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Multi-Scale Biosurfactant Production by *Bacillus subtilis* Using Tuna Fish Waste as Substrate

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Abstract: As one of the most effective biosurfactants reported to date, lipopeptides exhibit attractive surface and biological activities and have the great potential to serve as biocatalysts. Low yield, high cost of production, and purification hinder the large-scale applications of lipopeptides. Utilization of waste materials as low-cost substrates for the growth of biosurfactant producers has emerged as a feasible solution for economical biosurfactant production. In this study, fish peptone was generated through enzyme hydrolyzation of smashed tuna (*Katsuwonus pelamis*). Biosurfactant (mainly surfactin) production by *Bacillus subtilis* ATCC 21332 was further evaluated and optimized using the generated fish peptone as a comprehensive substrate. The optimized production conduction was continuously assessed in a 7 L batch-scale and 100 L pilot-scale fermenter, exploring the possibility for a large-scale surfactin production. The results showed that *Bacillus subtilis* ATCC 21332 could effectively use the fish waste peptones for surfactin production. The highest surfactin productivity achieved in the pilot-scale experiments was 274 mg/L. The experimental results shed light on the further production of surfactins at scales using fish wastes as an economical substrate.

Keywords: fish waste; biosurfactant; bacillus subtilis ATCC 21332; surfactin; multi-scale production; fermentation

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1. Introduction

Biosurfactants are surface-active macromolecules secreted by microorganisms through their secondary metabolism [1]. A biosurfactant has the amphoteric molecular structure with a hydrophilic head and a hydrophobic tail [2]. Biosurfactants have many advantages over chemical ones as surface active agents, such as a wider diversity of molecules with unique functional groups, higher biodegradability, better biocompatibility, and wider application under extreme temperature, pHs, and salinity conditions [3–7]. These environmentally-friendly macromolecules have recently been considered as potential candidates of biocatalysts grounded on their diverse and complementary functional groups and surface activities. Biosurfactants as biocatalysts to facilitate the phytoremediation of hydrocarbons in soils have been reported [8]. They could catalyze and promote the natural gas hydrate formation in seawater saturated sand/clay [9,10]. Emerging trending also focuses on the formation of biosurfactant-based hybrids for the biocatalysis process. Biosurfactant–inorganic hybrid nanoflower was synthesized with catalytic activity in degrading cationic dyes [11]. Lipopeptides are a group of the most effective biosurfactants that offer promising biocatalytic activities [12]. They are crystalline extracellular products mostly produced by *Bacillus subtilis* [13,14]. As one of the most widely studied lipopeptides, surfactin was first discovered in the fermentation broth of *Bacillus subtilis* IFO3039 [15]. They possess high surface activity, emulsification, foaming ability, and biocatalytic activity [16–18]. The surface tension of water could be reduced from 72 mN/m to 27 mN/m

with surfactin addition at a concentration of 0.005%. Because of these excellent properties, surfactin has been widely used in oil, environmental, pharmaceutical, food processing industries, and beyond [19–22].

Surfactin can be synthesized via microbial fermentation. However, low yield and high production cost hinder the industrialization of surfactin, resulting in an insufficient supply of this product with relatively high prices, and limited industrial application. To solve the previously mentioned problems, researchers try to explore the utilization of organic wastes as a rich source of hydrocarbons and nutrients for biosurfactant production. In the meantime, the pollution caused by the waste materials could be minimized. Till now, olive mill wastes [23], corn steep liquor, and sugarcane molasses wastes [24], animal fat and oil wastes [25], buttermilk and poultry-transforming wastes [26], and shrimp shell wastes [27] have demonstrated the feasibility to support biosurfactant production. Some of the previously mentioned waste materials proved to be feasible substrates for surfactin production with varied yields.

Fish wastes, such as fish head, fish skin, fish bones, red meat, and viscera generated from fish processing operations, account for 40% to 60% of the total weight of the fish [28]. Those wastes are likely to cause environmental pollutions, and even a series of health problems as a rich source of suspended solids, organic carbon, and nitrogen [29]. At the same time, such a high organic content (e.g., proteins, polyunsaturated fats, and minerals) can be utilized as nutrients before being discarded. Their utilization as fishmeal for animal feed has been commonly adopted with low economic returns and high environmental pollution. Previous studies indicated that fish peptones extracted from fish wastes exhibited the potential to support microbe growth [30–32]. To date, few attempts have been made on biosurfactant production from fish wastes compared to that of other waste materials. Therefore, further in-depth investigation on fish wastes-based biosurfactant production as an environmentally-friendly alternative to make full use of these fish wastes is highly desired.

Lab-scale investigations on biosurfactant production are necessary as preliminary investigations of the fermentation conditions. However, they cannot reflect the system complexity when produced on a large scale. The problem raised by the change of configuration of reactors, air input, and agitation type could lead to various operational challenges. For example, in the pilot-scale experiment, there would be plenty of foam in the production process. To further confirm the commercial application of fermentation production, technology practice on biosurfactant production on a large and pilot-scale is a clear necessity toward their industrialization and commercialization, yet it is tackled in a limited way [33]. A full-scale demonstration of biosurfactant production using fish wastes as substrate is, thus, highly desired.

Bonito (*Katsuwonus pelamis*), which is a tribe of medium-sized, ray-finned predatory fish in the family Scombridae, belongs to the tuna family. Though easily caught, bonitos are not popular because of the meat quality and the fishy smell and, thus, cheaper than other tuna in the East China Sea. Therefore, they are commonly processed to produce fish products. The proper utilization and treatment of the generated fish waste become a challenge. We tried to solve this problem by using tuna fish wastes as a substrate for biosurfactant production. Scale-up studies were also conducted to facilitate the industrialization of biosurfactants. To achieve the objectives, tuna fish wastes were processed using the enzymatic hydrolysis method to generate the fish peptones. These generated fish peptones were served as the substrate for biosurfactant production. *Bacillus subtilis* (ATCC® 21332™) was selected as a representative lipopeptide producer [34–36]. Surface tension (ST) and critical micelle dilution (CMD) were evaluated for monitoring the biosurfactant production. Electro Spray Ionization Mass Spectrometry (ESI-MS) and high-performance liquid chromatography (HPLC) were used to evaluate the production. Three scales (20 mL, 7 L, and 100 L) of production were conducted to achieve the system scale-up.

2. Results and Discussion

2.1. Characterization of Hydrolyzed Peptones

The enzymatic hydrolysis degree of fish wastes was around 44.2% using the trichloroacetic acid (TCA) method. The amino acid analysis proved the existence of Phe, Ala, Met, Pro, Gly, Glu, Arg, Lys, Tyr, Leu, Ser, Thr, Asn, Val, Ile, and His in generated fish meat peptone, and the results were shown in Figure 1. The freeze-drying process for each batch of concentrated tuna fish head hydrolysate required 3 to 4 days. A total of 89% weight loss was reported in the freeze-drying process, and, accordingly, 22.96% of tuna red meat can be converted to fish peptone through an enzymatic process.

Characterization of hydrolyzed peptones can be meaningful for the production analysis. The characteristics of different raw materials could cause significant effects on the properties of substrates [37]. In this research, the yield of the process related to hydrolysis was around 44.2%. The generated result was in accordance with the ones generated by Sun (2013) (42.9%) using the same method [38]. The hydrolyzed peptones contained materials to be a kind of comprehensive substrate.

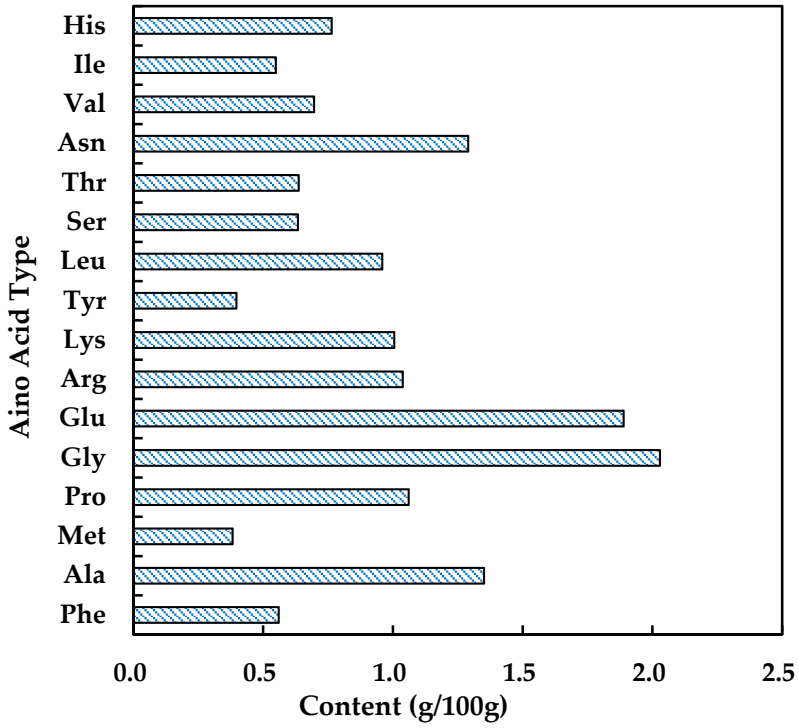

Figure 1. Composition of amino acid in tuna waste-based peptone after hydrolysis.

2.2. Bench-Scale Production of Biosurfactants

The results of batch-scale experiments of biosurfactant production using different fish waste peptone concentrations are shown in Figure 2. The ST of all the substrates were reduced to around 28 mN/m, which proved that ATCC 21332 could produce biosurfactants with comprehensive fish waste broth (with key supplement minerals). Moreover, the highest biosurfactant production rate was reported at a substrate concentration of 20 g/L, whose CMD values reached around 60. Thus, 20 g/L was selected as the optimized concentration.

An optimized fermentation medium at a concentration of 20 g/L proved that superfluous nutrients could have an inhibiting effect on surfactin production [39]. Furthermore, Pepi et al. (2013) [40] indicated that some fatty acids (e.g., palmitic acid and oleic acid) in fish peptone could also inhibit the biosurfactant production by Bacillus strains. Adding a small amount of manganese ion as a trace element was beneficial for the production of biosurfactants because the manganese ion could be the most important cofactor of glutamine synthetase, and glutamine synthetase is very important for the assimilation of

inorganic nitrogen by organisms [41]. Huang et al. (2015) found that a manganese ion could have a positive effect on nitrogen use and surfactin production by *Bacillus subtilis* ATCC 21332 [42]. Biosurfactant production by *Bacillus subtilis* ATCC 21332 using cod liver and head wastes was evaluated in our previous work (Zhu et al., 2020) [30]. The results showed that medium composition could significantly affect the structure and yield of produced biosurfactants. The generated fish waste peptones could substantially vary among different fish species and waste sources and affect biosurfactant production accordingly. The ST and CMD (i.e., 29.4 mN/m and 60.7, respectively) generated in this study were comparable to the ones generated by cod liver and head wastes peptones (i.e., 59.3 and 49.2, respectively). By using glycerol and waste frying oil as comparative carbon sources with *Bacillus subtilis* to produce biosurfactant, Ramirez et al. found olive mill wastes were potential substrates for biosurfactant production, which produced surfactin at a maximum concentration of 3.12 mg/L with 2% *w/v* of olive mill wastes in the medium [23]. The substrate can be optimized by adding additional nitrogen sources or carbon sources by conducting this step from bench-scale to pilot-scale. Sufficient carbon could facilitate the biosurfactant production process [43]. Therefore, additional carbon sources into this system, such as glucose and glycerol, or a continuous exploration of waste carbon sources (e.g., olive mill wastes) would be appealing and likely to improve the yield of biosurfactant production. Bench scale studies proved the feasibility to use tuna red meat wastes as a comprehensive substrate. Optimized fermentative conditions (e.g., concentrations of peptone and key supplement minerals) were determined.

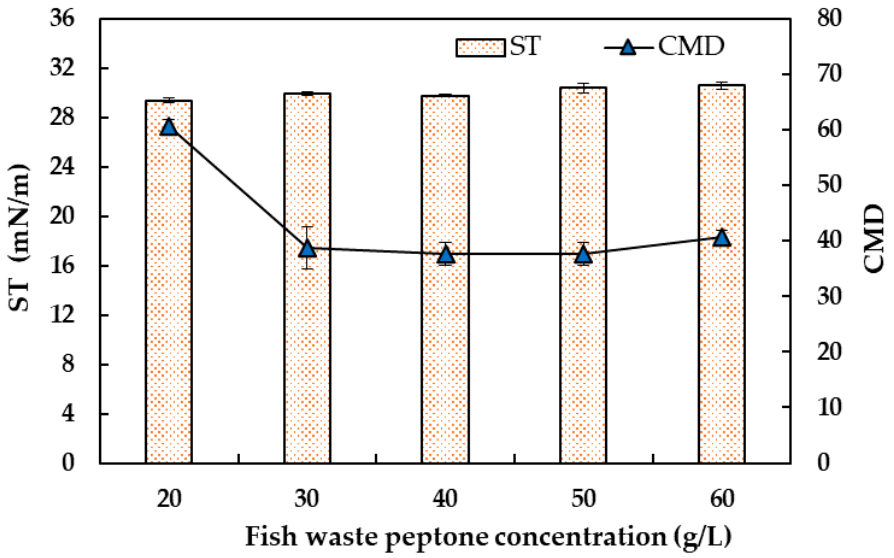

Figure 2. Bench-scale experiments exploring different fish waste peptone concentrations.

2.3. Batch-Scale Production of Biosurfactants

As shown in Figure 3, a reduction of ST occurred in the first 12 h, indicating a gradual secretion of biosurfactant products. A rapid ST drop and a CMD increase were found between the 12–24 h, implying a surge of biosurfactant production during this period. The ST and CMD values remained the same after 36 h. The highest biosurfactant concentration in the fermentation medium was achieved between 24–36 h. This result shed light on the fermentation time selected for pilot-scale experiments and a 48 h fermentation period was selected for the pilot-scale production.

To date, demonstration of biosurfactant production at scales (e.g., batch-scale and pilot-scale) using waste streams as substrate have been rarely explored, yet of great importance on their way to industrialization. Therefore, the antifoaming agent was added through the pump after foaming begins. After screening available antifoaming agents through a performance evaluation, the Foamdoctor® F2875 was chosen as the product applied in

batch/pilot studies. While ST values were similar between bench-scale and batch-scale experiments, a reduction of biosurfactant production (i.e., CMD values decreased from around 60 to 50) was reported during system scale-up.

In an amplification system, changes in the fermentation conditions such as pH, dissolved oxygen, and defoaming agents would affect the yield of surfactants [44,45]. These results generated by other studies could help explain the different CMD values between bench-scale and batch-scale products. Moreover, in a larger system, defoaming agents were essential because of the foam formation. All conditions that changed in a larger system could affect the metabolism activities and, thus, affect the yield of surfactants. Generally, the ventilation rate was 1 vvm, which made strains carry out cell metabolism activities in a suitable batch-scale condition. The volume of defoaming agents depended on the foaming situations, which was usually controlled by sensors on the fermenter. In addition, for continuous cell growth, the effect of inoculum age should also be considered [46].

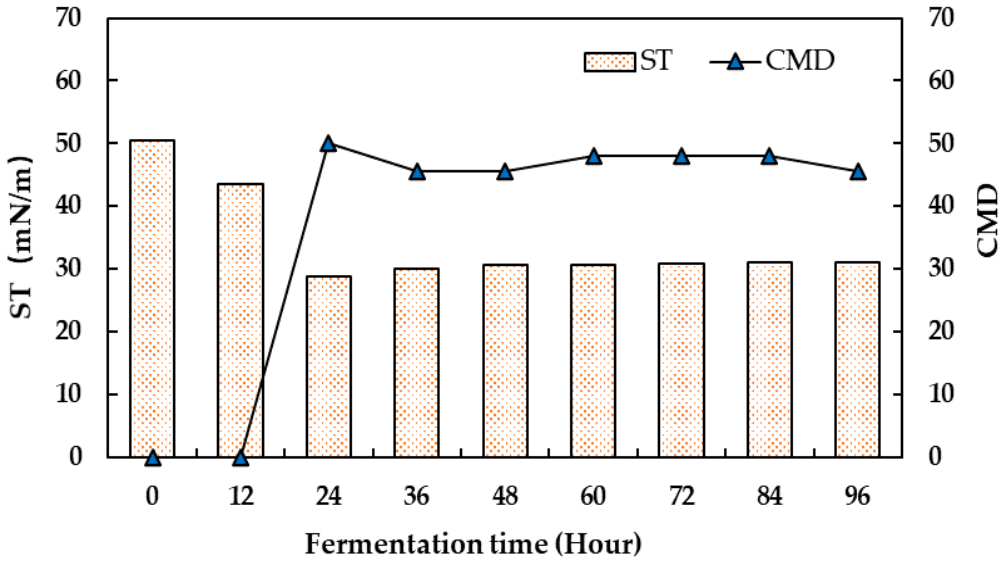

Figure 3. ST and CMD values in batch-scale experiments (7 L) with optimized concentration.

2.4. Pilot-Scale Biosurfactant Production Experiments

The results of fish wastes-based biosurfactant production in a pilot-scale reactor were shown in Figure 4, with a biomass result illustrated. The highest concentration was 129 mg/L in 24 h. The growth of bacteria was boosted from 6 h to 24 h and reached a peak after 24 h. The growth status of *Bacillus subtilis* ATCC 21332 in the culture medium was inferred because of sufficient materials. The fast metabolization of *Bacillus* strains led to an increasing bacterial colony concentration. Nutrient demand exceeded the supply after 24 h and limited the metabolic activities of strains and their reproduction. The content of bacteria went through a transitional plateau period, and the bacterial growth curve's plateau period existed between 12 h and 36 h. Compared with the results of batch-scale, the trends of ST values and biosurfactant contents were similar.

To date, no pilot-scale studies on lipopeptide production by *Bacillus substilis* ATCC 21332 have been reported using waste streams as substrate, so it was meaningful to explore the optimum conditions for surfactin fermentation scale-up. The surfactin production reduction was also reported in pilot-scale reactors compared to that of batch-scale studies using *Bacillus substilis* B006 for surfactin production [47], whose surfactin productivity reached 314.73 mg/L. The larger the system is, the more complicated the operation conditions are. Therefore, more emphasis should be given to the performance investigation for surfactin synthesis on a large scale.

The drop of surfactin content between 24 to 48 h could be explained by a rapid biosurfactant production and a spontaneous foam overflow occurred after that. To solve

this problem, a recovery tank was connected to the fermenter for foam collection. In this study, the foam collected in the recovery tank contained a surfactin concentration of around 274 mg/L, doubled its concentration in the sample collected at 24 h. Moreover, although the antifoaming agent was used during the fermentation period, there was still a lot of foam before 24 h. A novel bioreactor system based on integrated foam-control and a repeated fed-batch fermentation strategy has been applied to rhamnolipids production [48], which could help enhance biosurfactant production.

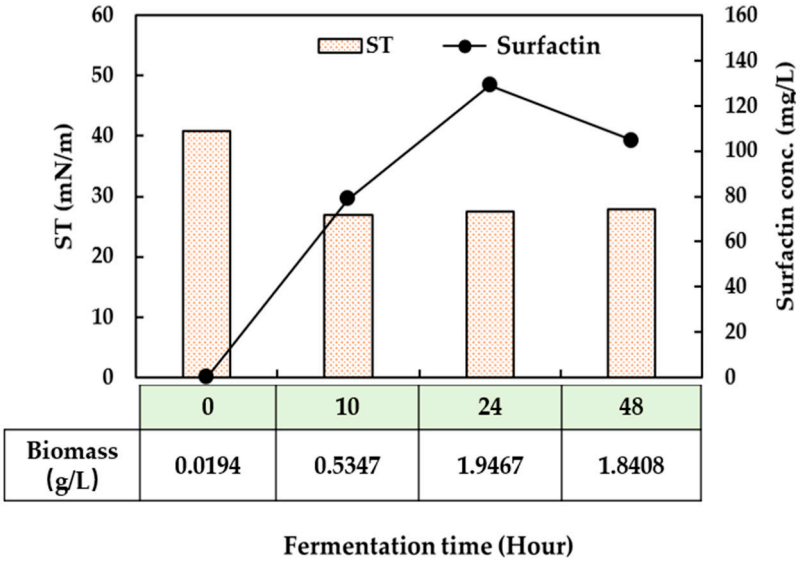

Figure 4. ST values, surfactin concentrations, and biomass in pilot-scale experiments (100 L).

2.5. Characterization of Biosurfactant Production

Electro Spray Ionization Mass Spectrometry (ESI-MS) analysis was conducted to characterize the structures of generated biosurfactant products. Results are shown in Figure 5. The surfactin standard exhibited five anion peaks around the mass-to-charge ratio of 1000. When compared to purified samples corresponding to the standard substance, surfactin was proven to be the product of fermentation.

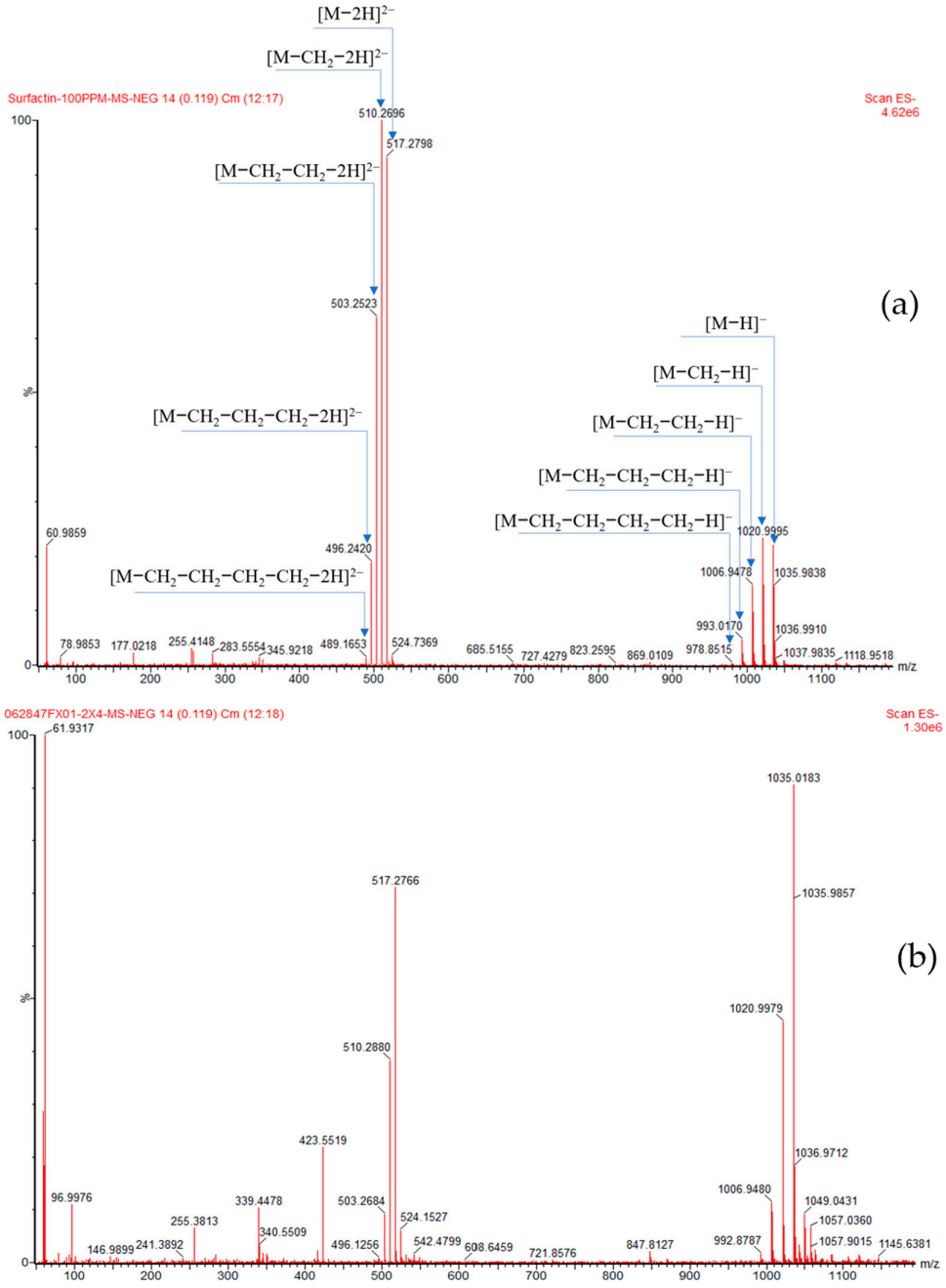

Figure 5. (**a**) Surfactin standard sample ESI-MS. (**b**) Biosurfactant production ESI-MS.

3. Methodology

3.1. Materials

Samples of tuna red meat wastes were from Ningbo Today Food Co. LTD, a fish processing plant in Zhejiang, China. Each sample was minced three times using a food processor at medium speed for 120 s. Fresh tuna red meat samples were taken for composition analysis prior to storage at −20 °C for subsequent experimentations. The red meat, which accounted for 13% to 15% of the weight of fish, was collected from tuna fish and subjected to proximate composition analysis. The results indicated that this meat had a moisture content of 58.6%, a protein content of 18.1%, and a fat content of 7.6 g/100 g.

Bacillus subtilis (ATCC® 21332™) was the strain used for this study, which was a kind of strain in the form of freeze-drying at Security Level 1. Nutrient agar medium or nutrient broth was used as a culture medium. The temperature of the growing condition was 30 °C. After 24 h of culture on solid medium, milk-white bacterial colonies can be seen. ATCC

21332 should be put into the freeze-drying tube in the cryogenic refrigerator at −80 °C for preservation and activation.

The Surfactin standard sample was purchased from Sigma (USA). Sodium chloride, protease peptone, beef extract, agar, ferrous sulfate, manganese sulfate, sodium hydroxide, concentrated hydrochloric acid, and methyl alcohol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). PB05 basic protein (200,000 U/g) and PB02 animal protein (100,000 U/g) were purchased from Naning Pangbo Biological Engineering Co., Ltd. (Guangxi, China). The anti-foaming agent Foamdoctor® F2875 was purchased from Shenzhen Dayang New Material Co., Ltd. (Guangdong, China).

3.2. Enzymatic Hydrolysis for Generating a Fish Waste-Based Substrate

The lab-scale enzyme hydrolysis of fish wastes followed the method developed by a technology developed by Sun (2013) [38]. PB05 basic protein and PB02 animal protein were selected as the hydrolysis enzymes. Generally, 200 g of the waste sample was added into a 1000-mL Erlenmeyer flask and mixed with equal volumes (200 mL) of distilled water (1:1 *w/v*). The ratio of animal protease and alkaline protease was 2:1 (*w/w*), and the volume of the enzyme was 1.5%. The pH was 7, which was regulated by HCl (2 mol/L) and NaOH (2 mol/L). The hydrolysis time was 6 h. The temperature was 50 °C with heating in the water bath. After finishing the hydrolysis period, the Erlenmeyer flask was put into another water bath at 90 °C for 15 min. The mixture in the flask was then centrifuged at 8000 *g* for 10 min. The supernatants were collected for the degree of hydrolysis (DH) measurement, and some were concentrated to one-third by the rotary evaporator, after which fish waste peptone was produced by the freeze dryer for three days. Figure 6 shows procedures during the enzyme hydrolysis of fish waste. Fish waste peptone was then stored at 4 °C for subsequent experiments in the laboratory. Procedures of enzyme hydrolysis generation for the scale-up testing were the same as those in the laboratory with enlarged amounts of all involved materials proportionally.

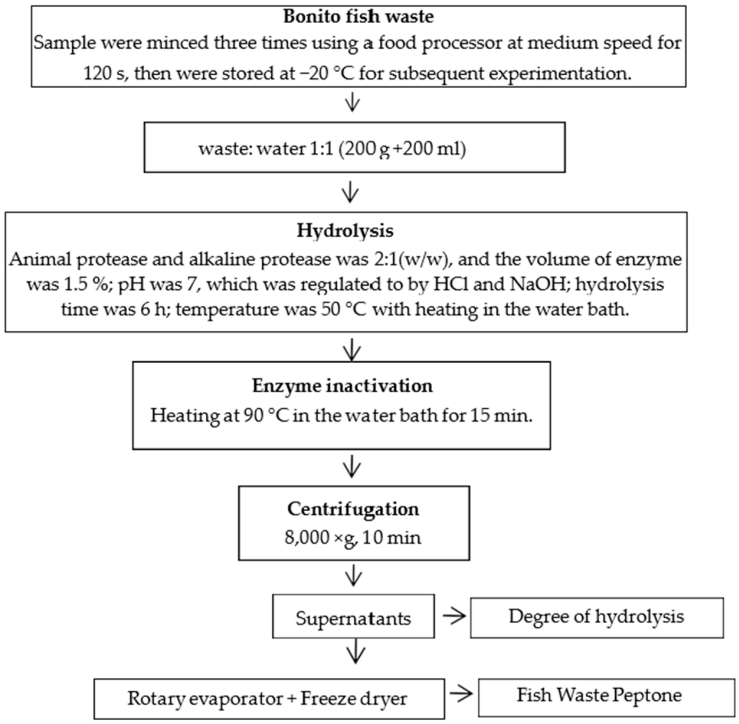

Figure 6. Flow chart of the enzyme hydrolysis in the laboratory.

3.3. Bench-Scale Biosurfactant Production (20 mL)

After strain activation, bacteria were first inoculated in a Petri dish and incubated at 30 °C for 24 h. The materials of the inoculation broth followed as: NaCl (5 g/L), protease

peptone (5 g/L), beef extract (3 g/L), and distilled water. After autoclaving at 121 °C for 20 min, a selected single strain colony was transferred into the Erlenmeyer flask.

The Erlenmeyer flask was incubated in a rotatory shaker at 120 rpm under 30 °C for 24 h. All experiments in this study used 2% (*v*/*v*) inoculum as a seed culture level during fermentation. After freeze drying for 3 days, fish waste peptones were prepared as comprehensive medium with distilled water and key supplement minerals: $FeSO_4$ and $MnSO_4$.

To obtain the optimized fish waste peptone concentration, these lab-scale experiments were conducted with a series of concentrations (g/L): 20, 30, 40, 50, and 60. Key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and $MnSO_4$ (0.15). Twenty milliliters of distilled water were added into each 50 mL Erlenmeyer flask. The strain in the flask was used as inoculum at the 2% (*v*/*v*) ratio. Erlenmeyer flasks were then incubated in a shaking incubator (130 rpm) at 30 °C for 7 days. After incubation, the supernatant was collected after centrifuging at 8000 *g* for 10 min. Biosurfactant production was evaluated with ST and CMD values. Each concentration had three parallel runs and all evaluations of the experiments were triplicate.

3.4. Batch-Scale Biosurfactant Production (7 L)

BioFlo 120 (Eppendorf, Germany) was the fermentation tank used in the batch-scale experiments (14 L total volume, 7 L working volume). Figure 7 shows the batch-scale experimental set-up for biosurfactant production. The fermenter was equipped with a paddle mixer, a heater band, and a set of sensors (i.e., foam, pH, temperature, dissolved oxygen, and revolving speed of stirring paddles), which could be controlled to set the experiments to desired conditions through the control panel under the screen. Air was injected through the gas pump during the fermentation. The antifoaming agent was added into a container through the pump after foaming. Optimized concentrations of fish waste peptones and manganese followed the results from the previous experiments. The key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and $MnSO_4$ (0.15). After autoclaving at 121 °C for 20 min and cooling down to room temperature, 2% inoculum of strains were added into the tank. Temperature was set as 30 °C, dissolved oxygen was set as 50%, and revolving speed of stirring paddles was set as 120 rpm. Samples were taken through the outlet every 4 h before 24 h to obtain detailed biomass change. After 24 h, samples were taken through the outlet every 12 h until the end of the fourth day. Biosurfactant production was evaluated with ST and CMD values. Samples at each time had two parallels and all evaluations of the experiments were triplicate.

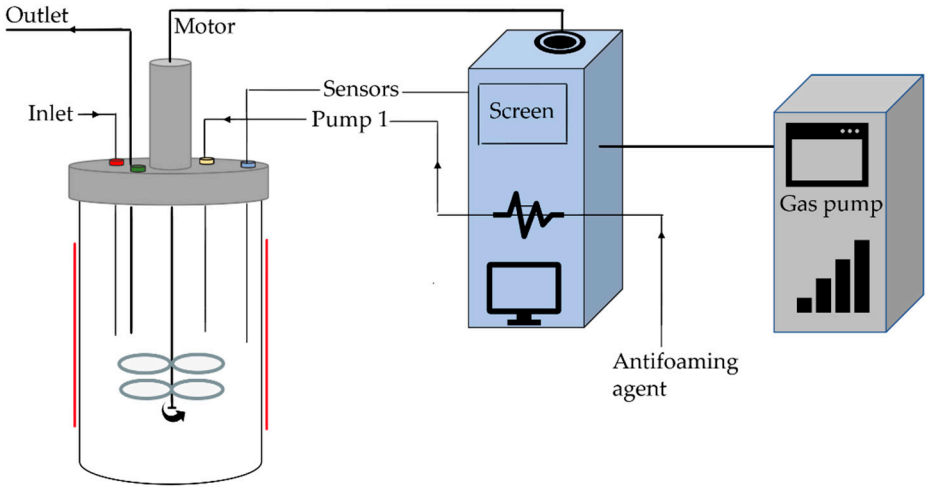

Figure 7. Lab-scale experimental set-up (7 L).

3.5. Pilot-Scale Biosurfactant Production (100 L)

The pilot-scale experiments were conducted in a 200 L fermentation tank (Zhenjiang Dongfang, Jiangsu, China) at a working volume of 100 L. Figure 8 shows the pilot-scale experimental set-up for biosurfactant production. As shown in the figure, this fermentation set-up was comprised mainly of a fermentation tank, a seed tank, and an agitator motor for agitation. There were several pumps connecting the seed tank with the fermentation tank: feeding and discharging production. Parameters, such as temperature, pH, and ventilation capacity, were controlled by a control system. The antifoaming agent was added into the container through the pump near the outlet after foaming. Optimized concentrations of fish waste peptones and manganese followed the results of bench-scale experiments. The key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and $MnSO_4$ (0.15). After autoclaving at 121 °C for 20 min and cooling down to room temperature, 2% inoculum of strains were added into the fermentation tank through the pump. Temperature was set as 30 °C, dissolved oxygen was set as 50%, and revolving speed of stirring paddles was set as 120 rpm. The ventilation rate was 1 vvm. Samples were taken through the outlet after 0, 10, 24, and 48 h. Biosurfactant production was evaluated with ST, biomass, ESI-MS, and HPLC.

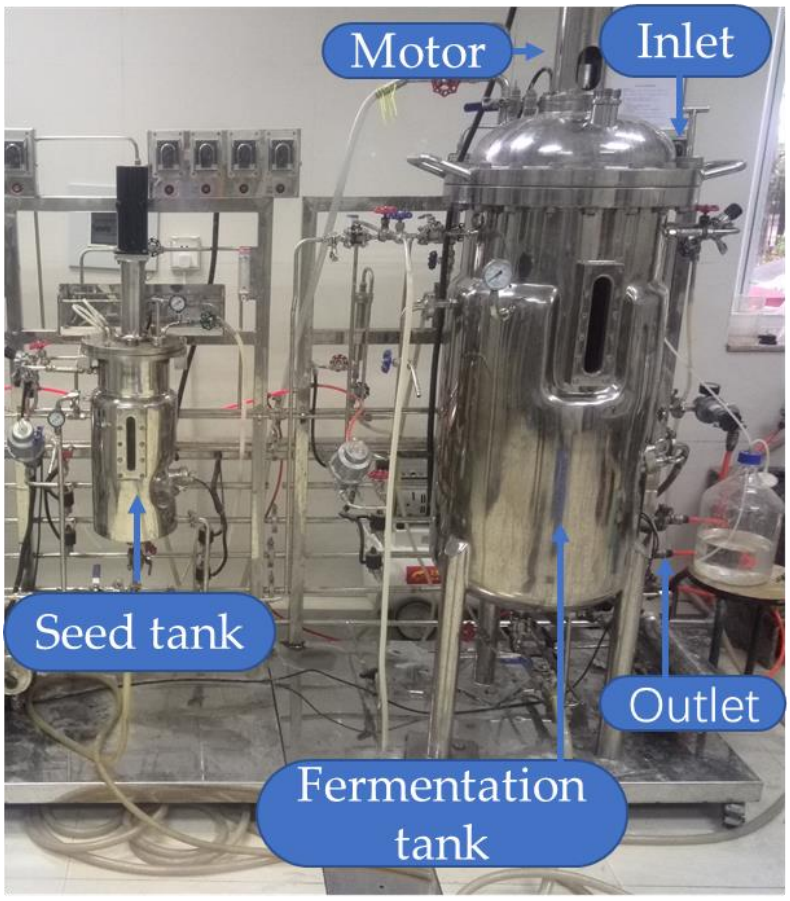

Figure 8. Pilot-scale experimental set-up (100 L).

3.6. Evaluation of Biosurfactant Production Performance

Surface tension: Surface tension was measured by the plate method using Sigma 700 surface tension meter (Biolin Scientific, Västra Frölunda, Sweden). Twenty-milliliter liquid was subjected to the determination of ST in a petri dish. To ensure the reliability of tested results, the average of three independent measurements was taken.

Critical micelle dilution (CMD): Critical micelle dilution was measured by the plate method using a SIGMA 700 surface tension meter. Critical micelle dilution could reflect the

concentration of biosurfactants in the medium and was determined following the method described by Cai (2017) [49]. When the ST just exceeded 40 mN/m, the dilution process stopped, and the dilution ratio was recorded as the CMD value for this culture broth. All the measurements were performed in triplicate.

Biosurfactant Purification and Characterization: Put the 7 L of fermentation broth in the centrifuge for 10 min at the rate of 8000× *g* to eliminate the thallus. The volume of the supernatant was defined as 30 mL. Then, 6 mol/L HCl was used to adjust pH to 2.0 as white flocculent precipitates formed. The supernatant was placed still for a while for more precipitates to gather. Then, put the supernatant with 5 mL in centrifuge for 15 min at the rate of 10,000× *g* to collect precipitates. The supernatant was shaken with 5 mL methyl alcohol and extracted for 1 h. Thereafter, a surfactin standard substance and supernatant were sent for inspection using qualitative and quantitative analysis through ESI-MS (Ningbo Institute of Oceanography, Ningbo, China) and HPLC (Ningbo Boao Bioengineering Co. LTD, Ningbo, China), respectively [50].

4. Conclusions

This study explored the conditions of using tuna fish wastes to generate surfactin. The research could help the local factories to dispose of the waste garbage and the environmental problem of wastewater. Fish wastes were first evaluated as a comprehensive substrate for strain growth and surfactin synthesis. The scale-up validation of surfactin production was attempted with a surfactin productivity of 274 mg/L in the fish-waste-based fermentation medium. Further works will be needed to further optimize the comprehensive fish waste substrate with a proper supplement of carbon or nitrogen source. This study demonstrated a cost-efficient approach for surfactin synthesis and paved the way for the industrialization of their production through an understanding of the metabolic mechanism and production kinetics of surfactin produced by strain ATCC 21332.

Author Contributions: Conceptualization, J.H., B.C., and P.Z. Methodology, J.H. and J.L. Validation, B.Z. and Z.Z. Resources, B.C. and P.Z. Data curation, J.H. and X.Y. Writing—original draft preparation, J.H. Writing—review and editing, J.L., Z.Z., and B.Z. Visualization, B.C. and X.Y. Supervision, B.Z., B.C., and P.Z. Project administration, B.Z. and Z.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Conflicts of Interest: The authors declare no conflict of interest.

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