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Screening of significant factors in collagen extraction from hybrid *Clarias* sp. using a statistical tool

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Abstract

Extraction of collagen from muscles of cultured catfish (*Clarias gariepinus* × *C. macrocephalus*) with the aid of pepsin digestion was investigated using a statistical tool. Fractional factorial design (FFD) was applied to evaluate the effects of eight process parameters: acetic acid concentration, acid extraction time, acid extraction temperature, acetic acid to muscles ratio, NaOH concentration, NaOH to muscles ratio, NaOH treatment time, and stirring speed. Contribution of every parameter in influencing the extraction efficiency was evaluated and factors that significantly affected the extraction were elucidated by employing experimental design and analysis of variance in FFD. The result of first order factorial design showed that acetic acid concentration, acid extraction time, acid to muscles ratio, and stirring speed had significant effect ($P < 0.05$) to the yield of pepsin soluble collagen (PSC) obtained at the end of the experiment. Effects of these process factors on the efficiency of collagen extraction were investigated, and are discussed in detail. Optimum conditions were found at 0.5 M acetic acid, 16 hr extraction period, solvent to muscles ratio at 25 ml/g, and stirring speed of 400 rpm, resulting in yield of PSC as high as 211.49 ± 15.51 mg/g.

Introduction

Cultured fish is an important protein source in Malaysia, especially in inland areas where marine fishes are not easily available and affordable (Yaakob and Ahyaudin, 1994). The main freshwater species cultured are Nile tilapia (*Oreochromis niloticus*), accounts for 44.7% of the total freshwater aquaculture production, followed by catfish (36.7%) and carps (10.08%) (FAO, 2012). The annual national production of cultured catfish in general, has steadily increased even though it is not the highest commodity of the total freshwater aquaculture production compared to tilapia. According to Kamarudin *et al.* (2011), the world catfish aquaculture production exhibited tremendous increase from 541, 883 tons in 1998 to 2.78 million tons (worth USD 3.92 billion) in 2008, resulted in approximately 6 folds of increment over the last decade. In Malaysia, the cultured catfish production showed significant improvement by 7 folds from 7, 158 tons in 1999 to 81,041 tons in 2009 (Anon, 2011). However, a key factor restricting the promotion of aquaculture practices, particularly for catfish aquaculture in Malaysia, is the poor economic return from investments. Advanced aquaculture techniques such as intensive pond and cage farming are well developed, but expanded investment is not preferable due to small profit margin.

Locally known as *Keli* in Malaysia, cultured hybrid catfish of *Clarias* sp. (*Clarias gariepinus* × *C. macrocephalus*) is one of the most popular freshwater fishes accepted by consumers contributing by its abundances and cheaper price as compared to other cultured fishes. Catfish is a good source of protein with a considerable amount of collagen exists in the muscles and skins (Sivakumar *et al.*, 2000). Demand for *Keli* in Malaysia is only meant for daily consumption so far, resulting in lower commercial value in contrast to deep sea species. Hence, it is as an interesting attempt to increase the commercial value of the cultured catfish by extracting collagen from the muscle (flesh), converting these sources into raw material for other applications, not restricted only to food industry. Development of these natural resources into value-added product such as collagen to yield additional income is expected to offer economic benefits to both the fisheries industry and local fishermen in Malaysia.

Collagen is gradually emerging as a popular biomaterial in cosmetic, biomedical and pharmaceutical industries following its unique characteristics such as high tensile strength, low antigenicity, bioresorbability, and good compability (Cliché *et al.*, 2003; Aukkanit and

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Garnjanagoonchorn, 2010). It is the major structural component of vertebrates and is the most abundant mammalian protein, representing almost 25 – 30% of total proteins in animal body (Wang *et al*., 2009). Collagen is recognized by three polypeptide α-chains, forming a triple helix structure which is able to form insoluble fibres with high tensile strength (Singh *et al*., 2011). Each polypeptide chain is characterized by the repeating structure of triplet (Gly-X-Y)n, where glycine residue is the structural prerequisite for the triple helix (Wang *et al*., 2009) while X and Y are often proline (Pro) and hydroxyproline (Hpy), respectively (Senaratne *et al*., 2006; Palpandi *et al.*, 2010; Singh *et al*., 2011). Collagen employed in commercial products is mainly derived from cows and pigs, but outbreak of mammalian diseases such as bovine spongiform encephalopathy (BSE) and foot/mouth diseases created anxieties among consumers on the risk of transferring the diseases to humans (Woo *et al.*, 2008). Therefore, development of aquatic-derived collagen has been intensified recently in an effort of searching replacement for the mammalian sources.

Catfish is a potential source of raw material due to its high availability, no risk of disease transmission as well as no religious barriers as in the case of Muslims who cannot consume porcine-derived collagen (Senaratne *et al*., 2006; Singh *et al.*, 2011). In fact, extraction of collagen from catfish aids in boosting up the commercial value of these cheaply abundant natural resources besides providing a new approach in finding alternative sources of safe collagen for industrial uses. There are multiple process variables that are possible in significantly affecting the yield of collagen in the extraction process, starting from the pretreatment step until the stage of dilute acid extraction. Due to the large number of factors studied, Fractional Factorial Design (FFD) was used in this study to screen the significant variables involving in the collagen extraction process from *Clarias* sp. Insignificant variables were eliminated in order to obtain a smaller and more manageable set of process variables. Following that, the objective of this work is also to investigate the effects of the significant variables on the yield of collagen. One-factor-at-a-time (OFAT) strategy was applied to study the yields' profiles and finding the proper working ranges of the variables which significantly influencing the extraction efficiency. This study would be beneficial in optimizing the extraction process variables for the highest (optimum) yield of collagen from *Clarias* sp.

Materials and Methods

Materials

Cultured catfish (hybrid of *C. gariepinus* x *C. macrocephalus*) were purchased from a local wet market in Parit Buntar, Perak, Malaysia. Upon arrival at the laboratory, the fishes were killed, dissected, deboned and the muscles were cleaned of adhering tissues before being cut into small pieces. Skin was manually removed by using a sharp knife. The muscles were then washed with distilled water and kept frozen at -20°C prior to collagen extraction.

Chemicals

Commercial pepsin from porcine gastric mucosa, sodium hydroxide, and acetic acid were purchased from Merck Sdn. Bhd. (Malaysia). All other chemicals used were of analytical grade.

Isolation of muscle Type I collagen

Extraction of pepsin soluble collagen (PSC)

All procedures were performed as previously described by Kimura *et al.* (1988) and Wang *et al.* (2009) with slight modifications. The extraction processes were carried out at 4°C. To remove non-collagenous proteins, the muscles were mixed with 10 volumes (v/w) of 0.1 M NaOH and stirred for 5 to 6 hr. The sample was then washed thoroughly with excessive distilled water until the pH was neutral or slightly basic. The residue was filtered using cheese cloth and actively stirred in 5 volumes (v/w) of 0.5 M acetic acid containing pepsin with an enzyme/substrate ratio of 1:40 for 20 hr to extract acid soluble collagen. The concentration of collagen extracted was measured using Lowry's modified method as reported by Komsa-Penkova *et al.* (1996). After centrifugation at 3,840 × g for 15 mins, soluble collagen solution was obtained from the supernatant. The collagen was precipitated by adding NaCl to a final concentration of 2.0 M in the presence of 0.05 M Tris-HCl buffer (pH 7.2). Resulting sediment was collected by centrifugating at 3,840 × g for 20 mins. The purified collagen was redissolved in minimal amount of 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, followed by distilled water and lyophilized. The freeze-dried product was designated as pepsin soluble collagen (PSC).

Collagen yield measurement

The yield of pepsin soluble collagen from muscle of *Clarias* sp. was calculated using Eq. (1) by as proposed by Li *et al.* (2009):

Table 1. Process Variables and levels for the fractional factorial design (FFD)

Process Variable	Code Value	
	-1	1
X_1 : Acetic Acid Concentration (M)	0.1	0.9
X_2 : Acid Extraction Time (hr)	4	8
X_3 : Acid Extraction Temperature (ºC)	5	15
X_4 : Acetic Acid to Muscle Ratio (ml/g)	5	20
X_5 : NaOH concentration (M)	0.1	0.9
X_6 : NaOH to Muscle Ratio (ml/g)	5	10
X_7 : NaOH Treatment Time (hr)	2	6
X_8 : Extraction Stirring Speed (rpm)	100	250

$$Y = \frac{V \times C}{W} \quad (1)$$

where Y is the yield of collagen in mg/g, V is the volume of extracted collagen solution in ml, C is the concentration of the same solution measured using spectrophotometer in mg/ml, and W is the wet weight of catfish muscle in g.

Experimental design and statistical analysis

Fractional factorial design (FFD)

Fractional Factorial Design is a popular experimental design method for two-level and is one of the most frequently applied fractional designs in engineering (Zhu *et al*., 2007). It made possible to consider multitudinous factors and identify the most important or relevant factors from a long list (Zhu *et al*., 2010). Screening of process variables aims at reducing determinations as to which few variables result in the greatest impacts on collagen extraction efficiency. In this study, 8 independent factors (Table 1) were tested at both high (+1) and low (-1) levels. Yield of extracted pepsin soluble collagen (PSC) expressed in milli-gram of collagen per gram of muscle (mg/g) was listed as the response variable. In order to evaluate the effect of the process variables, 16 experiments were performed in random order to cover all combinations of the factor levels in the experimental design. In addition, four center-point experiments were also conducted to investigate the curvature of the results and to indentify the reproducibility of the experiments. This was a proposed 2^{8-4} factional factorial design with a resolution of four. The experimental design protocol was contrived with the aid of the software Design Expert (Version 6.0.6, Stat-Ease Inc., Minneapolis, Minnesota USA). All experiments were done in triplicate and data presented were mean values of the triplicates. Data analyses were also performed using Design Expert for the selection of most influential factor(s) among the proposed ones.

Table 2. FFD with corresponding response (using coded variables)

X_1 (M)	X_2 (hr)	X_3 (ºC)	X_4 (mg/l)	X_5 (M)	X_6 (mg/l)	X_7 (hr)	X_8 (rpm)	Yield of Collagen (mg/g)
-1	-1	1	1	-1	-1	1	1	33.77±1.10
1	-1	-1	1	1	-1	1	-1	36.94±1.21
1	1	1	-1	-1	-1	1	-1	41.73±1.36
1	-1	1	1	-1	1	-1	-1	36.94±1.06
-1	1	1	-1	-1	1	-1	1	32.67±1.07
0	0	0	0	0	0	0	0	69.42±1.34
-1	-1	1	-1	1	1	1	-1	19.87±0.65
1	1	1	1	1	1	1	1	65.56±2.15
0	0	0	0	0	0	0	0	66.19±1.65
-1	1	-1	1	-1	1	1	-1	41.87±1.37
1	1	-1	1	-1	-1	-1	1	69.74±1.55
1	-1	-1	-1	-1	1	1	1	51.14±1.67
-1	-1	-1	-1	-1	-1	-1	-1	18.15±0.43
1	-1	1	-1	1	-1	-1	1	25.73±0.84
-1	1	-1	-1	1	-1	1	1	32.53±1.02
1	1	-1	-1	1	1	-1	-1	37.28±1.22
0	0	0	0	0	0	0	0	64.59±1.11
0	0	0	0	0	0	0	0	65.10±2.13
-1	1	1	1	1	-1	-1	-1	30.29±0.99
-1	-1	-1	1	1	1	-1	1	39.64±1.15

One-Factor-At-A-Time (OFAT) approach

One-Factor-At-A-Time approach is a conventional method for many industrial processes in optimizing process variables (Nei *et al*., 2009). It sequentially tunes each process parameter individually while holding all others fixed, assuming the various treatment parameters do not interact and that the response variable is a function of only the single varied parameter (Enda and Daniel, 2007). In this study, it is useful in identifying the proper working ranges of the chosen process variables from the results of FFD. Effects in variation of each significant variable towards the extraction efficiency and the influences reflected on the pattern of yields' profiles were also studied and investigated.

Results and Discussion

Screening of process variables by fractional factorial design (FFD)

The assumption in using a FFD is that higher order interactions are likely to be of little consequence, thus their corresponding aliasing can be safely ignored. Resolution is termed as the order at which such aliasing appears in a FFD (Enda and Daniel, 2007). In this study, Resolution IV was chosen where the main effects could be determined but two-factor interactions were aliased with one another. In order to elucidate factors inclusive of acetic acid concentration (X_1), acid extraction time (X_2), acid extraction temperature (X_3), acetic acid to muscle ratio (X_4), NaOH concentration (X_5), NaOH to muscle ratio (X_6), NaOH treatment time (X_7), and extraction stirring speed (X_8), that significantly affecting the yield of pepsin soluble collagen (PSC) from muscles of *Clarias* sp., FFD was arranged with such factors at different levels (Table 1). The corresponding results of the experiments are presented in Table 2.

Table 3. Analysis of variance of FFD

Source	*df*	Sum of squares	Mean square	*F* value	*Prob* > *F*
Model	8	2757.71	344.71	14.59	0.0001
Error	11	236.32	23.63		
C total	19	2994.03			

R^2=0.92; Dependent mean=44.09; Adj. R^2=0.86; *CV*=11.03; C total: Corrected total

Table 4. Regressive analysis of FFD

Variable	Coefficient Estimate	Standard error	*F* value	*Prob* > *F*	Significance
Intercept	38.53	1.22	14.59	0.0001	Highly significant
X_1	7.44	1.22	37.43	0.0001	Highly significant
X_2	5.42	1.22	19.92	0.0012	Significant
X_3	-2.38	1.22	3.83	0.0790	Non-significant
X_4	6.15	1.22	25.58	0.0005	Highly significant
X_5	-2.55	1.22	4.42	0.0619	Non-significant
X_6	2.42	1.22	3.98	0.0740	Non-significant
X_7	1.89	1.22	2.42	0.1506	Non-significant
X_8	5.31	1.22	19.11	0.0014	Significant

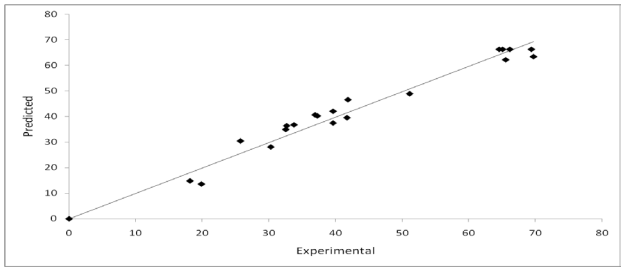

Figure 1. Comparison of experimental results and values calculated by the statistical model for extraction of PSC

Analysis of variance (ANOVA) was performed on the main effects and the results are summarized in Table 3. Results of the *F* value and the probabilities of $P_r > F$ are also shown. ANOVA for this experiment ($F = 14.59 > F_{(8,19,0.01)} = 6.75$) indicates that the variables significantly affect the yield of PSC extracted from muscles of *Clarias* sp. In addition, regressive analysis of the variables shown in Table 4 reveals that X_1 ($P_r > F = 0.01\%$), X_2 ($P_r > F = 0.12\%$), X_4 ($P_r > F = 0.05\%$), and X_8 ($P_r > F = 0.14\%$) influenced the extraction efficiency significantly, where as effects of other remaining variables with $P_r > F > 5.00\%$ could be neglected. Moreover, the deduced first-order multiple regressions as shown in Eq. (2):

$$Y = 38.53 + 7.44X_1 + 5.42X_2 - 2.38X_3 + 6.15X_4 - 2.55X_5 + 2.42X_6 + 1.89X_7 + 5.13X_8 \quad (2)$$

including all the above mentioned variables results in the linear correlation coefficient (R^2) values at 0.92 and Adj. R^2 at 0.86. According to the Annuar *et al*. (2008), the R^2 value is frequently used to judge whether the model correctly represents the data, implying that, if R^2 is close to one, then the regression model is correct This indicates that the predicted data are well fitted by the model which variations caused by the variables accounted for 92% of the variation in the extraction of PSC in this study. The experimental results and the model values of Eq. (2) are compared in Figure 1.

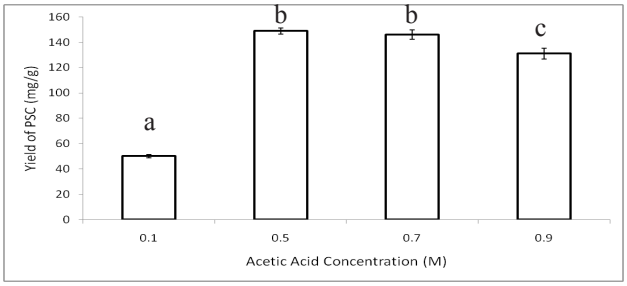

Figure 2. Effect of different concentration of acetic acid on the yield of pepsin soluble collagen (PSC) from muscles of *Clarias* sp. Condition: The extraction time, acid to muscles ratio and stirring speed were set at 24 hr, 10 ml/g, and 400 rpm, respectively. The column containing the same letter was not significantly different (P > 0.05).

Based on the results in Table 4, it can be concluded that the amount of extracted PSC in the extraction process is greatly influenced by acetic acid concentration, acid extraction time, acetic acid to muscles ratio, and the extraction stirring speed. They are the significant parameters which affecting the yield of collagen extracted in this study. In other words, parameters involving in the stage of dilute acid extraction are found to be predominant over those affecting the alkaline pre-treatment step. This finding is consistent with the work of Wang *et al*. (2009) which acetic acid concentration, time, and solvent to material ratio were identified as factors that imposed effects on the extraction efficiency of acid soluble collagen (ASC) from grass carp skin. They reported that each of these factors showed a significant role in the process.

Effect of acetic acid concentration

Acid extraction with pepsin digestion is a common method for collagen extraction nowadays. With great extractability towards collagen, acetic acid has been frequently used as a solvent for collagen extraction (Cheng *et al*., 2009). This was supported by the findings of Skierka and Sadowska (2007) and Cheng *et al.* (2009) who stated that the extraction of collagen from animal tissues through inorganic acid (e.g. hydrochloric acid) resulted in lower efficiency and yield than the organic acids. In fact, a number of collagen extraction studies from marine and land animals with acetic acid as the extracting medium had also been reported in the literature (Senaratne *et al.*, 2006; Nalinanon *et al*., 2007; Li *et al*., 2009; Aukkanit and Garnjanagoonchorn, 2010). Figure 2 shows the effect of different concentration of acetic acid on the yield of PSC from muscles of *Clarias* sp. Yield of PSC increased with the increase of acetic

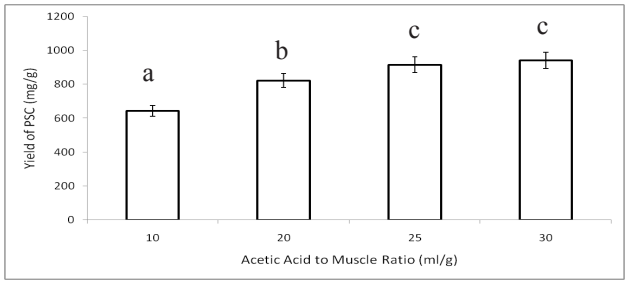

Figure 3. Effect of acetic acid to muscle ratio on the yield of pepsin soluble collagen (PSC) from muscles of *Clarias* sp. Condition: The extraction time, concentration of acetic acid and stirring speed were set at 24 hr, 0.5 M, and 400 rpm, respectively. The column containing the same letter was not significantly different ($P > 0.05$).

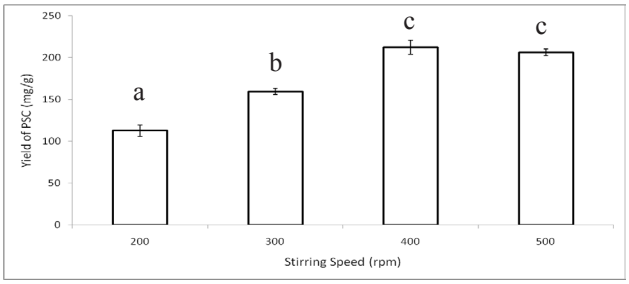

Figure 4. Effect of stirring speed on the yield of pepsin soluble collagen (PSC) from muscles of *Clarias* sp. Condition: The extraction time, concentration of acetic acid and acid to muscles ratio were set at 24 hr, 0.5 M, and 25 ml/g, respectively. The column containing the same letter was not significantly different ($P > 0.05$).

acid concentration to 0.5 M. However, a reverse trend was observed beyond this concentration of acetic acid. The highest yield was achieved when 0.5 M acetic acid was used as the extracting medium and PSC as much as 149.01 ± 6.84 mg/g was extracted. For higher concentration of acetic acid particularly at 0.9 M, the yield of PSC was found to be 130.92 ± 10.61 mg/g, which was significantly ($P < 0.05$) lower than that of 0.5 M.

Difference in the yield obtained through different concentration of acetic acid employed was probably due to different solubility of collagen in the acidic extracting medium. According to a few reports, pH value of the extraction bulk was completely dependent on the concentration of acid used. Hence, modification of the electrostatic interaction and structure of proteins might occur along the changes in acid concentration since pH value was in charge of the charge density of protein (Verheul *et al.*, 1998). In fact, Wang *et al.* (2009) recently stipulated that more positively charged amine groups of collagen were resulted at the pH when concentration of acetic acid used was at 0.5 M, leading to the highest yield among the studied concentrations. Denaturation of collagen at extremely low pH value however was also another possibility that lower yield was observed when acetic acid with concentration more than 0.5 M was utilized.

Effect of acetic acid to muscles ratio

Solvent to material ratio is an important variable affecting the efficiency of extraction. In this study, the effect of the amount of solvent (acetic acid) to *Clarias* sp. muscles ratio on the extractability is shown in Figure 3. An increasing acetic acid to muscle ratio could lead to a higher yield of PSC. The ratio was varied from 10 – 30 ml/g, but when it was raised to more than 25 ml/g, improvement in the yield of PSC was no longer significant. Higher solvent to material ratio increases the concentration gradient and diffusion rate of collagen particles from the fish muscles into acetic acid, thus enhancing the efficiency of extraction process (Wang *et al*., 2009). Nevertheless using a large amount of solvent is not cost-effective due to higher operating cost of solvent and waste handling at the end of the extraction process. Consequently, the ratio of acetic acid to the muscles ratio of 25 ml/g was appropriate for PSC extraction carried out in this study.

Effect of stirring speed

Mass transfer is another common phenomenon in any extraction processes, especially in the diffusion-controlled extraction. Stirring speed affects equilibrium time and the amount of analyte (in this study it refers to collagen) extracted in the extracting medium. Since mass transfer is limited by diffusion, in other words, the more efficient the stirring is, the better the mixing between solvent and raw material, the shorter the equilibrium time and the higher the amount of analyte would be extracted in pre-equilibrium conditions (Sanja *et al*., 2010). However, information on the variation of stirring speed in collagen extraction studies is still scarce in literature. Though collagen extraction was conventionally suggested to be carried out under vigorous stirring (Nagai, 2004), too high stirring speed could possibly lead to generation of excessive heat to the extraction process. Collagen is a thermal sensitive compound which is likely to be denatured easily under this circumstance (Wang *et al*., 2008). Excessive heat resulted from stirring could break the hydrogen bonds and Van der Waals interactions in the polypeptide chains, promoting the denaturation of collagen/enzyme, and consequently resulting in lower efficiency of the extraction process. In this study, 400 rpm was found to be the most appropriate stirring speed in obtaining the highest PSC yield at 212.45 ± 10.51 mg/g under the employed extraction conditions (Figure 4). A positive relationship was

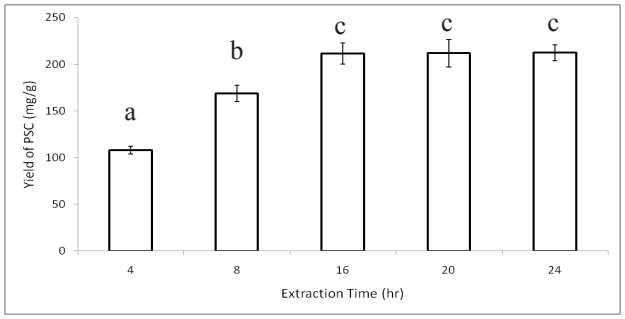

Figure 5. Effect of extraction time on the yield of pepsin soluble collagen (PSC) from muscles of *Clarias* sp. Condition: The concentration of acetic acid, acid to muscles ratio, and stirring speed were set at 0.5 M, 25 ml/g, and 400 rpm respectively. The column containing the same letter was not significantly different ($P > 0.05$).

found with the increase of stirring speed on the yield of PSC. The amount of PSC extracted increased significantly ($P < 0.05$) with the elevation of stirring speed from 200 rpm to 400 rpm. This could be due to the mass transfer between the fish muscles and acetic acid which was greatly enhanced with increasing stirring speed. Greater solubility of muscles in acetic acid was achieved, resulting in greater driving force for collagen particles to diffuse from the fish muscles into the medium. Further increase of the speed after 400 rpm however did not result in significant improvement of the yield.

Effect of extraction time

The effect of extraction time on the yield of collagen extracted is also another notable factor that needs to be properly investigated. A similar trend was observed, in comparison to that of stirring speed where a positive relationship was found between the extraction time and yield of PSC attained. The yield increased with the extension of time especially when the extraction time ranged from 4 - 16 hr (Figure 5). Though the extraction process was prolonged until 24 hr, no further significant improvement in the yield was observed. Similar remark was also reported by Wang *et al.* (2009) that when the time was longer than 24 hr, the yield of collagen extracted from grass carp skin was not improved. In fact, isolation of collagen from Baltic cod skin revealed that the collagen yield extracted for 72 hr was not significantly different from that for 24 hr (Sadowska *et al.*, 2003).

Mass transfer rate of collagen from the muscles matrix plays a key role in the efficiency of extraction in this study. As mentioned earlier, the mass transfer rate was controlled by the diffusion process which was time-concerned. Therefore the recovery of analyte (yield of collagen) would keep increasing along with the extension of time (Wang *et al*., 2009). However, in the presence of pepsin digestion, it worth to note that different time of extraction affected both the yield and properties of collagen obtained. Increasing extraction time was reported to result in loss of integrity of the collagenous materials (Aukkanit and Garnjanagoonchorn, 2010). At a higher temperature and longer time of pepsin digestion, larger amounts of telopeptide of tropocollagen would be digested and resulted in collagen with less fibril forming capacity. The fibril forming capacity is an important index of collagen molecular integrity and the denaturation of collagen caused a reduction in fibril-forming capacity (Lin and Liu, 2006). Higher extraction temperature and longer extraction time would lead to severe and serious pepsin digestion which caused the possibility of collagen to lose all their fibril forming capacity. Therefore, deciding on an appropriate extraction period is a crucial part in all collagen extraction processes in future.

Conclusion

Using a fractional factorial design and analysis of variance, the effective main parameters which significantly influenced the extraction efficiency of pepsin soluble collagen from muscles of hybrid catfish (*Clarias* sp.) were obtained by conducting the least number of experimental runs. Out of eight process parameters, only four of them which include acetic acid concentration, acetic acid to muscles ratio, stirring speed, and extraction time showed significant effect towards the extraction yield of collagen in this study. In addition, from the results of OFAT study, working ranges for these significant process variables were attained. Findings in this work were helpful in further optimizing studies of the extraction conditions, particularly to take into consideration of the interactions between the significant process variables and their corresponding effects on the yield of PSC extraction. Subsequently, it is possible to find out the best extraction conditions in order to maximize the yield of extracted collagen, with the minimization of the energy and cost of the process.

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