 5.0, E/S ratio of 0.45% and substrate depredated in 60 hours.

- The new technological procedure for cultivation of oyster mushroom using enzyme Ctec2 was developed; in which mushroom yield attained 65.1% over substrate dry weight (only 43% for the control). The incubation time was shortened from 06 days to 4.5 days

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