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Production of Cellulase and Pectinase by Using Aspergillus Oryzae in Molasses and Their Application for the Extraction of Soluble Solid Content from Coffee

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ABSTRACT: *Cellulase and pectinase are two enzymes which catalyze the hydrolytic reactions to convert cellulose and pectin into β-glucose and galacturonic acid, respectively. Cellulase and pectinase are important factors in improving the extraction of soluble solid content, especially for instant coffee. The objective of this research is to study on optimal conditions for the production of cellulase and pectinase by using Aspergillus oryzae and primarily applied to coffee treatment forsoluble solid content extraction. Both enzymes were produced by incubating A. oryzae in molasses with the supplement of $(NH_4)_2SO_4$ and substrates (CMC, pectin). Cellulase and pectinase activities were determined by measuring the absorbance at λ = 540 nm with 3,5-DNS reagent. As the result, the highest cellulase activity was 27.99 U/mL and achieved at 6°Brix of concentration of total soluble solid in molasses, 0.5% of CMC as substrate, 1% of $(NH_4)_2SO_4$ as nitrogen source, initial pH 5.0, incubated in 50 mL falcons at 35°C for 72 hours. The highest pectinase activity was 19.49 U/mL and obtained at 8°Brix of concentration of total soluble solid in molasses, 1% of pectin as substrate, other conditions were as same as cellulase production. Moreover, those enzymes were treated on coffee beans to enhance the extraction of soluble solid content which was 4.87°Brix, increased 24.60% compared to the control sample. Enzymes were used at 4% and at 35°C for 20 hours. The results could be used as references for further studies or applied in coffee industry.*

Keywords: *Aspergillus oryzae, cellulase, coffee treatment, pectinase, soluble solid content.*

I. INTRODUCTION

Vietnam is one of the largest producers of coffee all over the world. However, the exported coffee products from Vietnam are mainly green and raw beans which are very cheap. It is necessary to diversify kinds of coffee products, for example instant coffee, weasel coffee or fermented coffee to bring more added value for farmer and producer. For years, many companies in Vietnam have been trying to develop those products. However, thistechnologyis facing an obstacle. The mucilage layer in coffee bean which help to protect them from harmful effectsof the environment, such as biological hazards or physical damage, but this layer are easy to absorb the water and cause spoilage by fungi. It also inhibits the extraction of soluble solid content during coffee processing, increases the duration needed for coffee treatment, and lowers quality of final products [1]. Enzymatic treatment is an effective way to remove mucilage layer which is constructed mainly by cellulose and pectin. By removing this layer, the duration for treatment is shortened and increased the soluble solid extraction [2]. Moreover, enzymes also workdeeply in the cells of coffee bean to breakdown cellulose and pectin in the cell wall and release more soluble solid content. Cellulase is used to catalyze cellulolysis reaction by hydrolyses the linkage in cellulose structure, especially the β-1,4-glycosidic linkages, and breakdown those polysaccharides into monosaccharides or oligosaccharides. Besides, pectinase is an enzyme which plays a main role in hydrolysis of pectin into galacturonic acid.

Aspergillus oryzae has been applied for hundreds years in producing fermented foods in many East Asia countries, such as Japan, Korea, and China. In Vietnam, *A. oryzae* plays an important role in the process of making "tươngbần" which is a special sauce making from soy bean and glutinous rice. For along time, this strain has beenused in food processing in many countries and it is showed that*A. oryzae* is considered worldwide as an ingredient for making foods and safe for human health. Moreover, it is well-characterized industrial microorganisms and considered as GRAS (Generally Regarded As Safe) by FDA [3], [4].

Molasses is by – product of sugar production. The sugar industry in Vietnam produced around 800,000 tons of molasses per year [5]. This amount of waste needs to be treated to low down the environmental effects.

This research used molasses to create cultured medium. It supplies nutrients for the growth of microorganism in order to produce enzymes by submerged state fermentation [6]. The production of cellulase and pectinase by microorganism is significantly affected by the composition of the cultured medium and the fermentation conditions [7]. Therefore, in this study, the optimal conditions for producing cellulase and pectinase by using *A. oryzae* in molasses such as, the concentration of total soluble solid in molasses and duration for incubation will becarried out. Besides, the optimal conditions on the application of enzymes on coffee bean, such as the duration of enzymatic treatment and concentration of enzymes to achieve the highest soluble solid extraction from coffeewill also beinvestigated.

II. MATERIALS AND METHODS

2.1 Materials

Aspergillus oryzae used in this study was supplied by Food Engineering Laboratory at International University – Vietnam National University – Ho Chi Minh City. The strain was isolated and it had ability to grow and to be reproduced.

Molasses was supplied by Xanh A Chau Company, 134/1/5A CachMang Thang Tam Street, Ward 10, District 3, Ho Chi Minh city, Vietnam.

Robusta coffee was provided by Nguyen Huy Hung Coffee Company Ltd, 427 Hung Vuong Street, ĐắkHà District,Kon Tum Province, Vietnam. Coffee beans were qualified in size and shape uniform, and they were not contaminated by insects, strange smell or broken beans.

2.2 Methods

2.2.1 Pretreatment of molasses

Crude molasses was diluted by distilled water until it reached 24^{o}Brix, then added 3.5 mL of concentrated sulfuric acid per 1 liter of crude molasses before shaking it at 125 rpm and at room temperature (25^{o}C) for 24 hours. After that, it was filtered by filter paper two times and adjusted to pH 5.5 by NaOH 6M. Then, sterilized it at 121^{o}C for 20 mins before used [8].

2.2.2 Investigating optimal conditions for producing cellulase and pectinase

First of all, *A. oryzae* was cultured on malt extract agar at 30^{o}C for 7 days until the population reached around 10^7 cells/ml. Then, the optimal conditions for producing cellulase and pectinase were investigated one by one, including the concentration of total soluble solid in molasses and duration for incubation. After that, microbial enzymes were produced and determined enzyme activity. Then, cellulase and pectinase were applied on coffee beans with the ratio 1:1 to investigate the optimal duration for treatment and concentration of enzymes to achieve the highest soluble solid content extraction from coffee.

2.2.3 Producing enzymes by submerged state fermentation

Cultured media was contained in 50 mL falcon and included 10 mL of molasses, 1% $(NH_4)_2SO_4$ for nitrogen source, 0.5% CMC (carboxymethyl-cellulose) for cellulase or 1% pectin for pectinase production. Then, it was sterilized at 121^{o}C for 20 mins before inoculating 1 mL of spore suspension of *A. oryzae* with initial cell concentration around 10^7 cells/ml. Finally, it was incubated at 35^{o}C. The effect of concentration of molasses was prepared from 2 to 12 with the interval of 2^{o}Brix. The initial pH was adjusted from 4 to 6 with the interval of 0.5. The duration for incubation was set at 24, 48, 72, 96 and 120 hours.

2.2.4 Determination of enzyme activity

Crude enzymes were harvested by centrifuging cultured falcons at 10,000 rpm, at room temperature for 30 mins. The supernatant was filtered by sterilized filtered paper with the pore size 0.2 µm to eliminate all microorganism remained in the medium.

Cellulase activity was measured based on the hydrolysis of CMC 1% by cellulase into glucose at 40^{o}C for 15 mins. Then, the absorbance (OD) was measured at λ = 540 nm [9].

Pectinase activity was calculated based on the hydrolysis of pectin 1% by pectinase into galacturonic acid at 40^{o}C for 5 mins. Then, the absorbance (OD) was measured at λ = 540 nm [10].

2.2.5 Treatment of enzymes on coffee beans

50 grams of coffee bean was washed and soaked in water for 2 hours. Then, the bean was treated by enzymes (6%). The ratio between cellulase and pectinase was 1:1. Changing the durations for enzyme treatment from 16 to 24 hours, with the interval of 2 hours to investigate optimal duration for treatment. Then, enzymes concentration was changed from 2 to 10% with the interval of 2% to investigate the optimal concentration of enzymes. All control and samples were incubated at 35^{o}C.

2.2.6 Measurement of soluble solid content

After treatment, coffee beans was washed three times to remove all remaining viscous layer. Then it was dried in oven at 60^{o}C for 6 hours, until the moisture content of coffee beans reached the initial moisture content of coffee beans before soaking in water. After that, it was roasted at 240^{o}C for 20 mins by coffee roaster and ground into powder by the miller.

10 grams of coffee powder was boiled by 70 mL of distilled water for 5 mins. Then, the coffee extract was filtered two times by filter paper to eliminate all residues remained before measuring concentration of soluble solid content by using refractometer.

2.2.7 Statistical analysis

The SPSS statistical program with One-way ANOVA method and Duncan standard ($\alpha \leq 0.05$) was used to analyze means, standard deviation of replications and significance of samples.

II. RESULTS AND DISCUSSION

3.1 Influence of total soluble solid in molasses on cellulase and pectinase production

Influence of concentration of total soluble solid in molasses on cellulase and pectinase activity is showed in Figure 1.The concentration of total soluble solid in molasses played a vital role in the growth of microorganisms, in this case is *A. oryzae*. If the concentration of total soluble solid in molasses was too high, the hypertonic environment could be occurred, the cell of microorganism would be dehydrated and shrunk. In contrast, the low concentration of total soluble solid in molasses could cause hypotonic in which the cell would absorb solution from the outer environment. The cell would be swollen and busted. Therefore, the study about concentration of total soluble solid in molasses should be carried out to achieve the isotonic environment which was ideal for microorganism to grow and reproduce [11]. Moreover, the total soluble solid in molasses supplied sugar for microorganism as a source of energy. As too less sugar, microorganism do not have enough energy for growth and reproduction. On the other hand, as too much sugar, microbes will not produce the expected products, in this case is enzyme.

After incubating cultures at 35^{o}C for 72 hours, crude enzymes were harvested and tested for enzyme activity. It was shown that the highest cellulase activity was 9.15 ± 0.117 (U/mL) at 6^{o}Brix, and the highest pectinase activity was 8.51 ± 0.052 (U/mL) at 8^{o}Brix.

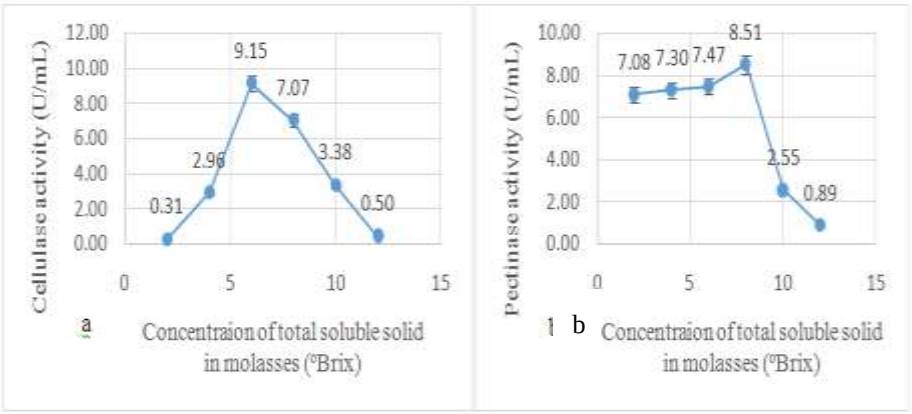

Figure 1: Influence of concentration of total soluble solid in molasses on cellulase production (a) and pectinase production (b).

3.2 Influence of duration of incubation on cellulase and pectinase production

The influence of duration of incubation on cellulase and pectinase activity is showed in Figure 2. The duration of incubation also played an important role in the production of cellulase and pectinase by using *A. oryzae*. The too short duration might be not enough for microorganism to adapt with new medium environment. Moreover, at early stage, microorganism just grew and increased in biomass, not producing enzymes [12]. The too long duration might cause the decreasing of nutrient sources, so microorganism had to use other nutrients, such as cellulose or pectin for growing. Thus, enzymes activity also decreased since they had to hydrolyze the substrates to create source of energy. Therefore, in both cases, the production of microbial enzymes was changed. It was necessary to find out the optimal duration for incubation to achieve the highest enzyme production.

This study found out that the optimal duration for incubation for both cellulase and pectinase production was 72 hours. The highest enzyme activity of cellulase was 27.99±2.33 (U/mL) and pectinase was 19.49±0.35 (U/mL). This duration was optimal for *A. oryzae* in both growing and producing enzymes.

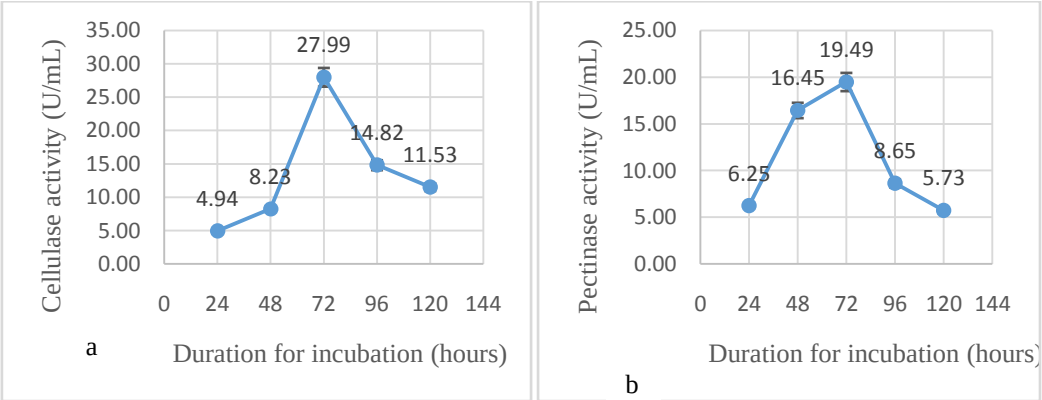

Figure 2: Influence of incubation duration on cellulase production (a) and pectinase production (b)

3.3 Application of cellulase and pectinase on coffee treatment for soluble solid content extraction

3.3.1 Influence of duration of enzymatic treatment on soluble solid content extraction

This experiment was designed to investigate the optimal duration for coffee treatment by enzymes on extraction of soluble solid content. Samples were prepared at constant 6% of crude enzymes with the ratio between cellulase and pectinase was 1:1, but the duration for enzyme treatment was changed from 16 to 24 hours with the 2 hours interval. All samples were incubated at 35^{o}C.

After incubation, coffee bean was washed three times, dried in oven at 60^{o}C for 6 hours, roasted and ground into powder before measuring soluble solid content by using refractometer. The result is shown in Figure 3, the highest soluble solid content extraction was 4.105^{o}Brix, obtained at 20 hours of treatment. The too short duration might be not enough time for enzymes to work. On the other hand, when the treatment happened too long, enzymes worked much and soluble solid content could be gone out and dissolved into the medium for a loss. Therefore, the soluble solid content in coffee was decreased.

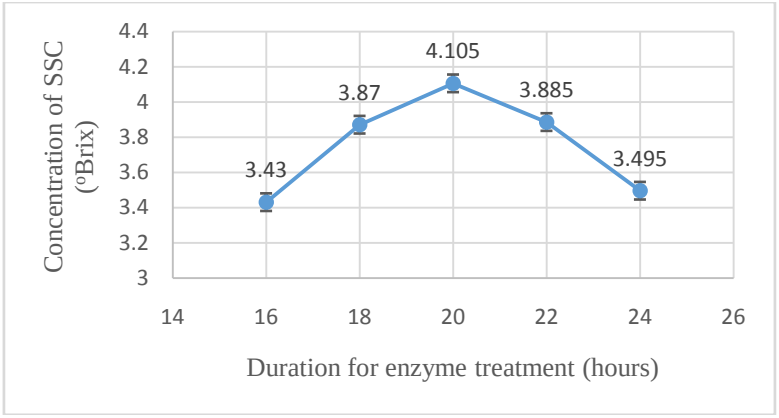

Figure 3: Influence of duration on enzyme treatment on soluble solid content extraction

3.3.2 Influence of concentration of enzymes on soluble solid content extraction

The optimal duration for enzyme treatment was obtained from the previous experiment. Samples were prepared at 2, 4, 6, 8, 10% of crude enzymes, with the ratio between cellulase and pectinase was 1:1. The control was sample without enzyme treatment. All controls and samples were incubated at 35^{o}C for 20 hours. After incubation, coffee bean was washed three times, dried in oven at 60^{o}C for 6 hours, roasted and ground into powder before measuring brix concentration. The result is showed in Figure 4, highest concentration of soluble solid content was 4.87^{o}Brix, increased 24.60% compared to the control sample and extracted in sample treated by 4% of crude enzymes. To explain for this result, less than 4% of crude enzymes might not enough quantity for the extraction of soluble solid content while more than 4%, enzymes would dissolved soluble solid content into the medium, the soluble solid content remained inside the coffee beans would be decreased.

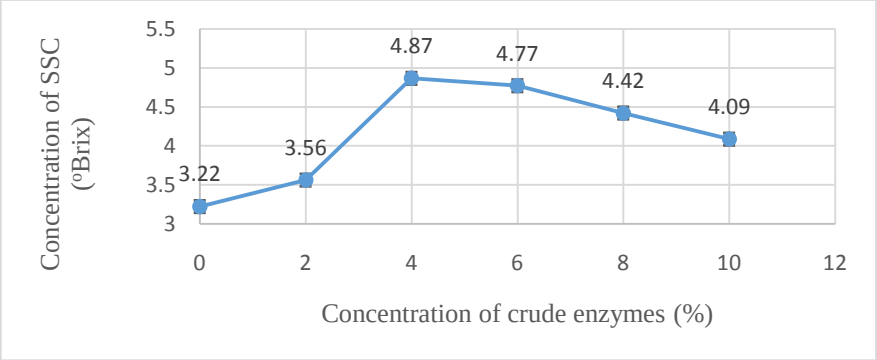

Figure 4: Influence of concentration of enzyme on soluble solid extraction

III. CONCLUSION

Optimal conditions for producing cellulase and pectinase by *A. oryzae* and optimal conditions on the application of enzymes on coffee bean to increase soluble solid extraction have been successfully investigated. The highest cellulase activity was 27.99 U/mL and achieved at 6°Brix of molasses concentration, 0.5% of CMC as substrate, 1% of $(NH_4)_2SO_4$ as nitrogen source, initial pH 5.0, incubated in 50 mL falcons at 35°C for 72 hours. The highest pectinase activity was 19.49 U/mL and obtained at 8°Brix of molasses concentration, 1% of pectin as substrate, 1% of $(NH_4)_2SO_4$ as nitrogen source, initial pH 5.0, incubated in 50 mL falcons at 35°C for 72 hours. The highest concentration of soluble solid content was 4.87°Brix, increased 24.60% compared to the control sample. This value was obtained at 4% of crude enzymes treated, incubated at 35°C for 20 hours. The results of this research can be used as references for further studies or applied in the coffee industry.

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