

EFFECT OF PECTIN BIODEGRADATION WITH *Aspergillus niger* ON TOTAL FLAVONOID CONTENT OF *Citrus limon* L.

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Received : April 19, 2019

Accepted : September 17, 2019

DOI: 10.15575/biodjati.v4i2.4537

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Abstract. Lemon peel and leaf as well as lower grade of lemon contain a considerable amount of flavonoid which can be extracted to produce valuable products. The presence of pectin in lemon hinders the extraction of flavonoid compound. This research was conducted to examine the effect of pectin biodegradation of lemon leaf, peel, and lower grade fruit using solid-state fermentation towards the yield of pectin, crude flavonoid, and total flavonoid content. Fermentation was carried out with the help of *Aspergillus niger* at 30°C for 3, 5, 7, and 9 days of cultivation time. Pectin was extracted from the fermentation broth using citric acid. Crude flavonoid was extracted using maceration and stirred about 500 rpm at 50°C for 40 minutes. Total flavonoid content was analyzed using a spectrophotometer. The optimum cultivation time of *Aspergillus niger* with leaf and peel was 9 days with a pectin yield from lemon leaf was 0.43% dry weight and the pectin yield from lemon peel was 0.04% dry weight. As for the lower grade fruit, a cultivation time for 7 days showed an optimal result with a pectin yield of 0.08% dry weight. The total flavonoid content in the leaf increased up to 94.3% (1.06 mg of the quercetin equivalents per g of substrate) whereas the total flavonoid content in the peel and lower grade fruit increased up to 42% (0.12 mg of the quercetin equivalents per g of substrate) and 48% (0.021 mg of the quercetin equivalents per g of substrate), respectively. These results highlighted the potential valorization of *Citrus limon* L. as an alternative source for producing flavonoid in food and pharmaceutical industry.

Keywords: *Aspergillus niger*, flavonoid, lemon, pectin, solid-state fermentation

Citation

Abduh, M. Y., Zuliansyah, W., Aprina, L. & Arazella, N. (2019). Effect of Pectin Biodegradation with *Aspergillus niger* on Total Flavonoid Content of *Citrus limon* L.. *Jurnal Biodjati*, 4(2), 194-203

INTRODUCTION

Lemon (*Citrus limon* L.) is a hybrid between other Citrus species from the same genus such as *C. aurantifolia*, *C. maxima*, *C. medica* and *C. aurantium*. Lemon can grow in almost all parts of the world, both tropical, subtropical, and temperate regions. The popu-

larity of lemon is relatively high and continue to grow in the community because lemon has been used in various applications such as food and beverages as well as cosmetics. According to Morey (2007), the consumption of Citrus fruits in Indonesia increased from 2.83 kg per capita in 1998 to 3.39 kg per capita in 2003. As a result, around 100.000 to 200.000 tons of

Citrus fruits are being imported to Indonesia every year (Citrus Australia Ltd, 2016).

The high rate of lemon consumption triggers many farmers in Indonesia to cultivate *Citrus limon* L. One of the major problems in lemon plantations, many lemon fruits are affected by pest disease make many of them have already rotten before being marketed (lower grade fruit). Lower grade fruits were produced when environmental conditions do not match the optimum conditions for the cultivation of lemon (Mulyanto, 2017). Apart from lower grade fruits, there are many leaves produced in a lemon plantation that have not yet being valorized. The abundant of lemon peel is also another problem that arises when the popularity of lemon fruit increases along with the development of lemon processing industries. This is because most lemon processing companies only extract the juice which results in abundant lemon peel that reaches up to 50-65% of the fruit mass (González-Molina et al., 2009).

Lower grade fruit, lemon leaf and lemon peel contain a considerable amount of flavonoid, one of the largest natural phenols in nature. Flavonoid contains two aromatic rings which are connected to three carbons either in the form of aromatic rings or not, forming a C6-C3-C6 arrangement. Flavonoid has been widely known for its benefit as an antioxidant, anti-inflammatory and antibiotic (Rohyami, 2008). The flavonoid can be extracted using an alkaline solvent but the presence of pectin in the structure of plant cell wall could bind with several types of flavonoids and obstruct the extraction process (Kanmani et al., 2014). This problem can be overcome by using an appropriate pretreatment so that the extraction process of flavonoid becomes more effective. The pretreatment could be carried out by destroying the cell wall physically by grinding or drying, chemically with the addition of

chemicals such as acids or bases, as well as biologically with the use of bacteria or fungi that produces pectin degrading enzymes (Ory & Angelo, 1977).

Pectinase can degrade pectin in the plant cell wall and can be produced by microorganisms such as *Aspergillus niger*. In a study by Kumar et al.(2011), the maximum production of pectinase and cellulase by *Aspergillus niger* can be achieved when *Aspergillus niger* was cultivated at optimal conditions using a solid-state fermentation with 5 days of cultivation time, pH 4.80, and moisture content of 65%. Therefore, a pretreatment using solid-state fermentation with *Aspergillus niger* as a biological agent was carried out in this research to degrade pectin in the plant cell wall before the extraction process to optimize the extraction of flavonoid and obtain a higher total flavonoid content. The outcome of this research will provide an insight on the potential valorization of *Citrus limon* L. as an alternative source for producing flavonoid in food and pharmaceutical industry.

MATERIALS AND METHODS

Chemicals used in this research were potato dextrose agar (PDA), distilled water, sodium chloride (NaCl), calcium hydroxide ($\text{Ca}(\text{OH})_2$), hydrochloric acid (HCl) 12 N, citric acid, 96% ethanol, methanol pro-analysis, and aluminum chloride (AlCl_3). All the chemicals were obtained from chemical warehouse at School of Life Sciences and Technology, Institut Teknologi Bandung. Quercetin was obtained from School of Pharmacy, Institut Teknologi Bandung.

Preparation of Lemon Substrate

Lemon leaves, peels and lower grade fruits were obtained from a lemon (cultivar Citra) plantation in Parongpong, West Java.

Lemon leaves used in this study were harvested from lemon plants (age 3-5 weeks). Lemon leaves were washed and dried out using a shade drying method until the moisture content was below 10%. Lemon peels were obtained from fresh lemon fruits. The peels were chopped to a size of 1 × 1 cm and dried out using an oven at 50°C until the moisture content was below 10%. Lower grade fruits were sliced into triangular shape and dried in an oven at 40°C until the moisture content was below 10%. The moisture content was measured using a moisture content analyzer. Dried lemon substrates were crushed using a blender and then screened using mesh No. 10, 35 and 60 to obtain a lemon powder (0.25 - 2 mm). The lemon powder was stored in a refrigerator at 4°C. The lemon powder was sterilized using a pasteurization method at 70°C twice for 30 and 15 minutes as suggested by Rangarajan et al. (2010) and Kim & Kim (2011).

Viability Assay of *Aspergillus niger*

Sterilized PDA was prepared in 30 petri dishes and confirmed that none of them were contaminated. Pure culture of *A. niger* was prepared into 6 test tubes and cultured at the same time under similar conditions. Spore suspension after 2 days of cultured was harvested from the PDA by adding 5 mL of NaCl 0.85% weight/volume (w/v) into the test tubes. The spores were harvested from the PDA until the spores dissolved in the NaCl solution. The suspension was homogenized using a vortex and 0.3 mL was taken to calculate the number of spores. The rest of the suspension was diluted into various concentrations of 10³, 10² and 10¹ spores/mL. After the homogenization, each of the suspension (0.1 mL) was added into a petri dish and spread until the surface of PDA became dry (Maturin & James, 2001). This was done twice for each concentration. The same procedures were carried out for the

spores on the third and sixth days. The viability curve was prepared following the procedures by Scott (2011).

Preparation of *Aspergillus niger* Spore Suspension

Aspergillus niger culture was obtained from School of Life Sciences and Technology, Institut Teknologi Bandung. The spores were sub-cultured on slant of PDA and maintained at room temperature (25°C) for 4 days. The spores were then stored at 4°C as a stock culture and then cultured to a new test tube containing PDA and incubated for 4 days at room temperature for preparation of spore suspension. Test tubes containing culture of *Aspergillus niger* that had been incubated for 4 days was added with 5 mL of 1% w/v NaCl. The preparation of spore suspension was carried out aseptically in the laminar air flow cabin following the procedures as suggested by Rangarajan et al. (2010).

Solid-State Fermentation of Lemon Substrate with *Aspergillus niger*

Sterilized lemon powder (10-30 grams) was added into the distilled water with a ratio of 1:1 w/v in roux bottles of 300 mL. The roux bottles were sterilized in an autoclave at 121°C for 15 minutes. The spore suspension was added into the bottles (1 mL/5 grams of substrate). All the work was carried out aseptically in a laminar air flow cabin. Incubation was conducted at 30°C for 3, 5, 7 and 9 days to investigate the effect of cultivation time using SSF method on the total flavonoid content of lemon substrates (Darah et al., 2013; Kumar et al., 2011; Mrudula & Anitharaj, 2011; Rangarajan et al., 2010).

Extraction of Pectin from Lemon Substrate

Ten grams of fermented substrates and biomass of *Aspergillus niger* was subjected

to a moisture content analyzer to determine its measure content. Thirty grams was placed into a 250 mL beaker glass and then mixed with 90 mL of distilled water and 10 mL of citric acid solution at pH of 3.2. The mixture was then heated using a hotplate stirrer at 60°C and stirred using a magnetic stirrer for 1 hour. The mixture was squeezed using batiste cloth. The liquid obtained was centrifuged and filtered using a Whatman No. 2 filter paper.

The filtrate was coagulated by adding 95% ethanol at a ratio of 1:1 volume/volume (v/v) and allowed to stand ± 2 hours to form pectin on the surface. The pectin was separated from the liquid and washed with ethanol 2-3 times. The pectin was then dried in the oven at 50°C until a constant weight was achieved (Kanmani et al., 2014). The yield of pectin can be calculated based on the percentage of dry weight (% dw) using the equation (1).

$$Y_p (\% \text{ dry weight}) = \frac{m_p (\text{gram})}{(m_{sl} \times (1 - mc)) (\text{gram})} \times 100\% \quad (1)$$

with, Y_p is the yield of pectin in dry weight (% dw), m_p is the mass of pectin in gram, m_{sl} is the mass of lemon powder in gram, and mc is the moisture content of lemon powder.

Extraction of Crude Flavonoid from Lemon Substrate

After solid state fermentation, thirty grams of substrates and biomass of *Aspergillus niger* were moved into Erlenmeyer flasks 250 mL. The mixture was added into 150-200 mL of distilled water and $\text{Ca}(\text{OH})_2$ powder was added until pH 11-11.5 followed by maceration using an incubator shaker at 50°C with a stirring speed of 500 rpm for 40

minutes. The mixture was squeezed using a batiste cloth. The liquid fraction was then centrifuged for 5 minutes with a stirring speed of 5000 rpm. The supernatant was filtered using a Whatman No. 2 filter paper. The pH of the filtrate was measured and then added HCl until pH 1.9-2.5. The liquid was allowed to stand for 12 hours to precipitate the flavonoid. The extract was centrifuged, and then filtered using a Whatman No. 2 filter paper. The solid extract was dried in an oven at 50°C until constant weight (Curto et al., 1992). The yield of crude flavonoid was calculated using equation (2).

$$Y_{cf} (\% \text{ dry weight}) = \frac{m_{cf} (\text{gram})}{(m_{sl} \times (1 - mc)) (\text{gram})} \times 100\% \quad (2)$$

with, Y_{cf} is the yield of crude flavonoid in dry weight, m_{cf} is the mass of crude flavonoid in gram, m_{sl} is the mass of lemon powder in gram, and mc is the moisture content of lemon powder.

Total Flavonoid Content of Lemon Substrate

Analysis of total flavonoid content was conducted using a spectrophotometer at a wavelength of 415 nm. A standard curve was prepared using a quercetin stand-

ard solution in the range of 0, 2.5, 5, 10, 15, 20, 25, 30, 35 and 40 ppm with the addition of 2% w/v AlCl_3 in methanol with a ratio of 1:1 volume/volume (v/v). The same procedures were used to measure the absorbance of flavonoid extracts. The total flavonoid content was expressed as a quercetin equivalent (Machado et al., 2016). All data was analyzed using one-way analysis of variance (ANOVA) using MINITAB 17 and Duncan's Multiple Comparison Test using SPSS.

RESULTS AND DISCUSSION

Viability Curve of *Aspergillus niger*

Figure 1 shows the percentage of viability of *A. niger* on a PDA medium. The viability was measured for five days (from day-2 to day-5). The highest viability was seen on day-4, about 45% of spores were able to germinate. The viability curve represents the amount of spores which was produced at a time. Asexual spores of *A. niger* would be released under two conditions, poor nutrients in the medium or unfavorable environmental conditions. PDA medium has enough nutrients for fungal growth, but limited oxygen supply resulting in production of the asexual spores. The process of sporulation would reach its peak when the fungi were in a stationary phase. At this phase, nutrient source started to be depleted and the

metabolism begins to be directed to the production of secondary metabolites. To continue the life cycle, *A. niger* would produce asexual spores as much as possible (Bruslind, 2018).

According to a study by Favella-Torres et al. (1998), the lag phase of *A. niger* cultivated on a PDA medium was at 20th hours after incubation. The logarithmic phase was seen when the incubation period reached 40-50 hours and after that, the stationary phase began to appear (Favella-Torres et al., 1998; Muzakkar & Winarsa, 2019). The findings supported the results obtained in this study which showed that the spores were produced on day-4 of incubation (after 50 hours of incubation). These spores also had a high viability (high germination percentage). Thus, it can be concluded that spores after 4-days of incubation were suitable to be inoculated for new substrates.

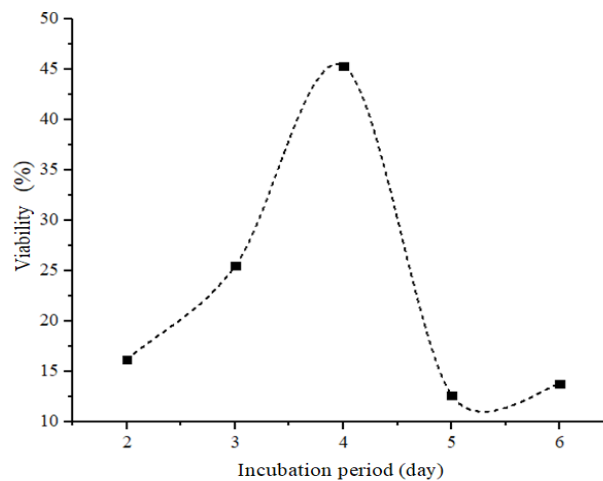


Figure 1. Viability of *A. niger* on Potato Dextrose Agar.

Determination of Pectin Yield

The yield of pectin from lemon leaf, peel, and lower grade fruit which had been pretreated with *A. niger* showed a decreasing trend as the pretreatment days increased as shown in Table 1. The average yield of pectin from the leaf was 2.52% dw at day-0 of pretreatment. The pectin yield decreased along with day of

pretreatment and reached a lowest amount of 0.43% dw after 9 days of pretreatment. Lemon peel and lower grade fruit also showed a decreasing trend of pectin yield. For samples without pretreatment, the pectin yield was 6.62% dw and 6.60% dw for lemon peel and lower grade fruit, respectively. Pectin yield decreased to 0.04% dw for lemon peel and 0.07%

for lower grade fruit. The decrement in pectin yield may be caused by pectin lyase enzyme activities that had been released by *A. niger*.

Determination of Crude Flavonoid Yield

The yield of crude flavonoid extract from lemon leaf, peel and lower grade fruit which had been pretreated with *A. niger* showed an increasing trend as the pretreatment days increased (Table 2). Average yield of crude flavonoid from leaf was 0.06% dw at day-0 of pretreatment. The crude flavonoid yield increased along with day of pretreatment and reached its highest amount of 1.17% dw after 7 days of pretreatment before slightly decreased to 0.83% dw after 9 days of pretreatment. Lemon peel and lower grade fruit also showed an increasing trend of crude flavonoid yield. The crude flavonoid yield in the samples without pretreatment, was 0.69% dw

and 0.62% dw for lemon peel and lower grade fruit, respectively. Crude flavonoid yield increased to a maximum value of 2.76% dw for lemon peel and 3.88% for lower grade fruit after 3 days of fermentation before eventually decreased as pretreatment time increased. According to Hay et al (1961), *A. niger* were able to metabolize some flavonoid compound when its environment contains high amount of flavonoid but lack of monosaccharides. Adetuyi & Ibrahim (2014) also reported that an increase in flavonoid content of okra seeds due to fermentation but the flavonoid content decreased as the fermentation period increased. As such may be attributed to the concentration of flavonoid compound in the samples and duration of the fermentation process (Ehsan et al., 2010; Adetuyi & Ibrahim, 2014).

Table 1. Pectin yield obtained from lemon peel, leaf and lower grade fruit

Pretreatment time (day)	Pectin yield (% dw)		
	Leaf	Peel	Lower grade fruit
0	2.52 ± 0.02 ^b	6.62 ± 0.18 ^a	6.60 ± 0.86 ^b
3	0.94 ± 0.3 ^{ab}	5.55 ± 3.67 ^a	0.54 ± 0.21 ^a
5	0.69 ± 0.3 ^a	0.09 ± 0.0004 ^a	0.2 ± 0.20 ^a
7	0.76 ± 0.5 ^a	0.07 ± 0.002 ^a	0.08 ± 0.06 ^a
9	0.43 ± 0.4 ^a	0.04 ± 0.007 ^a	0.07 ± 0.04 ^a

*% dw (dry weight) value is presented in mean ± SD (standard deviation). The 'a, b, c, d, e' value in the same column shows a significant difference (p < 0.05) (ANOVA "Duncan's Multiple Comparison Test")

Table 2. Crude flavonoid yield obtained from lemon peel, leaf and lower grade fruit

Pretreatment time (day)	Crude flavonoid yield (% dw)		
	Leaf	Peel	Lower grade fruit
0	0.06 ± 0.03 ^a	0.69 ± 0.001 ^a	0.62 ± 0.01 ^a
3	0.72 ± 0.05 ^{ab}	2.76 ± 0.92 ^b	3.88 ± 0.02 ^a
5	0.64 ± 0.48 ^{ab}	0.86 ± 0.76 ^a	3.01 ± 0.79 ^a
7	1.17 ± 0.48 ^b	0.53 ± 0.44 ^a	3.76 ± 1.99 ^a
9	0.83 ± 0.32 ^{ab}	0.71 ± 0.31 ^a	2.80 ± 1.26 ^a

*% dw (dry weight) value is presented in mean ± SD (standard deviation). The 'a, b, c, d, e' value in the same column shows a significant difference (p < 0.05) (ANOVA "Duncan's Multiple Comparison Test").

Determination of Total Flavonoid Content

Total flavonoid content in the substrate was measured using a quercetin standard and compared with the total flavonoid content

in the crude flavonoid extract. Analysis using ANOVA showed significant differences among the data (Table 3).

Table 3. Comparison of total flavonoid content in substrate (mg QE/g substrate) and crude flavonoid extract (mg QE/g crude) of lemon leaf, peel and lower grade fruit.

Pretreatment (days)	mg QE/g substrate			mg QE/g crude		
	Leaf	Peel	Lower grade fruit	Leaf	Peel	Lower grade fruit
0	0.06 ± 0.03 ^a	0.065 ± 0.007 ^b	0.011 ± 7 × 10 ^{-4a}	101.2 ± 24.1 ^{bc}	9.6 ± 1.0 ^c	28.3 ± 1.82 ^a
3	0.38 ± 0.03 ^a	0.035 ± 0.001 ^a	0.014 ± 1 × 10 ^{-3ab}	53.3 ± 10.9 ^a	1.3 ± 0.04 ^a	33.8 ± 2.42 ^{ab}
5	1.06 ± 0.80 ^a	0.043 ± 0.002 ^a	0.015 ± 4 × 10 ^{-4b}	165.7 ± 24.8 ^d	5.0 ± 0.3 ^b	53.3 ± 0.91 ^{bc}
7	0.94 ± 0.39 ^a	0.076 ± 0.006 ^b	0.021 ± 2 × 10 ^{-3d}	80.5 ± 23.6 ^{ab}	14.4 ± 0.1 ^d	43.5 ± 3.94 ^c
9	1.06 ± 0.41 ^a	0.123 ± 0.009 ^c	0.017 ± 7 × 10 ^{-4bc}	127.1 ± 19.6 ^c	17.3 ± 1.2 ^e	39.4 ± 4.54 ^d

*mg QE/ g value is presented in mean ± SD (standard deviation). The ‘a, b, c, d, e’ value in the same column shows a significant difference (p <0.05) (ANOVA “Duncan’s Multiple Comparison Test”).

Total flavonoid content of leaf, peel, and lower grade fruit showed an increasing trend along with the increasing pretreatment time. The total flavonoid content was 0.06, 0.065, and 0.011 mg QE/g substrate respectively for lemon leaf, peel and lower grade fruit. After 9 days of pretreatment, the total flavonoid content increased to 1.06 mg QE/g substrate for lemon leaf, 0.123 mg QE/g substrate for peel, and 0.017 mg QE/g substrate for lower grade fruit. These data are in line with the results obtained by Adetuyi & Ibrahim (2014) that fermentation of okra seeds increased the total flavonoid content and showed greater antioxidant activities as compared to unfermented okra seeds.

Statistically, total flavonoid content of lemon peel samples showed that there was a significant increase on the pretreated samples (P-value <0.05). The data also showed a significant decrease in samples from the 3rd and 5th days of fermentation. The highest content was obtained on the 9th day of fermentation.

The data for lower grade fruit also shows a significant difference with the highest increase on the 7th day (P-value <0.05). However, the leaf samples did not show significant effects on the total flavonoid content between the pretreated and non-pretreated samples.

The increasing total flavonoid content in the pretreated sample is caused by the ability *A. niger* to produce pectinase enzyme that can degrade pectin polymer so that the process of flavonoid isomeration with the addition of Ca(OH)₂ could take place (Curto et al., 1992). It is the isomeration process that makes flavonoids extractable. Due to the activity of pectinase enzymes increases along with the increasing of cultivation time, more and more pectin is degraded so that the total flavonoid content in lemon peel increases (Ahmed et al., 2016). As for the leaf, there was no increased in the total flavonoid content. This was supported by the absence of fermentation effect on the crude pectin content during the fermentation process. This was caused by the pectin

content in the leaf that was far less than the two substrates as mentioned in the previous section.

The total flavonoid content in the crude flavonoid extract was much higher as compared to the total flavonoid in the substrate which lies in the range of 53.3 to 165.7 mg QE/g for lemon leaf, 5.0 to 17.3 mg QE/g for lemon peel and 28.3 to 53.3 mg QE/g for lower grade fruit. The fluctuating trend of the total flavonoid content may be due to the inhomogeneity of the sterilization process that might have degraded some types of flavonoids (Doona et al., 2010). Statistically, the total flavonoid content in pre-treated samples were significantly difference as compared to the non-pretreated samples (P -value <0.05). In brief, the yield of pectin for all samples greatly decreased as the cultivation time increased. Consequently, the yield of crude flavonoid and total flavonoid content increased for all samples that were pre-treated with *A. niger*.

ACKNOWLEDGMENTS

This work was funded by a research grant from Ministry of Research, Technology, and Higher Education Indonesia (SITH.PN-1-28-2019). Acknowledgement to Mr. Asep for providing the lemon used in this study.

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