Growth of *Bacillus toyonensis* in Tofu Wastewater

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**Abstract.** *Bacillus toyonensis* has been isolated in Dumai mangrove ecosystem of Riau Province. One of factors affecting the growth of the bacteria is growth substrate. Tofu wastewater is rich in nutrition which can be used as substrate for bacterial growth. This research aimed to observe the growth of *B. toyonensis* in different concentration of tofu wastewater. The bacteria was grown in tofu wastewater at concentrations 8%, 10% and 12% was supplemented with 0.1 g K\textsubscript{2}HPO\textsubscript{4}, 0.15 g KH\textsubscript{2}PO\textsubscript{4}, 0.15 g NaCl and 0.5 g vitamin B12 in 100 mL distilled water. The bacterial growth was observed by using spectrophotometer at λ 610 nm and by analysis the total plate counts on plate count agar (PCA) at 0, 24, 48, 72 and 96 hour cultivation. Spectrophotometric observation showed that the highest bacterial growth of all tofu wastewater treatments indicated by the addition of 12% tofu wastewater, although the absorbance value was lower than culture in tryptic soy broth (TSB) as control. Exponential growth occurred between 0-24 hour incubation, and the highest growth indicated in substrate contained 12% tofu wastewater. Similarly, total plate count (TPC) analysis indicated that the highest bacterial growth of all treatment occurred at 24 hours incubation, and the highest count was also indicated by treatment of 12% tofu wastewater (2.42±0.06×10\textsuperscript{8} CFU/mL). In conclusion, tofu wastewater can be an alternative substrate for the bacterial growth.

1. Introduction

*Bacillus* is a genus of bacteria which is able to produce enzymes such as proteases, cellulase and amylase that have been used in many industries, biotechnology and environment. In feed industry, for example, *Bacillus toyonensis* has been used as probiotic in animal feed. Meanwhile, some species of *Bacillus* indicate an ability to degrade organic waste. *Bacillus toyonensis* found in mangrove sediment of the Dumai Marine Station of Riau Province had an ability to produce cellulase and protease [1, 2]. For the application, the bacteria need to be cultured in mass scale in cheaper and affordable medium containing nutritive components for the growth.

*Bacillus toyonensis* is a Gram-positive, spore-forming bacteria that forms a homogeneous independent branch within the *Bacillus* genus [3]. The species had been found in diverse environment. *B. toyonensis* strain AEMREG\textsuperscript{6} isolated from sediment samples of a marine environment in the Eastern Cape Province of South Africa produced a glycoprotein bioflocculant [3]. *B. toyonensis* strain PNTB1 has been shown to exist in neritic regions of Arabian Sea [4]. Strain P18 isolated from a deep-
sea sediment sample collected from the hydrothermal field in southern Okinawa Trough which is highly toxic to fish and mammalian cells and can invade into host tissues and cause mortality [5].

Growth of microorganism is influenced by some environmental factors and substrate as the growth medium, mainly rich in protein. Synthetic medium containing nutrient for bacterial culture is commercially available. However, the price is relatively expensive. Therefore, cheaper and easily obtained medium or substrate is required in order to produce bacteria in small and medium scales. Some researches had reported that tofu wastewater could be used as substrate for the growth of some microorganisms. The medium with tofu wastewater (TW) content of 6 v/v% was the best medium for biomass production of *Spirulina platensis* [6]. While, rapid growth in 10% volume and high concentration of alga in 90% volume shows that *Chlorella* sp. grows better in tofu wastewater than in seawater [7]. Two studies reported that the highest density and growth rate of *Chlorella pyrenoidosa*, in addition to the highest contents of lipid, carbohydrate and protein were found in the tofu wastewater treatment of 20% [8, 9]. Similarly, Cyanobacteria *Nostoc muscorum* grew well in the dilution of tofu wastewater 40% [10]. The best nitrogen source to increase the growth of lactic acid bacteria is sulphate ammonium because it contains higher total acid in comparison to urea-supplemented whey tofu [11]. However, *Bacillus amylobiquefaciens* subsp. *plantum* indicated lower biomass production in tofu wastewater compared to palm sugar medium [12].

Medium composition is the most important component in the bacterial cultivation. Tofu liquid waste contains high organic matter, those are protein 40-60%, carbohydrates 25-50%, oil and fat 10%, and nutrients of N, P, K, Ca, Mg, and Fe [13]. The high contents of organic substances can cause the rapid growth of microbes in the wastewater [14]. Our previous study identified *B. toyonensis* from mangrove sediment of Dumai Marine Station. However, optimum condition for growth of the bacteria was not studied, yet. Current research aims to observe the growth of *B toyonensis* in tofu wastewater.

2. Materials and Methods

Tofu wastewater was obtained from tofu home industry in Suka Karya, Pekanbaru. *B. toyonensis* used in this research was from culture collection in the Laboratory of Marine Microbiology which was obtained from previous study [1].

2.1. Preparation of Bacteria

*Bacillus toyonensis* was sub-cultured in nutrient agar (NA) medium by pour plate technique, and then was incubated at 30℃ for 48 hours.

2.2. Preparation of Culture Medium

Triplicate bacterial culture in 100 mL of each trial medium was prepared as follows:

A1B1 = 8% (8 mL) of tofu wastewater + 10% skimmed milk + 0.1 g K₂HPO₄ + 0.15 g KH₂PO₄ + 0.5 g vitamin B12, made up to 100 mL with distilled water.

A2B1 = 10% (10 mL) of tofu wastewater + 10% skimmed milk + 0.1 g K₂HPO₄ + 0.15 g KH₂PO₄ + 0.5 g vitamin B12, made up to 100 mL with distilled water.

A3B1 = 12% (12 mL) of tofu wastewater + 10% skimmed milk + 0.1 g K₂HPO₄ + 0.15 g KH₂PO₄ + 0.5 g vitamin B12, made up to 100 mL with distilled water.

Except skimmed milk, each trial medium was mixed, sterilized in autoclave at 121℃ for 15 minutes. While the skimmed milk was prepared by diluting 10 g of skimmed milk in 90 mL of
distilled water, then was pasteurized in shaker water bath 67°C for 30 minutes. After cooling down, the skimmed milk solution was added to the trial media. Tryptic soy broth (TSB) was used as control medium in the experiment.

2.3. Growth of Bacteria
After mixing each trial medium with skimmed milk, 10 mL of *B. toyonensis* culture (density of $10^6$ cells/mL) was added and mixed well. The suspension was then incubated at 37°C in shaker water bath. The bacterial growth was observed at hours 0, 24, 48, 72 and 96 of incubation time by measuring the absorbance (optical density, OD value) in spectrophotometer at wavelength ($\lambda$) 610 nm. At the same time, the total plate counts (TPC) of the bacteria on NA medium by using spread plate technique were also enumerated.

2.4. Data Analysis
Data of bacterial counts both observed from the OD value and analysed from the TPC were presented in tables and figures, and were analysed descriptively. The results were compared to previous related and similar studies.

3. Results and Discussion

3.1. Spectrophotometric Observation on the Growth of *B. toyonensis*
Figure 1 indicates the growth of *B. toyonensis* in medium containing different concentrations of tofu wastewater. The highest bacterial growth is shown in TSB medium (control positive) in comparison to the growth in medium containing 12%, 10% and 8% of tofu wastewater (A3B1, A2B1 and A1B1). Exponential growth occurred between 0-24 hours cultivation. Among the three trial medium, the best growth of *B. toyonensis* was shown in medium containing 12% tofu wastewater, although the increase was not significant during 96 hours cultivation.

![Figure 1. Absorbance value ($\lambda$=610 nm) of the growth of *B. toyonensis* within 96 hours observation](image)

In this finding, the growth of *B. toyonensis* in tofu wastewater was lower than that in TSB medium. This could be due to lower concentrations of the wastewater used. TSB was used as control medium because it has been widely used for the cultivation of a variety of microorganisms [15]. It contains nutritive components including pancreatic digest of casein (17.0 g), papain digest of soybean meal (3.0
g), sodium chloride (5.0 g), dextrose (2.5 g) and dipotassium phosphate (2.5 g). Similar finding was also reported [12] that lower growth of *B. amyloliquefaciens* subsp. *plantarum* in tofu wastewater in comparison to the growth in Luria broth (LB) medium.

The absorbance values indicate that increase on bacterial count in tofu wastewater occurred at 24 hours incubation. A slight significant increase of bacterial counts is indicated by bacteria grown in media containing 8% (A1B1) and 10% (A2B1) tofu wastewater within 0-24 hours cultivation, then the bacterial counts tends to decrease. While, the increase of bacterial count in 12% tofu wastewater (A3B1) is not significant from 0 to 96 hours. The increase of absorbance values of bacterial count is as a result of absorbance including life and death cell during the cultivation. More accurate development of count of *B. toyonensis* during the experiment was enumerated from total plate count of the bacteria on nutrient agar.

### 3.2. Enumeration of *B. toyonensis* Growth by Total Plate Count (TPC)

Total cell number of *B. toyonensis* during 96 hours cultivation in different concentration of tofu wastewater was enumerated by the total plate count (TPC) method. Table 1 shows that number of bacterial cells in all treatment increase during the cultivation from 0-24 hours. Afterward, the cell numbers decrease until 96 hours cultivation. The highest cell cumber is indicated by treatment by the addition of 12% of tofu wastewater (A3B1). The increase of bacterial number during the cultivation is shown in Table 2.

**Table 1.** Average number of cell (× 10⁸ cfu/mL ± standard deviation) of *B. toyonensis* during 96 hours cultivation

| Treatment code | Observation time (hour) |
|---------------|-------------------------|
|               | 0   | 24  | 48  | 72  | 96  |
| A1B1.1        | 2.19| 2.25| 2.12| 2.07| 2.01|
| A1B1.2        | 2.20| 2.23| 2.08| 2.05| 2.03|
| A1B1.3        | 2.21| 2.22| 2.11| 2.06| 2.02|
| Average       | 2.20 ± 0.01 | 2.23 ± 0.02 | 2.10 ± 0.02 | 2.06 ± 0.01 | 2.02 ± 0.01 |
| A2B1.1        | 2.19| 2.31| 2.21| 2.15| 2.10|
| A2B1.2        | 2.20| 2.31| 2.18| 2.13| 2.10|
| A2B1.3        | 2.21| 2.30| 2.16| 2.13| 2.07|
| Average       | 2.20 ± 0.01 | 2.31 ± 0.01 | 2.18 ± 0.03 | 2.14 ± 0.01 | 2.09 ± 0.02 |
| A3B1.1        | 2.19| 2.48| 2.39| 2.35| 2.29|
| A3B1.2        | 2.20| 2.36| 2.32| 2.29| 2.27|
| A3B1.3        | 2.21| 2.42| 2.37| 2.35| 2.31|
| Average       | 2.20 ± 0.01 | 2.42 ± 0.06 | 2.36 ± 0.04 | 2.33 ± 0.03 | 2.29 ± 0.02 |
Note: A1B1 = Culture medium + 8% tofu wastewater  
A2B1 = Culture medium + 10% tofu wastewater  
A3B1 = Culture medium + 12% tofu wastewater

Table 2. Increase of cell number of ($\times 10^8$ cfu/mL) of B. toyonensis during 96 hours cultivation

| Treatment code | Interval time (hour) | 0 - 24 | 24 - 48 | 48 - 72 | 72 - 96 |
|----------------|---------------------|-------|--------|--------|--------|
| A1B1           |                     | 0.03  | -0.13  | -0.04  | -0.04  |
| A2B1           |                     | 0.11  | -0.13  | -0.04  | -0.05  |
| A3B1           |                     | 0.22  | -0.06  | -0.03  | -0.04  |

Data in Table 2 shows that the highest increase of bacterial number occurs between 0-24 hours cultivation, indicated by bacteria cultured in 12% of tofu wastewater. Within 24-48 hours cultivation, number of bacterial cell decreases in which the highest decrease indicated by bacteria cultured in 8% and 10% of the wastewater. Then, the decrease of bacterial number is relatively lower (-0.03 to -0.05 $\times 10^8$ cfu/mL). The increase of cell number within 24 hours was due to the availability of nutrient in the culture medium. After that, number of bacterial cells tends to decrease could be due to the decrease of nutrient in the growth medium. Previous study [16] reported that Bacillus sp. cultured in the medium with the addition of 10% liquid whey tofu waste showed a higher growth increase compared to the 20% and 30% additions, that it began to increase from the 18th hour and reached the exponential optimum point at the 48th hour.

Bacillus spp. is a group of bacteria which require complex nutrients in culture medium for the growth. Present study uses tofu wastewater to cultivate B. toyonensis. Liquid whey tofu waste contains N 0.27%, P$_2$O$_5$ 228.85%, K$_2$O 0.29% and 1.68% protein [17]. The element P (phosphorus) plays an important role in the formation of nucleic acids and phospholipids in bacteria. The lack of P (phosphorus) or excess of P in a culture medium can slow down the bacterial growth process [18].

4. Conclusion  
Based on the research, the tofu wastewater can be an alternative substrate for the bacterial growth, especially in B. toyonensis culture

Acknowledgments  
Grateful thanks addressed to colleagues and student for the assistance and encouragement in conducting the research and in preparing this article.

References
[1] Nursyirwani N, Feliatra F, Tanjung A and Harjuni F 2020 The 8th International and National Seminar on Fisheries and Marine Science: IOP Conf. Series: Earth and Environmental Science 430 (2020) 012012 doi:10.1088/1755-1315/430/1/012012
[2] Nursyirwani N, Samiaji J, Tanjung A, Effendi I and Claudia K M 2021 The 9th International and National Seminar on Fisheries and Marine Science: IOP Conf. Series: Earth and Environmental Science 695 (2021) 012044 doi:10.1088/1755-1315/695/1/012044

[3] Okaiyeto K, Nwodo, U U, Mabinya L V and Okoh A I 2015 Molecules 20 (3) 5239–5259 doi: 10.3390/molecules20035239

[4] Tallur P N, Sajjan D B, Mulla S I, Talwar M P, Pragasam A and Nayak V M 2016 Biotech. 6 (1) 28 doi: 10.1007/s13205-015-0332-3

[5] Luo J C, Hao L, Jian Z, Zhao Y and Sun L 2021 Frontier in Cellular and Infection Microbiology 11 Article 629116 doi: 10.3389/fcimb.2021.629116

[6] Syaichurrozi I and Jayanudin J 2017 International Journal of Engineering 30(11) 1631-1638

[7] Widayat W, Philia J and Wibisono J E3S Web of Conferences 31 (2017) 04009 ICENIS (2017) doi.org/10.1051/e3sconf/20183104009

[8] Arsad S, Sari L A, Suherman S P, Cahyani D, Nadhira T, Yulinda E N, Musa M, Lusiana E D, Fiddy S and Prasetya F S 2020 AACL Bioflux 13(5) 2878-2885 http://www.bioflux.com.ro/aacl

[9] Musa M, Arsad S, Sari L A, Lusiana E D, Kasitowati R D, Yulinda E N, Nadhira T, Cahyani D 2021 Journal of Ecological Engineering 22(2) 70-76 https://doi.org/10.12911/22998993/130886

[10] Rusydi R and Yaqubtiyage A 2019 ICFAES 2019 IOP Conf. Series: Earth and Environmental Science 348 (2019) 012087 doi:10.1088/1755-1315/348/1/012087

[11] Safitri N, Sunarti T C and Meryandini A 2016 Jurnal Sumberdaya Hayati 2(2) 31-38 http://biologi.ipb.ac.id/index.php/jsdhayati

[12] Ali M, Karmi I, Amin M and Ichsan M 2018 Journal of Applied Biological Sciences Uygulamalı Biyolojik Bilimler Dergisi 12 (1): 46-50 www.nobel.gen.tr

[13] Amalia S N, Prihartanti E and Hastuti E D 2018 SNPINSA 2018 IOP Conf. Series: Journal of Physics: Conf. Series 1217 (2019) 012157 doi:10.1088/1742-6596/1217/1/012157

[14] Seroja, Effendi R H and Sigithariyadi 2018 Applied Water Science 8 2

[15] Anonymous 2014 Tryptic Soy Broth Dalynn Biological https://www.bd.com

[16] Zouari N, Dhouib A, Ellouz R and Jaoua S2000 J App Biochem Biotech 69:41-52