Utilization of tofu wastewater and sugar industry by-products as a medium for the production of antifungal metabolites by *Paecilomyces Marquand* Strain TP4

**D G S Andayani**, **D G T Andini**

1 Research Unit for Clean Technology, Lembaga Ilmu Pengetahuan Indonesia, Cisitu-Sangkuriang, Bandung 40135  
2 Faculty of Science and Technology, Universitas Airlangga, Mulyorejo, Surabaya 60115  

desakgedesa@gmail.com

**Abstract.** *Paecilomyces Marquand* strain TP4 is a saprophytic fungus isolated from soil taken from Tangkuban Perahu Mountain at West Java Province, Indonesia. This research aims to identify the growth profile of *P. Marquand* strain TP4 and the antifungal activity against *Fusarium sp.* The antifungal compound is produced by the liquid fermentation method using tofu wastewater and molasses media. The fermentation broth is extracted by liquid-liquid extraction using n-hexane, ethyl acetate and methanol solvents. The result shows that the optimum activity achieved after 120 hours of fermentation. The fermentation broth has antifungal activity against *Fusarium sp.*, where the inhibitory diameter was 20 mm. The total flavonoid content and total phenolic content of freeze-dried fermentation culture were 0.4839 mg/g and 1.171 mg/g, respectively. Ethyl acetate extract shows the highest antifungal activity with MIC 31.25 ppm and MFC 125 ppm. The FTIR spectrum shows the OH, aliphatic CH, C-C, aromatic C=C, CH and C-O. Characterization of an antifungal compound based on phytochemical test and FTIR determine to be a phenolic group.

1. Introduction

*Paecilomyces* is a saprophytic mold found in soil, humus, and air [1]. Saprophytic fungi have a high ability to decompose waste and cellulose. Apart from being decomposers, they also produce metabolites that can reduce pathogenic infections in plants [2]. *Paecilomyces Marquand* can produce leucine statin D compounds that are antagonistic to several fungi and positive bacteria, including *Staphylococcus sp.* resistant to benzylpenicillin macrolide antibiotics [3]. *P. Marquand* is suitable to be used as a biocontrol because it effectively reduces the proliferation of *Meloidogyne hapla*, a pathogenic worm of lettuce [4]. Molasses is a by-product of processing sugar cane (*Saccharum officinarum*). Molasses still contain high sugar (50 - 60%), amino acids, and minerals. The high sugar content in molasses can be used as a source of nutrition in the fermentation process. *Paecilomyces Marquand* Strain TP4 was isolated from Tangkuban Perahu Mountain at West Java Province, Indonesia has active against pathogenic bacteria, fungi, and breast cancer cell T47D [5].

There are 509 tofu artisans in Bandung with a scale of production of 15 kg to 7.5 tons of soybean seeds per day [6]. It is estimated that every 100 kg of soybean seeds will produce 1600 kg of tofu wastewater [7]. It will be very detrimental if potential wastewater is not utilized. Tofu wastewater can
be used as a fermentation medium because it contains nutrients such as amino acids and proteins [8]. Thus, tofu wastewater as an ingredient for fermentation media can reduce waste from tofu production, which is a problem for the environment because it has high acidity and pollutant content. Plant parasites, fungi produce some symptoms of plant diseases even though there are no disease-causing organisms. An example is *Fusarium sp.*, which causes wilt in plants [9].

According to BPS data in 2011, 76.87% of lowland rice crops experienced pest attacks, and from 89.39% of farmers who carried out pest control, 79.24% of farmers choosing pesticide [10]. On the one hand, the use of pesticides can indeed reduce the loss of results caused by pests. However, on the other hand pesticides can cause adverse effects, such as often causing poisoning in plants, can cause immunity to pests, produce unhealthy agricultural products because there are chemical pesticide residues, the emergence of new pests, the killing of natural enemies of pests and other non-target organisms, and not environmentally friendly. Alternatives to these pesticides include using fermented pesticides. Therefore, in this study, bio-control microorganisms, *Paecilomyces Marquand* strain TP4, were used to produce antifungal metabolites using molasses and tofu wastewater media. The fermentation results were tested against strawberry pathogenic fungi, *Fusarium sp*.

2. Methodology

2.1. Material

The materials used in this study were producing microorganisms, *Paecilomyces Marquand* strain TP4 and testing microorganism *Fusarium sp.* that was a laboratory collection of Research Unit for Clean Technology, Indonesian Institute of Sciences. Tofu wastewater and molasses were obtained from the homemade tofu industry, and a by-product of the sugar industry was located in West Java, Indonesia.

The microbiology media, chemicals and reagents used in this study were Potato Dextrose Agar (Difco), Potato Dextrose Broth (Difco), Nelson A and B, arsenomolybdat, Lowry A and B, Follin, magnesium powder, HCl, amyl alcohol, FeCl₃, NaOH, Liebermann-Burchard reagent, ethanol p.a (Merck, Darmstadt, Germany), methanol p.a (Merck), acetone (Merck), ethyl acetate (Merck), n-hexane (Merck), reagent Folin-Ciocalteu (Merck), gallic acid (Sigma-Aldrich, St Louis, USA), Quercetin (Sigma-Aldrich), Na₂CO₃ (Merck), Aluminum chloride (AlCl₃) (Sigma-Aldrich), CH₃COOK (Merck), distilled water, bi-distilled water. Merck 60 silica gel (0.2-0.5 mm), thin layer Chromatography (TLC) plates.

The instruments used in this study were Fermentor (New Brunswick BioFlo, USA), Freeze dryer, (Labocon LFD-BT-104), UV-Vis spectrophotometer (Hitachi U-2800, Marunouchi, Tokyo, Japan).

2.2. Methods

2.2.1. Fermentation. The fermentation process was started by inoculating 10% *P. Marquand* strain TP4 into the sterile media consisting of tofu wastewater and molasses with ratio C/N 0.0045 g/ml, arranged at pH 7. Fermentation was carried out for eight days at room temperature and 120 rpm. Sampling was done every 6 hours to determine the growth curve, pH during fermentation, liquid fermentation activity, and glucose and protein concentrations.

2.2.2. Growth curve. The microbial growth curve of producing metabolites was measured based on biomass dry weight [5] and fermented liquids' antifungal activity [11].

2.2.3. Freeze dryer. The fermented metabolites (FF) were dried for three days at -80°C using freeze dry.

2.2.4. Antifungal activity of fermentation broth. The antifungal activity of fermented liquids was carried out using the diffusion method [12].
2.2.5. **Glucose test.** The glucose level of the fermented liquid was determined by the Nelson-Somogy method. 1 mL of Nelson reagent was added to 1 mL of fermented liquid, then heated in the water-bath for 20 minutes. After cooling, the arsenomolybdat reagent was added to the solution, then mixed until homogeneous, then diluted using distilled water to the final volume of 10 ml. Absorbance was measured at a wavelength of 520 nm [13].

2.2.6. **Protein test.** The protein content of the fermented liquid was determined by the Lowry method. 5 mL Lowry C was added to 1 mL of fermented liquid, then mixed and let stand for 10 minutes. 0.5 mL follin reagent (Lowry D) was added to the solution, then mixed until homogeneous and let stand for 30 minutes. Absorbance was measured at a wavelength of 500 nm [14].

2.2.7. **Extraction.** Fermented products were filtered. The fermentation broth was extracted by liquid-liquid extraction using n-hexane, ethyl acetate, and methanol solvents. Then the extract was evaporated at 40 °C [11].

2.2.8. **Qualitative test of flavonoid content**
About 100 mg of magnesium powder was put into a test tube then added with 1 mL of 2 M HCl and 3 mL of amyl alcohol. A little FF was added and shook to the test tube, and then the color change was observed [15].

2.2.9. **Qualitative test of alkaloid content**
A mixture of 1% FeCl$_3$ and 1% potassium hexacyanoferrate (III) was added to the test tube's extract. Positive results of intense green, red, purple, blue, or black are formed [15].

2.2.10. **Total phenolic content assay (TPC)**
The determination of total phenol content was determined using the Folin-Ciocalteu method using gallic acid as standard with slight modifications [16].

2.2.11. **Total flavonoid content assay (TFC)**
The determination of total flavonoid content was carried out by spectrophotometric using Quercetin as the standard refers to some modifications [16].

2.2.12. **Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).** MIC and MFC of n-hexane, ethyl acetate, and methanol extracts were determined using microdilution methods. The MIC was performed in sterile, 96-well microplates with PDB medium, and MFC was determined by platting 100 μl from each adverse well and the positive growth control well onto metabolite-free PDB, with subsequent incubation at 30°C for 48 h or until subcultures started to grow from the growth control well. The MIC was defined as the lowest drug concentration with which there was a complete absence of growth. The MFC was defined as the lowest concentration of metabolite from which subcultures were negative or yielded fewer than two colonies, representing a killing factor of 99% [17].

2.2.13. **Thin-layer chromatography (TLC) test.** The TLC test was carried out using a mixture of n-hexane, ethyl acetate, and methanol as eluents. The crude extract is spotted on the TLC plate and then eluted using hexane, ethyl acetate and methanol from 0-100/100-0. The results were seen under ultraviolet light at wavelengths of 254 nm and 366 nm [18].
3. Results and discussion

3.1. Growth curve of \( P. \) Marquand strain TP4, \( \text{pH} \) during fermentation and antifungal activity of fermentation broth

\( P. \) Marquand strain TP4 fermentation was carried out in molasses medium and tofu wastewater. Molasses act as a carbon source because molasses still contain high sugar (50 - 60\%) \cite{6}. Tofu wastewater acts as a source of protein for microbes and molasses thinners. Protein in tofu wastewater is in ammonium ions and phosphate ions, needed for energy metabolism, cell membrane stability, carbohydrate biosynthesis, amino acid biosynthesis and cell replication. Dilution of molasses is needed to reduce sucrose levels because high sucrose will inhibit microbial growth.

Fermentation was carried out in a shaker fermenter with a shaking rate of 120 rpm in room temperature and \( \text{pH} \) 7. This condition is suitable for the growth of \( P. \) Marquand strain TP4. The extreme temperature limit of \( P. \) Marquand growth is between 5\(^\circ\)C - 35\(^\circ\)C, while the optimum temperature for growth, pathogenesis and survival is at a temperature of 20\(^\circ\)C - 30\(^\circ\)C. However, this fungus is not found to grow at 37\(^\circ\)C in vitro \cite{1,19,20}.

The growth curve of \( P. \) Marquand strain TP4 shows that the optimum time is reached after 120 hours of fermentation because at 120 hours, show the inhibition diameter of \( \text{Fusarium} \) sp. of 20 mm with a dry biomass weight of 0.3996 g (figure 1). It shows that the end of the stationary fermentation phase is reached at 120 hours. Secondary metabolites are produced when the stationary phase because in this phase competition starts getting nutrients so that to win the competition, microbes produce secondary metabolites such as antifungal to inhibit or combat the growth of other microorganisms \cite{32}.

The \( \text{pH} \) curve during fermentation also increases to 8.5 at 120 hours, which is the optimum \( \text{pH} \) of \( P. \) Marquand (figure 2). From the growth curve, it is known that the 0 to 30 hours fermentation is a logarithmic phase because there is no inhibitory activity. There will be no secondary metabolite production during the log phase, but microbes will actively synthesize new protoplasm and then divide continually.

When the fungi begin to enter the death phase, namely at 162 hours, there is an increase in biomass production again, and at 180 hours, there is a 24 mm inhibitory activity (figure 1). It shows that \( P. \) Marquand strain TP4 can produce metabolite compounds again after the stationary phase. In the death phase, cells are still possible to do division and metabolism, but more cells die than actively dividing, so there is a decrease in biomass production.

Based on these results, fermentation for antifungal metabolites was carried out at the optimum time of 120 hours.

![Growth curve of \( P. \) Marquandii strain TP4.](image.png)
3.2. **Glucose and protein concentration during fermentation**

P. Marquandii strain TP4 consumed glucose and protein are different during fermentation. Consumption of protein tends to be greater than glucose. It indicated that the curve of the protein had decreased more steeply. At 180 hours of fermentation, the protein concentration is lower than glucose, where the protein concentration is 0.024 mg/mL, while the glucose concentration is 0.038 mg/mL (Figure 3). Glucose consumption tends to be less and slower because most sugar sources in molasses are sucrose, which is a disaccharide. It will be hydrolyzed first to glucose to produce ethanol and carbon dioxide [5].

\[
C_{12}H_{22}O_{11} + H_2O \rightarrow 2 C_6H_{12}O_6 \tag{1}
\]

\[
C_6H_{12}O_6 \rightarrow 2 CH_3 - CH_2 - OH + 2 CO_2 \tag{2}
\]

3.3. **Extraction and phytochemical test**

The three extracts from liquid-liquid extraction have antifungal activity against *Fusarium sp.* Ethyl acetate extract has the highest antifungal activity with MIC, and MFC value is 31.25 ppm and 125 ppm, while the n-hexane and methanol extract has the same MIC and MFC value, 250 ppm and 500 ppm. From the phytochemical test results, the results were obtained n-hexane, ethyl acetate, and methanol extract and freeze-dry fermentation culture contain flavonoids and phenols. The results are shown in Table 1.

3.4. **Total phenolic content**

The total content of phenolic compounds in the sample was determined using the colorimetric method with gallic acid as a standard. Gallic acid is a hydroxybenzoic derivative and belongs to simple, stable and pure phenol acid. Reactions that occur can be seen from the color changes in the sample. The phenol compound in the sample reacts with a specific reagent Folin-Ciocalteu which produces complex blue compounds. The blue chromophore formation reaction involves the phosphotungstic phosphomolibdenum reaction [16]. After several calculations, we found that the total phenolic contained on FF was 1.171 ± 0.0995 mg GAE/g (Table 2).
3.5. Total flavonoid content
Determination of total flavonoid content using the spectrophotometer method with Quercetin as standard. The reaction that occurs can be seen from the sample solution’s color change when added to the AlCl\(_3\) solution. It occurs because there is the formation of complex compounds with flavonoids, which produce a more yellow color so that the absorbance can be read in the visible area. After that, a potassium acetate solution was added to maintain the wavelength shift in the visible area. After several calculation, FF contained 0.4839 ± 0.1192 mg QE/g (table 3).

### Table 1. Result of flavonoid and phenolic test on the extract and freeze-dry fermentation culture of *P.* marquandii strain TP4.

| Phytochemical Testing | Results | Positive reaction | Photo |
|-----------------------|---------|-------------------|-------|
|                       | Hexane  | Ethyl acetate | Methanol | Freeze Dry Fermentation Culture |
| Flavonoid             | +       | +                | +        | Presence of orange-colored amyl alcohol layer |
| Phenolic              | +       | +                | +        | Presence of an intense green, bluish-black color |

### Table 2. Total phenol content of *P.* marquandii strain TP4.

| Sample               | Absorbance λ 765 nm | Phenol concentration (mg/mL) | Total phenol content (mg GAE/g extract) | The average of total phenol content ± SD (mg GAE/g extract) |
|----------------------|---------------------|------------------------------|----------------------------------------|---------------------------------------------------------------|
| Freeze dry fermentation culture | 0.3745  | 3.2016                    | 1.2705                                 | 1.171 ± 0.0995                                                |
|                      | 0.3168              | 2.7003                      | 1.0715                                 |                                                                |

### Table 3. Total flavonoid content of *P.* marquandii strain TP4.

| Sample               | Absorbance λ 431 nm | Flavonoid concentration (mg/mL) | Total flavonoid content (mg QE/g extract) | The average of total flavonoid content ± SD (mg QE/g extract) |
|----------------------|---------------------|---------------------------------|------------------------------------------|----------------------------------------------------------------|
| Freeze dry fermentation culture | 0.0899  | 1.5198                      | 0.6031                                   | 0.4839 ± 0.1192                                                 |
|                      | 0.0565              | 0.9191                        | 0.3647                                   |                                                                |

3.6. Thin-layer chromatography (TLC)
TLC was conducted to confirm further the results obtained from phytochemical screening. Based on phytochemical results, the compounds in n-hexane extract tend to be non-polar, so n-hexane and chloroform eluent were used for TLC. The TLC results of n-hexane extract show that there are six stains were eluted with n-hexane: chloroform (5.5:4.5) with Rf 0.275 and 0.625. AlCl\(_3\) is a specific reagent for flavonoid; when sprayed on the TLC plate, then dried, it will show all 5-hydroxy-flavonoids as yellow spots when viewed under UV light of 366 nm. Based on the results obtained, it is assumed that Rf 0.275
and 0.625 are flavonoids because the spots are yellow after being sprayed with AlCl₃ and seen under UV light wavelength of 366 nm. The TLC results of ethyl acetate extract show two stains eluted using n-hexane: chloroform (7:3) with Rf 0.475 and 0.525. The black color stain was appeared by H₂SO₄ alcoholic spray that caused the chromophore group from the active substance is damaged because that reduced by sulfuric acid so that the wavelength shifts to a longer direction (UV becomes Vis). The results of TLC of methanol extract with n-hexane: ethyl acetate (6:4) eluent show that there is one stain with Rf 0.45. The eluent used does not characterize because it does not succeed in separating compounds characterized by the appearance of stains and tail-formed stains [22].

![Figure 4. Spectrum FTIR of antifungal compounds of P. Marquand strain TP4.](image)

3.7. Infrared spectrophotometry analysis
IR data shows a widening peak at 3269.53 cm⁻¹, indicating OH groups' presence supported by the appearance of sharp uptake at 1043.29 cm⁻¹, indicating the presence of C=O alcohol. Then there is a shoulder intensity band at 2973.70 cm⁻¹ showing CH aliphatic, and then there is C-C at the peak of 2159.66 cm⁻¹. The presence of aromatic rings is shown by the emergence of C=C aromatic uptake in the wavenumber area of 1584.59 cm⁻¹ and 1380.91 cm⁻¹ and strong intensity absorption at wave number 1260.59 cm⁻¹, which indicates the presence of an aromatic C-O group. The presence of aromatic compounds showed by the absorption presence bands at 776.39 and 666.26 cm⁻¹ resulting from CH bending out of the plane. The presence of functional groups OH, C=C aromatic, and C-O aromatics indicate that the antifungal extract contains a phenolic group (figure 4) [22].

4. Conclusion
Fermentation of *P. Marquand* strain TP4 in molasses and tofu wastewater media can produce secondary metabolites after 120 hours of fermentation. Fermentation broth shows antifungal activity against *Fusarium sp.* with 20 mm of inhibition diameter. The total flavonoid content and total phenolic content of freeze-dry fermentation culture were 0.4839 mg/g and 1.171 mg/g, respectively. Ethyl acetate extract shows the highest antifungal activity with MIC 31.25 ppm and MFC 125 ppm. Based on phytochemical and FTIR data, it is assumed that an antifungal compound is a phenolic group.

Acknowledgment
This research is funded by the Ministry of Research and Technology of the Republic of Indonesia and Higher Education in the National Innovation System Incentives Research 2019.

References
[1] Ilyas M 2006 *Biodiversitas* 7 216–220
[2] Sastrahidayat I R 2014 *The Role of Microbes for Plant Health and Environmental sustainability* (Malang: Universitas Brawijaya Press)
[3] Rossi C, Tuttobello L, Ricci M, Casinovi C G and Radics L 1987 The Journal of Antibiotics 40 130–3.
[4] Chen J, Abawi G S and Zuckerman B M 2000 Journal of Nematology 32 70–7
[5] Andayani D G S, Sukandar U, Sukandar E Y and Adnyana I K 2015 HAYATI Journal of Biosciences 22 186-190
[6] Sintawardani N 2012 Portrait of a tofu craftsman in Cibuntu http://www.lipi.go.id. [5 November 2017]
[7] Faisal M, Machdar I, Mulana F and Daimon H The 7th Int. Conf. of Chemical Engineering on Science and Applications 2013 Aceh
[8] Purnama S G, Pandey D S and Sudiana I G 2012 Indonesian Journal of Public Health 1 1–9
[9] Rao N S S 1994 Soil Microorganisms and Plant Growth (Jakarta: UI Press)
[10] BPS (Badan Pusat Statistik) 2011 Structure of Food Crop Farming Costs 2011 www.bps.go.id. [23 Oktober 2017]
[11] Wardani I G A A K, Andayani D G S, Sukandar U, Sukandar E Y and Adnyana I K 2013 Indonesia International Journal of Pharmacy and Pharmaceutical Sciences 5 713–6
[12] Khan N, Hidalgo P M, Ice T A, Maymon M, Humm E A, Nejat N, Sanders E, Kaplan D, Hirsch A M 2018 Front Microbiol 9 2363
[13] Somogyi M 1945 Journal of Biological Chemistry 160 61-68.
[14] Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 J Biol Chem 193 265-75
[15] Harborne J B 1998 Phytochemical Methods (London: Chapman & Hall)
[16] Andayani D G S, Lotulung P D N, Sulastwaty A, Qaanitaati N, Andini D G T, Nursyifa E 2019 Indonesia. J. Cancer Chemoprevention 10 159-168
[17] Rahmouini A, Saidi R and Khaddor M 2019 Euro-Mediterr J. Environ. Integr. 4 27
[18] Hajnos M W, Sherma J and Kowalska T 2008 Thin Layer Chromatography in Phytochemistry (New York: CRC Press)
[19] Susniaht N, Sumeno H and Sudarjad 2005 Plant Pest Science Teaching Materials (Bandung: Fakultas Pertanian-Universitas Padjadjaran)
[20] Rinaldi R, Samingan and Iswadi Pros. Sem. Nas. Biotik 2016 Aceh
[21] Gradisar H, Friedrich J, Krizaj J and Jerala R 2005 Applied and Environmental Microbiology 71 3420–26
[22] Padmawinata K and Soediro I 2005 Analisis Spektrum Senyawa Organik (Bandung: Penerbit ITB)