International Journal of Food Science and Nutrition
www.foodsciencejournal.com
ISSN: 2455-4898
Received: 14-08-2021, Accepted: 29-08-2021, Published: 14-09-2021
Volume 6, Issue 5, 2021, Page No. 14-18

Study on co-culture *(Aspergillus oryzae* and *Bacillus subtilis)* for husk removal in white pepper production

Huong T T Tran[1], Duy Q Nguyen[2], Quynh X T Nguyen[1], Phu H Le[3*]

[1] School of Biotechnology, International University, Vietnam National University in HCMC, Vietnam

[2] Department of Food Technology, Faculty of Chemical and Food Technology, Ho Chi Minh City University of Technology and Education (HCMUTE), Vietnam

[3] Department of Food Technology, School of Biotechnology, International University, Vietnam National University in Ho Chi Minh City, Vietnam

Abstract

This study was aimed to use microbial method to synthesize cellulase and pectinase by co-culture between *Aspergillus oryzae* and *Bacillus subtilis*. Then, bioproduct was appllied in white pepper production for highest percentage of husk removal. The co-culture between *A. oryzae* and *B. subtilis* resulted in various interactions that affect their growth, metabolism and differentiation. The most potential cellulase and pectinase producers were selected for studying their cellulase and pectinase productivities on rice bran and rice husk using solid state fermentation (SSF). The conditions for black pepper fermentation which included ratio between two microbes, temperature, fermentation duration and ratio of bioproduct were investigated. This application of bioproduct in pepper fermentation brought very high efficiency in peeling black peppercorn. Using 25% ratio of bioproduct with the ratio 1:3 for spores of *A. oryzae* and living cells of *B. subtilis*, moisture content was at 60%; black pepper was almost peeled totally up to 99.34 ± 0.48% during 60 hours fermentation.

Keywords: *Aspergillus oryzae, Bacillus subtilis*, cellulase, husk removal, pectinase, white pepper

Introduction

Peppers are known as the king of species and master of spice that can be processed to give four different types: black, white, green, and red pepper. White pepper is obtained by removing the outer skin, namely pericarp and outer portion of the mesocarp of the ripe or matured green berries or dried black pepper. White pepper is a value-added form of black one. The difference between the price of black pepper and white pepper is explained by the 30% of yield for the production of white pepper, against 70% yield for the production of black pepper [1]. It possesses a mild flavor and pungency as compared to black pepper [2]. Therefore, there is a growing demand for white pepper in the markets worldwide. Vietnam is the largest producer of pepper in the world followed by India and Indonesia. The principal of white pepper production is to remove skin from black pepper. Because the outer skin of black pepper is plant cell wall, its main component is polysaccharide (90%) consisting of cellulose, hemicellulose and pectin [3]. Based on this, some alternative processes including mechanical, chemical or biological methods that are used for removing the outer pepper from the seed. This study based on co-culture between *A. oryzae* and *B. subtilis* to degrade pectin and cellulose on the skin of black pepper during fermentation under aerobic condition. Interaction between microbes affects the growth, metabolism and differentiation of members of the microbial community [4]. These bacterial-fungal interactions often result in unique contributions to biogeochemical cycles and biotechnological processes. Thus, the interactions between bacteria and fungi are of central importance to numerous biological questions in agriculture, forestry, environmental science, food production, and medicine [5]. This interaction might subsequently enhance the cellulase and pectinase production from microorganisms to produce white pepper. Using co-culture cost effective and yield superior quality white pepper within a short duration of time as compared to the traditional method.

A. oryzae belongs to the *Aspergillus* which is a genus of mold species and defined as a group of conidial fungi. *A. oryzae* has ability to excrete large amount of correctly folded enzymes into extracellular medium [6] and plays a vital role in production of many commercial enzymes, such as α-amylase, lipase, protease, cellulase and pectinase. Since the ancient, *A. oryzae* has been applied in producing fermented foods in many East Asia countries such as Japan, Korea, and China. For a long time, *A. oryzae* was used in food processing in many countries and it is considered as a worldwide ingredient for making foods and safe for human health. Therefore, this species is well-characterized industrial microorganisms and considered as GRAS [7].

B. subtilis is a Gram-positive bacterium. The genus *Bacillus* comprises a diverse and commercially useful variety of species widely distributed in nature [8]. Due to its excellent fermentation properties, with high product yields (20 to 25 gram per liter) it is used to produce various enzymes, such as pectinase, amylase and proteases [9]. The Food and Drug Administration stated the enzymes derived from the *B. subtilis* strain were in common use in food and that no toxigenic and nonpathogenic strains of *B. subtilis* are widely available and have been safely used in a variety of food applications.

There are some previous researches which involved in the same field of this study. However, they focused on using

other microorganisms and pure culture [10], extracting enzymes [11]. The difference of this study is co-culturing between *A. oryzae* and *B. subtilis* leading to various interactions which alter their growth, metabolism, and differentiation; using bioproduct including living microorganism and enzymatic activity for white pepper production. The solid state fermentation was used to create the cultures medium for microbes. The optimal conditions on the application of bioproduct on black pepper, such as ratio between two microbes, temperature, duration, and ratio of bioproduct to achieve highest percent of husk removal will be investigated. Besides, the final product will be tested for piperine content as well as aflatoxins.

Materials and Methods

1. Materials

A. oryzae strain and viscozyme L in this study was supported by International University – Vietnam National University – Ho Chi Minh City. The *B. subtilis* strain was purchased from Vietnam Type Culture Collection (VTCC) of Institute of Microbiology & Biotechnology.

Germinated malt was collected from Du Hung Company, 71 Hai Thuong Lan Ong Street, Ward 10, District 5, Ho Chi Minh City, Viet Nam for making malt extract agar. Solid substrates were collected from local market including rice husk, rice bran used in solid state fermentation.

Black peppercorn used in this project was purchased from Phu Quoc Island, Kien Giang Province, Vietnam. Quality of peppercorn was nearly uniform in size and shape, and was not contaminated by insects, or strange smell.

2. Methods

2.1 Malt agar extract preparation for culture *A. oryzae and B. subtilis* inoculation [12]

Germinated malt was ground well and weighted at 200 g with distilled water in blender and was transferred to the 1000 ml beaker. After that, filtered all by cotton cloth and added more water until it reached 1000 ml then transferred into water bath at 52°C for 20 mins, then 63°C for 30 mins, 73°C for 30 mins and at last 100°C for 15 mins. The malt solution was cooled down and was adjusted to reach 10° Brix by refractometer and pH value should be 4.5-5.5. Then, 1.5% agar and 1% $(NH4)_2SO_4$ was added to the solution and transferred to 1000ml duran. The solution was sterilized at 121°C for 15 mins in autoclave.

This solution was volumed at 7ml and poured into test tube to make agar slant and covered by cotton wool. Lastly, *A. oryzae* and *B. subtilis* was cultured on the agar slant for 7 days.

2.2 Solid state fermentation [10]

The experiment was conducted in 250ml erlenmeyer flasks that contain constant solid substrate, nitrogen source, mineral salt and carbon source. The components of medium included: rice bran 75%, rice husk 20%, D-glucose 1%, ammonium sulfate 1%, CMC 1.5%, and pectin 1.5% that applied for both *A. oryzae* and *B. subtilis*. Volume of spores and living cells suspension would determine the volume of water needed to be added that maintain moisture content to reach 60%. Then, the erlenmeyer flasks was covered by wood cotton and sterilized at 121°C in autoclave for 15 mins. The flasks were cooled down at room temperature and inoculated with microbial suspensions with total volume was 10^7 cells/ml.

2.3 Investigating the ratio between spores of *A. oryzae* and living cells of *B. subtilis* on husk removal for black pepper fermentation

The suspension of *A. oryzae* and *B. subtilis* was added into prepared medium with inoculating 10^7 spores of fungi and living cells of bacteria and placed at room temperature for 6 days. Isolated microorganism was inoculated at different ratio between *A. oryzae* and *B. subtilis*: 0:1, 1:3, 1:1, 3:1, 1:0. After that, the cultured was mixed with rice flour in the ratio 1:1 to create bioproduct. Grinding the mixture well and storing them in the fridge for further uses.

2.4 Investigating the optimal temperature on husk removal for black pepper fermentation

Distilled water was volumed as 10 ml and added to 20g of black pepper, then soaked 7 hours to achieve 60% moisture content and poured into basket to remove water on the surface of pepper. After that, all of erlenmeyer flasks were put in incubators which were adjusted at different temperature: 31, 34, 37, 40 and 43°C. Other parameters such as fermentation time (48 hours) and 20% of bioproduct were fixed.

2.5 Investigating the optimal fermentation time on husk removal for black pepper fermentation

Black pepper was weighted as 20 g and soaked in 7 hours with 100ml of distilled water to reach 60% of moisture content. The black pepper was fermented at different durations: 24, 36, 48, 60, and 72 hours with the optimal temperature. Other parameters such as ratio between microbes and 20% ratio of bioproduct were fixed.

2.6 Investigating the optimal ratio of bioproduct on husk removal for black pepper fermentation

Black pepper was weighted as 20 g and put in erlenmeyer 250ml and 100ml of distilled water was added to erlenmeyer. The black pepper was soaked 7 hours to reach 60% moisture content and transferred into basket. Then, optimal ratio of bioproduct was added with high total activity of *A. oryzae* and *B. subtilis*. The process was carried out similarly to the part of determination optimal fermentation duration in pepper; however, the ratio of bioproduct was changed in five levels for 10, 15, 20, 25 and 30%.

2.7 Calculating percentage of husk removal [13]

Using pieces of washer to scape pepper until the outer part of the pericarp was not removed anymore. The clean pepper was dried in an oven at 60°C for 5 hours and weight of the outer shell removed was recorded. The clean pepper after drying must have the same moisture content. The percentage of skin removal for pepper products after fermentation was calculated by the following:

$$\frac{mf}{m} \times 100\% \qquad (1)$$

Where m: total weight of skin removed (g)

m_f: weight of outer shell removed by fermentation with viscozyme L (g)

2.8 Checking piperine content and aflatoxins

Blank sample as the control and white peppers after fermentation was sent to Quality Assurance & Testing

Center 3 (64- Le Hong Phong Street, Ward 2, District 5, HCMC, viet Nam) to test the piperine content as well as aflatoxins. The AOAC 2012 (987.07) and AOAC 2012 (991.31) methods were used to check piperine and aflatoxins, respectively.

2.9 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. The graphs were drawn by Microsoft Excel.

Results and Discussion
1. Fungal spores and living cells concentration
After 6 days of culture, fungal spores and living cells of *A. oryzae* and *B. subtilis* were counted by hemocytometer and concentration achieved $2x10^6$ (spores/ml) and $5x10^8$ (cells/ml), respectively.

Isolated microbial was inoculated at different ratio between spores of *A. oryzae* and living cells *B. subtilis*: 0:1, 1:3, 1:1, 3:1, 1:0 with the population of microorganisms were 10^7cells/ml. After that, the cultured was mixed with rice flour in the ratio 1:1 to produce bioproduct. This product was applied for white pepper processing.

2. Optimal conditions for black pepper fermentation
2.1 The optimal ratio between spores of *A. oryzae* and living cells of *B. subtilis* on pepper fermentation
In this section, black peppers were soaked in 7 hours to achieve 60% of moisture. Cellulase and pectinase are hydrolases, thus they cannot work if water is absent or too low [13]. From beginning 6%, the moisture of black pepper increased steadily and achieved 37% after 90 mins. The black pepper absorbed quite slow in the next 7 hours and saturated at 60% at that point for 7 hours [14]. After that, black pepper was fermented with different ratio between the spores of *A. oryzae* and living cells *B. subtilis.* Five different ratio (0:1, 1:3, 1:1, 3:1, 1:0) between *A. oryzae* and *B. subtilis* were test to choose which ratio was the optimal effect to husk removal.

The effect of different ratios between two microbes on percentage of husk removal was shown in Fig 1. Among them, the ratio 1:3 for *A. oryzae* and *B. subtilis* was the best ratio for the highest husk peeled of white pepper product at 94.17 ± 0.61%. The explanation is that interactions between the different microorganisms play a critical role in a co-culture compared to pure culture [15]. Co-cultures of different microorganisms may be also advantageous for the production of enzymes that leads to various interactions including the mutualism. Fungi and bacteria can form a range of physical associations that depend on various modes of molecular communication for their development and functioning [8]. During co-culturing, *B. subtilis* attach and grow on the hyphae of *A. oryzae* and both fungi and bacteria alter their metabolism. This interaction might subsequently enhance the cellulase and pectinase production from microorganisms to produce white pepper. Hence, the ratio 1:3 for *A. oryzae* and *B. subtilis* was the optimal choice for husk removal in pepper fermentation.

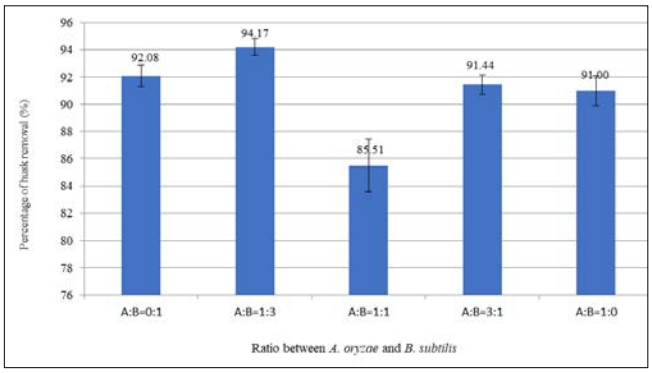

Fig 1: Ratio between *A. oryzae and B. subtilis* affecting percentage of husk remova

2.2 The optimal temperature on husk removal for black pepper fermentation
Temperature is other important factors for fermentation since it also influences fungi and bacteria growth, development and ability to synthesize enzymes. Each microorganism has a suitable temperature for growth and development. The temperatures studied in solid state fermentation were in the range of 27 to 47°C [14]. In this study, the percent of husk removal increased with temperature up to a certain limit. The effect of temperature to *A. oryzae* and *B. subtilis* for husk removal was clarified and the result was shown in Fig 2. The percent of husk removal was increased gradually and got highest; 94.06 ± 0.26% when temperature was increased to 37ºC but over this degree of heat, the percent of husk removal was reduced. That can be explained that above a certain temperature the percent of husk removal decreased with the increasing in temperature because enzyme denaturation led to the reduction of enzyme activity.

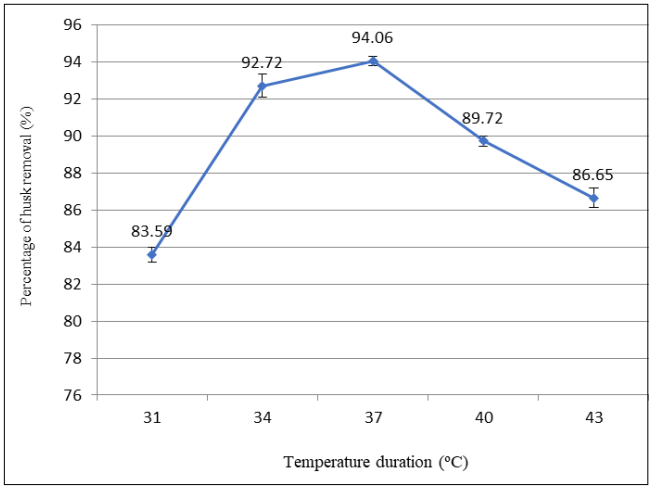

Fig 2: Temperature affecting percent of husk removal

2.3 The optimal duration on husk removal for black pepper fermentation
Optimal temperature was 37°C (in section 3.2.2) for white pepper production. However, it only removed 94.06 ± 0.26% of husk. Therefore, other factors for fermentation were considered. Duration plays a key role in growth of fungi and bacteria. Thus, it influences greatly on the quality of fermentation and economics aspect of white pepper production by bio-product. In the short duration of treatment, the enzymes cannot totally catalyze the substrate in pepper. Therefore, husk of pepper remained on it and

soluble solids could not be extracted from the cell of pepper. On the other hand, in a long duration of treatment, the fungi and bacteria could use the soluble solids remained in peppercorn as nutrient source and that led on reducing peppercorn quality. It could release toxic such as aflatoxins which causes hepatic carcinoma in human.

When optimal temperature (37°C) was determined, the optimal duration was checked by changing time from 24, 36, 48, 60 and 72 hours. The result was shown in Fig 3. Firstly, *A. oryzae* and *B. subtilis* require the time to synthesize enzymes needed (cellulase and pectinase) to adapt new environment. That was the reason why in first 24 hours percentage of husk removal was very little at 81.23 ± 1.26%. After that, *A. oryzae* and *B. subtilis* started to reproduce and produced a large quantity of enzymes to break down cellulose and pectin components of black pepper skin. The percentage of husk removal was got highest at 98.85 ± 0.51% after 72 hours fermenting. However, after fermenting 60 hours, the percent of husk removal was not significantly different to percentage of husk removal after 72 hours fermenting. So, duration at 60 hours was an appropriate choice for bringing high percentage of husk removal because it can reduce the time for treatment while still maintaining high efficiency and peppercorn quality.

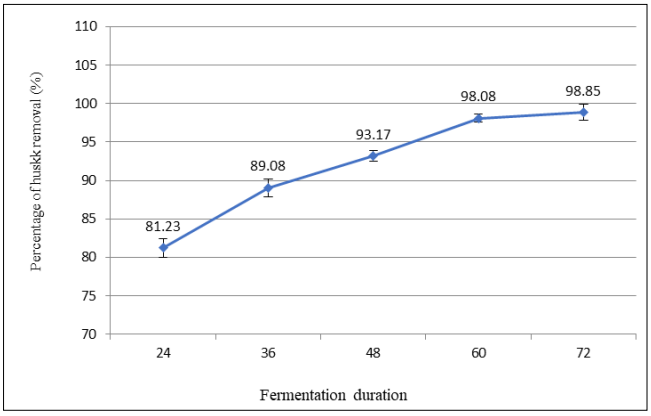

Fig 3: Fermentation duration affecting percent of husk removal

2.4 The optimal ratio of bio-product on husk removal for black pepper fermentation

Using the optimal temperature and duration for fermentation from previous experiments, in this study, bioproduct was used, the optimal duration for 60 hours at 37°C; a range from 5 to 15% was applied to check the pepper's skin removal. The bioproduct includes living microorganisms and rice flour in ratio 1:1 that brings more economic effect. Commonly, the more amounts of bioproduct would lead to the higher percentage of husk removal. When ratio of bioproduct is too limiting, *A. oryzae* and *B. subtilis* can grow and develop, but the enzymes products will be not enough to complete peeled pepper. In contract, too much amount of bioproducts that can simulate the development of the fungi and bacteria vigorously.

It also can lead to competition in nutrient and in space for growth so microorganisms cannot grow and develop as well as they can do. Moreover, using a lot of bioproduct does not bring high economic profit. Regarding to the graph (Fig 4) the black pepper was fermented with 2.5% addition of bioproduct every time of fermentation, percentage of bioproduct at 5% had too low effect to husk removal (86.15 ± 0.96%). Pectinase and cellulase still produced but not enough to remove the pepper skin. The percentage of husk removal was gradually increased and at its highest level at 15% of bioproduct, at 99.56 ± 0.45%. There was not significantly different among 12.5% and 15% of bioproduct; therefore, using the bioproduct at 12.5% could be optimal ratio for fermentation that would lead to achieve more appropriate result and economic profit.

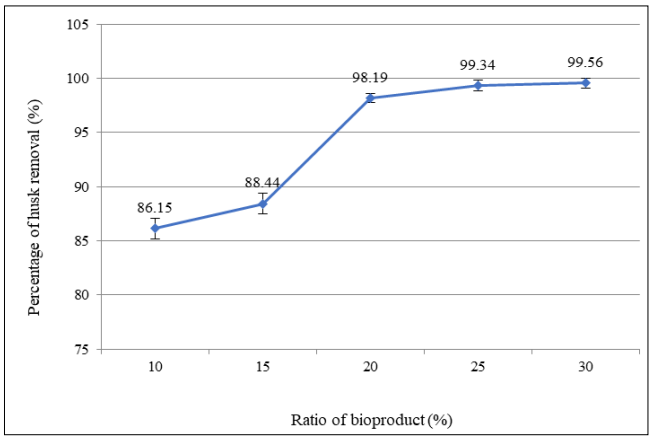

Fig 4: Ratio of bio product affecting percent of husk removal

3. Quality of peppers after fermentation

3.1 Piperine content of white pepper

Piperine is the standardized extract made from the fruit of Piper nigrum (black pepper) that is manufactured for use as a nutritional ingredient for both human and animal health. Piperine content of white pepper reached 3.6% (w/w) and satisfied requirement standard, increased 12.5% compared to black pepper.

3.2 Aflatoxins testing

Aflatoxins are mycotoxins produced by two species of *Aspergillus*, a fungus which is especially found in areas with hot and humid climates. Since aflatoxins are known to be genotoxic and carcinogenic, exposure through food should be kept allow as possible. It was necessary to test aflatoxins content in white peppers because it harmed to human health. Samples treated with bio-product would be tested by AOAC 2012 (991.31) method. The result from QUATEST 3 Company showed that aflatoxins content had not detected in white peppers.

Conclusions

In conclusion, white pepper production from black pepper by fermentation was affected by four factors: ratio between two microbes, temperature, duration, and ratio of bio-product. Among five different ratios of *A. oryzae* and *B. subtilis*, the ratio 1:3 was the best ratio for the highest husk peeled of white pepper product. Application of bio-product on white pepper fermentation gave high value for peppers with 25% bio-product, 99.34 ± 0.48% in 60 hours. This study found that bio-product of *A. oryzae* and *B. subtilis* were a good source to manufacture white pepper. Duration for white pepper production was reduced with 60 hours for fermentation compared to traditional method. Furthermore, the piperine content which is responsible for pungency was increased in the fermented pepper. The product of this study could be considered as free from aflatoxins.

Acknowledgements
We would like to express our special thanks to International University – Vietnam National University in Ho Chi Minh City – Vietnam for all the supports.

References

1. Webbjames. White pepper (*Piper nigrum*). Safe ingredients, 1973.
2. Viet Detal species. Pepper Morning cranial margin specialty of Phu Quoc. Viet Delta species News, 2013.
3. Nathalie J. Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci,2006:11:359-367.
4. Archer DB, Wood DA. The Growing Fungus. Chapman & Hall, London1, 1995.
5. Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A. Bacterial-Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food Microbiologist. Microbiol Mol Biol Rev,2011:75(4):583-609. [PubMed].
6. Zangirolami TC, Carlsen M, Nielsen J, Jorgensen SB. Growth and enzyme production during continuous cultures *o*f a high amylase-producing variant of *Aspergillus oryzae*, 2001.
7. Fogarty WM. Biotechnology Handbooks 7: Aspergillus. Plenum Press, New York, 1994.
8. Harwood CR. Introduction to the biotechnology of Bacillus. In: C.R. Harwood (Ed.). Biotechnology Handbooks *Bacillus* Plenum Press, London,1989:2:1-4.
9. Van Dijl JM, Hecker M. "*Bacillus subtilis*: from soil bacterium to super-secreting cell factory". Microbial Cell Factories,2013:12(3):3.
10. Tuyen Nguyen Kim. Study on white pepper production in phu yen province by bioproduct containing cellulase and pectinase. Bsc. Thesis. International HCMC, 2013.
11. Tram Nguyen Le Ngoc. Study on cellulase synthesis by fungi (A*spergillus oryzae* and *Pichia citrinum*) for white pepper production. Bsc. Thesis. International University HCMC, 2013.
12. Phu Hong Le. Production of microbial enzymes pectinase, cellulase and their application on production of green coffee by fermentation, 2012.
13. Ali S, Sayed A, Sarker RI, Alam R. Factors affecting cellulase production by *Aspergillus terreus* using water hyacinth. World J. Microbiol Biotechnol,1991:7(1):62-66.
14. Vu Ngo Hoang Huy. White pepper fermentation by *Aspergillus unguis* and *Wickerhamomyces edaphicus*: optimized fermented conditions. Bsc. Thesis. International University HCMC, 2012.
15. Baderet J. Relevance of microbial coculture fermentations in Biotechnology. The Society for Applied Microbiology, Journal of Applied Microbiology,2010:109:371-387.