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Production of Cellulases by *Penicillium citrinum* grown on agricultural waste in solid-state fermentation

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Abstract
The production of extracellular cellulases by *Penicillium citrinum*, on agriculture waste materials was studied in solid-state fermentation (SSF). The objective of this research is to study on optimal conditions for cellulase production by *Penicillium citrinum*. The study determined a different concentration raw materials of culture conditions such as carbon and nitrogen source, moisture content, duration, citrate buffer extraction. Cellulase activity was determined by measuring the absorbance χ = 540 nm with 3, 5 – DNS reagent. In the optimal culture conditions, cellulase activity of *Penicillium citrinum* obtained 87.83 U/mL in enzyme activity. The results showed that the high-level cellulase activity was produced at 40% rice husk, 59% rice bran, 1% urea and 60% moisture content, in 4 days with 70mL citrate buffer for extraction. Thus, the enzyme activity would be increased by purification.

Keywords: cellulase, *Penicillium citrinum*, rice husk, rice bran urea, enzyme activity

1. Introduction
Agricultural wastes are causes of environmental problem in the world. Using these products can reduce the issues they cause. These wastes including cereals, leaves, corncobs, especially, rice husk and rice bran are highly underutilized in Asia, particularly Vietnam. These materials are mainly used as animal feeds and the number of these product are left to be decomposed by microorganism such as bacteria and fungi (Immanuel, Dhanusha *et al*. 2006). Economically, industrial interest in cellulases because this enzyme has widely application in various filed such as animal feed production, starch processing malting and brewing, grain alcohol fermentation, extraction of fruit and vegetable juices, paper and textiles (Adsul, Bastawde *et al*. 2007) [1].

Cellulose forms about 45-50% of plant composition and it also contains in these wastes in the environment (Ng, Li *et al*. 2010) [11]. Cellulases are a family of enzymes breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units. A cellulase system consists of three major components: endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). Exoglucanases (1, 4-β-D-glucancellobiohydrolase, EC 3.2.1.9.1) are usually act on crystalline cellulose and cleave disaccharide units. Endoglucanases (endo-1, 4-β-D-glucanase, EC 3.2.1.4) are subject to product likely cellubiose, glucose inhibition (Ma, Nguyen *et al*.). They can also hydrolyze substituted cellulose such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC). B-glucoidases (EC 3.2.1.21) hydrolyze cellobiose and short (soluble) cellooliogosaccharides to glucose (Ma, Nguyen *et al*.). In view of biotechnology, microbial production of cellulases interest to scientist because the production of cellulase has been Reported from a wide variety of bacteria (Immanuel, Dhanusha *et al*. 2006) and fungi (Singh, Singh *et al*. 2009) [14], (Sarao, Arora *et al*. 2010) [13]. Solid state fermentation (SSF) is fermentation process occurring in the without or near-absence of water and employing a natural substrate as a solid support. Many microorganisms are employed on solid substrates, filamentous fungi, especially, can develop to significant extent in the without water (Ng, Li *et al*. 2010) [11]. SSF has many merits over liquid-state fermentation, containing high volumetric productivity and concentration of products, less effluent generation and low catabolic repression (Bayer, Lamed *et al*. 2007, Ng, Li *et al*. 2010) [2, 11]. This method also prefers for producing fungal enzyme due to concentrate enzyme and low cost. Therefore, SSF are widely used in commercial production enzymes (Camassola and Dillon 2007, Khan, Ali *et al*. 2007, Nair, Sindhu *et al*. 2008) [3, 8, 10]. *Aspergillus* and *Trichoderma* spp. are well known efficient production of cellulases (van Peij, Gielkens *et al*. 1998) [16]. However, there is a very little information regarding the production of cellulase from *Penicillium* spp in SSF. To our knowledge, the present study is the report on *P. citrinum* for cellulase production. And, they also were demonstrated for their improvement of efficiency in the SSF for production of cellulase using byproduct of agriculture such as rice husk and rice bran in Vietnam as well as raw materials. The influence of various conditions was evaluated in SSF. Besides, the optimal culture conditions for cellulase from *P. citrinum* will also be investigated.

2. Materials and Methods
2.1 Sample preparation
The raw materials (rice husk and rice bran) were bought from Tien Giang province, Vietnam. These materials are agricultural byproducts in Vietnam. These materials are high quality, proper price, plenty of source from rice processing factories. *Penicillium citrinum* was supported by Laboratory

of Cell Biotechnology. The chemicals used for the analyses including $(NH_4)_2SO_4$, yeast extract, urea and tryptone were purchased from Merk or Sigma-Aldrich.

2.2 Methods

2.2.1 Solid state fermentation (SSF)

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks, containing rice husk and rice bran was prepared to follow table 1. The flasks were sterilized at 121ºC for 20 min before inoculating *Penicillium citrinum* with initial cell concentration around $5*10^8$ spore/ ml. The culture was mixed by gentle shaking.

2.2.2 Enzyme extraction

Byproduct from microorganisms as *Penicillium citrinum* was ground with citrate buffer within 5 minutes. The suspension is filtered by cloth to eliminate solids and get liquid solution. After that, liquid solution was centrifuged at 6000 rpm for 15 minutes and clear supernatant was used as a source of extracellular enzyme.

2.2.3 Enzyme assay

Cellulase activity was measured according to the method described by (Ghose 1987). One unit of cellulase activity is defined as amount of enzyme that release 1µmole of reducing sugar per minute with glucose standard. The values of enzyme activity were expressed as U/ml for SSF

2.2.4 Optimization of culture conditions for maximum cellulase production

2.2.4.1 Effect of carbon source for *Penicillium citrinum* on cellulase production

The study fixed $(NH_4)_2SO_4$ (1%), and changed carbon source from rice husk and rice bran. *P. citrinum* was cultured separately in same culture conditions. There were six kinds of conditions with different proportions of rice husk and rice bran. They were named X_1, X_2, X_3, X_4, X_5 and X_6 in order to increasing of rice husk and decreasing of rice bran content. Oriented medium was changed as following:

Table 1: The component nutrition for *Penicillium citrinum* Unit: percent (%)

Culture	X1	X2	X3	X4	X5	X6
Rice husk	20	25	30	35	40	45
Rice bran	79	74	69	64	59	54
$(NH_4)_2SO_4$	1	1	1	1	1	1

Moisture content is fixed in 60%. *Penicillium citrinum* ($5x10^8$ spore) was added in culture medium at room temperature (28-30ºC). Cellulase production of *Penicillium citrinum* was studied in 3 days.

2.2.4.2 Effect of various nitrogen sources on cellulase production

The study was fixed carbon source at optimal carbon source And changed nutrients source of nitrogen: $(NH_4)_2SO_4$, yeast extract, urea and tryptone, and fixed 60%.

2.2.4.3 Effect of moisture content on cellulase production

Water is essential for the growth and metabolism of all cells. If it is reduced or removed, cellulase activity is decreased. The form in which water exist within the food is important as far as microbial activity is concerned. Thus, all optimal conditions were fixed and changed moisture content in ranging: 48- 52- 56- 60- 64-68 %.

2.2.4.4 Effect of duration on cellulase production

Time is main role on cellulase production. Both too short and too long duration affect cellulase produced by microorganisms. Similarly, all optimal condition was fixed, only duration was changing: 3 – 4 – 5 – 6 – 7 – 8 days, at 30ºC.

2.2.5 Effect of citrate buffer content on extracted cellulase

The experiment observed citrate buffer content affect to enzyme recovery. So, adding citrate buffer (0.05M and pH 4.8) content is different as following: 3/1 (30 ml citrate buffer/10g byproduct); 5/1 (50 ml citrate buffer/10g byproduct); 7/1 (70ml citrate buffer/10g byproduct); 10/1 (100ml citrate buffer/10g of byproduct).

3. Results and Discussion

3.1 Effect of carbon sources on cellulase production

Carbon sources (rice husk and rice bran) significantly affected the cellulase production as shown in figure 1. Cellulase production was found to be dependent on the nature of carbon source in order to use in the culture medium. Especially, carbon is the main of nutrition developing fungi. The composition of rice husk was identified according to by (Johar, Ahmad *et al*. 2012). The result showed that these composition is approximately of 35% cellulose, 33% hemicelluloses, 23% lignin, 25% silica ash (Johar, Ahmad *et al*. 2012). Therefore, enzyme production also depends on the chemical composition of the raw materials. At first, microorganism would be produced enzyme because the medium culture was lacked monosaccharides, so *P. citrinum* needed to excrete enzymes in order to break down polysaccharides to monosaccharides. If the substrate is lowed concentration, the catalytic site of enzyme is empty, product can be limited. In contrast, if culture medium is contained highly carbon concentration, it also affects to produce enzyme. Because of after a certain concentration, increasing of substrate will have no effect on cellulase activity because the enzymes are saturated. In the study, moisture content and nitrogen source would be fixed. The properties of two substrates are varied in total 99% content while the rice husk percentage was increased, the bran was decreased.

Based on figure 1, the X5 of *P. citrinum* is the highest cellulase activity for 3 days, which gave cellulase activity a 30.92U/mL.

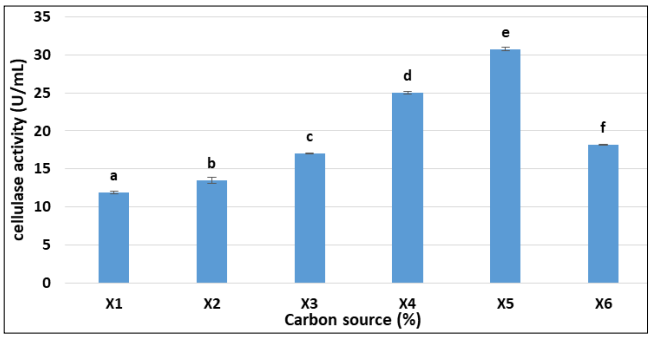

Fig 1: Effect of carbon source on cellulase production. Different letters (a, b, c, d, e, f) indicate statistically significantly differences ($p<0.05$).

3.2 Effect of various nitrogen sources on cellulase production

The effect of various nitrogen sources on cellulase production were variable, depending on the fungi and compound tested (Kachlishvili, Penninckx *et al*. 2006). Different concentration of nitrogen affected significantly cellulase production (Panagiotou, Kekos *et al*. 2003). In the sudy, observing different nitrogen sources to cellulase activity: yeast extract, tryptone, ammonium sulfate and urea. Maximum cellulase activity was obtained when urea was added. These data were in accordance to previous some reports (Veverka, Stolcova *et al*. 2007). Therefore, the optimal concentration of nitrogen source of *P. citrinum* was 1% of urea.

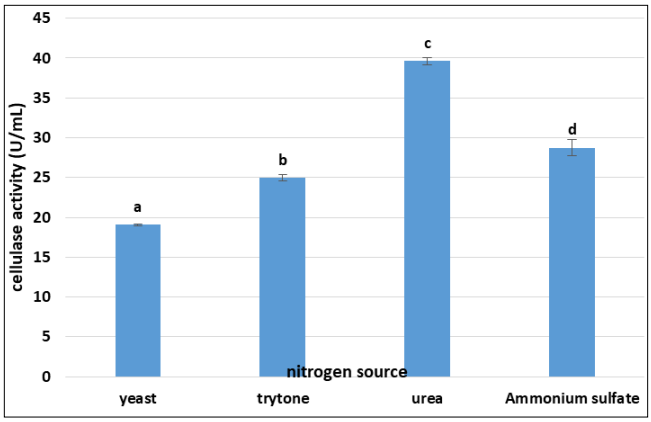

Fig 2: Effect of carbon source on cellulase production. Different letters (a, b, c, d) indicate statistically significantly differences ($p<0.05$).

3.3 Effect of moisture content on cellulase production

Moisture content is significantly important in solid - state fermentation because this necessary condition help microorganism's growth. Each species of microorganisms has their own specific moisture content to achieve the highest growth rate (Sukumaran, Singhania *et al*. 2009) [15]. The hydrolysis reaction between cellulose and cellulase requires the presence of water molecule. The determination of optimal moisture content for fungal activities is very necessary. In the study, at the beginning, cellulase activity is lower than other samples, *P. citrinum* need to more water in order to growth, the best moisture content for *P. citrinum* is 60%. However, too much water in the medium clumped and not benefit for growth of microorganism, which result decrease of enzyme production.

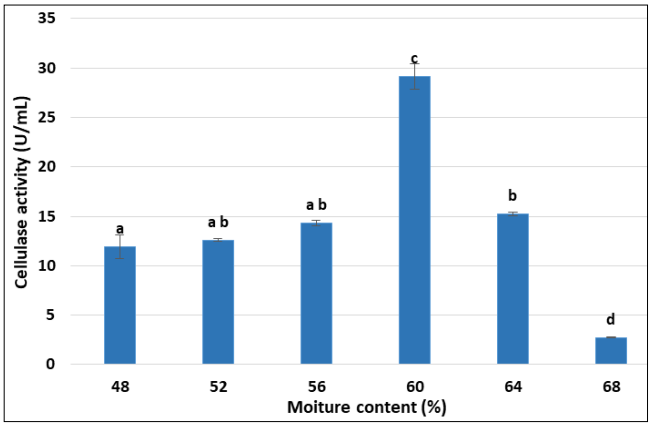

Fig 3: Effect of moisture content on cellulase production. Different letters (a, b, c, d) indicate statistically significantly differences ($p<0.05$).

3.4 Effect of duration on cellulase production

Similarly, by keeping the time and carbon, nitrogen sources at 30°C, the effect of the time on cellulase production is shown in figure 4. The result shows that the cellulase of the extracts with different durations were significantly different from each other. The highest cellulase activity of the extract was obtained at 4 days. At the beginning, the cellulase production of extract was lower than those of other samples because when fungi are added in culture medium, enzyme cannot produce at maximum level, they need to time to use minerals and monosaccharide in culture medium as well as nutrition source in lag phase. The first, fungi will try to adapt to culture and just a limited amount of enzyme was produced. In addition, the kind of enzyme depend on substrates. However, after the process reached a peak at a specific point of time, the cellulase activity decrease when the time continued to increase because this is a death phase containing some toxic effect to develop of fungi and lead to cell death.

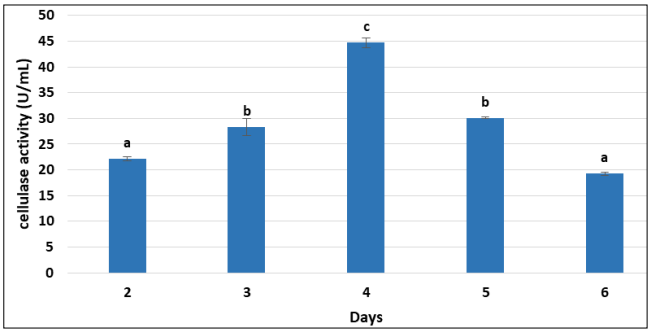

Fig 4: Effect of duration on cellulase production. Different letters (a, b, c) indicate statistically significantly differences ($p<0.05$).

3.5 Effect of buffer content on cellulase production

Citrate buffer (0.05M, pH = 4.8) is used to extract cellulase production. Same as many others aspect, citrate buffer should be added in a suitable proportion in order to maximize enzyme extracted. Enzyme would not be fully extracted if the citrate buffer is too low, on other hand, unnecessary high ratio of

citrate buffer could also prevent the enzyme to be extracted completely. By undertaking 5 experiments for each fungus with the ratio of citrate buffer over byproduct are varied on order of 3/1, 5/1, 7/1 and 10/1, the observed outcome has proven that optimum citrate buffer ratio for *P. citrinum* is 5/1 (50ml citrate buffer/ 10ml byproduct)

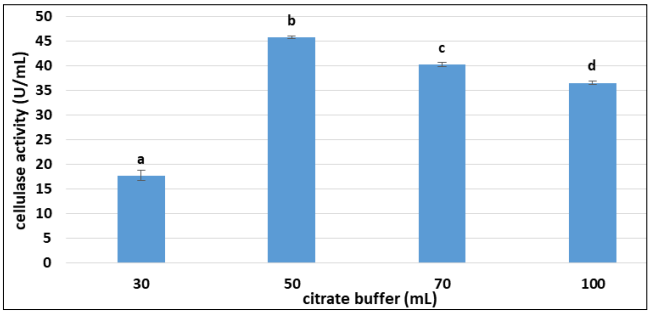

Fig 5: Effect of citrate buffer content on cellulase production. Different letters (a, b, c, d) indicate statistically significantly differences ($p<0.05$).

3.6 Optimization of culture conditions for a cellulase production *from P. citrinum*

With optimized incubation conditions, the enzyme activity of *P. citrinum* is 87.83 U/ml.

4. Conclusion

The study considered carbon, nitrogen source, moisture content, and duration, citrate buffer content that are significant influence to cellulase production. In the result, the optimum conditions for producing cellulase enzyme of *P. citrinum* including 4 days, 40% of rice husk and 59% of rice bran, 60% of humidity, 1% urea and ratio: 5/1 (50ml citrate buffer add 10g bio-product in order to extract highest enzyme production.).

5. Acknowledgements

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