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Effect of Type of Nitrogen Sources and Concentration on Protease Production: Fish Waste Hydrolysate as a Potential Low Cost Source

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Abstract. Fish waste hydrolysate is obtained from hydrolysis process of a fishery industry waste. It can be a good substrate source for the production of protease via a fermentation process. Protease covers above than 50% of the global industrial enzymes market and is regarded as an enzyme which is widely used in many applications. This research aims to determine the effect of the type of nitrogen sources and nitrogen source concentration on protease production from the fish waste hydrolysate. The experiments were done using one factor at a time (OFAT) of type of nitrogen sources and nitrogen source concentration in submerged fermentation (SmF) of 10% (v/v) *Bacillus cereus* (ATCC 10876). In the first stage, the variation of type of 1% (w/v) nitrogen sources such as fish waste hydrolysate, beef extracts, yeast extracts, tryptone, peptone and skim milk powder that mixed with the basal media were compared to evaluate the highest protease production. Then, the concentration of fish waste hydrolysate was varied from 1% to 3% (w/v) to analyse the production trend of protease. The protease activity and total protein content were then analysed by protease assay using tyrosine as a standard and Bradford method, respectively. Upon the observation, 1% (w/v) nitrogen sources on protease production revealed that the fish waste hydrolysates and beef extracts were capable to produce a high amount of protease (23.659 ± 0.38 U/mL and 28.7631 ± 0.59 U/mL, respectively). Among the concentration tested, 3% (w/v) of fish waste hydrolysate was found to be the highest protease production (190.85 ± 0.38 U/mL). Therefore, the fish waste hydrolysate has a potential as a low-cost source for protease production.

Keywords: Fish waste hydrolysate, protease, submerged fermentation (SmF), nitrogen sources, nitrogen source concentrations

1. Introduction

Proteases are known as proteinases, peptidases or proteolytic enzymes that can be found in all organisms which are very crucial for cell growth and maintenance [1]. The peptides can be formed using microbial proteases from fishing industry by-products [2]. The classification of proteases depends on the chemical groups that function in catalysis [3]. Regarding to [4], proteases constitute more than 65% of the worldwide industrial enzyme market. Proteases being regarded as versatile enzymes since their applications in many sector such as food, chemical, pharmaceutical, silk, detergent, and leather industries [2]. The proteases are included as additives in detergents to assimilate proteins [1]. Furthermore, wide utilization of microbial proteases in diseases prevention such as diabetes, obesity, osteoporosis, and arthritis shows the significant of the enzymes production [5]. Moreover, microbial proteases are renewable sources and can be formed in huge quantities compared to plant and animal proteases [6].

Since years back, many researchers tried to produce microbial proteases using low-cost media [7]. In order to achieve this purpose, they used fish processing by-products such as fish meat wastes [9], cephalopod wastes [10], chitinous materials [7, 11] and wastewater [12]. There are several re-utilization



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of fish waste can be adapted including production of hydrolysate, fermentation, fish oil formation and purification to prevent from wasting fish by-products [7, 8]. From the previous studies, the protease production gained using soy peptone is 373 U/mL [13]. Regarding to [6], the higher protease activity was recorded at 17.0 U/g-1 using several nitrogen sources. [14] stated that the highest protease activity of microbial media containing casein was achieved at 182.5 U/ml-1 using 3.5% of concentration. The protease activity might be huge in amount when it controlled by chitin and protein ratio [15]. Furthermore, the protease activity and the time of growth are influenced by the rate of preparation and chemical treatments.

The high demands of these applications need a large amount of setup costs for isolation and purification of biotechnological outcomes [16]. Based on [7], the microbial enzyme formations are controlled by the capitals and availability of raw materials. Moreover, some industrial methodologies are often accomplished in utmost conditions and use high-priced raw materials in enzymes production [17]. Therefore, the use of low-budget raw materials for protease production is more preferable. As reported from previous research, the production of value added product was converted from fish wastes, which can be obtained in abundant from the fisheries and market [16]. In this study, the protease was produced using inexpensive sources which was fish waste hydrolysate as a nitrogen source via submerged fermentation. The effect of different nitrogen sources were compared with the fish waste hydrolysate and the concentration of the nitrogen sources were varied to find the highest protease production. However, fermentation process has various parameters which could influence the performance. Thus, some optimization methods need to be done and the parameters should be monitored to obtain a maximum production of proteolytic enzymes. In addition, the production of proteases might be declined by times due to the depletion of nutrients [18] because they are capable to degrade themselves [19].

2. Materials and methods

2.1. Materials

The main material used for this research was fish waste isolated from all parts of Ikan Selayang collected from local food industry, located in Pahang, Malaysia. The *Bacillus cereus* (ATCC 10876) was supplied from Central Lab, Universiti Malaysia Pahang, Gambang, Kuantan. Meanwhile, Coomassie Brilliant Blue G-250, Bovine Serum Albumin (BSA), phosphoric acid, ethanol, sodium hydroxide (NaOH), hydrochloric acid (HCl), dipotassium phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and commercial nitrogen sources such as beef extracts, yeast extracts, skim milk powder, peptone and tryptone were purchased from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane, ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), anhydrous sodium carbonate ($NaCO_3$ anhydrous), copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), tyrosine [4-hydroxyphenylalanine], potassium sodium tartrate ($C_4H_4KNaO_6$), trichloroacetic acid (TCA) and casein from bovine milk sodium were purchased from Gardner Global Enterprise, Pahang, Malaysia. Folin-Ciocalteu phenol reagent was purchased from Merck Malaysia, Ltd.

2.2. Fish waste hydrolysate preparation

As shown in Figure 1, all parts of fish wastes were used to obtain fish waste powders. The by-products were cleaned with tap water to eliminate blood then steamed for 20 min. The steamed fish wastes were pressed with white cloth to withdraw water and then dried at 80°C. Next, the dried fish-minced were ground to obtain fine powders. The fish waste powders were then hydrolysed using acid hydrolysis where the powders were mixed with distilled water (1:2 w/v), 2N HCl was added until pH 3.3 and incubated at 85°C, 200 rpm for 2 h in a shaking water bath. The mixture was then neutralized using 2N NaOH until the pH 7.0 and centrifuged at 6700 x g, 10°C for 20 min. The supernatant was treated as fish waste hydrolysate and freeze dried at -80°C for 48 h to obtain a solid form of hydrolysates as shown in Figure 2.

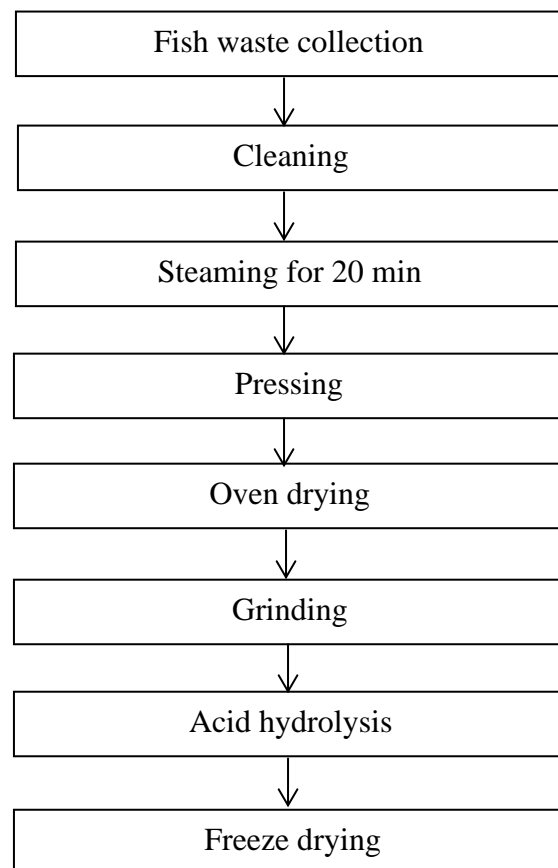


Figure 1. Preparation of fish waste powders and hydrolysates using acid hydrolysis.



Figure 2. Fish waste hydrolysate in solid form.

2.3. Culture Medium

Potato Dextrose Agar (PDA) was stirred until the powder was completely dissolved in distilled water and the mixture was then sterilized in the autoclave. Next, the mixture was cooled and poured into the

petri plates. The plates were labelled after the agar solidified and flipped the plates to avoid condensation of water when storing the media in a chiller at 4°C for further use.

2.4. Protease assay

The protease activity was examined using casein as a substrate according to [20] with a slight modification. Briefly, 1 mL of the supernatant from the fermentation was mixed with 5 mL of a 2% (w/v) casein solution and incubated in the water bath at 50°C and 100 rpm for 1 h. Then 5 mL of 15% (w/v) trichloroacetic acid, TCA was added to terminate the reaction. The samples were then centrifuged at 10 000 rpm for 10 min at 4°C before an aliquot of 0.5 mL of the supernatant was added into the alkaline reagent and 0.5 mL of 25% (v/v) Folin-Ciocalteu phenol reagent. The resulting solution was incubated at room temperature in the dark for 1 h. The absorbance was measured at 700 nm using a tyrosine standard [21]. One unit of protease activity was defined as the liberation of 1 g of tyrosine per minute under the assay conditions. All activity tests were performed in triplicate. The protease activity for each experiment was calculated using equation (1):

$$\text{Protease activity } \left(\frac{\text{U}}{\text{mL}} \right) = \frac{C \times 15}{1 \times t \times 0.5} \quad (1)$$

Where,

C	=	Concentration of nitrogen source (µg/mL)
15	=	Total volume of chemicals used during protease assay (mL)
t	=	Time of incubation (min)
0.5	=	Volume of supernatant (mL)

2.5. Total protein content determination

In the determination of protease-specific activity, the total protein content was observed by a Bradford method using 2% (w/v) bovine serum albumin (BSA) as a standard protein [20]. Briefly, 0.05 mL of cell-free supernatant was mixed with 1.5 mL Bradford reagent and the absorbance was measured at 595 nm in a UV-Vis Spectrophotometer. The amount of protein formed was determined using BSA as the reference standard. All activity tests were performed in triplicate. The protease-specific activity for each analysis was accounted using equation (2):

$$\text{Specific activity } \left(\frac{\text{U}}{\mu\text{g}} \right) = \frac{\text{Total protein}}{\text{Protease activity}} \quad (2)$$

2.6. Effect of nitrogen sources on protease production

Based on [22] and [23], the growth medium was initially added with different nitrogen sources that are 1% (w/v) skim milk powder, 1% (w/v) peptone, 1% (w/v) yeast extract, 1% (w/v) beef extract 1% (w/v) tryptone, and 1% (w/v) fish hydrolysate powder. None N source – containing basal medium was taken as a control in all experiments. All activity tests were conducted in triplicate. After screening, the protease content formed by nitrogen sources was then reported.

2.7. Effect of nitrogen source concentration on protease production

The screening process was then carried out by varying concentrations on nitrogen source chosen from the first OFAT factor (fish waste hydrolysate). The concentration was varied at 1%, 1.5%, 2%, 2.5% and 3% (w/v). The largest protease outcomes will be reported as the best concentration of nitrogen source for protease production. Each experiment was performed in triplicate.

3. Results and Discussion

3.1. Effect on nitrogen sources on protease production

In the present study, 1% (w/v) of different nitrogen sources such as beef extract, yeast extract, skim milk powder, peptone, tryptone including fish waste hydrolysate was added into each basal medium and the protease was produced after 48 h of incubation under submerged fermentation. Figure 3 shows the effect of different nitrogen sources for protease production. Based on the experiment, it is revealed that the beef extract was found to be the highest protease production (28.76 ± 0.59 U/mL), followed by skim milk powder (26.91 ± 0.14 U/mL), fish waste hydrolysate (23.66 ± 0.38 U/mL), peptone (13.09 ± 0.09 U/mL), yeast extract (11.91 ± 0.48 U/mL) and tryptone (10.75 ± 0.13 U/mL). It is obviously shown that the fish waste hydrolysate can produce a high amount of protease comparable with beef extract and skim milk powder. Thus, it has a potential to be exploited as a nitrogen source for protease production, plus it can be obtained at low-cost. A similar finding was also observed in the early study on protease production using acid hydrolysis with the isolation of *Bacillus cereus* by [23]. The protease production using fish waste hydrolysate (60.37 ± 0.59 U/mL and 74.77 ± 0.69 U/mL) were recorded. The enhancement of protease production in fish waste hydrolysate may be attributed to its high content of vitamins and omega-3 fatty acids [24]. In addition, the fish wastes may contain approximately 40% of proteins [25], minerals and carbohydrates that can provide beneficial peptides for protease synthesis or induction [26].

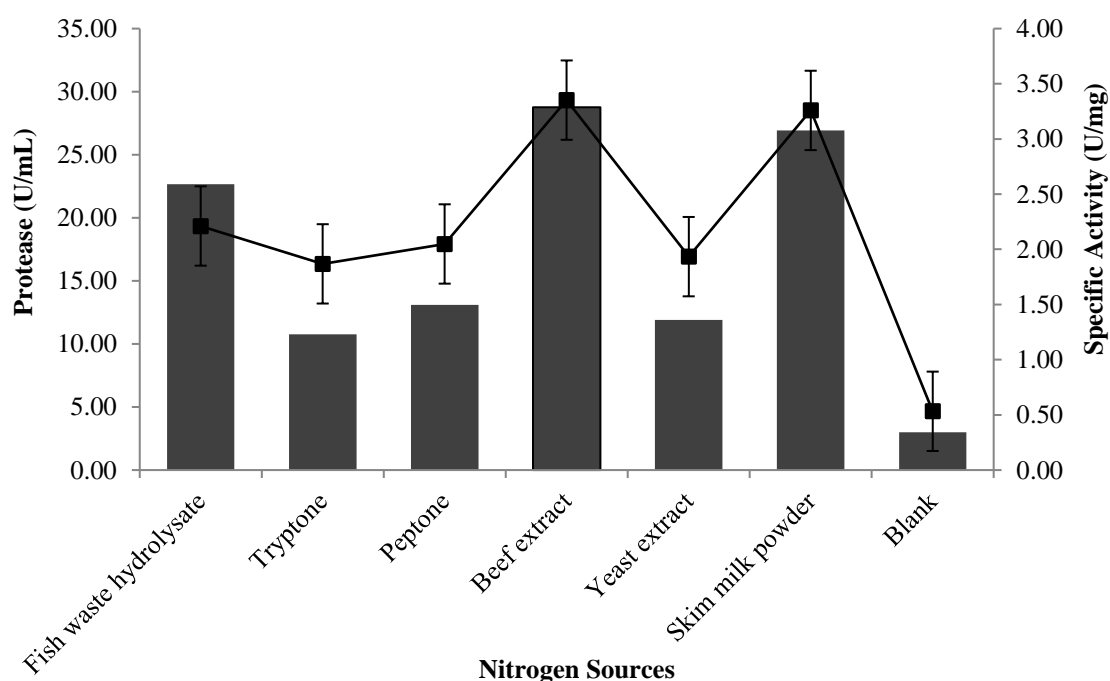


Figure 3. The determination of protease activity using different nitrogen sources.

According to Figure 4, it is clearly shows that the fish waste hydrolysate produced the highest protein content (10.25 ± 0.17 mg/mL) which was used to estimate protease-specific activity (2.21 ± 0.02 U/ μ g). This study also supports the previous findings by [27] where 15.48 ± 0.25 mg/mL of protein content was observed using fish waste hydrolysate as a substrate.

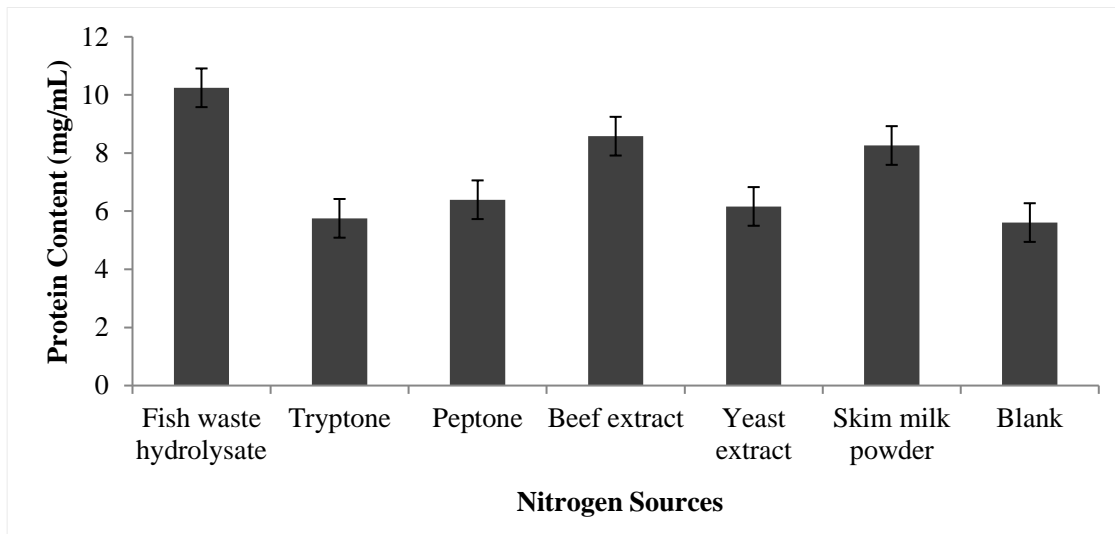


Figure 4. The determination of protein content using Bradford method.

From Figure 5, the fish waste hydrolysate shows that it produced 16.11 g/L of cell biomass concentration which the value was just a slightly different from beef extract. All the protease activities are obtained at different value regarding microorganism prepared in submerged fermentation and acid hydrolysis [28].

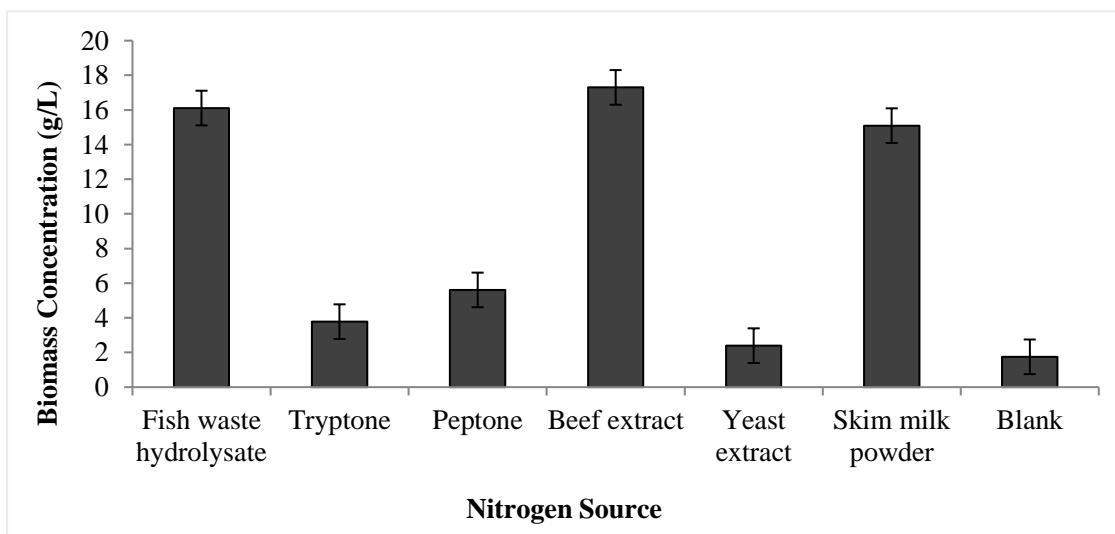


Figure 5. The determination of cell biomass concentration in producing protease.

3.2. Effect of nitrogen source concentration on protease production

In this study, 1% to 3% (w/v) of fish waste hydrolysate was used for further findings and the protease was determined after 48 h of incubation under submerged fermentation. Among the concentrations tested, 3% (w/v) was found to be the highest amount of protease (190.85 ± 0.38 U/mL) followed by 2.5% (w/v) (106.99 ± 0.03 U/mL), 2% (w/v) (59.97 ± 0.02 U/mL), 1.5% (w/v) (33.62 ± 0.01 U/mL) and 1% (w/v) (18.46 ± 0.01 U/mL) according to Figure 6. This study was supported by the previous findings of [23] where the protease production (178.50 ± 0.28 U/mL) was noticed. This study also supports

previous finding of [14] with the highest production was recorded at 3.5% (w/v) (182.50 U/mL). Based on the results obtained, the protease production was proportionally increased with the concentration of fish waste hydrolysate. The protease production increased at 3% (w/v) due to the growing phase by the bacteria linked with nutrient elevation in the culture media [29].

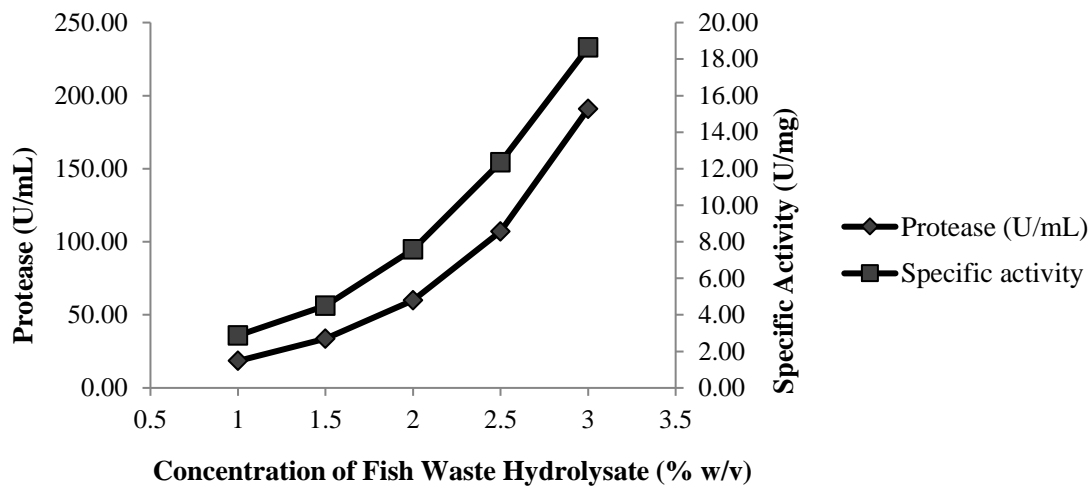


Figure 6. The determination of protease activity using different concentration of nitrogen source

Based on Figure 7 and Figure 8, the trends are proportionally elevated with the nitrogen source concentrations. At 3% (w/v) of fish waste hydrolysate, the protein content and cell biomass concentration were recorded 10.23 mg/mL and 16.11 g/L, respectively. The improvement of protease production could have been due to high lipid content that influenced by nutritional factors which is acting as inducers [30]. It can achieve a minimum colony count which indicates the higher microbial growth inhibition.

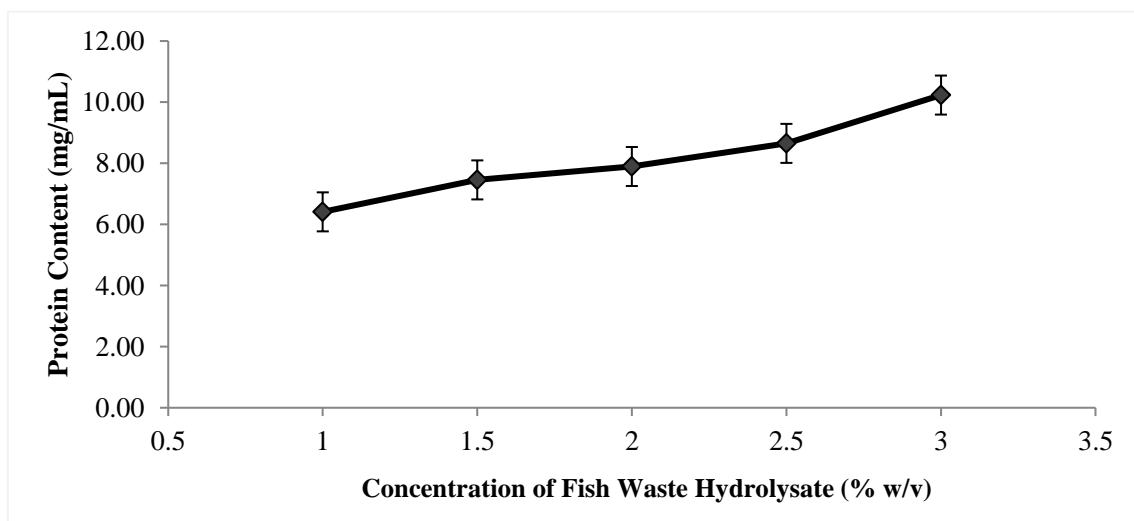


Figure 7. The determination of protein content using Bradford method.

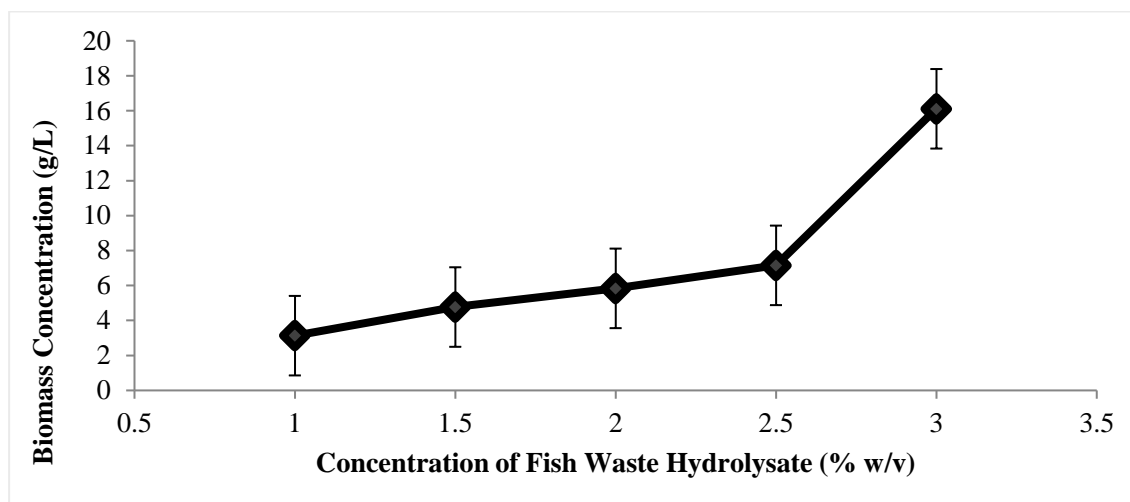


Figure 8. The determination of cell biomass concentration on protease production.

4. Conclusion

Fish waste hydrolysate is able to produce protease as much as beef extract. Therefore, it can be a potential low – cost of nitrogen source for protease production. The amounts of protease activity obtained by 1% (w/v) fish waste hydrolysate and 1% (w/v) beef extract were 23.66 ± 0.38 U/mL and 28.76 ± 0.59 U/mL, respectively. The highest protease production by fish waste hydrolysate was recorded at 3% (w/v) (190.85 ± 0.38 U/mL) and 1% (w/v) of fish waste hydrolysate was noticed to be the lowest protease production (22.67 ± 0.38 U/mL). Thus, fish waste hydrolysate has a potential to produce huge amounts of protease that can be used in many industrial applications. For the enhancement of this study, the different types of fish are recommended to be studied in order to optimize protease production. Besides, concentration above 3% (w/v) of nitrogen source is recommended in order to determine the concentration level of nutrient depletion and declining phase of bacteria.

Acknowledgments

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