Optimization of an effective growth medium for biomass production of *Bacillus cereus*

F Feliatra, U M Batubara*, I Effendi, A Adelina
Faculty of Fisheries and Marine Sciences, Universitas Riau, Pekanbaru, Indonesia

*Corresponding author: ummimardhiah@lecturer.unri.ac.id

Abstract. Protein is the main source of nutrition in fish feed. It is necessary to use bacterial single-cell protein (SCPs) as a substitute for conventional protein. This study aims to determine the effectiveness of tofu liquid waste as a growth medium for biomass production of *Bacillus cereus* as an SCPs. The isolates used were *B. cereus* SN7 and *B. cereus* consortium (combination of 5 isolates of *B. cereus* with different strains). The method used was experimental where isolates were cultured in a medium containing tofu liquid waste with different concentrations (8%, 10%, and 12%) enriched with 10% skim milk. Furthermore, the analysis of an effective growth medium was carried out by three methods, namely total plate count, optical density, and cell-dry weight. Cell biomass measurements were carried out for 24 hours with an observation time of every 6 hours. The results showed the best growth medium used in the culture of *B. cereus* SN7 and *B. cereus* consortium was tofu liquid waste at a concentration of 12% enriched with 10% skim milk. In conclusion, the cell mass of *B. cereus* SN7 and *B. cereus* consortium significantly increased at 6 to 12 hours.

1. Introduction
Feed is the main nutrient needed in aquaculture. Feed nutrients contain complex compounds consisting of proteins, carbohydrates, lipids, vitamins, water, and minerals. The composition of protein in feed formulations reaches 60% of all components contained in feed [1].

In decades, protein requirements are still very high. However, the amount of protein availability is very limited, resulting in losses in fishery products such as fish, shrimp, and lobster. In addition, another thing that causes losses in fishery production is the high selling price of protein or protein flour which is used as the main ingredient for making feed. As a result, many farmers have difficulty finding new protein sources that can replace commercial protein functions. Thus, it is necessary to search for natural protein sources but have the same function as commercial proteins.

One of the proteins that can be used as an alternative in the manufacture of feed is a single-cell protein (SCPs). The SCPs is a dry cell or single-cell microorganism biomass such as bacteria, yeast, algae, and fungi that can be used as a source of protein for food. The SCPs contains high protein and other nutrients such as carbohydrates, fats, vitamins, minerals, other micronutrients [2]. Based on the results of previous studies [3], five species of *B. cereus* were potential that could be developed as SCPs. The fifth species include *B. cereus* SN7, *B. cereus* SP4, *B. cereus* S5, *B. cereus* Xmb051 and, *B. cereus* BF2. [1] reported *B. cereus* SN7 has the potential to be developed as biological control agent especially in aquaculture.

To develop the potential of all strains of *B. cereus* as SCPs, an effective and efficient growth medium is needed, one of them by utilizing the industrial waste. Tofu liquid waste is an environmental problem...
caused by tofu industries and generated from the tofu production process. The tofu liquid waste contains very high organic substances and abundant in nature [4]. The utilization of tofu liquid waste is expected to increase the amount of B. cereus biomass that can be produced as a substitute protein source in the manufacture of fish feed. Therefore, this study aims to determine the effectiveness of tofu liquid waste as a growth medium for biomass production of B. cereus as an SCPs developed in the fish feed industry.

2. Material and Methods

2.1. Bacterial Strain
The bacteria used in this study consisted of two parts, namely B. cereus SN7 and B. cereus consortium (B. cereus SN7, B. cereus SP4, B. cereus S5, B. cereus Xmb051 and, B. cereus BF2). All bacterial strains were obtained from the collection of the Laboratory of Marine Microbiology, Department of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas Riau.

2.2. Culture Media and Growth Conditions
All bacteria were grown on modified media containing tofu liquid waste with different concentrations (8%, 10%, and 12%) enriched with 10% skim milk, KH2PO4, K2HPO4, and Vitamin B12. The mixture medium was dissolved in distilled water and put in a 120 ml glass bottle. B. cereus SN7 and B. cereus consortium were subsequently cultured in growth media that had been sterilized by autoclaving at 121°C for 15 minutes. Furthermore, these were incubated overnight at 37°C were performed under aerobic conditions on a water bath shaker at 150 rpm.

2.3. Analytical Methods

2.3.1. Optical density (OD)
Measurement of optical density is a method used to calculate the number of bacterial cells in growing media based on the absorbance value. About 2 ml of B. cereus and B. cereus consortium culture were incubated at 0, 6, 12, 18, and 24 hours subsequently was measured for its OD value using a spectrophotometer at a wavelength of 600 nm [5].

2.3.2. Total plate count (TPC)
TPC was used to count the number of viable cells on the modified media. This method was carried out by diluting 1 ml of B. cereus SN7 and B. cereus consortium suspensions start from 10-1-10-8 (serial dilution). The bacterial suspensions from the dilution were subsequently spread on the surface of Plate Count Agar (PCA) medium. PCA containing the bacterial suspensions was incubated for 24 hours at 37°C. Colonies growing on the surface of PCA were then counted and expressed in CFU/ml units. Sampling was carried out every 6 hours for 24 hours [5].

2.3.3. Cell-dry weight
The cell density can be quantified in cell-dry weight. Cultures of B. cereus SN7 and B. cereus consortium aged 0, 6, 12, 18, and 24 hours were taken as much as 1 ml each and put into micro-tubes of known weight. It was centrifuged for 25 minutes at 6,000 rpm and then the pellets were collected. The pellets obtained were then weighed and dried in an oven at 100°C for 30 minutes. Calculate the difference in the weight, and express the dry weight in g/l [6].

2.3.4. Data analysis
Data Analysis was performed using statistical calculations with ANOVA (Analysis of Variance) to determine the relationship between the treatment given and the results obtained. The results obtained will be discussed in descriptive statistics and compared based on existing literature.

3. Results and Discussions
3.1. Microbial Biomass Production

One of the most important roles of microbial biomass is the conversion of organic matter into materials that are easily absorbed and utilized by the microbes themselves. Optical density (OD) is used as a rapid proxy measurement of suspended biomass concentration. It is used qualitatively as the turbidity of B. cereus SN7 and B. cereus consortium culture (Table 1). Based on the data in Table 1, two groups of bacteria were able to grow in the three concentrations of tofu liquid waste enriched with 10% skim milk. It was seen from the increase of absorbance value during the incubation period (0 to 24 hours).

Further analysis showed that the most effective growth medium for increasing biomass based on OD values was a 12% concentration of tofu liquid waste enriched with 10% skim milk. It was indicated the higher concentration of waste that the more sources of nutrients that can be used for bacteria growth.

| Bacterial strains | Medium Concentrations | Incubation Period (hours) (10^8 cell/ml) |
|-------------------|-----------------------|----------------------------------------|
|                   | 0                     | 6                                      |
|                   | 12                    | 18                                     |
|                   | 24                    |                                         |
| B. cereus SN7     | 8%                    | 2,67                                   |
|                   |                       | 4,46                                   |
|                   |                       | 4,23                                   |
|                   |                       | 2,37                                   |
|                   |                       | 1,96                                   |
|                   | 10%                   | 2,65                                   |
|                   |                       | 4,44                                   |
|                   |                       | 4,38                                   |
|                   |                       | 2,37                                   |
|                   |                       | 1,97                                   |
|                   | 12%                   | 2,76                                   |
|                   |                       | 4,84                                   |
|                   |                       | 4,51                                   |
|                   |                       | 2,42                                   |
|                   |                       | 1,97                                   |
| B. cereus consortium | 8%       | 2,67                                   |
|                   |                       | 4,71                                   |
|                   |                       | 4,44                                   |
|                   |                       | 2,44                                   |
|                   |                       | 1,99                                   |
|                   | 10%                   | 2,67                                   |
|                   |                       | 4,67                                   |
|                   |                       | 4,35                                   |
|                   |                       | 2,44                                   |
|                   |                       | 1,98                                   |
|                   | 12%                   | 2,78                                   |
|                   |                       | 4,88                                   |
|                   |                       | 4,69                                   |
|                   |                       | 2,49                                   |
|                   |                       | 2,01                                   |

In the production of microbial biomass, the measurement of cell dry weight is a fundamental thing that needs to be done. Measurement of the cell-dry weight of B. cereus SN7 and B. cereus consortium aims to determine the total weight of cells measured each incubation period (0-24 hours). Based on the results of cell-dry weight measurements in the two groups of bacteria were not significantly different (Table 2). The highest cell-dry weight was obtained during the incubation period of 12 and 24 hours. An increase in the number of cell weights at 12 hours was thought because the cells were in the exponential phase, while the increase in cell weight at 24 hours was thought because of the accumulation of dead cells in the death phase [7].

In the exponential phase, the cells are in a state of balanced growth. During this phase, the mass and volume of cells increased according to the previous conditions, so that the average composition of bacterial cells and the relative concentrations of metabolites remained constant for a certain period. In the death phase, the number of living cells decreases exponentially. However, the accumulation of residues into the environment occurs due to lysis or rupture of dead cells. [8] reported dry weight of cells is expressed as a physical value and metabolic value consisting of OD, turbidity and capacitance, oxygen absorption, and CO₂ evolution. It was indicated that there is a relationship between dry weight and cell metabolism, turbidity, and growth phase.

| Bacterial strains | Medium Concentrations | Incubation Period (hours) Cell-dry weight (g/ml) |
|-------------------|-----------------------|--------------------------------------------|
|                   | 0                     | 6                                      |
|                   | 12                    | 18                                     |
|                   | 24                    |                                         |
| B. cereus SN7     | 8%                    | 0,06                                   |
|                   |                       | 0,13                                   |
|                   |                       | 0,15                                   |
|                   |                       | 0,06                                   |
|                   |                       | 0,11                                   |
|                   | 10%                   | 0,06                                   |
|                   |                       | 0,12                                   |
|                   |                       | 0,16                                   |
|                   |                       | 0,12                                   |
|                   | 12%                   | 0,09                                   |
|                   |                       | 0,08                                   |
|                   |                       | 0,08                                   |
|                   |                       | 0,12                                   |
|                   |                       | 0,14                                   |
| B. cereus consortium | 8%          | 0,06                                   |
|                   |                       | 0,04                                   |
|                   |                       | 0,14                                   |
|                   |                       | 0,09                                   |
|                   |                       | 0,12                                   |
| 10%               |                       | 0,13                                   |
|                   |                       | 0,07                                   |
|                   |                       | 0,07                                   |
|                   |                       | 0,12                                   |
|                   |                       | 0,13                                   |
| 12%               |                       | 0,11                                   |
|                   |                       | 0,03                                   |
|                   |                       | 0,07                                   |
|                   |                       | 0,08                                   |
|                   |                       | 0,08                                   |

The increase of B. cereus SN7 and B. cereus consortium biomass in SCPs production was a critical success factor. Based on the visible count data showed that there was an increase in the number of living...
cells of the *B. cereus* SN7 and *B. cereus* consortium in all concentration media (8%, 10%, and 12%) significantly at the 6th and 12th hours (Table 3). It was shown that the logarithmic phase began to occur at 6 to 12 hours. The logarithmic phase is a growth phase that bacteria have adapted and used the medium for cell division so the cell number increases rapidly and constantly (exponentially). Cell division is influenced by several factors, including nutrient content, pH, temperature, and oxygen abundance.

| Bacterial strains | Medium Concentrations | Incubation Period (hours) | (10^6 CFU/ml) |
|-------------------|-----------------------|---------------------------|---------------|
|                   |                       | 0 | 6 | 12 | 18 | 24 |
| *B. cereus* SN7   | 8%                    | 0.82 | 1.89 | 1.79 | 1.70 | 1.43 |
|                   | 10%                   | 0.93 | 1.81 | 2.33 | 1.46 | 1.71 |
|                   | 12%                   | 1.11 | 2.28 | 2.27 | 1.83 | 1.72 |
| *B. cereus*       | 8%                    | 0.42 | 1.50 | 1.90 | 1.46 | 1.22 |
| consortium        | 10%                   | 1.02 | 1.24 | 1.80 | 1.43 | 1.11 |
|                   | 12%                   | 1.12 | 2.11 | 1.93 | 1.41 | 1.26 |

3.2. Potential of tofu liquid waste as growth medium
The effectiveness of tofu liquid waste as a growth medium for *B. cereus* SN7 and *B. cereus* consortium was analyzed based on the increase in the amount of biomass. Based on medium concentrations parameter shows that an increase in the amount of biomass occurred in the media with a concentration of 12% tofu liquid waste enriched with 10% skim milk (Figure 1.)

![Figure 1. The growth curves of *B. cereus* SN7 and *B. cereus* consortium](image)

Tofu liquid waste is an industrial waste product obtained during the production of tofu. The main component in tofu liquid waste is protein. The protein content in tofu liquid waste is 1-6 g/L. Under anaerobic conditions, protein releases ammonia gas (NH₃) and hydrogen sulphide (H₂S) which can cause a foul-smelling aquatic environment [9]. Tofu liquid waste modified media contains the appropriate nutritional composition needed for *B. cereus* SN7 and *B. cereus* consortium as SCPs. The production of SCPs is highly profitable and has prospects [10, 11]. [12] reported that there are many advantages to be gained when developing SCPs is including a) it does not require a large area, b) fast and high production yield, and c) produces less waste generally because SCPs bacteria are heterotrophic.

The nutrients contained in tofu liquid waste are very complex. About 60% of the composition of tofu liquid waste contains protein which is degraded into organic compounds and is useful as a macronutrient and micronutrient for heterotrophic microorganism. [13] reported that a group of heterotrophic bacteria that has the potential to improve wastewater with high nitrate and organic matter content. Tofu liquid
waste also contains a high phosphate of 1.74%. For some bacteria, phosphate serves as an ATP-forming agent which increase bacterial growth [14]. Skim milk is used as an additional source of nitrogen for B. cereus SN7 and B. cereus consortium growth culture because of the content of lactose, casein, and other nutrients needed for the growth. Casein collects in colloidal structures in the form of casein micelles and makes up about 10% of the volume in skim milk. The modified medium containing tofu liquid waste enriched with 10% skim milk showed the growth phase of B. cereus SN7 and B. cereus consortium was formed after overnight incubation (0 to 24 hours). The growth phases were formed include the lag phase (adaptation phase), the exponential phase, the stationary phase, and the death phase. Microbial growth curves can provide useful information for understanding microbial growth and selecting optimal growth [15].

4. Conclusion
Based on the results and discussion, it can be concluded that tofu liquid waste could be used as an alternative growth medium for B. cereus SN7 and B. cereus consortium. The best-modified media formulation for B. cereus SN7 and B. cereus consortium growth was tofu liquid waste at a concentration of 12% enriched with 10% skim milk. Cell biomass of B. cereus SN7 and B. cereus consortium increased significantly at 6 to 12 hours. Thus, the production of B. cereus SN7 and B. cereus consortium biomass as SCPs can be developed in the fish feed industry.

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