Distribution of protein fractions in tofu whey wastewater and its potential influence on anaerobic digestion

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Abstract. Wastewater from tofu (soybean curd) production contains a high organic concentration that might pollute the environment if it is not treated properly. Among the major organic constituents of tofu wastewater are proteins. Degradation of proteins generates ammonia and H₂S gases, and if this occurs in an anaerobic digester might hamper the digester’s performance. On the other hand, proteins can potentially be recovered as value-added products. This research aimed to identify protein amounts in different fractions of tofu wastewater, as a basis of designing a process to recover the proteins. Furthermore, the influence of a lower protein concentration to anaerobic digestion of tofu wastewater was calculated. Tofu wastewater was fractionated by gravity settlement and filtration into settleable, suspended, and dissolved fractions. Subsequently, total solids, protein, and COD concentrations of each fraction were analysed. Our results show that tofu wastewater contained 2.9 g-protein/L, equivalent to 4 g-COD/L. While most of the proteins were dissolved, 9% of the proteins were settleable. This might be recovered via a settling chamber. Using the protein-separated wastewater in anaerobic digestion might reduce NH₃ and H₂S by 10% while only reduce biogas production by 2%.

1. Introduction

Tofu or soybean curd is a popular food in Asia, including Indonesia. There are around 80,000 tofu factories in Indonesia, processing a total of 2.6 million tonnes of soybean per year [1]. Tofu production requires 18–30 kg of water per kg of soybean [2,3], some of which are discharged as wastewater.

Wastewater from tofu production is generated from soaking, washing, and coagulation-pressing processes, as well as housekeeping. The whey generated from coagulation and pressing processes is of importance due to the high organic concentration. Coagulation of soybean curd is the most important step in the tofu-making processes that can influence the chemical composition of the wastewater. Coagulation can be achieved by adding salt, acid, or enzymatic coagulants [4]. Commonly-used salt-type coagulants are calcium chloride, calcium sulphate, magnesium chloride, and magnesium sulphate. Commonly-used acid-type coagulants are glucono-δ-lactone, L-ascorbic acid, and various types of food-grade acids. The use of enzymatic coagulant is still novel but recently gains attention as it produces tofu with different texture and aroma compared with the other coagulants. Acid-type coagulants reduce the soymilk pH into the isoelectric point of soybean protein, which is required in a smaller concentration compared with salt-type coagulants [4,5]. Most Indonesian tofu factories used fermented whey as coagulant [3]. Due to the addition of the coagulant, the whey normally has a pH between 3.5–6.5 [6,7].

Tofu whey wastewater contains varying COD concentration between 5–37 g-COD/L; up to 76% is in the form of easily degradable BOD [2,6,8]. Direct discharge of whey wastewater into water bodies
creates water pollution and other environmental problems such as odour and unpleasant scenery. Therefore treatment of the wastewater is necessary. One of the methods to treat tofu whey wastewater is anaerobic digestion. Anaerobic digestion has the advantage of requiring less energy and nutrient inputs while producing biogas as a by-product [9].

Protein is a major component in tofu wastewater. From the input soybean, 68–76% protein is recovered in tofu as the main product while 4–6% protein end up in the whey [10], therefore, protein content in the tofu whey wastewater ranges from 1 to 6 g-protein/L [7,8,10–12]. In anaerobic condition, degradation of protein releases ammonia (NH₃) and hydrogen sulphide (H₂S) gases. Ammonia is toxic and an inhibitor in the anaerobic wastewater treatment, particularly in methanogenesis [13]. H₂S has a rotten egg smell and is corrosive to piping systems. Sulphide/sulphate species in wastewater inhibit anaerobic wastewater treatment due to the sulphide toxicity to various anaerobic microorganisms and the competition with sulphate reducing bacteria [14].

While its presence may hamper anaerobic wastewater treatment, the protein itself has many potential applications. Tofu wastewater contains soy proteins that have good functional properties including solubility, water absorption, viscosity, gel formation, emulsification, etc [15]. Soy proteins contain high and balanced essential amino acids for animal feed [16]. Therefore, separation and recovery of protein from the wastewater may present two benefits: as a pre-treatment before anaerobic wastewater treatment and generating valuable products.

Recovery of proteins from wastewater has been long investigated as an approach to simultaneously remove protein from the wastewater and recover valuable products. The challenges in protein recovery from the wastewater are the dilute stream and the complex composition of the wastewater that often interferes the recovery process [17]. Processes that can be used for protein recovery include centrifugation, alkaline/acid precipitation, flocculation and coagulation, foaming, membrane filtration, electrical processes, and adsorption chromatography [18]. Some of these methods had been applied in protein recovery from tofu or soybean processing wastewater, for instance, flocculation-coagulation and foaming [19,20], while a combination of electrodialysis and bipolar-membrane electro-acidification processes was applied in the recovery of protein and magnesium [21].

The aim of this research was to identify protein amounts in different fractions of tofu whey wastewater. Tofu whey wastewater was fractionated by gravity settlement and filtration into settleable, suspended, and dissolved fractions. Subsequently, the protein content in each fraction was measured. The results can be used as a basis of designing a process to recover proteins from tofu whey wastewater. Since this process can be used as a pre-treatment in the tofu wastewater treatment, the influence of low protein concentration to subsequent process—namely anaerobic digestion—was calculated.

2. Material and Methods

2.1. Materials

Tofu whey wastewater, henceforth referred to as (tofu) wastewater, was obtained from tofu factory TT, located in West Bandung Regency, West Java, Indonesia. The factory is a traditional small factory that uses one-day fermented whey as the coagulant for tofu production. Upon collection, the wastewater was stored at 4 °C until used.

2.2. Separation of tofu wastewater fractions

Before the separation procedure (Figure 1), the wastewater container was immersed in water until reaching room temperature (21 ± 2 °C).
One litre of tofu wastewater was transferred into a pre-weighed Imhoff cone to allow the wastewater to separate into settled and non-settled fractions. After 1 h, the non-settled fraction was transferred into an empty container using a pipette. Following the settlement, the non-settled fraction was vacuum-filtered through 8 μm filter paper. The filtrate was subsequently vacuum-filtered through 0.45 μm membrane filter.

All fractions were weighed in each step. The fractions obtained from the separation were classified as shown in Table 1.

Table 1. Tofu wastewater fractions [22].

| Fraction       | Classification                          |
|----------------|-----------------------------------------|
| Dissolved      | Size < 0.45 μm                          |
| Suspended-2    | Size 0.45 – 8 μm                        |
| Suspended-1    | Size > 8 μm but not settled after 1 h   |
| Settled        | Undetermined size, settled after 1 h    |

2.3. Analyses and calculations

2.3.1. Solids and COD determinations. Total, settled, non-settled, 8 μm-filtrate, and 0.45 μm-filtrate fractions were analysed for solids content using gravimetric method [23]. Total, non-settled, 8 μm-filtrate, and 0.45 μm-filtrate fractions were analysed for Chemical Oxygen Demand (COD) concentration using closed reflux titrimetric method [23].

2.3.2. Protein determination. Total, settled, non-settled, 8 μm-filtrate, and 0.45 μm-filtrate fractions were analysed for protein concentration using a modification of the Lowry method [24]. To 1 mL of sample, 0.05 mL of 5% SDS and 0.95 mL of 1 N NaOH were added. The mixture was heated at 105 °C for 5 mins. Subsequently, 2 mL Lowry reagent (58.4 g/L Na2CO3, 0.3 g/L CuSO4·5H2O, 0.6 g/L KNaC4H4O6·4H2O) was added and the mixture was incubated at 37 °C for 30 mins. Folin-Ciocalteu reagent (20%) was added and after 20 mins, the resulting colour was measured with a spectrophotometer (Merck SQ118, Germany) at 660 nm.

2.3.3. Calculations. Solids, COD, and protein contents in the suspended-1 fraction were determined using the mass balance approach via the difference between non-settled fraction and 8 μm-filtrate. Likewise, the contents in the suspended-2 fraction were determined via the difference between 8 μm- and 0.45 μm-filtrates.

Total suspended solids (TSS) was calculated as [22]:

\[
TSS = \text{Total solids} - \text{Solids in dissolved fraction}
\]
Empirical protein formula of C_{46}H_{77}O_{17}N_{12}S was used in calculations [25]. Protein was converted to COD based on the following theoretical reaction:

\[ C_{46}H_{77}O_{17}N_{12}S + 48.75 O_2 + 12 H^+ \rightarrow 46 CO_2 + 20.5 H_2O + 12 NH_4^+ + SO_2 \]

Based on the reaction, the calculated conversion factor was 1.4 g-COD/g-protein.

Anaerobic hydrolysis and acidogenesis of proteins were assumed to occur based on the following reaction [25,26]:

\[ C_{46}H_{77}O_{17}N_{12}S + 43.5 H_2O \rightarrow 15.75 CH_3COOH + 14.5 CO_2 + 31.5 H_2 + 12 NH_3 + H_2S \]

Based on the reaction, anaerobic degradation of protein would generate 0.185 g/g-protein of total ammonia and 0.031 g/g-protein of total H_2S.

The concentrations of NH_3 and H_2S in the biogas were calculated using the dimensionless distribution coefficient for air/water system at 25 °C for NH_3 (0.0007) and H_2S (0.3996) [22].

2.3.4. Statistical analysis. Experiments and measurements were performed in duplicates. The results are presented as average ± standard deviation.

3. Results and Discussion

3.1. Characteristics of tofu wastewater

The characteristics of tofu wastewater used in this study before fractionation are presented in Table 2. The concentrations of total solids and total suspended solids were 14 g/kg and 2 g/kg, respectively (Table 2). Previous studies using calcium sulphate as a coagulant reported total solids concentration of 19-39 g/kg [7,10]. Solids concentration in the wastewater is influenced by process conditions and the types of soybean and coagulant; calcium sulphate and fermented maize yielded a lower solid concentration in the tofu (a higher concentration in the wastewater) compared to magnesium sulphate, alum, and lemon juice [27]. Studies focusing on the treatment of tofu wastewater from Indonesian origin mostly report TSS concentration—which is a requirement in the wastewater quality standard—in the range of 0.9-3.1 g/kg [2,8]. TSS concentration of wastewater used in this study is within the range found in previous studies.

| Parameter          | Unit   | Concentration |
|--------------------|--------|---------------|
| Total solids       | g/kg   | 13.8 ± 0.1    |
| Total suspended solids| g/kg    | 2.2 ± 0.6   |
| COD                | g/L    | 7.0 ± 0.1     |
| Proteins           | g/kg   | 2.9 ± 0.2     |
| pH                 | -      | 4.4 ± 0.1     |

COD concentration of the tofu wastewater used in this study was 7 g/L (Table 2), within the low range of values 5–37 g-COD/L found in previous studies [2,6,8]. Nevertheless, the concentration was high enough to cause an environmental problem and within the range where anaerobic treatment is advantageous compared with other treatments [22].

The protein concentration of the tofu wastewater was 3 g/kg (Table 2), within the range of 1–6 g/kg found in previous studies [7,8,10–12]. On a dry matter basis, the protein content was 21% of the total solid, higher than 14–19% found in previous studies [7,10,11] but still within the same order of magnitude. The protein content was equivalent to 4 g-COD/L, which means more than half of organic content in the tofu wastewater constituted of proteins.

3.2. Mass distribution of solids, COD, and protein

Mass distribution of solids, COD, and proteins in tofu wastewater fractions is presented in Table 3. Figure 2 shows the amount of protein in different fractions as percentages of solids and COD.
Table 3. Distribution of solids, COD, and proteins in the settled, suspended, and dissolved fractions.

| Fraction   | Mass distribution (%) |
|------------|------------------------|
|            | Solids  | COD     | Proteins |
| Dissolved  | 80 ± 4  | 86 ± 11 | 67 ± 1   |
| Suspended-2| 5 ± 3   | 6 ± 7   | 1 ± 2    |
| Suspended-1| 10 ± 2  | 3 ± 0   | 17 ± 2   |
| Settled    | 5 ± 0   | 5 ± 4   | 9 ± 1    |

Figure 2. Protein fraction as a percentage of (a) solids and (b) COD.

The settled fraction retained 5% of solids and 9% of proteins (Table 3). This means that after settlement by gravity for one hour, the settled fraction contained 43% protein on a dry weight basis or twice the concentration in the unfractionated wastewater (Figure 2a). Protein concentration in the settled fraction is similar to soybean meal from soybean oil production [16]. Referring to the tofu making process [12], the settled fraction would consist of coagulated soymilk that was not recovered in the tofu product. Soymilk properties, coagulant type, and process conditions would influence the amount and properties of the settled fraction. In the tofu factory where we obtained our sample, the coagulant used was one-day fermented whey, an acid-type coagulant. Acid type coagulants reduce soymilk pH to the isoelectric point of soybean proteins between pH 4.5 and 5.5, followed by precipitation facilitated by hydrophobic and electrostatic interactions [4,28]. The pH of tofu wastewater was 4.4 (Table 2), still close to the isoelectric point. Protein precipitation is also influenced by temperature [29], however, less than 10% difference in solubility could be expected between experimental condition (21 ± 2 °C) and the range of normal process conditions (25-35 °C).

Settlement by gravity for one hour removed 5% of the COD from the wastewater (Table 3). Figure 2b shows that in the settled fraction, the calculated protein fraction to COD was 166 ± 130%; the error might result from the uncertainty of the measurement. However, the solid, protein, and ash contents in the settled fraction were 21, 9, and 7 g/kg, respectively. Therefore it can still be concluded that at least 60% of the organic fraction in the settled fraction was protein.

Suspended-1 was the wastewater fraction that did not settle after one hour and did not pass through the 8 µm filter. This fraction contained 10% of solids, 3% of COD, and 17% of proteins (Table 3). Suspended-2 was the wastewater fraction that passed through the 8 µm filter but did not pass through the 0.45 µm filter, containing 5% of solids, 6% of COD, and 1% of proteins. In total, both suspended
fractions still comprised 15% of the total solids and 18% of the proteins. Given more settling time or with the addition of coagulants, it might be possible to recover these fractions with the physical process.

The large constituents of solids (80%), COD (86%), and proteins (67%) were still present in the dissolved fraction. This is expected since the dissolved protein could not easily be coagulated, therefore remained in the wastewater after the pressing of the coagulated soymilk into tofu. The dissolved fraction contained soluble proteins, peptides, and free amino acids, as well as sugars, glycerol, organic acids, and isoflavones [12]. When coagulation was not optimum, SDS-PAGE showed clear bands of protein with molecular weights of 14 and 40 kDa. On the other hand, at the maximum coagulation, multiple bands at molecular weights lower than 43 kDa were shown [30]. The amino acid composition of proteins in the tofu wastewater was quite similar to that of soybeans [10], however, free amino acid composition showed a high concentration of arginine and proline (20% and 21%, respectively, from total free amino acids) [12]. Functional peptides and free amino acids can be applied in food, pharmaceuticals, or bulk chemical productions [31,32]. Further identification of proteinaceous constituents in the dissolved fraction is necessary.

3.3. Influence of protein separation on subsequent process
Fractionation of tofu wastewater suggests that one-hour gravity settlement could remove 5% solid, 5% COD, and 9% proteins from the wastewater. If the wastewater is further processed anaerobically, the lower COD and protein contents in the wastewater might influence CH₄, NH₃, and H₂S productions. Table 4 shows the theoretical gas production from anaerobic digestion of tofu wastewater, assuming all COD could be converted to biogas and no nitrogen is used for microbial growth and maintenance.

| Table 4. Theoretical gas production. |
|--------------------------------------|
| Wastewater used | COD concentration g/L-wastewater | Biogas production b L/L-wastewater | NH₃ production | H₂S production |
|------------------|----------------------------------|-----------------------------------|----------------|----------------|
|                  | Total (unfractionated)           | Non-settled wastewater a          | - Concentration in | - Concentration in |
|                  |                                  |                                   | g/L-wastewater | ppm            |
|                  |                                  |                                   | 103            | 4854           |
|                  |                                  |                                   | 0.53           | 0.09           |
|                  |                                  |                                   | 0.47           | 0.08           |
|                  |                                  |                                   | 4345           | 4854           |

a Wastewater fraction that is not settled after 1 h, consisted of suspended and dissolved fractions
b Based on the theoretical methane production of 0.38 L-CH₄/g-COD (25 °C, 1 atm), assuming 50% CH₄ in the biogas

Unfractionated tofu wastewater would produce 103 ppm NH₃ and 4854 ppm H₂S in the biogas from the protein degradation (Table 4). Fortunately, this will not be the case since complete degradation cannot occur and part of the nitrogen is consumed by anaerobic microorganisms. Furthermore, the formula of C₆₆H₇7O₁₇N₁₂S was only used as a simplification of protein structure and deviation from the actual protein in the tofu wastewater would certainly occur. However, it is also worth noting that while soybean contains 2% sulphur-containing amino acids methionine and cystine that equals to 0.5% sulphur in the protein, higher contents of methionine and cystine (5%, equivalent to 1.2% sulphur) are present in tofu wastewater [10].
Based on Table 4, using the non-settled wastewater would only reduce biogas production by 2%, while reducing NH$_3$ and H$_2$S production by 10%. Separation of large particles might also speed hydrolysis, which often is the rate-limiting step [33].

In a wastewater treatment plant, gravity settlement can be performed in simple and low-cost unit operations. Optimisation of design and operation parameters might increase protein recovery in the settled fraction. Removing the solids also aids wastewater treatment operation, by reducing the clogging risk from particulates.

4. Conclusions
Our results show that 9% of protein from tofu wastewater can be recovered using gravity settlement, resulting in a protein product with 43% protein content on a dry weight basis. Using the non-settled wastewater fraction might increase anaerobic digester performance by reducing clogging risk and generation of unwanted NH$_3$ and H$_2$S. Optimisation of design and operation parameters might increase protein recovery in the settled fraction.

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