Preliminary study to investigate cellulose biodegradability of Bacillus - Aspergillus and Neurospora Crassa on cassava peels (Manihot Esculenta)

T Tran1,2*, H H Loc2, N T V Ha3, L V Tan1,4, D A Le1 and L T A Hong5

1NTT Hi-Tech Institute, Nguyen Tat Thanh University, 300A Nguyen Tat Thanh, Ward 13, District 4, HCMC, 70000, Viet Nam.
2Faculty of Food and Environmental Engineering, 331 QL1A, An Phu Dong Ward, District 12, HCMC, 70000, Viet Nam
3Faculty of Environmental, Ho Chi Minh City University of Natural Resources and Environment, 236B Le Van Sy Street, Ward 1, Tan Binh, HCMC, 70000, Viet Nam.
4Department of Science and Technology of Ben Tre Province, 280 Street 3/2, Ward 3, Ben Tre. 86000, Viet Nam.
5Bioconversion technology, Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Hanoi Highway, Linh Trung Ward, Thu Duc District, HCMC, Viet Nam.

*Corresponding author: thanhtran2710@gmail.com

Abstract. Cassava peel holds an abundance of nutrients that could be utilized as fertilizer in agriculture. However, high cellulose contents make it hard for organic digestion, thus slow down the absorption for plants. In this study, we investigate microorganisms for effective cellulose degradation of cassava peel waste collected directly from Hung Duy Tapioca Starch Factory in Tay Ninh Province, Viet Nam. Experiments evaluate the decrease of mass cassava peel of the two groups of Bacillus - Aspergillus and positive control strains such as Neurospora Crassa. The sample was tested on Czapek - Dox liquid culture medium, a method of assessing the dry mass difference of samples used to assess biodegradability in 28 days. Besides, tests to assess the production of products and environmental parameters such as glucose and pH were recorded. Initial results showed that the combination of Bacillus and Aspergillus provides the highest glucose concentration, reached 16.52 mg/ml and digests 53% of the cassava mass compared with a combination of Neurospora Crassa reached 11.45 mg/ml and 46% of the cassava mass. Comparison results also showed that the action potential of microorganisms was stronger when combined and provided evidence that the formation of combinations of microorganisms would work well in probiotics, which brings better environmental treatment efficiency.

1. Introduction
Cassava (Manihot esculenta) is one of the most important crops in many tropical countries, including Vietnam. Cassava area in Viet Nam reached 560,000 ha in 2014, which nearly doubled the figure in 2000 [1]. The constant expansion has changed cassava role in Viet Nam from food crops to industrial crops. After the process of starch production, the plant has generated a huge amount of solid waste such as cassava peel which will be discarded everywhere without treatment. The volume of them so large because peel weight accounts for 10% of total volume so that the increase of the annual amount of peel waste and their accumulation is a major source of pollution which generates bad odor and increases the risk of disease outbreaks.
The problem of pollution from the industrial sector, agriculture or other sectors of the economy growing, the number of research that aims to improve the use of waste also occurs more conversion of cassava peels by the organism isolation means that these waste could be used as raw materials in industries for the value-added product. The proper use of these wastes as raw materials will significantly reduce generated waste and bring additional economic benefits. Although it is economically and technologically possible, emphasis should be put on the utilization of waste materials for recycling and upgrading purposes.

On the other hand, the involvement of agriculture perspective, cassava peel waste utilization as organic fertilizer would be very good while chemical fertilizer prices are increasing. Some studies show that organic amendments could be used for enhancing soil fertility, planting nutrient status, saving the cost of secondary and micro-nutrients requires for obtaining good yields and leads to less environmental pollution. Unlike chemical inorganic fertilizers, fertilizers, or decomposition products from agricultural residues often carry a large number of support microorganisms such as cellulose, soluble phosphorus, dissolved silicon should add more biodiversity or restore soil better. Consequently, it was proved to a significant increase in yield application on the rice crop [2, 3]. Thus, base on demand for organic fertilizers, the overall aim of the study is on the treatment and the utility of cassava peel waste into organic fertilizer. However, during the re-use of fertilizers, high cellulose content from the cassava peels (nearly 45%) is a major obstacle as it will significantly increase the treatment and biodegradation time, so the priority is to degrade the cellulose component in cassava peels.

Some studies show that cellulase enzymes from microorganisms such as fungi, bacteria have cellulose-degradation ability to cause significant changes in the nutritional composition of cassava peel waste like increasing glucose and nitrogen [4]. Beside, cellulase enzymes also help to reduce the breakdown time of cellulose than normal in the environment [5]. The ability of microbiology to generate cellulase enzymes depends on a very complex relationship involving many factors such as temperature, pH, inducer, environmental additive, agitation and culture time [6]. In addition, for practical application, the use of the activity of one strain may not be sufficient, but the combination of strains, Aransiola’s study also shows combination of other strains will generate variations efficacy including negative and positive [4], which can bring about an increased microbial activity such as more efficient treatment, better biomass growth, more enzyme generation ... due to its supportive action, however, it can also is less effective than when acting alone due to mutual antagonisms such as fungi secreting antibiotics that adversely affect the bacterial association.

Therefore, this study aims to look for bioprocessing solutions to treat and decompose cassava peels in the best possible time. As such, it is the next step after the isolation and selection of the best microorganism strains. More specifically, we tested the breakdown of cellulose by the cellulase enzyme activity of selected strains of microorganisms on the cassava peel with Neurospora Crassa, which is one of the best biodegradation microorganisms [7].

2. Materials and methods

2.1. Materials
Cassava peel was taken from Hung Duy 2 Cassava Starch Plant in Chau Thanh District, Tay Ninh Province. They were cut into pieces, which were crushed and sifted through a filter 0.154 mm. The substrates were then separated at room temperature in different foam boxes for further use.

The liquid medium used in the experiment is Czapek - dox (2015) [8] were prepared following the manufacturer’s specification, homogenized and after that sterilized by autoclaving at 121°C for 15 mins.

2.2. Biodegradation of substrates.
Thermostatic shaker IKA- Germany KS 4000 is used for the incubation of experimental samples. Biodegradation was proceeded by weighing 3g crushed cassava peel added 150 ml liquid medium in 250 ml flask. Each flask sterilized at 121 °C for 30 mins, then allowed to cool and added 100 µl liquid biomass of microorganisms, incubated at 28 ± 2 °C within 28 days. Finally, we have three flasks each
containing one microorganism is \textit{Aspergillus niger} (N), \textit{Aspergillus fumigatus} (F), \textit{Neurospora Crassa} (P), one flask has a pair of microorganism are \textit{Aspergillus fumigatus} and \textit{Bacillus Amyloliquefaciens} (FB) (there are 50 µl of Aspergillus and 50 µl of Bacillus enrichment solution), and one control flask (without microorganism added).

The evaluation of biodegradable by filtering sediment (cassava peels) and dried in an oven at 103 – 105 °C for 1 hour, 1 hour in the extraction hood and then weighed to determine the mass of cassava peels after biodegradation. The experiments were evaluated based on a cycle of 1 week for a total of 4 weeks, besides that, the test samples had 3 times repeatability (5 × 3 = 15 Erlenmeyer for a week, 60 Erlenmeyers for total four weeks).

2.3. \textit{Analytical method (Enzyme assay)}

Reducing sugar content was determined by Dinitrosalicylic Acid (DNS) colorimeter method of Miller (1959) [9]. A reaction mixture composed of 0.2 ml of crude enzyme solution was added to 1.8 ml of 0.5 % CMC in 50 mM sodium phosphate-buffered (pH 7). The mixture was incubated at 37°C in a shaking water bath for 30 mins. The reaction was terminated by adding 3ml of DNS reagent. The mixture was boiled for 5 mins, and the optical density of samples was measured at 540 nm against a blank containing all the reagents minus the crude enzyme. The process of culturing microorganisms generated cellulase enzymes which accelerated the degradation of cellulose to glucose. pH values were measured using a portable pH meter (HI98129).

3. \textit{Results and discussions}

3.1. \textit{Assessment of the change of pH and glucose}

Figure 1 shows that after 21 days the pH values of each sample tend to decrease. At week 4, while the pH value of FB sample still down, three remaining samples such as N, F, PMO have the pH value tends to increase. In which, sample F had the highest increase (from 2.45 to 5.24). Considering the beginning of the experiment, sample F is also the sample with the greatest ability to reduce pH value in the first week (from 7.08 to 3.57). This may be explained by when the fungus grows, the environment becomes acidified and the pH value decreases [10].

![Figure 1. pH of different tested microorganisms vs time](image)

\textit{(F = Aspergillus fumigatus, N = Aspergillus niger, FB = Aspergillus fumigatus and Bacillus amyloliquefaciens, PMO = Neurospora Crassa)}

The amount of glucose produced as a by-product of cellulose digestion in materials. Therefore, in this experiment, to assess the ability of cellulose substrate to decompose in cassava peel material, the amount of glucose generated as an indicator to evaluate and compare the ability of the microorganisms involved in the process decompose.
Figure 2. Glucose concentration of different tested microorganisms vs time

\( F = Aspergillus fumigatus, N = Aspergillus niger, PMO = Neurospora Crassa, FB = Aspergillus fumigatus and Bacillus amyloliquefaciens \)

After the first week, the glucose produced from different treatments had a difference, while the glucose from the FB combination gave the best results, the N sample that used Aspergillus niger was a strain with average cellulose degradation, showed the lowest results.

Sample FB after 28 days generated the largest amount of glucose, 16.52 ± 0.05 mg/ml, while sample Aspergillus niger (N) would produce the lowest glucose concentration only was 3.79 ± 0.03 mg/ml.

Additionally, the guesswork is glucose reductio at week 4 (after 28 days) which might accrue from the fermentation of glucose. Sample FB after four weeks still no sign of the decline of glucose concentration maybe it could not take part in the process of fermentation. Experimental results show that the pH value is inversely proportional to the amount of glucose produced by the decomposition of cellulose by the study of Aransiola et al [4]. Neurospora Crassa (PMO) is one of the microorganism strain capable of maximum biodegradation means that produces the relatively large amount of enzyme. Strains Aspergillus fumigatus (F) are also capable of average biodegradation, and the ability to produce the enzyme is also equal to the common strain as Trichoderma [7]. However, when combined with the Bacillus strain, it yielded a surprisingly mutually supportive outcome. As a result, cellulose substrate digestibility was improved over positive control strains over the same period.

3.2. Assessment the biodegradable cassava peel of selected microorganisms

The study had studied the mass of the cassava peel decreased after four-week experiments to assess the biodegradation effectiveness of the selected microorganism. The biomass loss is calculated based on the difference of dry weights of experimented cassava peel before and after four weeks of the experiment.

Figure 3 shows the decline of cassava peel weight each week. After four weeks, the biodegradation of sample FB containing was the best reached approximate 53% (at the beginning of the experiment, the weight was 3 g, and after four weeks the remained weight was 1.41 ± 0.04g). Three remaining samples containing PMO, F was 46% (the remained cassava peel weight was 1.63 ± 0.05g), 39% (which was 1.84 ± 0.07g) and Aspergillus niger (N) was the worst of the selected microorganism which biodegradation was about 33% (which was 1.99 ± 0.05g). Considering our previous experiments [11] with the volume of cassava peel input is 1g, the degradation of 3 samples PMO, F, N is 62%, 52%, 34%, respectively. Strain F are also capable of average biodegradation (12% biomass loss) compared to the commonly used as Trichoderma strain (14% biomass loss). But the research needs to be expanded because the difference in the rate of biodegradation is due to the change in the input volume.
According to the research of Shrestha et al. showed that *Neurospora Crassa* (PMO) is one of the microorganism strain capable of maximum biodegradation (18% biomass loss) [7]. However, it is the independent decomposition of each strain, which means that if in a combination of strains, the degradation capacity of the strains will be able to decrease or increase. We believe that the projects that use microorganisms strains to apply to accelerate the processing time in processing agricultural waste materials in the future will be very promising. And in practical terms, even though our efforts are often for a particular dominant species, there is a high possibility that there will be many microbial strains in which we have a hard time controlling it. Therefore, instead of using just one strain, we have the flexibility to combine a variety of different strains that are potentially more beneficial.

4. Conclusions
The combination of two types of fungi and bacteria FB (*Aspergillus fumigatus* and *Bacillus Amyloliquefaciens*) gave the best results that were higher than the positive control strains *Neurospora Crassa* (PMO). Besides, the combination strains *Aspergillus fumigatus* and *Bacillus Amyloliquefaciens* (FB) also proved to be better than single samples *Aspergillus fumigatus* (F) and *Aspergillus Niger* (N). With 3 g cassava peel input, the biodegradation ability of samples FB, PMO, F, N respectively 53%, 46%, 39%, 33% and reducing sugar reached 16.52, 11.45, 7.86, 6.52 mg/ml. The purpose of this manuscript henceforth is limited to a primary report on the interactions between the strains. The ongoing experiments include investigating the interactions by changing the rates of microorganisms to learn more about the interoperability and application of a combination of microorganisms in environmental treatment.

References
[1] Agromonitor 2015 Annual Report of Cassava starch industry in 2014 and prospects for 2015.
[2] Balescaron S, Đukić V, Marinković J, Dozet G, Petrović D K and Tatić M 2011 *Afr. J. Microbiol. Res.* 5 4909-16
[3] Bloemberg G V, Wijffjes A H, Lamers G E, Stuurman N and Lugtenberg B J 2000 *Mol. Plant. Microbe. In.* 13 1170-6
[4] Aransiola Michael N and Fagade Obasola E 2015 *Int. J. Plant. Sci. Ecol.* 1 124-30
[5] Schwarz W 2001 *Appl. Microbiol. Biot.* 56 634-49
[6] Immanuel G, Dhanusha R, Prema P and Palavesam A 2006 *Int. J. Environ. Sci. Te.* 3 25-34"
[7] Shrestha P, Ibáñez A B, Bauer S, Glassman S I, Szaro T M, Bruns T D and Taylor J W 2015 *Biotechnol. Biofuels.* **8** 38

[8] Abildgren M, Lund F, Thrane U and ELMHOLT S 1987 *Lett. Appl. Microbiol.* **5** 83-6

[9] Dashtban M, Maki M, Leung K T, Mao C and Qin W 2010 *Crit. Rev. Biotechnol.* **30** 302-9

[10] Narendranath N V and Power R 2005 *Appl. Environ. Microb.* **71** 2239-43

[11] Tran T, Minh V D, Son D V T, Hong L T A, Do H N, Duc N D, Huyen N T B and Ha N T V 2016 *J. Environ. Eng. Sci.* **B** 342-7