# The Study of Antibacterial and Antioxidant Activities of Styrax Leaves Fermentation by *Aspergillus niger*

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**Abstract.** Styrax leave is a part of Styrax plant that can be used as medicine because it contains potential antibacterial and antioxidant bioactive components. Fermentation has been known increasing antibacterial and antioxidant activity of herbal plants. *Aspergillus niger* is one of fermentation agents that can increase the activity of antibacterial and antioxidant from natural ingredients. Therefore, this study aimed to test the activity of antibacterial and antioxidant of Styrax leaves Fermentation by *Aspergillus niger* using Solid State Fermentation and Submerged Fermentation methods. Cultures of *Aspergillus niger* aged 32 h in concentration of 0.00192 g/mL was inoculated as much as 10% of working volume and was used for fermentation up to 56 h (logarithmic phase, L) and 64 h (stationary phase, S). The unfermented and fermented Styrax leaves were extracted with ethanol using maceration method. The total phenol content was increased significantly in submerged fermented styrax leaves until stationary phase to 40.834 ± 0.356 mg GAE/g. The antioxidant activity represented by $IC_{50}$ value with a significant increasing in styrax leaves fermented by submerged fermentation until stationary phase to value 50.2201 ± 2.2633. The test of antibacterial activity showed that submerged fermented of styrax leaves until stationary phase increased significantly the inhibitions diameter zone against *Staphylococcus aureus* and *Pseudomonas aeruginosa* to 29.167 ± 0.763 mm and 25.4 ± 0.360 mm, respectively. Based on this research, submerged fermented process until stationary phase is proven can increase antioxidant and antibacterial activity of styrax leaves.

**Keywords:** Styrax leaves · Fermentation · Antioxidant · Antibacterial · Aspergillus niger

## 1 Introduction

Styrax is one of the most widely grown plants in Indonesia, especially on the island of Sumatera. Styrax leaves are widely consumed by the public as tea which is believed to


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have benefits for the body. Styrax leaves have been studied to contain bioactive compounds, namely glycosides, anthraquinones, saponins, flavonoids, tannins, and triterpenoids which are known to be active compounds as antibacterials [1–3]. Bioactive components are components found in plants or food that are useful for human health. However, bioactive components cannot always be extracted using simple diffusion processes such as maceration, soxhletation and hydrodistillation [4–6].

Fermentation is considered as an option to produce new, active and less toxic bioactive components. The fermentation process can destroy plant cell walls which are useful for liberating or synthesizing various types of bioactive components that can be useful as antioxidants or antibacterials. Bioactive compounds are secondary metabolites produced from plants that have antioxidant and antibacterial properties. One of the microorganisms that is widely used to produce or increase the content of secondary metabolites that are useful for the pharmaceutical and industrial fields is *Aspergillus niger*. *A. niger* is one of the microorganisms that can produce many types of secondary metabolites that are useful in the fermentation process [7] and shows that the antioxidants activities increase through fermentation [8].

The use of solid fermentation (SSF) and submerged fermentation (SmF) methods does not always result in a similar increase in antibacterial or antioxidant activity on various types of substrates, therefore, the aims of this study was to compare the antibacterial and antioxidant activities of styrax leaves produced without the fermentation process, using the SSF and SmF which was stopped in the final logarithmic phase and the final stationary phase.

## 2 Materials and Methods

### 2.1 Preparation of Styrax Leaves

The styrax leaves used in this research were harvested from 5–10 leaves from the tip of the tree with 7–7.5 cm length and 2–2.5 cm width which where light green in colour and in good leaf condition. Styrax leaves were washed with water and then dried at 50 °C using an oven for 24 h. After that, the leaves were ground to 60 mesh.

### 2.2 Microorganism Preparation

*Aspergillus niger* was rejuvenated by inoculating one loop of colonies on sterile Potato Dextrose Agar (PDA) media, then incubated at 37 °C for 3 days. The standard curve was made by making eight variations of the dilution of *Aspergillus niger* inoculum using 1% NaCl with a volume of 15 mL. The growth curve was made by inoculating 3 loops of *Aspergillus niger* on YPG media and sampling every 1 h. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 600 nm. Sampling was carried out until the growth of *Aspergillus niger* reached the stationary phase. Absorbance data was converted to cell concentration ($g.mL^{-1}$) using the equation of the line obtained from the standard curve. Then, a growth graph between the concentration of cells against time was made.

### 2.3 Fermentation Process

Fifty gram of styrax leaves was used as a fermentation substrate. For Solid State Fermentation (SSF), water was added to achieve humidity of ±60%. For Submerged Fermentation (SmF), water was added to achieve humidity of ±90%. *Aspergillus niger* added as much as 10% (v/v) for both SSF and SmF and fermentation was stopped when it reached logarithmic phase (54 h) and stationary phase time (64 h). 200 mL Ethanol was used as an agent to extract bioactive compounds in styrax leaves without fermentation and with fermentation (SSF and SmF).

### 2.4 Total Phenol Determination Process

The determination of the total phenol content was carried out by taking a 1 mL aliquot of the extract mixed with 1 mL of Folin-Ciocalteau phenol reagent. After reaction, then 1N $Na_2CO_3$ was added. The absorbance was measured at a wavelength (λ) of 725 nm using a spectrophotometer [6].

The standard gallic acid curve was determined by making 8 concentrations of standard solutions of gallic acid with a concentration range of 0–100 ppm. Next, 0.01 g of gallic acid was added to 100 mL of ethanol. Then dissolved again into several concentrations up to 100 ppm. From each concentration of standard gallic acid solution, 1 mL was taken and 1 mL of Folin-Ciocalteu reagent was added which was then homogenized. After homogenization, 4 mL of 1N $Na_2CO_3$ was added which was then measured using a spectrophotometer with a wavelength (λ) of 725 nm. The absorbance value of gallic acid is plotted against the concentration of gallic acid.

### 2.5 Determining Antioxidant Activity Using DPPH Method

The sample was diluted into 5 concentrations, namely 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. The absorbance measurement of the sample was carried out by mixing 2 ml of each sample solution and 2 ml of 50 ppm DPPH. Both solutions were incubated at a temperature of 25 °C for 30 min and then the absorbance was measured at a wavelength of 517 nm using a spectrophotometer. The blanko used was a mixture of 2 ml of 70% ethanol and 2 ml of 50 ppm DPPH. The percentage of DPPH inhibition is shown in the following equation:

$$\text{Inhibition percentage } (\%) = \left( \frac{\text{Blanko Absorbance} - \text{Sampel Absorbance}}{\text{Blanko Absorbance}} \right) \times 100 \quad (1)$$

After being calculated using the above equation, the percentage of inhibition (%) of each sample concentration will be obtained.

### 2.6 Determination of Antibacterial Activity

Antibacterial activity test was conducted against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The bacteria were rejuvenated on NB media and incubated at

37 °C. The logarithmic phase of *Staphylococcus aureus* started from the 2nd hour to the 8th hour and *Pseudomonas aeruginosa* from the 4th hour to the 18th hour. A total of 0.1 mL of bacterial suspension was inoculated into sterile nutrient medium and spread evenly using L rods. Sterile disc paper was dipped in 5 L of unfermented or fermented styrax leaves extract, nebacetin (positive control) and ethanol (negative control), then placed on agar media. The agar medium was incubated at 37 °C for 24 h and then diameter of the clear zone around the disc was measured using a calliper.

## 3 Result and Discussion

Different treatment in Styrax Leaves show different yield in total phenolic compounds, antioxidant activity and antimicrobial activity. Figure 1 showed there was an increase in total phenol from fermented styrax leaves compared to unfermented styrax leaves.

From above the results, it can be concluded that there was an increase in total phenol from fermented styrax leaves compared to unfermented leaves. The most significant increase in total phenol was obtained in styrax fermented using the submerged fermentation in stationary phase based on statistical analysis of Turkey's HSD Test. Some researches [9–13] reported that fermenation can increase phenolic content through β-glucosidase enzyme activities. This enzyme hydrolyze β-glucosidic bond to produce free phenolic.

The antioxidant activity of styrax leaves with fermentation and without fermentation is shown in Table 1 in scavenging 50% of free radicals (*diphenylpicrylhydrazyl*) into non-radical compounds (*diphenylpicrylhydrazine*).

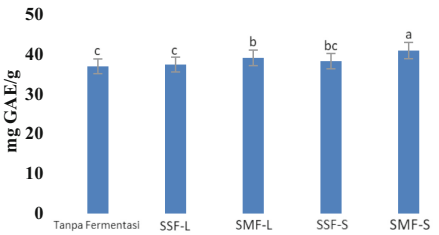


**Fig. 1.** Total phenol concentration of each test sample.

**Table 1.** Comparison of the $IC_{50}$ levels of each sample and its analysis statistical

| Sample | $IC_{50}$ | *Turkey HSD Inference* |
|---|---|---|
| Non Fermentation | 439.7362 ± 3.302 | |
| SSF-L | 264.6443 ± 10.288 | *Significant* |
| SmF-L | 106.7296 ± 1.271 | *Significant* |
| SSF-S | 150.5107 ± 5.993 | *Significant* |
| SmF-L | 50.22014 ± 2.263 | *Significant* |

The $IC_{50}$ value is the value used to indicate the effectiveness of a sample in counteracting 50% of free radicals. Based on Table 1, it was shown that the half-maximal inhibitory concentration ($IC_{50}$) of the fermented sample is compared to the unfermented sample. Based on the statistical analysis of Tukey's HSD Test, the comparison of the $IC_{50}$ significance value was shown to be the greatest for styrax leaves fermented by the submerged fermentation method to the stationary phase [14].

Antibacterial activity test of unfermented and fermented styrax leaves using SmF and SSF methods to logarithmic and stationary phase was carried out against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in vitro. The antibiotic Nebacetin was used as a positive control in this experiment and showed high inhibitory response activity in both types of bacteria used. Ethanol was used as a negative control. The inhibition from all of component is shown on Fig. 2 for antibacterial activity against *Staphylococcus aureus* and Fig. 3 is shown antibacterial activity against *Pseudomonas aeruginosa.*

The fermented styrax leaves extract showed a significant difference in antibacterial activity for *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Based on the statistical analysis of Turkey's HSD Test, it was concluded that all fermented samples showed a significantly differences in forming diameter inhibitory zones and SmF at stationary phase showed the greatest result to forming Inhibitory zones for both *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Fermented styrax leaves showed and proving an increase in total phenol, antioxidant activity, and antibacterial activity with help of *A. niger* as a fermentation agent. *Aspergillus niger*, as a large fungus has the characteristics of vigorous growth, short

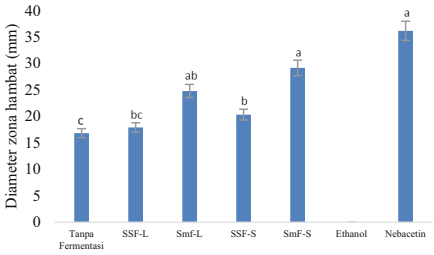


**Fig. 2.** Inhibitory zone of antibacterial activity against *Staphylococcus aureus.*

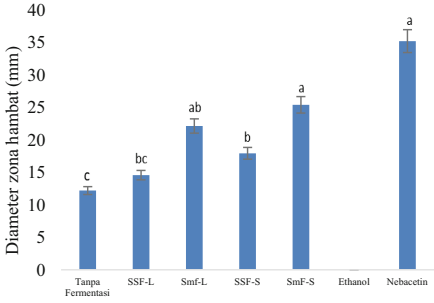


**Fig. 3.** Inhibitory zone of antibacterial activity against *Pseudomonas aeruginosa.*

fermentation cycle, and no toxin production. It is one of the safe strains certified by the Food and Drug Administration (FDA). It can secrete amylase, cellulase, glucosidase, and endoglucanase. Fermentation of *Aspergillus niger* is an effective method of biotransformation of Chinese medicinal materials by using enzymes produced by *Aspergillus niger* [15–16]. It has advantages of improving curative effect, reducing toxicity, and producing new active ingredients. Fermentation serves to break down or change the substrate that exists in plants with the help of *Aspergillus niger* that produce enzymes, one of which is the β-glucosidase enzyme so that it can increase the bioactive components present in the substrate or facilitate the ethanol used for extraction to bind the bioactive compounds present in incense leaves [16]. In this study, styrax leaves contain several bioactive compounds, namely glycosides, anthraquinones, saponins, flavonoids, tannins, and triterpenoids, which have been proven by Pharmacological studies have introduced the use of this compounds in anticancer, antioxidant, anti-inflammation and antimycrobacterial.

Antibacterial activities assayed to determine the zone of inhibitions towards *S. aureus* and *P. aeruginosa.* Fermented styrax leaves was found to have significant inhibition zone and Styrax Leaves in SmF at stationary phase has the most significant inhibition zone which was shown in Table 1. This activity was equally the same compared to antibacterial activity of Styrax Resin which was famously known and widely used in the world for the effectiveness to inhibit antibacterial [17].

Based on test on *S. aureus* and *P. aeruginosa* it was shown that the SmF method to the stationary phase was also able to significantly increase the zone of inhibition in these two types of bacteria. These results are in line with the increase in the total phenol content and antioxidant test of styrax leaves extract fermented using the submerged fermentation method to the stationary phase.

The criteria for antibacterial strength based on the diameter of the inhibition zone formed on agar media that has been overgrown with test bacteria are as follows:

a. Diameter >20 mm: Has a very strong inhibition
b. Diameter 10–20 mm: Has a strong inhibition
c. Diameter 5–10 mm: Has moderate resistance
d. Diameter 0–5 mm: Has a weak inhibition.

Based on the results of the antibacterial test against *S. aureus*, the diameter of the inhibition zone of the unfermented styrax leaves extract was 16.833 ± 0.2886 mm which was classified as a strong inhibition zone, as well as the solid-state fermentation extract up to the stationary phase and logarithmic with the diameter of the inhibition zone. Respectively were 20.333 ± 0.57735 mm and 17.9 ± 0.17321 mm. The submerged fermentation extracts up to the stationary and logarithmic phases showed a very strong inhibition zone with the diameters of the inhibition zones being 29.1667 ± 0.76376 mm and 24.8 ± 0.34641 mm, respectively. Likewise, the results of the antibacterial test against *P. aeruginosa*, the diameter of the inhibition zone of the unfermented styrax leaves samples, and fermented by solid state fermentation until the stationary and logarithmic phases showed strong inhibition with diameters of 12.2 ± 0.2645 mm, 17.933 ± 0.20817 mm and 14.5667 ± 0.51316 mm, respectively. Extracts fermented by submerged fermentation to stationary and logarithmic phases also showed very strong

inhibition with inhibition zone diameters of 25.4 ± 0.360 mm and 22.133 ± 0.152 mm, respectively.

The antioxidant activities were assayed by DPPH free radical scavenging. The DPPH free radical is a simple and acceptable method to evaluate the antioxidant activity of plant extracts. The DPPH forms a stable molecule on accepting an electron or a hydrogen atom and thus has applications in the determination of radical scavenging activity of natural products as well as synthetic compounds. The fermented styrax leaves exhibited high antioxidant activity by DPPH free radical scavenging assay with styrax leaves with SmF at stationary phase showed the most significant $IC_{50}$ value compared to unfermented styrax leaves [14].

In this study, extracts fermented by submerged fermentation showed the most significant results compared to extracts that were not fermented and fermented by solid state fermentation. This is because submerged fermentation is very effective in increasing bioactive compounds. Submerged fermentation has several advantages such as high microbial productivity, lower risk of contamination and easy handling of fermentation conditions so that more bioactive compounds are produced. This statement is in line with the results of this study that the antioxidant and antibacterial activity of incense leaves experienced the most significant increase when fermented by submerged fermentation [2].

Based on these results, it can be concluded that styrax leaves extracts have great potential as antimicrobial compounds againts Gram-negative and Gram-positive bacteria and they can be used in the treatment of infectious diseases caused by Gram negative and Gram-positive bacteria. They can also be a source of natural antioxidants with styrax leaves with SmF at stationary phase shown the most significant difference compared to unfermented styrax leaves.

Due to their antibacterial and antioxidant activities, fermented styrax leaves extracts have promising potential as a source of antioxidant and antimicrobial agents. Antibacterial and antioxidant activities found in these extracts might make styrax leaves have the same antibacterial and antioxidant activity compared to styrax resin that has been used around the world as natural antioxidant and antibacterial agent and make styrax leaves to be developing as antibacterial and antioxidant agents in future days.

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