Effects of chemical hydrolysis operating parameters on the production of antioxidant from fish waste

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Abstract. Instead of being inappropriately disposed and causing sanitary and environmental problems, underutilised fish waste can be processed into value-added products such as antioxidants which can be used for pharmaceutical, medicine and agriculture purposes. In this study, chemical hydrolysis was chosen as it was more suitable for industrial scale, shorter time and cheaper compared to enzymatic hydrolysis. However, information on the operating parameters of fish waste chemical hydrolysis was scarce. Therefore, the objectives of this study were to investigate the effect of types of solvents used, the concentration of HCl, demineralization time and deproteinization time on the production of antioxidant from fish waste via One Factor in Design Expert. Demineralization was carried out by immersing 10 g of fish waste in 0.2 M to 1.0 M of Hydrochloric acid (HCl) for 30 min to 150 min at room temperature and continued stirring at 150 rpm. Deproteinization occurred by introducing the sample into 1.0 M of Sodium Hydroxide (NaOH) for 30 min to 150 min at 60 °C and continued stirring at 150 rpm. These samples were then washed with distilled water and oven-dried at 60 °C for 5 hours. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and Total Phenolic Compound (TPC) assays were used to analyze the availability of antioxidant by calculating the percentage of antioxidant activity and Gallic Acid Equivalent, GAE (g/L). The best concentration of HCl used was 0.72 M at 92 min of demineralization, 70 min of deproteinization and ethanol as the suitable solvent used in DPPH and TPC assays. These valuable findings are beneficial for further works on enhancing the production of antioxidant from marine sources like fish waste.
Introduction

Fish waste is defined as different kinds of fish species having no or low commercial value, undersized or damaged condition [2]. There are about 70% of the fish being processed which included stunning, scaling, cutting of fish before the final sale and these generated 20-80% of fish waste [3]. The statistics showed that 57% of the fish waste was directly disposed to the sea and led to water quality problems and affected the sheltered environment of marine life [3]. This improper handling of fish waste resulted in cloudy water problems and strong odours as a result of bacterial decomposition and reduction of oxygen level in the seawater [4]. Fish waste can be processed into value-added products such as amino acids, collagen and gelatin, oil and enzymes as well as antioxidant by using certain processes [3].

Antioxidants as food preservatives had gained increasing attention as they prevent food from deterioration through oxidation and reduce the loss of nutrition [5]. Antioxidants are compounds that inhibit oxidation end and also known as ‘free radical scavengers’ [6]. Antioxidant activity can be measured by different types of mechanisms which included hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, metal chelation and Reactive Oxygen Species (ROS) scavenging assays such as 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay [7]. There were also a few studies that investigated chemical hydrolysis in producing antioxidants from fish scales [8] [9]. Unfortunately, these studies only provided certain information on the temperature, type of treatment and type of solvent [10]. However, there are other important parameters that need to be considered such as the concentration of acid solvent used and reaction time. In this study, the effects of type of solvent used to dissolve the final sample, concentration of HCl, demineralization time and deproteinization time were investigated to obtain the maximum antioxidant from fish waste.

Materials and Method

2.1. Materials

Fish waste was collected from a local supplier of Keropok Lekor Ghani Black, Kuantan. Hydrochloric acid, sodium hydroxide, 2,2-Diphenyl-1-picrylhydrazyl, Folin-Ciocalteu reagent and Sodium Carbonate were purchased from Sigma Aldrich.

2.2. Pre-treatment of fish waste

The fish waste was washed with distilled water and oven dried at 180 °C for 30 minutes. Next, the dried sample was crushed into a smaller surface area or powder form by using blender [9].

2.3. Demineralization

The sample was soaked with hydrochloric acid (HCl) at different concentrations (0.2 M - 1.0 M), reaction time (30min – 150min) at room temperature with constant stirring at 150 rpm. The ratio of solid to liquid was 1:10. Next, the sample was rinsed with distilled water and oven dried at 60°C for 5 hours [10]. The best concentration of hydrochloric acid and time of demineralization were selected by one factor ANOVA method in Design Expert Version 7.1.6.
2.4. Deproteinization
The sample was slowly added into 1.0 M of sodium hydroxide solution. The temperature was maintained at 60 °C with constant stirring within the range of 30 min to 150 min. Next, the sample was washed with distilled water until the pH value became neutral which was pH 7 [9]. The best time of demineralization were selected by one factor ANOVA method in Design Expert Version 7.1.6.

2.5. Analysis of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay
In DPPH assays, 25 μl of sample was mixed with 975 μl of DPPH methanol solution and incubated for 4 hours in the dark at room temperature. The reduction of DPPH radical was measured at 517 nm using UV-vis spectrophotometer. The control was conducted in the same way but distilled water was used to replace the sample [9]. This procedure was carried out in triplicates. Equation (1) shows the formula of antioxidant activity [11].

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\text{Antioxidant activity, } \% = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100\% \quad \text{Equation (1)}
\]

2.6. Total phenolic content (TPC) assay
The sample was analyzed by total phenolic content (TPC) assays. 10 μL of sample was mixed with 50 μL of 10% v/v of Folin-Ciocalteu reagent, 150 μL of 30% w/v of sodium carbonate and 790 μL of distilled water. After 1 hour of incubation in the dark at room temperature, the absorbance value of sample was read at 765 nm using UV-vis spectrophotometer [9]. This assay were carried out in triplicates. Standard calibration curve of TPC assay was carried out and Gallic acid was used as standard. The results were quantified in term of Gallic Acid Equivalent (GAE), g/L.

3. Result and Discussion
3.1. Effect of different types of solvent used in DPPH and TPC assays
Ethanol, methanol, acetic acid, distilled water and toluene was used to determine the best solvent used in DPPH and TPC assays. Solvent is used to dissolve the sample after demineralization and deproteinization for the DPPH and TPC assays. Figure 1 showed the effect of different types of solvent on percentage of antioxidant activity by DPPH assays. Based on the figure, ethanol showed the highest percentage of antioxidant which was 44.10%. Meanwhile, toluene showed the lowest value of percentage of antioxidant activity which was 29.52%. For methanol, acetic acid and distilled water, different percentage of antioxidant activity were obtained which were 40.02%, 34.94% and 33.92% respectively.
Figure 1. Effect of different types of solvent on percentage of antioxidant activity by DPPH assays, distilled water as control.

Ethanol is known as a good solvent for antioxidant extraction and is safe for human consumption. Sultana and Anwar [11] reported that ethanol had been extensively used to extract antioxidant compounds from various materials such as plant, plant-based foods and marine because of its ability to extract the antioxidant from the sample or materials. The extract yields and resulting antioxidant activities of the materials were strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent [13]. Polar solvents were frequently used for recovering the antioxidant from plant matrices, marine waste and etc [13]. Therefore, in this work, ethanol which is a polar solvent succeeded in enhancing the production of antioxidant from fish waste. However, toluene which is a non-polar solvent showed the least percentage of antioxidant activity in DPPH assay. This is because it is unable to recover the antioxidant from the sample. Thus, in the present study, ethanol was selected as the solvent to dissolve the sample and extract the maximum antioxidant by using DPPH assays.

Figure 2 showed the effect of different types of solvent on GAE, g/L by TPC assays. Based on the result, ethanol had the highest GAE which was 0.032 g/L but toluene had the lowest GAE which was 0.010 g/L. Meanwhile, methanol, acetic acid and distilled water obtained 0.024 g/L, 0.020 g/L and 0.017 g/L GAE.
Similar finding was reported by Do and Angkawijaya [12]. They reported that ethanol was frequently used for recovering polyphenols from a variety of materials such as plant, marine waste, and food sources. Moreover, they also reported that ethanol was used to obtain the maximum total phenolic content from various methods of extraction such as Soxhlet extraction, subcritical water extraction, maceration and etc. Other than that, Sultana et al. [11] demonstrated the efficacy of ethanol to extract the phenolic compound from onion and citrus peel for which the ethanol had the highest efficiency compared with methanol and acetone. As shown in Figure 1 and Figure 2, high antioxidant activity from DPPH assay has a positive relationship with TPC activity, where high TPC gives a high antioxidant capacity due to the linear correlation between DPPH and TPC assay [14]. Therefore, in the present study, ethanol was selected as the best solvent to dissolve the sample and extract the phenolic compound from the sample.

3.2. Effect of different concentrations of Hydrochloric acid (HCl) on antioxidant activity

Figure 3 showed the effect of different concentrations of HCl from 0.2 M to 1.0 M on the percentage of antioxidant activity using DPPH assays. The maximum antioxidant activity was found at 0.8 M of HCl which was 23.00%. Meanwhile, the minimum value of antioxidant activity was 20.81% at 0.2 M of HCl. The best concentration of HCl using DPPH assay obtained from Design Expert by ANOVA was 0.72 M.

The findings of Tokatli et al. [13] showed similar trend with this study but shrimp waste was used as the substrate in the antioxidant hydrolysis process. Tokatli et al. [13] reported that the demineralization step removed the mineral content in the sample such as calcium carbonate resulted in the improvement on the purity of antioxidant which was shown in equation (2).

$$\text{CaCO}_3 + 2\text{HCl} \rightarrow \text{CO}_2 + \text{CaCl}_2 + \text{H}_2\text{O}$$

Equation (2)
Figure 3. Effect of concentration of HCl on percentage of antioxidant activity by DPPH assays. Red dot denotes the design point, dotted line denotes the range of minimum and maximum amount and the black line denotes the trend of graph.

Avelelas and Horta [14] also reported that chemical treatment especially high concentration of acid hydrolysis resulted in changes to the physiochemical properties of antioxidant. This includes the hydrolysis of antioxidant chain that reduced the average molecular weight of the biopolymer, consequently producing harmful effluent wastewater and contributing to the cost of the antioxidant purification process. They also reported that antioxidant with low impurity tolerance and mineral free was usually used in biomedical and nutrition. Therefore, several concentrations of HCl were tested to minimize the chain damage [16]. In this work, HCl was selected in demineralization because it has higher potential for complete calcium carbonate removal from fish waste compared to other acids such as sulphuric acid, nitric acid and acetic acid [17]. Here, the best concentration of HCl obtained by ANOVA was 0.72 M. At a concentration above this value, the antioxidant activity diminished due to the unfavourable operating condition.

Figure 4 showed the effect of concentrations of HCl from 0.2 M to 1.0 M on GAE, g/L by TPC assays. Based on the figure, the GAE was increased from 0.0026 g/L at 0.2 M of HCl to 0.0040 g/L at 0.8 M of HCl but decreased to 0.0036 g/L at 1.0 M of HCl. The maximum and minimum GAE were 0.0040 g/L and 0.0026 g/L at 0.8 M and 0.2 M of HCl respectively. The best concentration of HCl with TPC assay obtained from ANOVA, Design Expert was 0.72 M.
Tokatlı et al. [13] stated that the higher value of the GAE indicated the higher amount of the antioxidant obtained. As the concentration of HCl increased, more reactions occurred and more mineral content was removed which is as shown in equation (2) [10]. However, elevated concentration of HCl affected the availability of antioxidants as well, therefore optimum concentration of acid is essential [10]. Moreover, the excess of concentration of HCl used might lead to the reduction of cost effectiveness during antioxidant extraction [18]. Therefore, the best concentration of hydrochloric acid is needed in order to remove the mineral content and to obtain the antioxidant. In the present study, 0.72 M was obtained by ANOVA which resulted in better hydrolysis that led to higher antioxidant activity.

### 3.3 Effect of demineralization time on antioxidant activity

Figure 5 showed the effect of demineralization time from 30 to 150 min on the percentage of antioxidant activity by DPPH assays. The highest percentage of antioxidant activity of 28.70% was achieved when demineralization process was carried out at 90 min. Conversely, the lowest percentage of antioxidant activity of 25.13% was achieved at 30 min. Thus, the best demineralization time selected by ANOVA, Design Expert was 91.8 min.
Figure 5: Effect of demineralization time on percentage of antioxidant activity by using DPPH assays. Red dot denotes the design point, dotted line denotes the range of minimum and maximum amount and the black line denotes the trend of graph.

The results of this study showed a close resemblance with the results of Percot et al. [10] who investigated on the demineralization time for antioxidant activity within the range of 30 min – 1440 min, and 95.8 min was found to be the best in their case. However, in this study, smaller range of demineralization time was studied which was 30 min to 150 min. Percot, Christophe [10] also reported that the molecular weight of antioxidant decreased with the increased time of hydrolysis. Antioxidant with a low molecular weight favoured the scavenging activity and antioxidant activity than that of high molecular weight of antioxidant [19]. However, the antioxidant would be degraded if the process were carried out at a certain lengthened time [10]. Therefore, several demineralization times were carried out to obtain the maximum antioxidant activity. In the present study, the best demineralization time obtained by ANOVA was at 91.8 min. Above this value, the antioxidant significantly reduced, might be due to degradation [10].

Figure 6 showed the effect of demineralization time from 30 to 150 min on the GAE, g/L by using TPC assays. Based on the figure, it clearly showed a symmetrical bell shape with a peak at 0.0276 g/L at 90 min while the lowest GAE was 0.0141 g/L at 30 min. There was an obvious change from 0.0141 g/L at 30 min to 0.0225 g/L at 60 min and continuously increased to 0.0276 g/L at 90 min but decreased to 0.0225 g/L at 120 min and further decreased to 0.154 g/L at 150 min. Thus, the best demineralization time selected by ANOVA, Design Expert was 91.8 min.

According to Percot et al. [10], they reported that the demineralization times reported in the literature were too long and antioxidant would be degraded if the process was carried out at extended time. Therefore, Hajji and Younes [18] suggested that optimum demineralization time was needed in order to remove the mineral content effectively. Besides, Fadhi et al. [19] also reported that reaction
time was one of the factors to reduce the calcium concentration because reaction time would affect the ability of HCl to diffuse into the antioxidant. Thus, in the present study, the optimum time of demineralization obtained by ANOVA, Design Expert was 91.8 min or 1 hour and 39 min.

![Graph](image)

Figure 6: Effect of time of demineralization on GAE, g/L by using TPC assays. Red dot denotes the design point, dotted line denotes the range of minimum and maximum amount and the black line denotes the trend of graph.

3.4. Effect of deproteinization time on antioxidant activity

Figure 7 showed the effect of deproteinization time from 30 to 150 min on the percentage of antioxidant activity by DPPH assays. Based on the figure, 27.14% of antioxidant activity was obtained at 90 min. Meanwhile, the lowest value of antioxidant activity was obtained at 150 min which were 19.80%. Thus, the best deproteinization time selected by ANOVA, Design Expert was 70.1 min.

The result of a recent study by Tokatli et al. [13] who used shrimp waste as raw material was in contrast with the result of this work. The deproteinization time in that study ranged from 30 min to 1440 min [13], while in this study, the time ranged from 30 min to 150 min. According to Bellali et al. [20], deproteinization of the sample was significantly influenced by the treatment time. They demonstrated that optimum deproteinization was achieved at relatively short process time. This was because extremely long treatment time might result in the loss of yield and reduced antioxidant quality through hydrolysis of the antioxidant [22]. This concurred with the results of this study where the antioxidant activity increased up to 90 min but at extended time, the antioxidant activity significantly dropped. Thus, in the present study, the optimum time of deproteinization obtained by ANOVA was 70.1 min.
Figure 7. Effect of deproteinization time on the percentages of antioxidant activity by using DPPH assays. Red dot denotes the design point, dotted line denotes the range of minimum and maximum amount and the black line denotes the trend of graph.

Figure 8 showed the effect of deproteinization time from 30 to 150 min on the GAE, g/L by TPC assays. Based on the figure, it showed a bell shape with a peak of 0.0682 g/L of GAE at 90 min. The lowest GAE value was obtained at 150 min which was 0.0110 g/L.

Figure 8: Effect of deproteinization time on GAE, g/L by using TPC assays. Red dot denotes the design point, dotted line denotes the range of minimum and maximum amount and the black line denotes the trend of graph.
The TPC value of these results correlates linearly with the radical scavenging or antioxidant activity as can be seen in Figure 7 and Figure 8. The higher the TPC value, the higher the ability to extract the phenolic compound out from the sample [23]. Pereira and Valentão [24] reported that phenolic compound was also considered one of the antioxidants because they are good hydrogen donors. Hydrogen–donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of generation of new radicals [24]. The higher the GAE indicated more antioxidant obtained [15] as proven in this study. Bellali et al. [20] also stated that the higher the stability of temperature would increase the rate of hydrolysis and resulted in a shorter time of deproteinization. However, Vázquez and Rodríguez-Amado [23] declared that the deproteinization process using 1.0 M of NaOH with a temperature and a reaction time below 70 ℃ and 24 hours like in this work, had no significant influence on the antioxidant activity.

4. Conclusion

This study discovered that the best solvent used for DPPH and TPC assays was ethanol, since it had the highest potential to exchange the electron with the sample and extract more antioxidant (44% antioxidant activity, 0.032 g/L GAE) from the fish waste. As for the operating parameters, the best demineralization and deproteinization conditions obtained by one factor ANOVA were 0.72 M at 92 min at room temperature and 1.0 M NaOH at 70 min at 60 ℃.

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