Preliminary Study of *Hyptis pectinata* (L.) Poit Extract Biotransformation by *Aspergillus niger*

D S Rejeki¹, A L N Aminin ¹, M Suzery ¹
¹Chemistry Department, Faculty of Sciences and Mathematics, Diponegoro University, Indonesia.

E-mail: meiny.suzery@live.undip.ac.id

Abstract. One alternative approach to increase the content of bioactive compounds is fermentation. *Hyptis pectinata* (L.) Poit is a plant that can be found in tropical area and potentially as anticancer, anti-inflammatory, insect repellant, antiviral and antioxidant. In this research, efforts have been made to increase bioactive plant capacity of *Hyptis pectinata* (L.) Poit through submerged fermentation using *Aspergillus niger*. The study was performed by adding methanol extract of *Hyptis pectinata* (L.) Poit on two conditions, that was added at the beginning of fermentation and while entering a phase of death. *Aspergillus niger* growth rate in both conditions was observed by determining the dry weight of cells every 24 hours. The transformation profil of extract was observed after 24 hours of extract addition in early death phase by the TLC method. The results show that the addition of *Hyptis pectinata* (L.) Poit extract at log phase triggers the cells to growth faster, whereas the addition at the early death phase precisely accelerates cell death. TLC profile shows the emergence of new compounds suspected as the products of transformation of Hyptis pectinata (L.) Poit extract on day 8 after addition of extract.

1. INTRODUCTION

Nowadays, the discovery of bioactive compounds from the herbs is very developed and be the best choice for many people. The people have various alternatives to treat some diseases. Because of the phenomena, it is needed a certain effort to explore the natural materials well; not only for inhibiting, but also for providing effective cures without causing excessive side effects.

One effort that can be used to increase the content of bioactive compounds in plants is by fermentation [1]. This process can cause the decomposition and or biotransformation of complex substrates into compatible components by changing the properties of the product or altering the quantity of certain bioactive compounds [2]. Biotransformation is considered an economically competitive technology in synthetic organic chemistry because it provides new production routes for fine chemical, pharmaceutical, and agrochemical compounds [3].

Microorganisms are known to perform a variety of reactions, even if they are not chemically manageable [4]. The efficiency of using microorganisms as one of the biocatalyst agents is known to be more effective and productive, due to its ability to metabolize substrates. Biotransformation by using microbes is more specific than ordinary chemical compounds, because it allows the addition, deletion or modification of certain functional groups. Catalyzed reactions include oxidation,
dehydrogenation, hydroxylation, dehydration, condensation, decarboxylation, deamination, amination and isomerization [5].

Aspergillus niger considered as a safe microorganism and the FDA (Food and Drug Administration) in the United States classifies it as a GRAS (Generally Recognized As Safe) microbe for not producing mycotoxins and in its metabolism Aspergillus niger is able to produce citric acid [6]. In some studies, Aspergillus niger is used in the biotransformation process [7]. Among them, it is a biotransformation in isoflavone compounds, namely 7,4’-dimethoxyisoflavon and 7,4’ diacethoxyflavon which can produce a new metabolite that is daidzein. Potentially as antihemolytic, antioxidative, and antitumor [8]. Biotransformation research was developed against Budlein A lactone compound, which can produce 3 new metabolites that can reduce tumor cell growth and cell viability in low doses [9]. The next 13 metabolites derived from 20 (S)-Protopanaxatriol compounds that potentially inhibit the growth of 7 cancer cells with an average IC$_{50}$ below 100 (μmol/Liter) [10] catechin biotransformation [11] and flavonoid compounds that have higher antioxidant biotransformation results [12].

Hyptis pectinata Poit is a plant that thrives in wild land and has not been widely used in Indonesia [7]. In Brazil, the farming of this plant has developed, and is known to treat inflammation, pain, injury, cancer, bacterial infections, and for healing wounds [13] anti-inflammatory and the decoction of leaves used as tea drinks [14]. While in India and Mexico, this plant is known as an herbal plant that has a versatile drug quality in the treatment of fever, skin diseases and stomach disorders. In the development of potency of Hyptis pectinata (L) Poit, research has been done on isolation, transformation, activity test of the pure compound and essential oil isolation from Hyptis pectinata Poit extract [15]. Until now no research has reported on the effect of fermentation on the methanol extract of Hyptis pectinata Poit using Aspergillus niger. This research is very important to do, considering it can be used as an alternative to increase the content of bioactive compounds.

This research was conducted at the Laboratory of Organic Chemistry and Biochemistry, Faculty of Science and Mathematics, Chemistry Department of Diponegoro University. This research starts with the preparation of Hyptis pectinata Poit leaf extract, Aspergillus niger rejuvenation, Aspergillus niger growth profile measurement, Hyptis leaf extract fermentation with Aspergillus niger as preliminary test and and transformation profile analysis using TLC method.

2. METHOD

2.1. Extraction

The 108 grams of Hyptis pectinata Poit leaves were collected from Kanayakan Village, East Dago-Bandung. The first step was covering the leaves with black cloth and drying under the sunlight for three days. Then, the dried leaves have been crushed and made into powder. The extraction process was performed by maceration. Hyptis pectinata Poit powder was macerated with 2 L methanol for 3 days and filtered. The filtrate was concentrated with a rotary vacuum evaporator up to one-third of volume, then added warm aquadest (1:1) and left for 1 x 24 hours until it formed into two layers. The top layer was a yellow methanol-water, while the bottom layer was a green chlorophyll deposit. The top layer was concentrated again with a rotary vacuum evaporator until the liquid extract was obtained. To remove the residual solvent, evaporation was performed over the water bath, and measured rendement to the extract [16].

2.2. Preparation of Aspergillus niger Culture

The black colonized Aspergillus niger stock was preserved on PDA (Potato Dextrosa Agar) medium which is made with tilted agar and incubated for 2-3 days. For inoculum preparation, culture is rejuvenated in PDB (Potato Dextrosa Broth) media as a starter, and observations are made until the spores looked solid (1 x 10$^7$) spores/mL [9].
2.3. Measurement of Aspergillus niger Growth Profile

The mold isolates from the culture stock of PDB medium can be rejuvenated by inoculating each 10 mL of starter solution into 10 flask containing 100 mL of PDB medium. Then they were incubated in a shaker incubator at 30°C Celsius and 125 rpm speed. The measurement of Aspergillus niger mold growth profile was performed on the basis of dry cell weight and determined once every 24 hours on screening results. Because of the increasing acid content in the media, thus the mycelium was added with 1% Sodium hydroxide and washed back with distilled water until neutral pH was obtained. The mycelium was dried in an oven at 80 °C for 24 hours, the weighs the mycelium to obtain a constant weight. The relationship between the weight of the dry cell and the incubation time was used to make the growth curve.

2.4. Fermentation of Hyptis Pectinata Poit leaf methanol extract with Aspergillus niger

An amount of 10^7 spores / mL of Aspergillus niger in 100 mL PDB of 1% was added by 1 gram of the Hyptis pectinata Poit leaf extract. Then it was incubated at 30°C and at 125 rpm speed. Measurement of Aspergillus niger mold growth profile in PDB medium with the addition of Hyptis leaf extract, was performed by measuring the weight of dry cells determined every 24 hours. Furthermore, the same steps were done as in the Aspergillus niger growth profile measurements.

2.5. Identification of Biotransformation Compounds

To observe the biotransformation process on the fermented production, identified using TLC. The filtrate was separated by centrifugation at 2500 rpm for 25 minutes. To remove the remaining solvent, a rotary vacuum evaporator was applied to get concentrated solvent. Crude extract was analyzed further at every 24 hours once using the Gel 60 F_{254} gel plate on 4 various eluents. The measurement of stain spots was performed under UV detector lamps with λ_{366} nm. The Rf svalue generated from the incubation time variation would be compared. The presence of changes in Rf values indicated the occurrence of transformation of compounds in fermentation products.

3. RESULTS AND DISCUSSION

3.1. Aspergillus niger Growth Profile

The Aspergillus niger growth profile shows information about growth phases that occurred in Aspergillus niger, which can be used to determine the exact time for fermentation (Figure 1).
From the resulting data, it is seen that the initial phase of adaptation (lag) occurs on days 0-1, in this phase there is an adjustment of cells with the enzyme environment to break down the substrate. The next phase of acceleration (logarithmic) occurs on days 1-3, because in this phase the cells begin to divide and become more active. Then the exponential phase occurs on days 3-5. During this phase, the cell can divide maximally due to the availability of many nutrients, so that cell activity is greatly increasing, and this phase is an important phase for fungi life. At the beginning of this phase many enzymes can be produced. Meanwhile, on day 5-7, there is a stationary phase seen from the dry cell period remains. In that phase the number of cells that grow is relatively the same as the number of dead cells. The profile in this phase is a horizontal straight line and there are many secondary metabolites. Furthermore, during days 8-11, it is the death phase of *Aspergillus niger*, which can be seen from the decline in the dry cell mass. In this phase, the nutrients have begun to decrease and the presence of other metabolites that are toxic and can inhibit cell growth, so that the growth rate of *Aspergillus niger* begins to decline.

3.2. Fermentation Profile Methanol Extract of *Hyptis pectinata* Poit Leaves with *Aspergillus niger*

From the pattern formed between the addition of *Hyptis* extract and without the addition of *Hyptis* extract, there is a prominent difference at the beginning of the growth phase. It is characterized by an increasing number of cells in the logarithmic phase. While at the end of the stationary phase, the addition of *Hyptis* extract actually decreases the number of cells sharply. As shown in Figure 2:

![Figure 2. Aspergillus niger Growth Curve](image)

The results show that the addition of *Hyptis* extract at the beginning of the growth phase provides a significant increase in the number of cells in the logarithmic phase compared to the number of cells without the addition of *Hyptis* extract. The increasing number of cells at the beginning of the growth phase is assumed because of the molecules secreted by *Aspergillus niger*. In general, in the logarithmic phase of enzyme production is abundant. The ability of hydrolytic extracellular enzymes in *Aspergillus niger* can decompose the polymer into its monomers, thus in this phase facilitating the absorption of nutrients well.

In addition, when viewed from its nature, *Aspergillus* lives as a decomposer and a heterotrophic symbiont that gets its nutrients or nutrients by absorption. In general, the surface area in liquid culture also supports very rapid growth and adapts *Aspergillus niger* to obtain the perfect absorptive nutrients. The addition of *Hyptis* leaf extract to the liquid inoculum of *Aspergillus niger*, suppresses the hormone synthesis that promotes the addition of cells. According to another report that *Aspergillus utus* in a liquid culture treatment can synthesize the presence of auxin and gibberellin hormones that can promote the growth and development of host plants [17].
According to its phytochemical content, the compounds of *Hyptis pectinata* Poit plants are composed of α,β-unsaturated lactone skeletons [8]. Whereas if it is seen from the structure of the major compound lactone, there is an atom C in the cyclical position which is easily replaced by another atom (e.g. atom O). The existence of lactones resembles the structure of kinetin hormone which is thought to be synthesized by *Aspergillus niger*. Kinetin hormone is a derivative of cytokinin hormone that serves to spur cell division. Thus, in the presence of similar structures, it can induce the occurrence of faster cell division and can increase the number of cell densities.

![Figure 3. Aspergillus niger Growth Profile on the early addition of extract at 8 hours](image)

Unlike the case at the beginning of the death phase, the addition of *Hyptis* extract can decrease the number of *Aspergillus niger* cells. The decline in the number of cells at the beginning of the dead phase is because the supply of nutrients that have been exhausted and also because the formed transformant can increase the toxicity for the growth of *Aspergillus niger*. According to another report, one component of *Hyptis pectinata* (L.) Poit is an essential oil (Pectinolina), which consists of Pectinolida A-G. The bioactive compound of pectinolide in *Hyptis* extract is potential as an antimicrobial [18].

### 3.3. Identification of Biotransformation Compounds

Table 1. Rf values of the biotransformation of the compound

| Observation Day to Eluent | n-heksan | Chloroform Ethyl acetate | Methanol |
|---------------------------|----------|--------------------------|----------|
|                           | Rf       |                          |          |
| 8                         | -        | 0.80                     | -        | 0.87     |
| 9                         | -        | 0.82                     | -        | 0.86     |
| 10                        | -        | 0.85                     | -        | 0.87     |
| 11                        | -        | 0.84                     | -        | 0.84     |
| 12                        | -        | 0.82                     | -        | 0.86     |
| 13                        | -        | 0.82                     | -        | 0.84     |
| 14                        | -        | 0.78 (0.31; 0.34)         | -        | 0.83     |
| 15                        | -        | 0.66 (0.73; 0.77; 0.82)   | -        | 0.87     |
| 16                        | -        | 0.97                     | -        | 0.83     |
| 17                        | -        | 0          (0.87)          | -        |          |
On the 15th day observation, it is seen a change of stain from some eluent. Of the chloroform eluents there are two different stains. While on the ethyl acetate eluent, which initially cannot elute at all, but on 15th day, it has formed three different stains. Similarly, on the 16th day, chloroform and ethyl acetate eluents also still develop a stain with different Rf. While on the next day, in the chloroform eluent, there is no change of stain, the distance of the stain that appears is proportional to the distance in the eluent. Based on the qualitative TLC test it can be concluded that the start formation of the transformational result is at the 15th day, so the fermentation process can be stopped at that time. The process of fermentation in liquid culture generally has a shorter time than fermentation in solid medium, but the influence of adding a certain substrate, can increase the length of fermentation time. This is due to the more complex substrate degradation process because of the addition of substance to the environment where microbe grows [17].

4. CONCLUSION
In Addition of Hyptis pectinata (L.) Poit extract at early growth increase the cell growth of Aspergillus niger cells that are affected by the molecular secretion in the form of hormone cells growth. While the significant decline of cells in early death phase is influenced by secondary metabolites that are pathogenic to Aspergillus niger growth. It is assumed that the transformation on the 8th day after the addition of Hyptis pectinata (L.) Poit extract was marked by a change in the number of stains formed.

ACKNOWLEDGMENTS
Research of Development and Application (RPP) of PNBP UNDIP.

BIBLIOGRAPHY
[1] Martins, S., Mussato, S.I., Mart nez avula, G., Monta ez-Saenz, J., Aguilar, C.N., & Teixeira, J.A (2011). Bioactive phenolic compounds: Production and extraction by solid–state fermentation. Arview. Biotechnology Advances, 29 (3), 365-373.
[2] Hussain, A., Bose, S., Wang, J. H., Yadav, M. K., Mahajan, G. B., & Kim, H. (2016). Fermentation, a feasible strategy for enhancing bioactivity of herbal medicines. Food Research International, 81, 1–16.
[3] Adelin.E., Martin, M.T., Bricot, M.Francoise., Cortial, S., Retailleav, P., Ouazzani, J. (2012). Unexpect thio conjugation of Sch-642305 with 3-merc aptolactate catalyzed by Aspergillus niger ATCC 16404, Biotransformation of natural compounds, 84, 135-140.
[4] Falcao, R.A., L.A, Patricia., Nascimento, do., De Souza, S.A., Da Silva, T.M.G., De Queiroz, A.C., Da Matta, C.B.B., Moreira, M.S.A., Camara, C.A., Silvan, T.M.S, 2013, Antileishmanial Phenylpropanoids from the Leaves of Hyptis Pectinata (L) Poit, (1-7).
[5] Walker, J.M. and Rapley, R. 2002. Molekular Biologi and Biotechnology Britain: Athenacum Pr.
[6] Sasmitaloka, K. S. (2017). Produksi asam sitrat oleh Aspergillus niger pada kultivasi media jamur, Integration Journal Process,3(6), 116-122.
[7] Parshikov, i.A., & Sutherland, J.B (2014). The use of Aspergillus niger cultures for biotransformation of terpenoid. Proces Biochemistry, 49 (12), 2086-2100.
[8] Miyazawa, M., Ando, H., Okuno,.Y., Araki, H., (2004), Biotransformation of isoflavones by Aspergillus niger as biocalalyst, Journal of Molecular catalysis, B : Enzymatic, 27, 91-95.
[9] Arakawa,N.S., Neto,L.G., Ambrosio, S.R., Antonvcci, G.A., Sampaio, S.V., Pupo, M.T., Sard.S., Schmidt, T.J., Da Costa, F.B, (2013) Unusual biotransformation Products of the sesquiterpene lacton Budlein A by Aspergillus species, Journal of Phytochemistry, 96, 92-100.
[10] Cao, H., Chen, X., Reza, A., & Xiao, J. (2015). Microbial biotransformation of bioactive flavonoids. Biotechnology Advances, 33(1), 214–223
[11] Ni, H., Chen, F., Dong, Z., Ying, M., & Fan, Y. (2015). LWT - Food Science and Technology Biotransformation of tea catechins using Aspergillus niger tannase prepared by solid state fermentation on tea by product. LWT - Food Science and Technology, 60(2), 1206–1213.
[12] Chen, G., Song, Y., Ge, H., Ren, J., Yang, X., and Li, J., (2015), Microbial Biotransformation of (20) S-protopanaxatriol by Aspergillus niger and the cytotoxicity of the resulting metabolites. *Phytochemistry Letters*, 11 (19), 111-115.

[13] Bispo M., Moura R., Franzotti E., Bomfim K., Arrigoni-Blank M., Moreno M., Marchioro M., Antoniolli A., (2001), Antinociceptive and antiedematogenic effects of the aqueous extract of experimental animals, *Journal of Ethnopharmacology* 76, 81-86.

[14] Arigoni, B.F., Antoniolli A., Caetano, L.C., Campos, D.A., A.F.Blank., 2008, Antinociceptive activity of the volatile oils of *Hyptis pectinata* Poit. (Lamiaceae) genotypes, *Phytochemistry*, 334-339

[15] Suzery, M., & Cahyono, B. (2014). Jurnal Sains dan Matematika Evaluation of Cytotoxicity Effect of Hyptis pectinata Poit (Lamiaceae) extracts using BSLT and MTT methods *Jurnal Sains dan Matematika*, 22(3), 84–88.

[16] Suzery M., Gultom M., and Cahyono B. (2013). Senyawa Hiptolida dan Pektinolida dalam fraksi Diklorometana dari daun Hyptis pectinata Poit. *Jurnal Sains & Matematika*, 21(2), 31–34.

[17] Marina, S., Angel, M., Silva, M.-Flores., Cervanter, M.G-Badillo, Rosales, M., T-Saavedra, Islas, M.A-Osura., Casas, S.-Flores (2011), The Plant Growth Promoting Fungus Aspergillus ustus Promotes Growth and Induces Resistance Against Different Lifestyle Pathogen In Arabidopsis thaliana. *J. Microbiol. Biotechnol*, 21 (7), 686-696

[18] Pereda-Miranda, R., Hernandez, L., Villavicencio, M.J., Novelo, M., Ibarra, P., Chai, H., Pezzuto, J.M., (1993). Structure and stereochemistry of pectinolides A–C, novel antimicrobial and cytotoxic 5,6-dihydro-a-pyrones from *Hyptis pectinata*. *J. Nat. Prod.* 56, 583-593.