OPEN ACCESS

International
Journal
Of Modern Engineering Research (IJMER)

Pectinase Production By Bacillus Subtilis on Different Fermentation Modes And Its Application on Coffee Treatment

Quynh X. T. Nguyen [(1)], Huong T. T. Tran [(2)], Duy Q. Nguyen [(3)], Nhu B. Ma [(4)], Phu H. Le [(5)]

[1,2,3,4,5](School of Biotechnology, International University, Vietnam National University in HCMC, Vietnam)
[5](Center of Research and Technology Transfer, International University, Vietnam National University in HCMC, Vietnam.
Corresponding Author: Quynh X. T. Nguyen

ABSTRACT: *Pectinase is a group of enzymes that degrades pectin substances. This enzyme can be produced by microbial fermentation. Pectinase is important factor in improving the extraction of soluble solid content, especially for instant coffee. The objective of this research is to study on optimal mode of fermentation to produce pectinase by using Bacillus subtilis and investigate conditions of coffee treatment with this enzyme for the highest soluble solid content extraction. Pectinase was produced by bacterial fermentation on solid, semi-solid and submerged states. The enzyme activity was determined by anthrone assay. As the result, the highest pectinase activity at 125.60±6.547 (U/ml) achieved from molasses submerged mode with the supplement of 1% of $(NH_4)_2SO_4$ and 2% of pectin, initial pH 4.5 incubated at 37°C for 6 days. For investigation on optimal conditions for coffee treatment, the highest soluble solid content was 4.26±0.02 (°Brix) with 55% moisture content of coffee beans, 8% of enzyme ratio and incubated at 35°C for 24 hours. This result could be used as reference for further studies of applied on coffee production.*

Keywords: *Bacillus subtilis, coffee treatment, semi-solid, submerged fermentation, solid state, soluble solid content, pectinase.*

I. INTRODUCTION

Coffee is the second most consumed beverage all over the world after tea. Hence, for health and economic benefits, coffee should be researched and applied to increase the quality and diversity of coffee products. There are about 3% of pectin content in green coffee beans [1], this component inhibits extraction of total soluble solid content and absorbs the water easily, causing spoilage by fungi during storage duration. To solve this problem, during fermentation, green coffee beans are treated with pectinase that catalyze s the degradation of pectin structure inside the beans. Pectinase is a group of enzyme that can hydrolyze pectin in the cell wall into galacturonic acid through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions [2] leading to release more extraction of soluble solid content. The production of pectinase has been reported from a wide variety of bacteria, yeast, and fungi [3] by fermentation method. Fermentation has been classified into two main kinds based on the type of substrate used during fermentation: submerged fermentation and solid-state fermentation [4].

Bacilli are one of major importances in the fermentation industry since they elaborate a variety of useful enzymes and antibiotics [4]. Hence, this study observed *B. subtilis* for pectinase production. The pectinase production by filamentous fungi in solid-state fermentation and submerged fermentation has been studied extensively [5]. From this print of view, the study used fermentation for production of pectinase using cheap byproducts of agriculture (rice husk, rice bran). And molasses is a byproduct of sugar production in Vietnam as raw material. Besides, the optimal culture conditions for pectinase from *B. subtilis* will also be investigated.

II. MATERIALS AND METHODS

2.1 Materials

Vietnam Type Culture Center from Ha Noi, Vietnam supplied Bacillus subtilis. The accession number was VTCC-B-1322 and scientific name was *Bacillus subtilis subsp. subtilis*. This bacterium is Gram-positive soil microbe. It was activated and growth at 35°C.

Molasses, rice bran and rice husk were collected from manufacturing factories at Ho Chi Minh city. Robusta coffee beans were purchased from Nguyen Huy Hung Company at Dak Ha district, Kon Tum province, Vietnam. Qualities of the beans were uniform about size and shape. There was no contamination of insects and broken beans.

2.2 Methods

2.2.1 Pretreatment of molasses.

Crude molasses was diluted to 9^o Brix, and then added 3.5 mL of concentrated sulfuric acid per 1 liter of crude molasses by shaking it at 25^oC, 125 rpm. After 24 hours, filtering molasses twice and adjusting the filtrate with NaOH 6M until pH was 4.5. Finally, the treated molasses was sterilized at 121^oC for 15 minutes.

2.2.2 Submerged fermentation mode (molasses).

In 50 mL falcon, cultured media contained 10 mL of molasses, 1% $(NH_4)_2SO_4$ for nitrogen source, pectin concentration 2% and initial pH was at 4.5. After sterilizing at 121^oC for 15 minutes, adding concentration of starter culture (around 10^7 cells/ml) into the culture. It was incubated at 37^oC and duration was 6 days.

2.2.3 Solid-state fermentation mode (rice bran and rice husk).

In 250 mL flask, culture media contained 75% rice bran, 21% rice husk, 1% D-glucose, 1% ammonium sulfate, 2% of pectin. Moisture content was fixed at 60%. The Erlenmeyer flask was sterilized at 121^oC for 15 minutes. The concentration of starter culture was 10^7 cells/g and then the culture was incubated at 37^oC for 6 days.

2.2.4 Semi-solid fermentation mode (rice bran, rice husk with molasses).

In 250 mL flask, culture media included 70% rice bran, 17% rice husk, 10% treated molasses, 1% ammonium sulfate, 2% of pectin. Moisture content was fixed at 80%. The Erlenmeyer flask was sterilized at 121^oC for 15 minutes. The concentration of starter culture was around 10^7 cells/g and it was incubated at 37^oC for 6 days.

2.2.5 Crude enzyme extraction.

Solid and semi-solid state fermentation modes were mixed with citrate buffer (0.05M, pH 4.8 and ratio: 1:7 w/v), shaken, and then ground well by mortar and pestle for 10 minutes. The mixture was filtered by filter paper 3 times to eliminate the biomass. The centrifuge was followed at 6.000 rpm for 20 minutes at 4^oC. The clear supernatant broth is used as crude enzyme and stored at 4^oC in fridge for coffee treatment [8].

In submerged fermentation, after finishing the incubation, all the falcons were centrifuged at 10,000 rpm and at room temperature for 30 minutes. The crude enzyme was harvested by filtering the supernatant by sterilized membrane filter with the pore size 0.2 µm to eliminate all microorganisms remained in the medium. The supernatant was collected as a source of crude enzyme [9].

2.2.6 Determination of pectinase activity. [10]

Pectinase activity was determined by anthrone assay. One unit (U) is defined as an amount of pectinase, which converts 1 mL pectin into galacturonic acid for 60 minutes under standard conditions. Standard conditions for determining the pectinase activity were: 60 minutes; 30°C; pH 3.9 – 4.1 (substrate + enzyme). Pectinase activity was determined by spectrophotometer with anthrone reagent at OD (584 nm).

2.2.7 Investigation on optimal conditions for highest soluble solid content extraction.

After experiment 2.2.6 was done, the highest pectinase activity was chosen to treat coffee beans for increasing the extraction of soluble solid content.

- ***Effect of initial moisture content of coffee beans.***

50 grams of coffee beans was soaked in water until moisture content reaches: 40, 45, 50, 55 and 60%. Then the beans were treated by 6% pectinase (v/w) and incubated at 35^oC for 32 hours.

- ***Effect of the crude enzyme ratio in coffee treatment.***

50 grams of coffee beans was soaked in water to get the optimal moisture content (result from previous experiment). The beans were treated by pectinase at 5 different ratios: 2, 4, 6, 8, 10%. The control was sample without enzyme treatment. All control and samples were incubated at 35^oC for 32 hours.

- ***Effect of treatment duration on soluble solid content extraction.***

50 grams of coffee beans was soaked in water to get the optimal moisture content then treated by pectinase with the optimal ratio (result from previous experiments). Then the samples were incubated at 35^oC in 5 different durations: 16, 24, 32, 40, 48 hours.

2.2.8 Measuring the total soluble solid and caffeine content.

After the treatment, washed coffee beans were dried in oven at 60^oC until reaching initial moisture content (7.7%). Then dried coffee beans were roasted at 240^oC for 15 minutes and ground into powder. 10 grams of coffee powder was put into a beaker and 70 mL of boiled distilled water was added. The mixture was shaken at 175 rpm for 10 minutes. After the samples cooled down, they were filtered by filter paper to get supernatant. Finally, the soluble solid content of extracted solution was measured Brix degree by refractometer.

After all, coffee powder of 3 samples (no enzyme treatment, fermentation by the crude pectinase and fermentation by Viscozyme L) was sent to QUATEST 3 (64 Le Hong Phong street, District 5, HCMC) to check for caffeine content by the TCVN 9723 : 2013 (Vietnamese standard).

2.2.10 Statistical analysis.

The SPSS statistical program with One-way and Duncan Standard were used to analyze the data ($p < 0.05$)

III. RESULTS AND DISCUSSIONS

3.1 Influence of different fermentation modes on pectinase production.

In this study, the pectinase activity of Viscozyme L - a commercial enzyme was positive control. As shown in table 3.1, the result showed that mode of fermentation had significant effects on pectinase activity. The pectinase activity of Viscozyme L was smaller than Islam et al., 2013 (139.5 U/mL) [11] but the difference was not much. Beside that crude enzyme of molasses medium had pectinase activity at 125.60±6.55 (U/mL). The lower pectinase activity was 48.84±2.62 (U/mL) from semi-solid state fermentation mode. The solid state mode produced crude enzyme which its pectinase activity was 105.96±8.97 (U/mL).

This result was explained based on different modes of fermentation. In submerged mode, substrates were free flowing liquid utilized rapidly and the enzyme was secreted into the fermentation broth. High water content was ideal for bacteria since bacterial cells were evenly distributed throughout the broth. On the other hand, solid and semi-solid were surface fermentation. Those fermentation techniques were suitable for fungi and microorganisms that require less moisture content, which was particularly appropriated for mold. Hence, the control of moisture content was an important parameter in solid and semi-solid fermentation modes. In the result, the crude pectinase of semi-solid state fermentation had lowest enzymatic activity. In could be explained that too high moisture content had negative consequences for growth, as the porosity of the medium and oxygen diffusion are reduced [12]. In conclusion, the optimal moisture content could help to bring the highest pectinase activity from solid state fermentation mode.

Table 3.1: Pectinase activity of crude enzyme from 3 different modes of fermentation and Viscozyme L

No	*Fermentation types*	*Pectinase activity (U/mL)*
1	Solid state	$105.96±8.97^{b}$
2	Semi-solid state	$48.84±2.62^{a}$
3	Submerged state	$125.60±6.55^{c}$
4	Viscozyme L (possitive control)	$134.93±1.84^{d}$

^{a,b,c,d}Values are means ± standard deviations of duplicate measurements. Different letter in the same column indicate a significant difference ($p < 0.05$)

3.2 Influence of initial moisture content of coffee beans on extraction of total soluble solid content.

As the result shown in figure 3.2, the soluble solid content increased significantly and gradually from 3.25^{o}Brix at 40% moisture content to 3.80^{o}Brix at 55% moisture content. After reaching the highest point, which is 3.80^{o}Brix, soluble solid content decreased to 2.72^{o}Brix at 60% moisture content, which was the lowest one. To explain this result, pectinase is a hydrolysis enzyme thus coffee beans could not absorb enzyme if the moisture content of the beans is too low. Besides that, with high moisture content, molecular structure of pectin is loosen leading to these molecules are broken down easily. However, if the moisture content is too high, the soluble solid content will be released to the medium during soaking duration. In conclusion, after reaching the optimal moisture content the soluble solid content will be decreased.

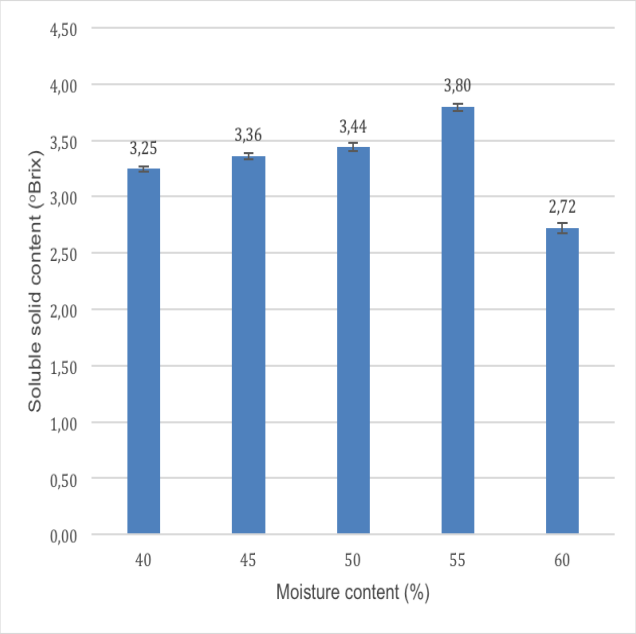

Figure 3.1: *Effect of changing moisture content to soluble solid content.*

3.3 Influence of the crude enzyme ratio on extraction of total soluble solid content.

Figure 3.2 shows that soluble solid content was highest with 8% of pectinase ratio, which was 3.97°Brix. On the other hand, with 10% concentration of pectinase, soluble solid content was lowest at 3.68°Brix. From 2% to 6% concentration of pectinase, there was a significant increase ($p<0.05$) in soluble solid content, which was corresponding to 3.48, 3.61 and 3.77°Brix. Pectinase plays an important role in degrading pectin structure to bring more soluble solid content during coffee fermentation. If there was low concentration of pectinase, it would not enough to hydrolyze pectin contents in coffee beans. However, with a high pectinase ratio, the enzyme could degrade and dissolve soluble solid content inside coffee beans in the water. Therefore, after reaching the optimal ratio, the soluble solid content went down.

In this study, optimal concentration of crude enzyme was 2% instead of 8%. Because, at 2% ratio, the achieved soluble solid content was high enough for commercial uses. In comparison with 8%, 2% brings more economic effect, which is saving a huge sum of money for the manufacturer.

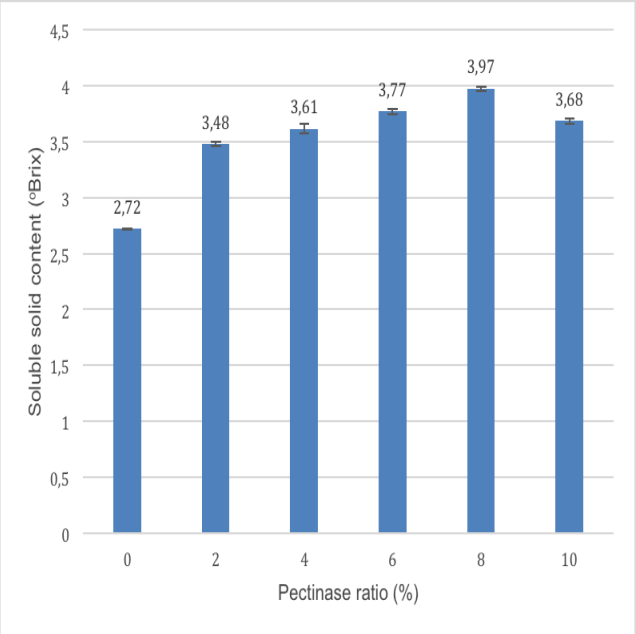

Figure 3.2: Effect of crude enzyme ratio to soluble solid content.

3.4 Influence of treatment duration on extraction of total soluble solid content.

As the results, the soluble solid content of treated samples at 16 hours and 24 hours were 4.07 and 4.26^{o}Brix. After that, trend of decreasing soluble solid content was observed, as at 3.94, 3.39, 2.72^{o}Brix corresponding to 32, 40 and 48 hours. It can be explained by the effect of reaction time to soluble solid content. If the duration is too short, the enzyme will not have enough time to degrade pectin molecules leading to lower concentration of extracted soluble solid content. On the contrast, with too long fermentation duration, the amount of soluble solid content can be released and dissolved to the medium. Therefore the optimal duration brings highest soluble solid content during coffee fermentation.

In other research, the optimal fermentation duration was 20 hours for highest extracted soluble solid content (Duy Q. N., 2016). It could be explained in that research, there was not only pectinase, but also cellulose that played a role in the treatment.

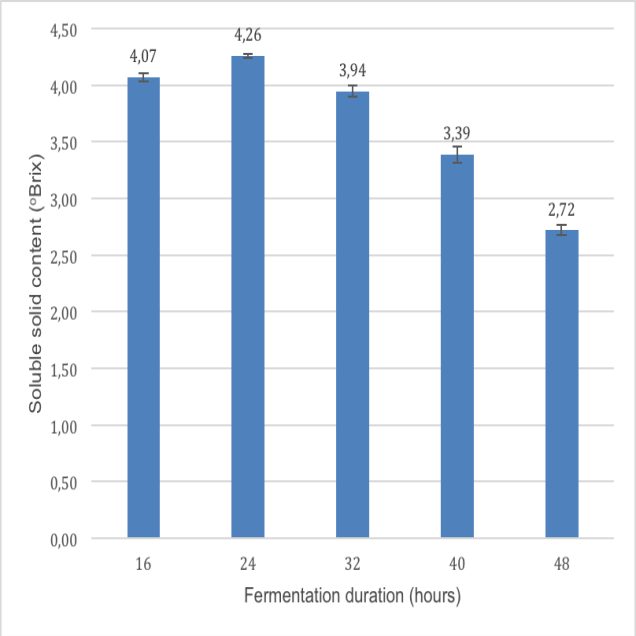

Figure 3.3: Effect of changing fermentation duration to soluble solid content.

IV. CONCLUSION

The optimal mode of fermentation for pectinase production by *Bacillus subtilis* was successfully defined. The highest pectinase activity was 125.60±6.55 (U/mL) at submerged fermentation by using molasses. The survey on optimal conditions for coffee fermentation by using the crude pectinase were carried as the highest soluble solid content was 4.26^{O}Brix(respectively, $p<0.05$) from the sample treated at 55% moisture content of coffee beans, 8% (v/w) concentration of enzyme at 35^{o}C for 24 hours. The product had standard caffeine content which was 1.6 (g/100g). The green coffee beans were treated by enzyme extracted from *Bacillus subtilis* so there was no concern about Aflatoxin. Therefore, the crude pectinase can be applied to produce coffee products with improvement of quality, safety and reducing cost, environmental problems.

ACKNOWLEDGEMENTS

We would like to express my gratitude to International University – Vietnam National University in Ho Chi Minh City and Kontum province for helping and supporting in this project.

REFERENCES

[1]. Clarke R. J., Coffee, volume 2: Technology, 64,1987
[2]. Pedrolli D. B., Monteiro A. C., Gomes E. and Carmona E. C., Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes, The Open Biotechnology Journal, 2009, 3, 9-18.
[3]. Sunnotel and Nigam P., Pectinolytic activity of bacteria isolated from soil and two fungal strains during submerged fermentation, School of Biomedical Sciences, University of Ulster, Coleraine-BT52 1SA, Northern Ireland, UK, 2002.
[4]. David Dubnau, Molecular Biology of the Bacilli: *Bacillus subtilis*. Published by Academic Press Inc, ISBN 10: 0122227018, 1982
[5]. Subramaniyam R. and Vimala R., Solid state and submerged fermentation for the production of bioactive substances: a comparative study, International Journal of Science and Nature, 2012, 3, 480-486.
[6]. Dao Q T. T., Phu H. L., Enzyme and Food fermentation – Laboratory manual. Food Technology department, School of Biotechnology, International University – Vietnam National University – Ho Chi Minh city, 2014

[7]. Duy Q. N., Study on the production of cellulase and pectinase by using *Aspergillus oryzae* in molasses and their application for the extraction of soluble solid content from coffee. Food Technology department, school of Biotechnology, International University – Vietnam National University – Ho Chi Minh city, 2016.
[8]. Phu H. L., Production of microbial enzymes pectinase, cellulase and their application on production of green coffee by fermentation PhD. Thesis, University of Science – Vietnam National University in Ho Chi Minh City, Vietnam, 2012
[9]. Islam S., Feroza B., Alam A.K.M. R. and Begum S., Pectinase production by *Aspergillus niger* isolated from decomposed apple skin. Bangladesh J. Sci. Ind. Res, 2013.
[10]. Dênis Silva, Kivia Tokuioshi, Eduardo da Silva Martins, Roberto Da Silva, Eleni Gomes, Production of pectinase by solid-state fermentation with *Penicillium viridicatum* RFC3. Laboratorio de Bioquımica e Microbiologia Aplicada, Instituto de Biocieˆncias Letras e Cieˆncias Exatas (IBILCE), Universidade Estadual Paulista (UNESP), Rua Cristovao Colombo, Sao Paulo, 2014.
[11]. Duy Q. N., Study on the production of cellulase and pectinase by using *Aspergillus oryzae* in molasses and their application for the extraction of soluble solid content from coffee. Food Technology department, school of Biotechnology, International U *Corresponding Author* niversity – Vietnam National University – Ho Chi Minh city, 2016 *Corresponding Author*

Quynh X. T. Nguyen . "Pectinase Production By Bacillus Subtilis on Different Fermentation Modes And Its Application on Coffee Treatment." International Journal Of Modern Engineering Research (IJMER), vol. 07, no. 09, 2017, pp. 50–55.