Laporan Akhir Projek Penyelidikan Jangka Pendek

Collagen Extraction From Freshwater Fish: Kinetics, Mechanism And Characterization Studies

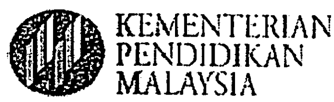
by
Prof. Dr. Mashitah Mat Don
Prof. Azlina Kamaruddin @ Harun
Dr. Mohamad Hekarl Uzir
Dr. Khairiah Abdul Karim
Mr. Kiew Peck Loo

2015

X0068

BORANG ERGS – P3(R)

Code Project : ERGS/1/2012/TK05/USM/02/12



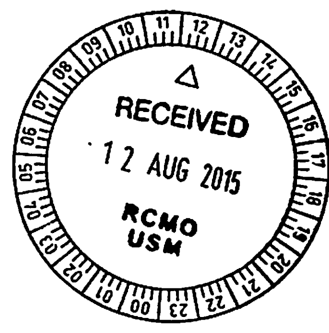
FINAL REPORT
EXPLORATORY RESEARCH GRANT SCHEME (ERGS)

Laporan Akhir Skim Geran Penyelidikan Eksploratori (ERGS) IPT
Pindaan 1/2015

A

RESEARCH TITLE: Collagen Extraction from Freshwater Fish: Kinetics, Mechanisms and Characterization Studies

PHASE & YEAR: 1/2012



START DATE: 1/8/2012
END DATE: 31/7/2014
EXTENSION PERIOD (DATE): RMC LEVEL: 1/8/14 – 31/7/15
KPM LEVEL:

PROJECT LEADER: Prof. Dr Mashitah Mat Don
I/C / PASSPORT NUMBER: 600124085608

PROJECT MEMBERS: (including GRA)
1. Prof. Azlina Kamaruddin @ Harun
2. Dr. Mohamad Hekarl Uzir
3. Dr. Khairiah Abdul Karim
4. Kiew Peck Loo

PROJECT ACHIEVEMENT *(Prestasi Projek)*

B

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	**0 - 50%**	**51 - 75%**	**76 - 100%**
Percentage			100%

RESEARCH OUTPUT		
Number of articles/ manuscripts/ books *(Please attach the First Page of Publication)*	**Refereed Journal**	***Non-Refereed Publication***
	6	0
Conference Proceeding *(Please attach the First Page of Publication)*	**International**	***National***
	2	0
Intellectual Property *(Including Paten,Copyright, Industrial Design,layout Design of Integrated Circuit & Trademarks)*		

HUMAN CAPITAL DEVELOPMENT					
Human Capital	Number				Others (please specify)
	On-going		Graduated		
Citizen	Malaysian	Non Malaysian	Malaysian	Non Malaysian	
No. PHD STUDENT	-	-	1	-	
Student Fullname: IC / Passport No: Student ID:	-	-	Kiew Peck Loo 871023035358 PJD0099	-	
No. MASTER STUDENT	-	-	-	-	
Student Fullname: IC / Passport No: Student ID:	-	-	-	-	
No. UNDERGRADUATE STUDENT	-	-	-	-	
Student Fullname: IC / Passport No: Student ID:	-	-	-	-	
Total	0	0	1	0	

EXPENDITURE *(Perbelanjaan)*

C

Budget Approved *(Peruntukan diluluskan)* : RM 70,000.00
Amount Spent *(Jumlah Perbelanjaan)* : RM 65,450.55

Balance *(Baki)* : RM 4,549.45
Percentage of Amount Spent *(Peratusan Belanja)* : 93.5 %

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS *(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)*

D

International		
Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)		
1) International Conference on Science, Technology & Social Sciences (ICSTSS) 2012	20 – 22 November 2012	Universiti Teknologi Mara (UiTM) Pahang Malaysia
2) International Conference of Environment (ICENV) 2012	11 – 13 December 2012	School of Chemical Engineering, Universiti Sains Malaysia (USM)

National		
Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit) -	-	-

E PROBLEMS / CONSTRAINTS IF ANY *(Masalah/ Kekangan sekiranya ada)*

Non-applicable.

F RECOMMENDATION *(Cadangan Penambahbaikan)*

i. In the present study, it was found that the properties of the collagen extracted from the skin of hybrid *Clarias* sp. resembled the characteristics of the desired mammalian type I collagen, in contrast to the muscle collagen. Optimization study on the extraction of skin collagen is recommended to obtain the highest yield at the most optimum extraction conditions.

ii. For decades, collagen emerged 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. Nevertheless, current trend of researches focused only on finding alternative sources for mammalian derived collagen. In vivo and in vitro assessments on the identified potential applications are recommended to investigate the efficacy of the extracted collagen.

iii. Extraction of collagen from the skin of hybrid Clarias sp. in a larger scale is recommended. Prior to the up-scaling of the extraction process, proper equipment design is a prerequisite to achieve the desired yield. There is also a need to evaluate the economic potential to ensure profitability of the process based on the equipment design.

G RESEARCH ABSTRACT – Not More Than 200 Words *(Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan)*

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the muscle and skin of six different species of Malaysian freshwater fishes, namely a hybrid catfish of *Clarias* sp. (*C. gariepinus* X *C. macrocephalus*), red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasianodon hypophthalmus*), Sultan fish (*Leptobarbus hoevenii*), and labyrinth fish (*Trichogaster trichopterus*). The extracted muscle collagens were designated as M-ASC and M-PSC, whilst skin collagens were termed S-ASC and S-PSC, for the acid and pepsin soluble collagen, respectively. The concentration of extracted collagen was determined using Lowry's method with slight modification. Results showed that such modification was possible for measurement of collagen concentration directly from the extracting medium. The yields of extracted collagens for all selected species were higher for PSC as compared to ASC. The highest muscle collagen yields of 8.54 ± 0.98 % (wet basis) were extracted from the hybrid catfish of *Clarias* sp. Artificial Neural Network (ANN) and Genetic Algorithm (GA) were applied to compare the estimation capabilities between the statistical- and artificial intelligence-based optimization techniques. The ANN/GA approach was superior over the conventional Response Surface Methodology (RSM), with lower average yield error at the range of 0.24 – 3.43 % and higher training R^2 (0.9997 < R^2 < 1), respectively. From the characterization studies, S-

UNIVERSITI SAINS MALAYSIA
UNIT KUMPULAN WANG PENYELIDIKAN/RU
JABATAN BENDAHARI KAMPUS KEJURUTERAAN
PENYATA KUMPULAN WANG
TEMPOH BERAKHIR 6/2015

6/25/2015

Tajuk Projek : COLLAGEN EXTRACTION FROM FRESHWATER FISH: KINETICS, MECHANISM AND CHARACTERIZATION STUDIES
PROF. MADYA MASHITAH MAT DON

Pusat Pengajian : Pusat Pengajian Kejuruteraan Kimia

Penyelidik : MASHITAH MAT DON

Status Projek : AKTIF

No Projek (Agensi) KEJURUTERAAN

Tempoh Projek : 2012 / 8 - 2015 / 7

No Akaun : 203 / 6730068

Vot	Keterangan	Peruntukan Asal	Perbelanjaan Tahun Lalu	Peruntukan Semasa	Tanggungan	Belanja	Jumlah Belanja	Baki	%
11000	Gaji	(8,200.00)	$0.00	$0.00	$0.00	$0.00	$0.00	($8,200.00)	0.00
		($8,200.00)	**$0.00**	**0.00**	**$0.00**	**$0.00**	**$0.00**	**($8,200.00)**	0.00
21000	PERJALANAN DAN SARA HIDUP	7,342.70	$0.00	$0.00	$0.00	$0.00	$0.00	$7,342.70	0.00
23000	PERHUBUNGAN DAN UTILITI	(23.40)	$0.00	$0.00	$0.00	$0.00	$0.00	($23.40)	0.00
24000	SEWAAN	712.36	$0.00	$0.00	$0.00	$341.70	$341.70	$370.66	0.00
25000	BEKALAN MAKANAN	(322.30)	$0.00	$0.00	$0.00	$0.00	$0.00	($322.30)	0.00
27000	BEKALAN DAN ALAT PAKAI HABIS	801.42	$0.00	$0.00	$1,710.00	$5,658.00	$7,368.00	($6,566.58)	0.00
28000	PENYELENGGARAAN DAN PEMBAIKAN KECIL	2,500.00	$0.00	$0.00	$0.00	$0.00	$0.00	$2,500.00	0.00
29000	PERKHIDMATAN IKTISAS DAN HOSPITALITI	16,600.40	$0.00	$0.00	$1,705.00	$5,447.03	$7,152.03	$9,448.37	0.00
		$27,611.18	**$0.00**	**0.00**	**$3,415.00**	**$11,446.73**	**$14,861.73**	**$12,749.45**	0.00
35000	HARTA-HARTA MODAL LAIN	0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	0.00
		$0.00	**$0.00**	**0.00**	**$0.00**	**$0.00**	**$0.00**	**$0.00**	0.00
		$19,411.18	**$0.00**	**$0.00**	**$3,415.00**	**$11,446.73**	**$14,861.73**	**$4,549.45**	0.00

6/26/2015

Nama Projek

Penyelidik

Bil	Kod Dok	No Dok.	Tarikh Dokumen	Kod Akaun	Amaun	Keterangan
VOT 111						
1	WR	3946	11/22/2013	203.111.PJKIMIA.6730068.	$2,100.00	GAJI PROJEK BLN 11/2013
2	WR	3994	12/23/2013	203.111.PJKIMIA.6730068.	$2,100.00	GAJI PROJEK BLN 12/2013
3	WR	4052	2/3/2014	203.111.PJKIMIA.6730068.	$2,000.00	GAJI TAMBAHAN JAN
4	WR	4079	2/24/2014	203.111.PJKIMIA.6730068.	$2,000.00	GAJI BULAN PROJEK
5	WR	4132	3/24/2014	203.111.PJKIMIA.6730068.	$4,000.00	GAJI STAF PROJEK MAC 2014
6	WR	4180	4/23/2014	203.111.PJKIMIA.6730068.	$4,000.00	GAJI PROJEK BULAN 4/2014-1
7	WR	4224	5/22/2014	203.111.PJKIMIA.6730068.	$4,000.00	GAJI PROJEK BULAN 5/2014-1
8	WR	4267	6/23/2014	203.111.PJKIMIA.6730068.	$4,000.00	GAJI PROJEK BULAN 6/2014-1
9	WR	4326	7/21/2014	203.111.PJKIMIA.6730068.	$2,000.00	GAJI PROJEK BULAN 7/2014-1
					$26,200.00	
VOT 221						
10	JR	21288	12/31/2012	203.221.PJKIMIA.6730068.	$371.20	AC000000002092
11	BV	42285	1/14/2013	203.221.PJKIMIA.6730068.	$81.00	T/P 11/2012 - KIEW PECK LOO
12	BV	42285	1/14/2013	203.221.PJKIMIA.6730068.	$95.90	T/P 11/2012 - KIEW PECK LOO
13	BV	42286	1/14/2013	203.221.PJKIMIA.6730068.	$12.60	T/P 12/2012 - KIEW PECK LOO
14	BV	42286	1/14/2013	203.221.PJKIMIA.6730068.	$36.00	T/P 12/2012 - KIEW PECK LOO
15	BV	42286	1/14/2013	203.221.PJKIMIA.6730068.	$63.80	T/P 12/2012 - KIEW PECK LOO
16	BV	42392	1/17/2013	203.221.PJKIMIA.6730068.	$371.20	Bayaran:PHPJKIMIA3000000000077
17	JR	21444	12/31/2012	203.221.PJKIMIA.6730068.	($371.20)	AC000000002092
18	IV-TP	20130100001·	1/29/2013	203.221.PJKIMIA.6730068.	($3.20)	TUNTUTAN BAYARAN PENGINAPAN MELEBIH
					$657.30	
VOT 223						
19	BV	63351	3/12/2014	203.223.PJKIMIA.6730068.	$12.00	Bayaran:PFPJKIMIA3000000002628
20	JR	26229	10/31/2014	203.223.PJKIMIA.6730068.	$11.40	Bayaran:PDPJKIMIA3000000002250
					$23.40	
VOT 224						

Nama Projek

6/26/2015

Penyelidik

Bil	Kod Dok	No Dok.	Tarikh Dokumen	Kod Akaun	Amaun	Keterangan
21	BV	62381	2/21/2014	203.224.PJKIMIA.6730068.	$165.24	Bayaran:PFPJKIMIA300000 0002579
22	BV	70953	8/4/2014	203.224.PJKIMIA.6730068.	$122.40	Bayaran:PFPJKIMIA300000 0002795
23	BV	80063	2/9/2015	203.224.PJKIMIA.6730068.	$125.46	Bayaran:PFPJKIMIA300000 0002991
24	BV	81561	3/19/2015	203.224.PJKIMIA.6730068.	$124.44	Bayaran:PFPJKIMIA300000 0003223
25	BV	81564	3/19/2015	203.224.PJKIMIA.6730068.	$60.18	Bayaran:PFPJKIMIA300000 0003291
26	BV	85613	6/8/2015	203.224.PJKIMIA.6730068.	$31.62	Bayaran:PFPJKIMIA300000 0003485
					$629.34	

VOT	225					
27	BV	39251	11/26/2012	203.225.PJKIMIA.6730068.	$210.00	Bayaran:PFPJKIMIA300000 0001736
28	BV	47710	5/3/2013	203.225.PJKIMIA.6730068.	$112.30	Bayaran:PFPJKIMIA300000 0002015
					$322.30	

VOT	227					
29	BV	36934	10/12/2012	203.227.PJKIMIA.6730068.	$720.09	Bayaran:PSPJKIMIA300000 0002041
30	BV	37605	10/24/2012	203.227.PJKIMIA.6730068.	$332.00	Bayaran:PSPJKIMIA300000 0002042
31	BV	37974	11/2/2012	203.227.PJKIMIA.6730068.	$354.09	Bayaran:PSPJKIMIA300000 0002115
32	BV	39204	11/26/2012	203.227.PJKIMIA.6730068.	$1,507.00	Bayaran:PSPJKIMIA300000 0002117
33	BV	39294	11/2/2012	203.227.PJKIMIA.6730068.	$450.50	Bayaran:PSPJKIMIA300000 0002182
34	BV	51602	7/16/2013	203.227.PJKIMIA.6730068.	$195.00	Bayaran:PSPJKIMIA300000 0003031
35	BV	54670	9/24/2013	203.227.PJKIMIA.6730068.	$455.00	Bayaran:PSPJKIMIA300000 0003226
36	BV	54923	9/27/2013	203.227.PJKIMIA.6730068.	$909.15	Bayaran:PSPJKIMIA300000 0003267
37	JR	23064	9/27/2013	203.227.PJKIMIA.6730068.	$38.62	BAYARAN:PSPJKIMIA3000 000003267
38	JP	8215	10/22/2013	203.227.PJKIMIA.6730068.	$228.31	PEMBATALAN TELEGRAPHIC TRANSFER
39	JP	8215	10/22/2013	203.227.PJKIMIA.6730068.	($947.77)	PEMBATALAN TELEGRAPHIC TRANSFER
40	BV	56628	11/4/2013	203.227.PJKIMIA.6730068.	$909.15	Bayaran:PSPJKIMIA300000 0003314
41	JR	23400	11/4/2013	203.227.PJKIMIA.6730068.	$12.46	BAYARAN:PSPJKIMIA3000 000003314
42	BV	62843	3/3/2014	203.227.PJKIMIA.6730068.	$110.00	Bayaran:PSPJKIMIA300000 0003646

6/26/2015

Nama Projek

Penyelidik

Bil	Kod Dok	No Dok.	Tarikh Dokumen	Kod Akaun	Amaun	Keterangan
43	BV	62844	3/3/2014	203.227.PJKIMIA.6730068.	$985.00	Bayaran:PSPJKIMIA300000 0003674
44	BV	63351	3/12/2014	203.227.PJKIMIA.6730068.	$108.40	Bayaran:PFPJKIMIA300000 0002628
45	BV	64789	4/14/2014	203.227.PJKIMIA.6730068.	$767.56	Bayaran:PSPJKIMIA300000 0003733
46	BV	65339	4/22/2014	203.227.PJKIMIA.6730068.	$419.95	Bayaran:PSPJKIMIA300000 0003737
47	JR	24655	4/28/2014	203.227.PJKIMIA.6730068.	$18.13	BAYARAN:PSPJKIMIA3000 000003812
48	BV	65791	4/4/2014	203.227.PJKIMIA.6730068.	$1,270.04	Bayaran:PSPJKIMIA300000 0003812
49	BV	66953	5/20/2014	203.227.PJKIMIA.6730068.	$481.00	Bayaran:PSPJKIMIA300000 0003920
50	BV	70307	7/18/2014	203.227.PJKIMIA.6730068.	$323.60	Bayaran:PSPJKIMIA300000 0004126
51	BV	70312	7/18/2014	203.227.PJKIMIA.6730068.	$1,220.00	Bayaran:PSPJKIMIA300000 0004125
52	BV	72404	9/4/2014	203.227.PJKIMIA.6730068.	$432.00	Bayaran:PSPJKIMIA300000 0004174
53	BV	72408	9/4/2014	203.227.PJKIMIA.6730068.	$232.00	Bayaran:PSPJKIMIA300000 0004181
54	BV	73118	9/23/2014	203.227.PJKIMIA.6730068.	$360.00	Bayaran:PSPJKIMIA300000 0004155
55	BV	74092	10/16/2014	203.227.PJKIMIA.6730068.	$990.00	Bayaran:PSPJKIMIA300000 0004245
56	JR	26229	10/31/2014	203.227.PJKIMIA.6730068.	$226.30	Bayaran:PDPJKIMIA300000 0002250
57	BV	75107	11/5/2014	203.227.PJKIMIA.6730068.	$917.00	Bayaran:PSPJKIMIA300000 0004331
58	BV	77445	12/11/2014	203.227.PJKIMIA.6730068.	$684.00	Bayaran:PSPJKIMIA300000 0004328
59	BV	77445	12/11/2014	203.227.PJKIMIA.6730068.	$684.00	Bayaran:PSPJKIMIA300000 0004328
60	BV	77445	12/11/2014	203.227.PJKIMIA.6730068.	($684.00)	Bayaran:PSPJKIMIA300000 0004328
61	BV	78210	12/22/2014	203.227.PJKIMIA.6730068.	$990.00	Bayaran:PSPJKIMIA300000 0004501
62	BV	79940	2/4/2015	203.227.PJKIMIA.6730068.	$1,497.00	Bayaran:PSPJKIMIA300000 0004500
63	BV	80021	2/9/2015	203.227.PJKIMIA.6730068.	$351.00	Bayaran:PSPJKIMIA300000 0004552
64	BV	81292	3/11/2015	203.227.PJKIMIA.6730068.	$1,520.00	Bayaran:PSPJKIMIA300000 0004624
65	BV	81293	3/11/2015	203.227.PJKIMIA.6730068.	$350.00	Bayaran:PSPJKIMIA300000 0004583
66	BV	81295	3/10/2015	203.227.PJKIMIA.6730068.	$70.00	Bayaran:PFPJKIMIA300000 0003249
67	BV	82767	4/1/2015	203.227.PJKIMIA.6730068.	$280.00	Bayaran:PSPJKIMIA300000 0004654
68	BV	85949	6/12/2015	203.227.PJKIMIA.6730068.	$1,590.00	Bayaran:PSPJKIMIA300000 0004786
					$21,356.58	

Nama Projek

6/26/2015

Penyelidik

Bil	Kod Dok	No Dok.	Tarikh Dokumen	Kod Akaun	Amaun	Keterangan
VOT	229					
69	BV	35495	9/14/2012	203.229.PJKIMIA.6730068.	$800.00	Bayaran:PFPJKIMIA300000 0001652
70	BV	38590	11/12/2012	203.229.PJKIMIA.6730068.	$900.00	Bayaran:PSPJKIMIA300000 0002181
71	BV	38747	11/19/2012	203.229.PJKIMIA.6730068.	$2,900.00	Bayaran:PSPJKIMIA300000 0002250
72	JR	20602	11/19/2012	203.229.PJKIMIA.6730068.	$500.00	Bayaran:PDPJKIMIA300000 0001069
73	JR	21461	1/23/2013	203.229.PJKIMIA.6730068.	$60.00	Bayaran:PDPJKIMIA300000 0001138
74	JR	21506	1/30/2013	203.229.PJKIMIA.6730068.	$500.00	Bayaran:PDPJKIMIA300000 0001075
75	JR	21622	2/19/2013	203.229.PJKIMIA.6730068.	$150.00	Bayaran:PDPJKIMIA300000 0001146
76	JR	23567	12/6/2013	203.229.PJKIMIA.6730068.	$200.00	Bayaran:PDPJKIMIA300000 0001574
77	BV	63351	3/12/2014	203.229.PJKIMIA.6730068.	$185.20	Bayaran:PFPJKIMIA300000 0002628
78	JR	25375	7/16/2014	203.229.PJKIMIA.6730068.	$120.00	Bayaran:PDPJKIMIA300000 0001972
79	JR	25712	8/25/2014	203.229.PJKIMIA.6730068.	$60.00	Bayaran:PDPJKIMIA300000 0001957
80	JR	26229	10/31/2014	203.229.PJKIMIA.6730068.	$124.40	Bayaran:PDPJKIMIA300000 0002250
81	BV	75741	11/18/2014	203.229.PJKIMIA.6730068.	$900.00	Bayaran:PFPJKIMIA300000 0003040
82	BV	75741	11/18/2014	203.229.PJKIMIA.6730068.	$900.00	Bayaran:PFPJKIMIA300000 0003040
83	BV	75741	11/18/2014	203.229.PJKIMIA.6730068.	($900.00)	Bayaran:PFPJKIMIA300000 0003040
84	BV	80022	2/9/2015	203.229.PJKIMIA.6730068.	$3,614.00	Bayaran:PSPJKIMIA300000 0004269
85	BV	81295	3/10/2015	203.229.PJKIMIA.6730068.	$55.00	Bayaran:PFPJKIMIA300000 0003249
86	JR	27805	4/8/2015	203.229.PJKIMIA.6730068.	$250.00	Bayaran:PDPJKIMIA300000 0002449
87	JR	27806	4/8/2015	203.229.PJKIMIA.6730068.	$500.00	Bayaran:PDPJKIMIA300000 0002448
88	BV	85610	6/8/2015	203.229.PJKIMIA.6730068.	$1,028.03	Bayaran:PFPJKIMIA300000 0003504
					$12,846.63	
				Jumlah	$62,035.55	

Proof of Publications

Journals/Manuscripts

1) Screening and Empirical Kinetic Models of Collagen Extraction from Selected Malaysian Freshwater Fish: *Journal of Food Process Engineering*

Journal of Food Process Engineering

Journal of Food Process Engineering ISSN 1745-4530

SCREENING AND EMPIRICAL KINETIC MODELS OF COLLAGEN EXTRACTION FROM SELECTED MALAYSIAN FRESHWATER FISH

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ABSTRACT

Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were extracted from the muscles of selected cultured catfish (hybrid of *Clarias gariepinus* × *Clarias macrocephalus*), red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasius sutchi*), sultan fish (*Leptobarbus hoevenii*) and labyrinth fish (*Trichogaster trichopterus*), freshwater fishes that are widely consumed in Malaysia. The extracted yields for the tested species were higher for PSC as compared with ASC. The catfish contained the highest amount of collagen (PSC) at 368.36 ± 12.05 mg collagen/g muscles. A mathematical description of the principle kinetics involved in collagen extraction was attained using four two-parametric models, namely the power law, parabolic diffusion, Peleg's and Elovich's models. The power law model was found to be the best model capable of predicting the extraction data with R^2 = 0.993, P value < 0.033% and root mean square deviation = 2.605%, respectively.

PRACTICAL APPLICATIONS

Utilization of fish collagen has been favorable over that of mammalian sources, mainly in making more effective use of underutilized resources. The application of acid-extraction technology to recover and isolate collagen from Malaysian freshwater fish muscles would be able to boost up the commercial values of these cheap and underutilized protein sources while assisting in conservation of these natural resources in Malaysia. Mathematic models proposed in this study were also proven to be successful in governing and describing the kinetics of collagen extraction process.

INTRODUCTION

Collagen has long been found and isolated among members of the kingdom Animalia, owing to the fact that it is the principal component of the animal extracellular matrix, serving as the most primary protein in animals (Celerin *et al.* 1996). Sadat *et al.* (2010) reported that collagen is the cement that holds everything together at the layer of animal skins. It served as the primary mortar between the bricks of all smooth-muscle tissues such as bones, cartilage, skin tendons, ligaments, blood vessels, muscles, teeth and other organs of the vertebrate. Representing approximately 30% of the total protein in animal bodies, collagen is made up of three polypeptide α chains, forming a triple helix structure, which is able to form insoluble fibers with high tensile strength (Prabjeet *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; Prabjeet *et al.* 2011).

Generally, in its purified form, collagen finds wide application in cosmetic, biomedical and pharmaceutical industries as a biomaterial (Cliche *et al.* 2003). Few unique characteristics such as low antigenicity, high tensile strength, bioresorbability, easily purified and good biocompatibility (Palpandi *et al.* 2010) leads to rapid expansion and utilization of collagen in the cartilage reconstruction, production of wound dressings, skin substitutes for burn patients, vitreous implants and carriers in drug delivery (Senaratne *et al.* 2006).

2) Extraction of Acid and Pepsin Soluble Collagen from Selected Malaysian Freshwater Fish Muscles: Modified Lowry's Measurement Method: *Journal of Agrobiotechnology*

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Extraction of Acid and Pepsin Soluble Collagen from selected Malaysian Freshwater Fish Muscles: Modified Lowry's Measurement Method

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ABSTRACT

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) are extracted from the muscles of selected cultured freshwater fishes that are widely consumed as food in Malaysia. The species tested were *Clarias* species (hybrid of *Clarias gariepinus* × *C. macrocephalus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasius sutchi*), and sultan fish (*Leptobarbus hoevenii*). The concentrations of extracted collagen were determined using Lowry's method with slight modification. Results showed that such modification was possible for measurement of collagen concentration directly from the extracting medium. The highest difference between the yields of ASC and PSC was found in *Clarias* spp. which was approximately 3.8-fold though no significant increase in the yields of both collagens was achieved after 12 hours of the extraction period. At the end of the extraction process, the range of yields based on dry weight of muscles of ASC and PSC for all of the selected fish muscles were between 97.52 ± 0.97 to 139.71 ± 0.18 mg/g and 299.93 ± 0.45 to 368.36 ± 1.05 mg/g, respectively. The presence of collagen in fish muscles as reported in this study showed that these Malaysian freshwater fishes have a potential to be utilized as alternative sources of mammalian collagens.

Keywords: Collagen, extraction, freshwater fish, modified Lowry's method, muscle

3) Collagen Extraction from Malaysian Cultured Catfish (Hybrid Clarias sp.): Kinetics and Optimization Using Response Surface Methodology: *ISRN Chemical Engineering*

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Research Article

Collagen Extraction from Malaysian Cultured Catfish (Hybrid *Clarias* Sp.): Kinetics and Optimization of Extraction Conditions Using Response Surface Methodology

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A central composite design (CCD) was used for the experimental design and results analysis to obtain the optimal processing parameters (acetic acid concentration, liquid to solid ratio, and stirring speed) for the extraction of pepsin soluble collagen (PSC) from muscles of cultured hybrid catfish of *Clarias* sp. (*Clarias gariepinus* × *C. macrocephalus*). Statistical analysis showed that the linear and quadratic terms of these three independent variables had significant effects on the yield of PSC. There was also an interaction between the ratio of liquid to solid and the stirring speed in affecting the extraction efficiency. Optimal conditions for a higher yield of PSC were an acetic acid concentration of 0.67 M, a liquid to solid ratio of 24.65 ml/g, and the stirring speed of 423.64 rpm. The verification of the optimization showed that the percentage error differences between the experimental and predicted values were in the range of 0.22–1.42%. The experimental values agreed with the predicted values, indicating an excellent fit of the model used and the success of the response surface methodology in modeling the extraction of PSC from the muscles of catfish. The experimental results were also fitted to the power law model and it was proven to be appropriate in describing the kinetics of collagen extraction process.

1. Introduction

For decades, collagen has been exploited in medical applications owing to its extraordinary biocompatibility and safety, particularly the ability to form fibers with extra strength and high stability by self-aggregation in the drug delivery system [1]. It serves as the most popular precursor of gelatin, which is widely applied to commercial products [2]. In fact, the application of collagen also diversifies in food, cosmetics, pharmaceutical, and cell culture industries. It is gradually emerging as another popular food additive in ham, sausage, and other food for mouth feel improvement, and is recently regarded as an effective edible condiment for skin care [3]. Consumption of collagen has increased with the development of new industrial applications, leading to an increasing demand for this biomaterial nowadays. Sources of collagen in the industries are primarily derived from cattle hides, beef bones, and pork skin. However, health conscious consumers are opposing the utilization of collagen extracted from these land animals due to health concerns. Besides the risks of transferring bovine spongiform encephalopathy (BSE), foot-and-mouth disease, and avian flu, porcine-derived collagen is not permitted to be used by Muslims and Jews for religious reasons, while collagens extracted from bovine sources are prohibited for Sikhs and Hindus [4]. Therefore, raw materials from fishery products have attracted attention as alternative sources of consumer-friendly collagen [5].

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 musclesand skins [6]. In our preliminary study, it was found that this hybrid *Clarias* catfish contained the highest amount

4) **Screening of significant factors in collagen extraction from hybrid *Clarias* sp. using a statistical tool: *International Food Research Journal***

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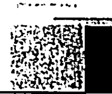

Screening of significant factors in collagen extraction from hybrid *Clarias* sp. using a statistical tool

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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 h 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.

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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 Aliyaudin, 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 (Cliche *et al.*, 2003; Aukkanit and

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5) A Hybrid of Back Propagation Neural Network and Genetic Algorithm for Optimization of Collagen Extraction from Malaysian Cultured Catfish (Hybrid *Clarias* sp.): *Biotechnology and Bioprocess Engineering*

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RESEARCH PAPER

A Hybrid of Back Propagation Neural Network and Genetic Algorithm for Optimization of Collagen Extraction from Malaysian Cultured Catfish (Hybrid *Clarias* sp.)

Peck Loo Kiew, Zainal Ahmad, and Mashitah Mat Don

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Abstract Fractional factorial design (FFD) was applied to evaluate the effects of various process parameters in influencing the extraction efficiency of pepsin soluble collagen (PSC) from muscles of cultured catfish (*Clarias gariepinus* × *C. macrocephalus*). Result of the first order factorial design showed that acetic acid concentration, acid extraction time, acetic acid to muscles ratio, and stirring speed posed significant effect ($P < 0.05$) on the yield of PSC obtained at the end of the extraction process. Two different artificial intelligence techniques namely artificial neural network (ANN) and genetic algorithm (GA) were then integrated for optimizing the extraction conditions to obtain the highest yield of PSC. The ANN was trained using the back propagation algorithm. A model was successfully generated with R^2 value of 0.9527 and MSE value of 0.1672 for unseen data set, implying a good generalization of the network. Input parameters of the established ANN model were subsequently optimized using GA. The hybrid of ANN-GA model predicted a maximum extraction yield of PSC at 238.25 mg/g under the following conditions: an acetic acid concentration of 0.70 M, the acetic acid to muscles ratio of 25.78 mL/g, and the stirring speed of 432.50 rpm. Verification of the optimization showed the percentage error differences between the experimental and predicted values were less than 5%, indicating excellent modeling, predicting ability and optimization by the ANN-GA model

Keywords: collagen, fractional factorial, artificial neural network, genetic algorithm, extraction

1. Introduction

Representing almost 25 ~ 30% of total proteins in animal body, collagen is known as the most abundant mammalian protein and major structural component of vertebrates [1]. It 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 compatibility [2,3]. In fact, collagen has been exploited in medical applications for decades owing to its extraordinary biocompatibility and safety, particularly the ability to form fibers with extra strength and high stability by self-aggregation in drug delivery system [4]. Besides serving as the most popular precursor of gelatin, Han *et al.* [5] also recently reported it as an effective edible condiment for skin care. Consumption of collagen has increased with the development of new industrial applications, leading to increasing demand for this biomaterial nowadays. Sources of collagen in the industries are primarily derived from cattle hides, beef bones, and pork skin. However, health-conscious consumers are opposing the utilization of collagen extracted from these land animals due to health concerns.

Development of aquatic-derived collagen has therefore been intensified in an effort of searching replacement for the mammalian sources. 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

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ORIGINAL ARTICLE

Isolation and Characterization of Collagen from the Skin of Malaysian catfish (Hybrid *Clarias* sp.)

Peek Loo Kiew · Mat Don Mashitah

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Abstract Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were isolated from the skin of hybrid *Clarias* sp. with the yields of 18.11±0.32 and 26.69±0.54% (wet weight basis), respectively. Both collagens were characterized as type I collagen, containing α1 and α2 chains. Presence of high molecular weight crosslinks were observed in the gel electrophoresis of both collagens. Fourier transform infrared spectra of both collagens were almost similar, suggesting that pepsin hydrolysis did not disrupt the triple helical structure. The amino acid analysis showed glycine was the most abundant, with 207/1000 and 223/1000 residues present in ASC and PSC, respectively. The amounts of imino acids were 185/1000 residues for both. Thermal denaturation temperatures were determined to be 31.5 and 31.0°C, respectively. Both collagens exhibited high solubility in acidic pH (1−5) and below 4% (w/v) NaCl concentration

Keywords acid soluble collagen · characteristics · *Clarias* sp. · pepsin soluble collagen · skin

Introduction

The term "collagen" is no longer a new word in our daily lives. Bansal (2001) described that collagen was actually derived from the Greek word "kola" for glue and was initially known as "that constituent of connective tissue, which yields gelatin upon boiling". Not only widely known as the most abundant protein in mammals, collagen is also being recognized as the major structural protein, which form molecular cables to strengthen the tendons and the resilient sheets that support the skins and internal organs of mammals and fishes (Bansal, 2001; Quereshi et al., 2010). Recently, numerous reports pointed out that the global demand for collagen has been increasing over the years. It is gradually emerging as a popular biomaterial in both bio related and non bio related industries, following its unique characteristics such as high tensile strength, low antigenicity, bioresorbability, and good compability (Cliche et al., 2003). Rapid expansion and wide utilization of collagen is currently observed in pharmaceutical applications particularly in the cartilage reconstruction, production of wound dressings, skin substitutes for burn patients, vitreous implants, and carriers in drug delivery (Wang et al., 2008). In addition, it is also regarded as an effective edible condiment for skin care and is extensively utilized as food additive or production of edible casings in meat processing industries as well (Singh et al., 2011). Therefore, collagen *per se* is considered as one of the most useful biomaterials nowadays.

Collagen basic structure, known as tropocollagen, is made up of three polypeptide chains, with each twisted in a left-handed helix (α-chain) and coiled around each other to form a right-handed triple helix structure (Mario Hiram et al., 2010; 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). On the other hand, X and Y are often proline (Pro) and hydroxyproline (Hpy) (Senaratne et al., 2006; Palpandi et al., 2010; Singh et al., 2011), respectively. For industrial purposes, the conventional main sources of collagen are limited to land-based animals such as skins and bones of bovine or porcine(Mario Hiram et al., 2010). Even though collagen offers a wide range of useful applications, pessimism and strong concerns still persist among consumers with regard to its usage. This is mainly due to religious sentiments since collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews (Muyonga et al., 2004; Senaratne et al., 2006; Singh et al., 2011). Conventional derivation of collagen from cattle and pigs has also been called into question ever since the

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ISOLATION OF COLLAGEN FROM THE SKIN OF *KELI*: POTENTIAL ALTERNATIVE FOR MAMMALIAN TYPE I COLLAGEN

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ABSTRACT

Utilization of fish collagen has been favourable over that of mammalian sources in recent years, mainly in making more effective use of under-utilized marine resources. In this study, initial extraction of the skin of cultured *Clarias* species (hybrid of *Clarias gariepinus x C. macrocephalus*), a freshwater catfish known as *Keli*, in acetic acid yielded 18.11±0.32% (wet weight basis) of acid soluble collagen (ACS). With the aid of pepsin digestion, the yield of pepsin soluble collagen (PSC) was found to be 26.69±0.54% (wet weight basis), which was approximately 1.47-fold higher than ASC. The isolated collagens were confirmed as type I collagen based on the amino acid composition. Both ASC and PSC had glycine as the major amino acid and no cysteine was found in any of the isolated collagen. This was consistent with reported characteristics of type I collagen in the literature. Presence of collagen in the skin of *Keli* showed the possibility to convert this natural resource into value-added products, thus improving its commercial value whilst assisting in ensuring the sustainability of Malaysian freshwater fishes.

Keywords: Catfish; Collagen; Extraction; Amino Acid Composition.

INTRODUCTION

Collagen, one of the extracellular matrix constituents of multi-cellular animals, serves as the major structural component of vertebrates and is the most abundant mammalian protein where it constitutes approximately 25 - 30 % of total protein in animal body [1]. In layman's term, it is described as cement that holds everything together at the layer of animal skins. In addition, collagen also acts as the primary mortar between the bricks of all smooth muscle tissues such as bones, cartilage, skin, tendons, ligaments, blood vessels, muscles, teeth and other organs of vertebrate [2]. It is recognized by three polypeptide α-chains, forming a triple helix structure which is able to form insoluble fibres with high tensile strength [3]. Each polypeptide chain is characterized by the repeating structure of triplet $(Gly\text{-}X\text{-}Y)_n$, where glycine residue is the structural prerequisite for the triple helix [4] while X and Y are often proline (Pro) and hydroxyproline (Hpy) [3, 5, 6], respectively.
In its purified form, 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 [7]. Rapid expansion and high utilization of collagen is currently observed in pharmaceutical applications particularly in the cartilage reconstruction, production of wound dressings, skin substitutes for burn patients, vitreous implants, and carriers in drug delivery [8]. In addition, it is recently regarded as an effective edible condiment for skin care and is also widely utilized as food additive or production of edible casings in meat processing industries [3]. For decades, this fibrous protein has been extracted and isolated from members of the kingdom

2) International Conference on Science, Technology & Social Sciences 2012

IKAN KELI (*Clarias Gariepinus X C. Macrocephalus*): A POTENTIAL SOURCE OF TYPE I COLLAGEN

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Keywords: Catfish, collagen, extraction, sustainability, under-utilized.

Introduction

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 compatibility [1]. Rapid expansion and high utilization of collagen is currently observed in pharmaceutical applications particularly in the cartilage reconstruction, production of wound dressings, skin substitutes for burn patients, vitreous implants, and carriers in drug delivery [2]. In addition, its good moisturizing property also leads to high demand of collagen in the production of cosmetics [3]. Skins and bones of mammals, particularly cattle and pigs are the most common souces of collagen for these industrial purposes [4]. This is owing to the fact that collagen stands as the most abundant animal protein polymer, representing for almost 25 – 30 % of total protein in animal body [5]. Being one of the extracellular matrix constituents of multi cellular animals, it can be found in connective tissues and served as major component of bones, cartilage, skin, tendons, muscles, teeth, and other organs of vertebrate[2, 6].

Conventional derivation of collagen from cattle and pigs however has been called into question ever since the emergence of the mad cow disease which is also known as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD), resulting in declining and limited usage of collagen derived products from these sources [7] [8]. There are concerns and anxieties among consumers on the risks of transferring these diseases to humans. Moreover, collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews, creating religious barriers in its applications and utilization [2, 9, 10, 11]. Therefore, the search for safer and alternative sources of collagen has intensified in the effort to attain worldwide marketability without utilization and import restrictions for collagen-derived products. Few alternative sources of collagen have been sought. Among them, extensive researches have been concentrated in the development of aquatic-derived collagen as a replacement to mammalian collagen. Skin, bone, scale, fin, muscles, and cartilage of freshwater and marine fish, muscle layer of the ascidians and adductor of pearl oyster were reported as the potential alternative sources of collagen in literature [4, 10-12].

Cultured fish is an important fish and protein source in Malaysia, especially in inland areas where marine fishes are not easily available and affordable [13]. As reported by See et al. [14], the major freshwater species cultured in Malaysia include red tilapia (26,175.33 tons), catfish (21,891.55 tons), black tilapia (5,848.98 tons) and pangasius catfish (5,748.44 tons). However, promotion of aquaculture practices is greatly restricted mainly due to the poor economic return from investments. Even though advanced aquaculture techniques such as intensive pond and cage farming are well-developed, expanded investment is not preferable since the profit margin is not high [13]. One of the main causes for this situation is lower commercial values of cultured freshwater fishes in

FINAL REPORT

EXPLORATORY RESEARCH GRANT SCHEME (ERGS)

COLLAGEN EXTRACTION FROM FRESHWATER FISH: KINETICS, MECHANISMS AND CHARACTERIZATION STUDIES

PHASE : 1/2012 (1/8/2012 – 31/7/2015)

PROJECT LEADER : PROF. DR. MASHITAH MAT DON

INSTITUTION : UNIVERSITI SAINS MALAYSIA
(SCHOOL OF CHEMICAL ENGINEERING)

TABLE OF CONTENTS

TABLE OF CONTENTS i

EXECUTIVE SUMMARY viii

CHAPTER 1 INTRODUCTION 1

1.1 Research Background 1

1.2 Problem Statement 6

1.3 Research Objectives 9

CHAPTER 2 LITERATURE REVIEW 10

2.1 Collagen 10

2.1.1 Types of Collagen 11

2.1.2 Structure of Collagen 13

2.1.3 Physico-chemical Composition of Collagen 17

2.1.3 (a) Electrophoretic Patterns 17

2.1.3 (b) Amino Acid Composition 18

2.1.3 (c) Viscosity 19

2.1.3 (d) Solubility 21

2.1.4 Sources of Type I Collagen 22

2.1.4 (a) Human 22

2.1.4 (b) Land Animals 23

2.1.4 (c) Aquatic Lives 26

2.1.4 (d) Recombinant Collagen 30
2.1.5 Type I Collagen Extraction Processes 32
2.1.5 (a) Sample/Raw Material Preparation 32
2.1.5 (b) Neutral-Salt Extraction 33
2.1.5 (c) Dilute Acid Extraction 34
2.1.5 (d) Enzyme Aided Acid Extraction 36
2.1.5 (e) Recovery of Collagen 39
2.1.6 Process Parameters Affecting Type I Collagen Extraction Efficiency 39
2.1.6 (a) Acetic Acid Concentration 40
2.1.6 (b) Extraction Temperature 40
2.1.6 (c) Extraction Time 42
2.1.6 (d) Solvent to Material Ratio 43
2.1.6 (e) Enzyme Dosage 44
2.1.7 Applications of Collagen 44
2.1.7 (a) Biomedical Applications 45
2.1.7 (b) Non-Biomedical Applications 48
2.2 Malaysian Freshwater Fishes 49
2.2.1 Aquaculture in Malaysia 49
2.2.2 Applications of Malaysian Freshwater Fishes 53
2.2.2 (a) Food Source 53
2.2.2 (b) Therapeutic and Nutritive Source 54
2.2.3 Potential Source of Collagen 56

2.3 Optimization of Process Parameters 59

2.3.1 Statistical Based Optimization 59

2.3.1 (a) Design on Experiment (DOE) 59

2.3.1 (b) Fractional Factorial Design (FFD) 60

2.3.1 (c) Response Surface Methodology (RSM) 61

2.3.2 Artificial Intelligence-Based Optimization 64

2.3.2 (a) Artificial Neural Network (ANN) 64

2.3.2 (b) Bootstrap Re-Sampling Neural Network 67

2.3.2 (c) Genetic Algorithm (GA) 68

2.3.2 (d) Artificial Neural Network/Genetic Algorithm (ANN/GA) 69

2.4 Collagen Extraction Kinetics and Modeling 70

2.4.1 Empirical Kinetic Model 70

2.4.1 (a) Power Law Model 72

2.4.1 (b) Parabolic Diffusion Model 72

2.4.1 (c) Peleg's Model 73

2.4.1 (d) Elovich's Model 74

2.5 Summary 75

CHAPTER 3 MATERIALS AND METHODOLOGY 76

3.1 Materials and Chemicals 76

3.2 Equipments 78

3.3 Research Methodology Flow Chart 79

3.4 Screening of Collagen from Malaysian Freshwater Fishes 80

3.4.1 Preparation of Raw Material 80

3.4.2 Extraction of Type I Collagen 81

3.4.2 (a) Extraction of Acid Soluble Collagen (ASC) 81

3.4.2 (b) Extraction of Pepsin Soluble Collagen (PSC) 82

3.4.2 (c) Collagen Concentration Measurement by Lowry's Modified Method 82

3.5 Optimization of Pepsin Soluble Collagen (PSC) Extraction Process 84

3.5.1 Screening of Significant Process Parameters Using Fractional Factorial Design (FFD) 84

3.5.2 Optimization of Process Parameters Using One-Factor-At-One-Time (OFAT) Method 85

3.5.3 Optimization of Process Parameters Using Response Surface Methodology (RSM) 86

3.5.4 Optimization of Process Parameters Using Artificial Neural Network (ANN) and Genetic Algorithm (GA) 87

3.5.4 (a) Development of Feedforward Artificial Neural Network (FANN) 87

3.5.4 (b) Evaluation of Model Predictability 89

3.5.4 (c) Genetic Algorithm (GA) Based Optimization 90

3.6 Characterization of Extracted Collagen 91

3.6.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) 91

3.6.2 Amino Acid Composition 92

3.6.3 Functional Group Analysis 92

3.6.4 Determination of Collagen Denaturation Temperature 93

3.6.5 Collagen Solubility Study 93

3.6.5 (a) Effect of pH 93

3.6.5 (b) Effect of Sodium Chloride (NaCl) 94

3.7 Kinetics and Modeling of Collagen Extraction 94

CHAPTER 4 RESULTS AND DISCUSSIONS 96

4.1 Determination of Collagen Concentration Using Modified Lowry's Method 96

4.2 Screening of Collagen from Selected Malaysian Freshwater Fishes 99

4.3 Optimization of Collagen Extraction Process 103

4.3.1 Screening of Process Parameters Using Fractional Factorial Design (FFD) 103

4.3.2 Optimization of Process Parameters Using One-Factor-At-One-Time (OFAT) Method for Extraction of Muscle Collagen 107

4.3.2 (a) Effect of Acetic Acid Concentration 107

4.3.2 (b) Effect of Acetic Acid to Material Ratio 109

4.3.2 (c) Effect of Extraction Time 110

4.3.2 (d) Effect of Stirring Speed 112

4.3.3 Optimization of Process Parameters Using Response Surface Methodology (RSM) for Extraction of Muscle Collagen 114

4.3.4 Optimization of Process Parameters Using Artificial Neural Network (ANN) and Genetic Algorithm (GA) for Extraction of Muscle Collagen 122

4.3.4 (a) Artificial Neural Network Topology 122

4.3.4 (b) Prediction Accuracy of Trained Network 125

4.3.4 (c) Genetic Algorithm Based Optimization 126

4.3.4 (d) Validation of Predictive Model 127

4.3.5 Comparison of Collagen Yield between Response Surface Methodology (RSM) and Artificial Neural Network and Genetic Algorithm (ANN/GA) 128

4.4 Characterization of Collagen from Hybrid *Clarias* sp. (*C. gariepinus* X *C. macrocephalus*) 130

4.4.1 Characterization of Fish Muscle Collagen 130

4.4.1 (a) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 130

4.4.1 (b) Amino Acid Analysis 131

4.4.2 Characterization of Fish Skin Collagen 134

4.4.2 (a) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 134

4.4.2 (b) Amino Acid Analysis 136

4.4.2 (c) Functional Group Analysis 139

4.4.2 (d) Determination of Collagen Denaturation Temperature 142

4.4.2 (e) Collagen Solubility Study 145

4.4.3 Comparison Between Muscle Collagen and Skin Collagen 149

4.4 Kinetics and Modeling of Skin Collagen Extraction from Hybrid *Clarias* sp. (*C. gariepinus* X *C. macrocephalus*) 150

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS 154

5.1 Conclusion 154

5.2 Recommendations for Future Work 158

REFERENCES 159

APPENDICEES 173

Appendix A Selected Freshwater Fishes for Collagen Extraction 173

Appendix B Optimization by Response Surface Methodology (Suggested optimum process conditions for the extraction of M-PSC by CCD) 174

Appendix C Optimization by ANN/GA 175

LIST OF PUBLICATIONS 181

COLLAGEN EXTRACTION FROM FRESHWATER FISH: KINETICS, MECHANISMS AND CHARACTERIZATION STUDIES

EXECUTIVE SUMMARY

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the muscle and skin of six different species of Malaysian freshwater fishes, namely a hybrid catfish of *Clarias* sp. (*C. gariepinus* X *C. macrocephalus*), red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasianodon hypophthalmus*), Sultan fish (*Leptobarbus hoevenii*), and labyrinth fish (*Trichogaster trichopterus*). The extracted muscle collagens were designated as M-ASC and M-PSC, whilst skin collagens were termed S-ASC and S-PSC, for the acid and pepsin soluble collagen, respectively. The concentration of extracted collagen was determined using Lowry's method with slight modification. Results showed that such modification was possible for measurement of collagen concentration directly from the extracting medium. The yields of extracted collagens for all selected species were higher for PSC as compared to ASC. The highest muscle collagen yields of 8.54 ± 0.98 % (wet basis) were extracted from the hybrid catfish of *Clarias* sp. Artificial Neural Network (ANN) and Genetic Algorithm (GA) were applied to compare the estimation capabilities between the statistical- and artificial intelligence-based optimization techniques. The ANN/GA approach was superior over the conventional Response Surface Methodology (RSM), with lower average yield error at the range of 0.24 – 3.43 % and higher training R^2 ($0.9997 < R^2 < 1$), respectively. From the characterization studies, S-ASC and S-PSC from the skin of hybrid *Clarias* sp. exhibited similar properties and characteristics to the

CHAPTER ONE

INTRODUCTION

1.1 Research Background

Approximately 0.80 % of the Earth's surface and 0.01 % of the World's water are made up of freshwater (Dudgeon et al., 2006). Though this sounds like a very tiny fraction of global water, yet freshwater is supporting at least 100 000 species out of 1.8 million of the identified aquatic species worldwide (Dudgeon et al., 2006). Inland waters and freshwater biodiversity constitute valuable natural resources in terms of economics, cultures, scientific knowledge and education. Having close to hundred river systems, two natural lakes and an enormous amount of freshwater water bodies as well as peat swamps, Malaysia is well-known worldwide as one of the countries that being bestowed with mega-diverse bioresources. In term of fish biodiversity, it was reported that approximately 616 species of freshwater fishes were discovered in Malaysia (Chew and Zulkafli, 2012).

Freshwater or cultured fish was an important protein source in Malaysia, especially in inland areas where marine fishes were not easily available and affordable (Yaakob and Ahyaudin, 1994). Apart from essential fatty acids, minerals and vitamins, it was estimated that fish contributed about 60 – 70 % of total protein requirements or intake in Malaysian daily diet (Osman et al., 2001; Shafri and Abdul Manan, 2012). The main freshwater species cultured were Nile tilapia (*Oreochromis niloticus*), accounted for 44.7 % of the total freshwater aquaculture production, followed by catfish (36.7 %)

and carps (10.08 %) (FAO, 2012). Even though freshwater fishes in Malaysia were diverse and intriguing, inland or freshwater fishery still remained at its infancy with little knowledge on the ecological, biological, genetics background and applications of these fishes. With the aids of technology advancement and government support, the freshwater fish industry in this country was blooming and emerging gradually since the past decades. Numerous efforts had been done by the Department of Fisheries (DOF) Malaysia to encourage the expansion of freshwater industry so as to improve the income of the agriculturist and fishermen (Airina and Jamaludin, 2012). Nevertheless, demand for freshwater fish had remained low in the past few years. Dey et al. (2008) reported that fish consumers were unevenly distributed in Malaysia, with large concentration in urban areas of the west coast of Peninsular Malaysia. Marine fishes were more preferable to freshwater fishes among these urban consumers (Endinkeau and Tan, 1993; Dey et al., 2008), resulting the later to have little or lesser commercial value for human consumption. This might be caused by the lack of information and knowledge on the nutritional values of freshwater fishes among Malaysians (Endinkeau and Tan, 1993).

Several studies in recent years concentrating on evaluation of the fatty acid composition especially on the omega-3 and omega-6 of some commonly cultured Malaysian freshwater fishes such as *Channa striatus* ("Haruan"), *Pangasius hypothalamus* ("Patin"), and *Clarias macrocephalus* ("Keli"), had shed some lights on the nutritional importance of freshwater fish consumption (Endinkeau and Tan, 1993; Airina and Jamaludin, 2012). Omega-3 and omega-6 polyunsaturated fatty acids (PUFA) particularly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) had been proven to exhibit positive effects on cardiovascular diseases and cancers (Zuraini et al.,

2006). PUFA was also showed to lower serum triglyceride and cholesterol levels, and assist in preventing blood clotting (Endinkeau and Tan, 1993). An appreciable amount of omega-3 PUFA was reported in Malaysian freshwater fishes by Endinkeau and Tan (1993), Suriah et al. (1995), Zuraini et al. (2006) and Airina and Jamaludin (2012), making them suitable for inclusion in the formulation of highly unsaturated low-fat diets and production of fish oil. Apart from that, snakehead fishes of the Channidae family included *C. striatus, Channa micropeltes* (*toman*) and *Channa lucius* (*bujuk*) were also claimed to possess anti-inflammatory properties (Zuraini et al., 2006). *C. striatus* was highly valued for its medicinal properties and was popular among Malaysian as a therapeutic agent which its efficacy in treating wounds, relieving pain and boosting energy among the sick and elderly were proven (Shafri and Abdul Manan, 2012).

See et al. (2010) reported that there was an increasing trend in the utilization of fish by-products including fish skin and bone to produce collagen and gelatin. Collagen, in particular, had been extracted from the skin of land animals or mammals such as pig and bovine for industrial applications which included functional food ingredients, cosmetics, biomedical and leather industry (Nagai et al., 2010; Jamilah et al., 2013). Currently, fish collagen has been gaining increasing interest and attention as an alternative for mammalian collagen. In Malaysia, collagen products derived from animal tissues such as skins and bones were available in various forms in the market, yet most of them were imported and of questionable origin as to the "Halalness" (Babji et al., 2011). A number of research works have shown the possibility to extract this biomaterial from parts of freshwater fishes as a replacement to mammalian collagen. Not only carrying lower risk of disease transmission, fish collagen would be able to cater for

increasing demand of "Halal" and "Kosher" collagen products for the Muslim and Jewish communities, respectively. Commercial value of freshwater fishes in Malaysia is much lower in contrast to those of marine species. Consequently, conversion of these natural resources into value-added products to generate additional income has both economics and conservation benefits to the freshwater industry in Malaysia.

One of the biggest health concerns for millions of people today is about keeping their youthful appearance and beauty even when they reach their late 30s or 40s. However, skin, like other organs, ages with the passage of time. Fine wrinkles, dryness, sagginess, flakiness and greater visibility of skin pores were among the indications of skin aging, which frequently associated with natural biological phenomenon known as collagen degradation (Hsu and Chiang, 2009). Collagen constituted about 25 – 30 % of total protein in animal bodies, making it the most abundant protein polymer in animals (Bae et al., 2008; Bama et al., 2010). Type I collagen was the main constituent of the extracellular matrix (ECM) of skin dermis which served to maintain the structural integrity and provide strength to the skin dermis (Bae et al., 2008). Skin aging phenomena was associated with the degradation of collagen, regulated by matrix metalloproteinase (MMPs), an enzyme that was able to break down collagen and was normally found in the spaces between tissues (Hsu and Chiang, 2009). Therefore, it was believed that aging process could be improved by promoting collagen synthesis and reducing MMPs activities in the skin. Generally, in its purified form, collagen found wide application in food, cosmetic, biomedical and pharmaceutical industries as a biomaterial (Bae et al., 2008).

Collagen had long been found and isolated from members of the kingdom Animalia owing to the fact that it was the principal component of the animal extracellular matrix and was the most primary protein in animals (Pati et al., 2010a). Skins and bones of mammals, particularly cattle and pigs, were the most common sources of collagen for industrial purposes (Senaratne et al., 2006; Bae et al., 2008). Apart from that, type I collagen had also been extracted from the skins, bones, fins and scales of freshwater and marine fishes, chicken skin and a number of marine lives such as squid, octopus, jellyfish and starfish (Pati et al., 2010a). Few reports on isolation and characterization of polysaccharides and collagen from sea cucumbers also suggested that the major edible component of sea cucumber consisted of collagenous fiber (Saito et al., 2006; Cui et al., 2007). Collagen extraction studies from these sources, especially from aquatic environment had been carried out extensively to evaluate their potentials as alternatives to mammalian collagen (Pati et al., 2010a).

Recently, there have been a lot of interests in investigating possible means of making more effective use of under-utilized resources and by-products from fish industry, particularly in collagen extraction researches. Extensive efforts are focusing in extraction and isolation of collagen from aquatic environment so as to provide a new platform in finding alternative sources of collagen in various applications, not only restricted to skin care and biomaterials.

1.2 Problem Statement

The global demand for collagen has been increasing over the years. Nevertheless, the conventional sources of collagen for various industrial applications have been limited to only those of vertebrates, which most of them were originated from pig skin (80 %), cattle split (15 %), and the remaining were from other sources such as pig and cattle bones, poultries, and fishes (5 %) (Liu et al., 2010). Though collagen offers a wide range of useful applications, pessimism and anxiety still persist among health-conscious consumers with regard to its usage. There are increasing concerns among researchers on the possibility of animal tissue-derived collagens to transmit pathogenic vectors, particularly prions (Karim and Bhat, 2009). Outbreak of highly contagious epizootic infections such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), food and mouth disease, mad cow disease, *Streptococcus suis*, and H1N1 flu etc. in pigs and cattle intensified the situation (Senaratne et al., 2006; Wang et al., 2011). Also, in religious point of view, collagen extracted from bovine sources are prohibited for the Sikhs and Hindus, while porcine related products cannot be consumed by the Muslims and Jews, creating much restrictions and limitations in its usage (Sadowska et al., 2003; Kittiphattanabawon et al., 2005; Singh et al., 2011). Due to these constraints in safe food practice and religious sentiments, the conventional derivations of collagen from cattle and pigs have therefore been called into question.

Scientific reports and literature associated with alternative sources of collagen have experienced a boom in the last 10 – 15 years since alternatives to mammalian collagens are strongly desired (Gómez-Guillén et al., 2011). Researchers reported that skin, bone, scale, fin and cartilage of freshwater and marine fish, scallop mantle, muscle

layer of the ascidians and adductor of pearl oyster were the potential new sources of collagen (Kittiphattanabawon et al., 2005; Bae et al., 2008; Rodziewicz-Motowidło et al., 2008; Yan et al., 2008). In this regard, fish collagen was highlighted as the most promising alternative due to its high availability, lower risk of disease transmission, lack of dietary restriction and the possibility of high collagen yield (Senaratne et al., 2006; Liu et al., 2010). Even though fishes are one of the main protein sources for Malaysians, the demand for freshwater fish in Malaysia however is only meant for human daily consumption and other minor uses, such as meal production and bait, resulting in lower commercial value in contrast to deep-sea species. Development of such 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. However, utilization of these natural resources is less being exploited. In fact, not much has been done on the isolation of acid- and pepsin-soluble type I collagen derived from freshwater fishes, particularly from the Malaysian species.

The efficiency of the collagen extraction process from the raw materials can be influenced by many process factors such as acid concentration, extraction time, stirring speed, and liquid to solid ratio (Wang et al., 2008b; Woo et al., 2008). However, detailed study on the effects of these process parameters on the kinetics and mechanisms of the extraction process is still scarce in the literature. In engineering point of view, despite many studies reported on the possibility of various raw materials as potential alternatives for mammalian collagen, a suitable kinetic model for collagen extraction is not yet available in the literature.

In addition, collagen extraction process presents many challenging control problems which include nonlinear dynamic behavior and multivariable interactions between the process variables and response variables. Even though Response Surface Methodology (RSM) has been widely applied for process development, neural network-based models have recently been shown by several researchers to exhibit superior performance in terms of both accuracy and predictive capability (Chou and Chen, 2012). A number of drawbacks associated with RSM have been pointed out by few researches. Among them, Lou and Nakai (2001) and Desai et al. (2008) stated that RSM was useful only for quadratic approximations and the sensitivity analysis of input variables was difficult to perform using RSM due to the presence of cross interactions. Collinearity problems between the studied process factors were also among the problems being highlighted.

Artificial neural network (ANN) on the other hand, has emerged as an attractive tool for non-linear multivariate modeling in the last two decades. Genetic algorithm (GA) based on ANN model as an objective or fitness function has been applied successfully in optimizing the input space of various bioprocess studies, involving modeling of fermentation process (Desai et al., 2005) and biomass as well as production of various bio-products from bacteria (Lou and Nakai, 2001). Even though ANN has been recognized as a powerful and practical approach to model extremely complex non-linear process while GA was used in various diverse optimization systems in biochemical engineering, application of these soft computing intelligence techniques in collagen extraction studies is still very limited.

1.3 Research Objectives

The primary objective of this research is to investigate the possibility of extracting type I collagen from the muscle of selected Malaysian freshwater fishes and characterize the extracted collagen. The kinetics and modeling study of the extraction process were also conducted. The measurable objectives are as follow:

1) To propose a suitable and reliable routine collagen measurement method so as to determine the yield or amount of extracted collagen.
2) To screen the presence of collagen concentration in different identified species of freshwater fish in Malaysia.
3) To optimize the collagen extraction process parameters using statistical tool and artifical intelligence technique.
4) To characterize the extracted collagen.
5) To study the mathematical kinetics and modeling of the collagen extraction process.

CHAPTER TWO

LITERATURE REVIEW

2.1 Collagen

The term "collagen" is no longer a new word in our daily lives. Bansal (2001) described that collagen was actually derived from the Greek word "kola" for glue and was initially known as "that constituent of connective tissue, which yields gelatin on boiling". Not only widely known as the principal structural protein in the vertebrate body, collagen was also being recognized as the major structural protein which formed molecular cables to strengthen the tendons and the resilient sheets that supported the skins and internal organs of animals and fishes (Goodsell, 2000; Bansal, 2001). Collagen provided structure to animal bodies by protecting and supporting the soft tissues and connecting them with each other as well as to the skeleton (Goodsell, 2000). Approximately 90% or more collagen contributed to the extracellular proteins in tendon and bone, whilst more than 50% could be found in the skin (Meena et al., 1999; Nair et al., 2010). Bones and teeth could be formed by adding mineral crystals to collagen (Goodsell, 2000). Nevertheless, this protein was absent in unicellular or single-celled living bodies and in plants in which cellulose and related polysaccharides served a similar role (Meena et al., 1999).

Though collagen could be found in most parts of animals, but Nakamura et al. (2003) reported that type I collagen was especially concentrated in the skin-associated tissues and bones. In layman term, collagen was the cement that held everything together

at the layer of animal skins. It served as the primary mortar between the bricks of all smooth-muscle tissues such as bones, cartilage, skin, tendons, ligaments, blood vessels, muscles, teeth and other organs of the vertebrates (Quereshi et al., 2010). This naturally occurring matrix polymer was highly conserved across species and was a vital constituent of the body of animals and fishes, so as to keep the body in shape and flexible (Cheema et al., 2011). In addition, collagen aided to build connections, especially over the scars, which helped connective tissue to grow in order to make up for the loss of tissues (Zahrani, 2011). Ever since the discovery of its structure at the molecular level in 1930s, collagen had been extensively studied as a potential biopolymer in manufactured materials and the human effort of exploiting its potentials and applications had marked success in both medical and industrial sector (Meena et al., 1999; Zahrani, 2011).

2.1.1 Types of Collagen

In human, vertebrates and many other protein-contained collagenous domains, 29 distinct types of collagen have been indentified to date; on the basis of protein or DNA sequence information (Shoulders and Raines, 2009). The first 5 types identified collagens represented almost 90 % of the total collagens in animal bodies (Zahrani, 2011). The collagen family had been well described by Prockop and Kivirikko (1995) who divided it into several classes based on the structural features and the polymeric structures that they formed. They were classified as: (a) fibril forming collagens (types I, II, III, V, and XI), (b) network-like structure forming collagens (types IV, VIII and X), (c) fibril-associated collagens with interrupted triple helices which referring to those found on the surface of collagen fibrils (types IX, XII, XIV, XVI, and XIX), (d) beaded

filaments forming collagen (type VI), (e) anchoring fibrils forming collagens for basement membrane (type VII, XIII and XVII), and (g) other collagens that had been only partially characterized (recently discovered types). A list of types of collagen and some tissues in which they were found is provided in Table 2.1.

Table 2.1: Different types of collagen and their distributions in vertebrates or animal bodies (Shoulders and Raines, 2009; Kasoju et al., 2010)

Type	Distribution
I	Most connective tissues, i.e. bones, skin, tendon, blood vessels and ligaments
II	Cartilage and vitreous of the eye
III	Blood vessels, skin and intestines
IV	Basement membranes in all organs
V	Tendons, interstitial tissues, bone, dermis and placenta
VI	Liver, kidney and bladder
VII	Epidermal/ dermal junction and bladder
VIII	Endothelial cells, heart and eye
IX	Cartilage, cornea and vitreous
X	Cartilage
XI	Cartilage and intervertebral disc
XII	Tendons and fibril associated tissues
XIII	Epidermis, hair follicles, nail root cells, eye and heart
XIV	Similar to type I
XV	Capillaries, testis, kidney and heart
XVI	Dermis and kidney
XVII	Hemidesmosomes in epithelia
XVIII	Basement membrane and liver
XIX	Basement membrane
XX	Cornea (chick)

XXI	Stomach and kidney
XXII	Tissue junctions
XXIII	Heart and retina
XXIV	Bone and cornea
XXV	Brain, heart and testis
XXVI	Testis and ovary
XXVII	Cartilage
XXVIII	Dermis and sciatic nerve

About a quarter of the total proteins in vertebrates was collagen (Goodsell, 2000). Not only marked its presence in the scaffoldings in mammals, collagen could also be found from the Achilles tendon to the cornea (Kasoju et al., 2010; Nair et al., 2010). Type I collagen was predominant in higher order animals, particularly in the skin, tendon and bone. Type II collagen on the other hand was unique to cartilage, while type III collagen presented in trace amount (approximately 5 – 10 %) in association with type I. Apart from that, type III collagen was mainly found in the blood vessels. Type IV occurred in another highly specialized form in which it only presented as a loose fibril network in the basement membranes. The remaining types of collagen however presented only in a small amount and were often associated with specific biological structures (Meena et al., 1999; Kasoju et al., 2010; Zahrani, 2011).

2.1.2 Structure of Collagen

The presence of collagen in all connective tissues made it one of the most studied bio-molecules of the extracellular matrix (ECM) (Parenteau-Bareil et al., 2010). To this day, all indentified types of collagen had been characterized and they were found to share a unique basic structure in which three polypeptide (protein) α-chains assembled

together to form a triple helix (Gómez-Guillén et al., 2011; Zahrani, 2011). The first correct model of the structure of collagen was proposed by Ramachandran and Kartha and was popularly known as the *Madras Model* (Sasisekharan and Yathindra, 1999; Kasoju et al., 2010). Lee et al. (2001) recently stated the molecular structure of collagen had been firmly established on the evidence from earlier studies, such as amino acid composition analysis, X-ray diffraction analysis, electron microscopy and physicochemical examination of solutions. Each type of collagen was represented by different combination of α-chains that was a unique gene product with a unique amino acid sequence. Generally, these α-chains were intertwined around a central axis, forming a triple helix structure known as tropocollagen (Figure 2.1). It was a cylindrical protein that was about 280 – 300 nm in length and 1.5 nm in diameter (Zahrani, 2011; Ezquerra-Brauer et al., 2012). Each polypeptide α-chain, on the other hand, contained approximately 1000 amino acids and had an approximate molecular weight of 100 kDa (Kasoju et al., 2010; Nair et al., 2010; Ezquerra-Brauer et al., 2012).

Collagen was organized in tissues and organs in a hierarchical fashion. For this organization, collagen molecules were first assembled into fibrils, which were collected into bundles and these in turn were organized into tissue-specific non-aggregates. For instance, they formed regular layers in bone and cornea, irregular layers in skin and were found in aggregated forms in tendons and ligaments. The diameter, orientation and packing density of such fibrils and fibers varied from tissue to tissue and within a single tissue itself. This variation continued even at the molecular level, where there were tissue-specific differences in the lateral packing of individual molecules into fibrils. The

diversity in fibrillar structure related to anisotropy with specific mechanical and structural properties required for different connective tissues (Pati et al., 2010b).

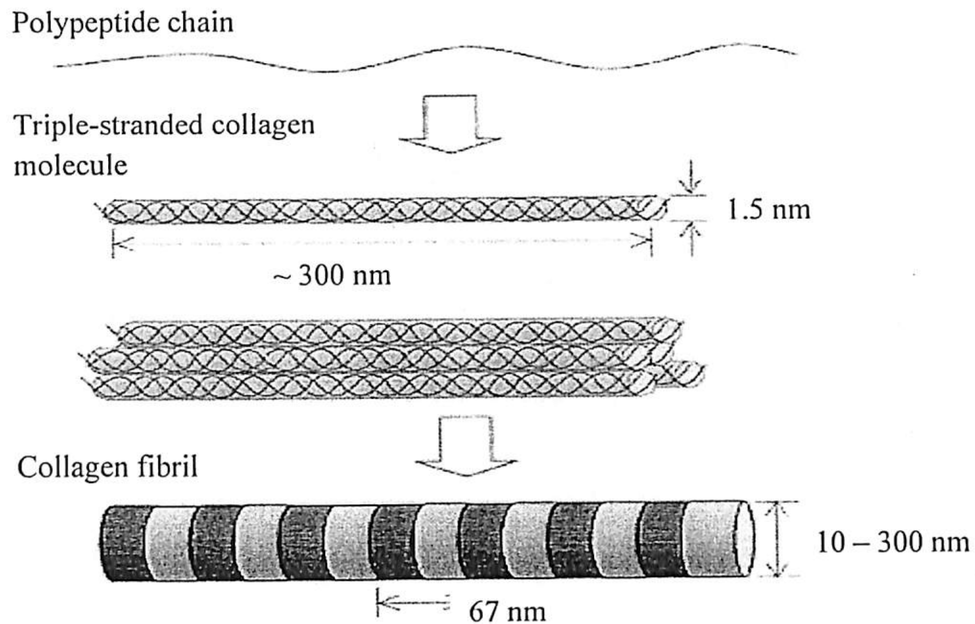

Figure 2.1: Schematic diagram of Type I collagen fibril structure. Helical collagen molecules formed from three polypeptide chains, and these associated laterally to form collagen fibrils with a characteristic banded structure (Nair et al., 2010).

Tropocollagen molecules may be formed by homotrimers (three identical α-chains) such as collagen type II and III or by heterotrimers (two or more different α-chains) as in type I, IV and V (Figure 2.2) (Ezquerra-Brauer et al., 2012). Mainly stabilized by intra- and inter-chain hydrogen bonding, the collagen helical structure was the product of an almost continuous repeating of the triplet amino acid (Gly-X-Y), where X and Y were often proline and hydroxyproline, respectively (Figure 2.3) (Parenteau-Bareil et al., 2010; Gómez-Guillén et al., 2011). Therefore, the most distinct

characteristic of collagen to differentiate it from other proteins was its amino acid composition which was very high in glycine residues (approximately 33 % of total amino acid content) and the imino acid residues (proline and hydroxyproline) (Bansal, 2001). At the end of the triple helix (telopeptide region), where helical behavior was absent, intermolecular cross-linking with other adjacent molecules was associated with the presence of globular proteins (Engel and Bächinger, 2005).

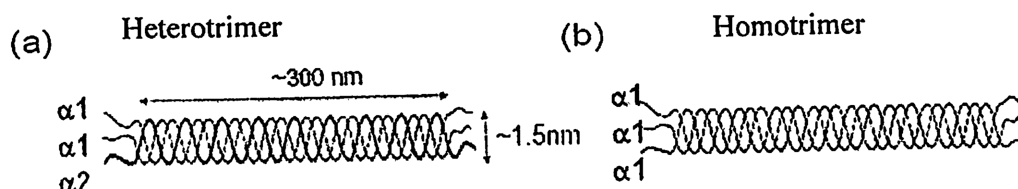

Figure 2.2: Schematic representation of type I collagen triple helix. (a) heterotrimer and (b) Homotrimer (Han, 2009).

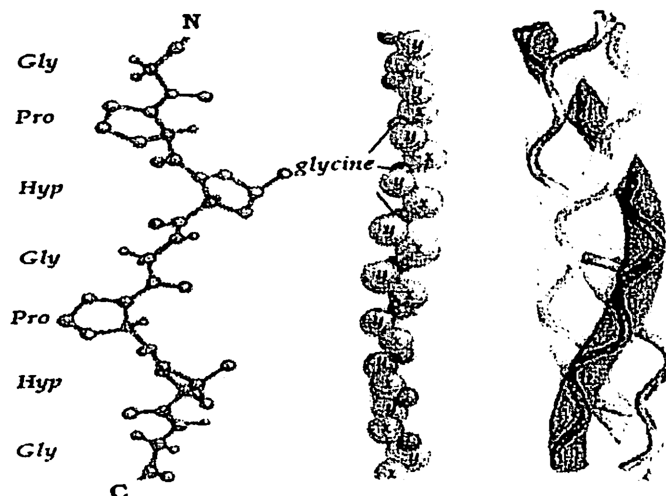

Figure 2.3: Amino acid sequence of a typical collagen molecule (Ezquerra-Brauer et al., 2012).

Another special characteristic of collagen was the existence of the unusual amino acid hydroxylysine which was produced from lysine in the endoplasmic reticulum through enzymatic hydroxylation by lysyl hydroxylase. Hydroxylysyl residues were the main contributor to the attachment of sugar components for the formation of the triple helical structure of the collagen molecule (Kasoju et al., 2010). According to Parenteau-Bareil et al. (2010) and Ezquerra-Brauer et al. (2012), the presence of glycine at every third amino acid position was vital in order to achieve tight and firm coiling of the three polypeptide α-chains in the tropocollagen molecule. This was made possible by the features of glycine which was the smallest amino acid and did not come with any side chain. Easily accommodated in the middle of a superhelix, glycine therefore aided in forming a stable packed structure which would be difficult to achieve with other bulkier residues (Ezquerra-Brauer et al., 2012). The –NH groups of Gly formed hydrogen bonds with the –C=O groups of the X position residues on the adjacent chain. These hydrogen bonds (one per Gly-X-Y triplet) were aligned perpendicular to the helical axis. They tied α-chains together and provided the main stabilizing force for the triple helix structure (Han, 2009). Apart from that, imino acids also stabilized the triple helix structure by stiffen the α-chains and permitted sharp twisting of the collagen helix (Kasoju et al., 2010), which were normally associated with the stability and thermal behavior of the triple helical conformation.

2.1.3 Physico-Chemical Characteristics of Collagen

2.1.3 (a) Electrophoretic Patterns

Qualitative analysis by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is one of the most common characterization methods to

reveal the subunit compositions of collagen or identify the components exhibiting electrophoretic mobility corresponding to the chains present in the tested collagens (Zhang et al., 2011). The typical patterns of type I collagen were consisted of the bands of α chains (α1 and α2) and their dimers (β-components) (Huang et al., 2011). Collagens extracted from the skin of numerous fish species had been reported to be type I as the major component. Among them, the skin collagens of bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon et al., 2005), brown backed toadfish (*Lagocephalus gloveri*) (Senaratne et al., 2006), largefin long barbel catfish (*Mystus macropterus*) (Zhang et al., 2009), brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010a), and striped catfish (*Pangasianodon hypophthalmus*) (Singh et al., 2011) were reported to be made up of two α chains, β and γ components. The protein bands of type I collagen subunit were normally detected at approximately 120 kDa for α1 and 112 – 114 kDa for α2, and higher than 200 kDa for β and γ components, respectively (Bae et al., 2008).

2.1.3 (b) Amino Acid Composition

The amino acid composition is one of the crucial parameters that would affect the physico-chemical properties of the collagen such as thermal stability, cross-linking ability and solubility in NaCl (Nurul, 2011). The analysis of amino acid content in collagen from different sources indicated that the amino acid composition of type I collagens were different for different species (Lin and Liu, 2006a). Thermal stability of the collagen was closely related to the relative abundance of imino acids (hydroxyproline and proline) (Pati et al., 2010a). Higher amount of these imino acids increased the thermal stability of collagen due to higher cross-linked density.

Additionally, the difference in imino acid content amongst collagens from different species was associated with the difference in the living environments of the respective sources, especially temperature of the habitat (Kittiphattanabawon et al., 2010a). Generally, glycine is the most abundant amino acid in type I collagen, which normally accounts for approximately 30 % of the total amino acid content (Bae et al., 2008). This observation was reported by many researchers for the extraction and characterization results of various aquatic-derived type I collagens such as brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010a), balloon fish (*Diodon holocanthus*) (Huang et al., 2011), cobia (*Rachycentron canadum*) (Zeng et al., 2012) and barramundi (*Lates calcarifer*) (Jamilah et al., 2013) etc. In addition, these collagens were also rich in hydroxyproline, alanine and proline. Another distinguished characteristic of the amino acid composition of type I collagen was the absence of cystein and relatively low percentage of methionine (Rodziewicz-Motowidło et al., 2008; Pati et al., 2010a).

2.1.3 (c) Viscosity

Viscosity measurement is useful to determine the thermal stability of collagen owing to that fact that the loss in viscosity with heating is attributed to the denaturation of collagen (Kittiphattanabawon et al., 2005; Nurul, 2011). Ogawa et al. (2004) mentioned that high viscosity was one of the physico-chemical characteristics of collagen because it was made up by structured systems that great electrostatic repulsion was observed among the collagen molecular chains in solution even at low concentrations, led to a high degree of viscosity. As reported in the literature, most of the extracted collagens studies revealed that the relative viscosity of the collagens

decreased continuously upon heating up to 30 °C, and subsequently, rate of decrease in viscosity reduced in the temperature range of 35 – 50 °C (Senaratne et al., 2006; Huang et al., 2011). Kittiphattanabawon et al. (2005) therefore explained that heat treatment of collagen under high temperature could cause the breaking of hydrogen bonds, which were responsible in stabilizing the collagen structure. Continuous heating up to 40 °C will then led to the denaturation of collagen with the degradation of collagen triple helical strands into a mixture of random coil (single or double strands) (Nurul, 2011).

Thermal denaturation measurement of collagen provided useful information regarding the thermal stability of collagen in relation to the living environment of the collagen sources and the amino acid content (Li et al., 2008). For fish collagens, the denaturation temperature of collagen solution from ocellate puffer fish (*Takifugu rubripes*) was reported to be approximately 28 °C (Nagai et al., 2002), grass carp (*Ctenopharyngodon idella*) at about 28.4 °C (Zhang et al., 2007b), surf smelt (*Hypomesus pretiosus*) was about 32.5°C (Nagai et al., 2010), balloon fish (*Diodon holocanthus*) at 29 – 30 °C (Huang et al., 2011) and nile perch (*Lates niloticus*) was between 36 – 36.5 °C (Muyonga et al., 2004). The results suggested that denaturation temperatures varied significantly amongst fish species. Collagens derived from fish species living in warm environments contained higher content of hydroxyproline and exhibited higher thermal stability than those living in cold environments (Singh et al., 2011).

2.1.3 (d) Solubility

As collagen is made up of protein, its solubility is greatly influenced by the pH of the solution. When the pH is lower or higher than the isoelectric point (pI), solubility of collagen is improved because net charge residues of the collagen molecules are greater, resulting in repulsive forces between the collagen chains (Zeng et al., 2012). pI for collagen was in the pH ranged 6 – 9. Consequently, lowest solubility of either ASC or PSC was often found to fall within this range (Nalinanon et al., 2007; Skierka and Sadowska, 2007; Bae et al., 2008; Jamilah et al., 2013). Solubility was high in the pH ranging 1 – 4 and collagen molecules started to coagulate and precipitate when the pH was near to pI, due to increased hydrophobic interaction among the molecules (Bae et al., 2008; Nurul, 2011). Apart from that, addition of sodium chloride (NaCl) was also useful in analyzing solubility of the extracted collagen. An increase in NaCl concentration could lead to reduction in collagen solubility in the solution. This was due to the induced hydrophobic-hydrophobic interactions and aggregation between collagen chains, thereby resulting in precipitation of the molecules which was also known as the salting out effect (Jongjareonrak et al., 2005b; Huang et al., 2011). Often, the solubility of ASC and PSC was maintained in the presence of NaCl up to 1 – 2 % but drastic decrease was reported when the concentration was further increased to more than 3 or 4 % (Zeng et al., 2012). Similar findings were being highlighted for the collagens from the skin of dusky spinefoot (*Siganus fuscescens*), sea chub (*Kyphosus bigibbus*), eagle ray (*Myliobatis tobijei*), red stingray (*Dasyatis akajei*), balloon fish (*Diodon holocanthus*), brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus*), striped catfish (*Pangasianodon hypophthalmus*) and barramundi (*Lates calcarifer*) (Jongjareonrak et al.,

2005a; Kittiphattanabawon et al., 2005; Bae et al., 2008; Huang et al., 2011; Singh et al., 2011; Jamilah et al., 2013).

2.1.4 Sources of Type I Collagen

Collagen can be extracted from various sources, even from almost every living animal, owing to the fact that it is one of the most abundant proteins on earth (Parenteau-Bareil et al., 2010). All collagens are extracellular proteins made up by specific amino acid composition. Skin collagens from animals are rich in glycine and alanine (constitute about 50 % of total amino acids), as well as proline and hydroxyproline (almost 20% of total amino acids). Likewise, human collagen contains approximately 33% of glycine, 21 % of proline and hydroxyproline and 11% of alanine (Neklyudov, 2003). However, bovine skin and tendons, as well as porcine skin are the common sources of collagen for different applications in the industry since decades ago. In recent years, aquatic lives gradually attracted much interest among researchers as potential sources of collagen since a considerable amount of collagen have been successfully extracted from sponges, squid, fish, sea cucumber, jellyfish and etc. In addition, recombinant collagen has also spurred interest in the development of new and non-animal sources of collagen (Olsen et al., 2003). These collagens are worth investigating because their properties differ from one source to another (Lin and Liu, 2006a).

2.1.4 (a) Human

Human originated collagens are available in the market either in the form of autologous (obtained from the patient) or isogenic (obtained from the donor or even cadavers) (Catherine, 2004). Cultures of human tissue were used as a source of collagen

and prepared for intra-dermal injection (Olsen et al., 2003). For instance, in 2003, Food and Drug Administration (FDA) approved a collagen product known as *Cosmoderm*, which was extracted and purified from tissue cultures of human foreskin fibroblast to treat facial wrinkles (Bauman, 2004). There was also collagen filler such as *Autologen* and *Dermalogen*, which were dermal matrix dispersion obtained from autologous skin of the patient and tissue banks, respectively (Cheema et al., 2011). Before it could be introduced as an intra-dermal injection product, the dermis was mechanically pulverized and solubilized with different chemical modifiers to form a dispersion with type I collagen as the major constituent, traces of type III and VI collagens, elastin, chondroitin sulfate, and other proteoglycans (Cheema et al., 2011). Also, a method along with apparatus had been reported and documented for extraction of collagen from human adipose tissue (Ftaiha, 1993).

Even though the application of autogenic human collagen eliminates any risk of virus or prion transmission and virtually no risk of an immune response, the major disadvantages, however, are the need of a prior surgery to acquire the skin and a processing time of 3 – 4 weeks (Catherine, 2004; Cheema et al., 2011). As in the case of isogenic collagen, concerns over availability and the theoretical risk of disease transmission are the main issues associated with its usage (Olsen et al., 2003).

2.1.4 (b) Land Animals

Type I collagen is commonly found in the connective tissues, including tendons, bones and skins of animal origins (Muyonga et al., 2004). Various body parts of land animals such as bovine, porcine and chicken (bone, skin, cornea and placenta)

have been traditionally exploited as sources of collagen for its wide application in the leather and film industries, in cosmetic and biomedical materials as well as in food industry (Kittiphattanabawon et al., 2005). Generally, bovine and porcine skins and bones are the main sources for these purposes. However, according to Cliche et al. (2003), biomaterials were made mainly with bovine and to a lesser extent of porcine. It was characterized by the absence of tryptophan and a low content of aromatic, heterocyclic and sulphur-containing amino acids (Neklyudov et al., 2003). Extraction and isolation of collagen from different animal skins had been attempted in literature. In fact, investigation on different extraction methods of collagen from chicken, goat, buffalo and human skin tissues was described in the work of (Quereshi et al., 2010).

The research on bovine collagen started to shed some lights in the literature around 1970s, when researchers managed to extract collagen successfully from cow skin and process it into liquid form for cosmetic or beauty industry (Armstrong, 2010). Injectable bovine collagen was then created from sterile and purified collagen from cow skin (Zahrani, 2011). As for porcine, type I collagen was extracted from pig rind or hides which was widely used in processing products such as films and sausage casings (Zahrani, 2011). A recent research by Shevchenko et al. (2008) reported that a paste formulation from porcine collagen was successfully integrated into full-thickness wounds without showing rejection and excessive inflammation to the patients, suggesting the possibility of collagen from pig hides as an effective alternative to current dermal substitutes in full-thickness wounds.

In Thailand, wastes from poultry processing industry in the form of tracheas of chicken, duck and ostrich were also evaluated as potential sources of collagen. Type I and II collagen were found as a major component of duck trachea and larynx along with small proportion of type XI collagen. The peptide patterns obtained by SDS-PAGE showed that pepsin-solubilized collagen from all samples comprised of type I and type II with different proportions (Jaroenviriyapap and Vittayanont, 2009). In earlier work by Cliche et al. (2003), it was suggested that chicken skin could also serve as a good source of high quality collagen. Available as poultry by-product, chicken skin was claimed to contain approximately 75 % of type I and 15 % of type III collagens. Extraction yields varied with the solubilization process where 38.9 % of the collagen content in the solid phase was extracted with pepsin while 25.1 % with ethylene diamine (Cliche et al., 2003). In another attempt by Deskmukh and Nimni (1969), rat skin collagen was successfully extracted using penicillamine in NaCl solution.

Not only focusing on multi-cellular animals, the tissues from the Archaeogastropod, *Nerita crepidularia*, which was more commonly known as snails or slugs, were extracted and characterized for collagen. Guanidine hydrochloride and pepsin were used to solubilize and digest the tissues of *N. crepidularia*. Yields on dry weight basis were reported to be 0.48 % and 1.28 %, respectively. The Fourier Transform Infra Red Spectrum analysis (FTIR) of the extracted collagen showed more or less same number of peaks, lying within the same range values of the commercial collagen (human placenta collagen) that was used as a standard (Palpandi et al., 2010). Similar investigations were also done on the cuticles of *Ascaris lumbricoides* and earthworm of *Lumbricus* sp. (Baccetti, 1967). Presence of collagenous connective

tissues was also reported in arthropods, in which the extracellular system was dominated by chitinous skeleton (Palpandi et al., 2010).

Nevertheless, the most significant news of the recent time is that the scientists have developed a method of extracting collagen from the alligator (*Alligator mississippiensis*) bones. Type I alligator bone collagen was proven to exhibit biochemical and thermal properties similar to type I collagen from the skin and bone of those warm-blooded animals (Bogren, 2007). In addition, Nagai et al. (2012) recently reported on the preparation of collagen from the tendon of Yezo sika deer by limited pepsin digestion method. It was proven to exhibit similar characteristics with porcine collagen via various characterization studies such as SDS-PAGE, subunit composition and thermal behavior analysis. Although type I collagen is extractible from many different sources, increasing attentions have been paid to those of aquatic-derived due to various limitations of land-animal derived collagens in terms of the availability, as well as health and religion constraints.

2.1.4 (c) Aquatic Lives

Fish processing by-products contribute as much as 70 – 85 % of the total weight of the catch, and these has typically included processing wastes from fish, shellfish, and by-catch of unutilized species, as well as underutilized species (Gómez-Guillén et al., 2011). They are normally discarded as wastes. Therefore, various efforts have been done in order to explore the possible use of these processing by-products since a lot of valuable components, especially protein, can be recovered of which collagen is inclusive. Almost 30 % of the wastes are in the form of bones and skins with

high collagen content, particularly type I collagen (Jongjareonrak et al., 2005b; See et al., 2010). Though conventional sources of collagen brought up conflicts and limitations in its applications, the demand for this biomaterial is still in the rise. Therefore, aquatic-derived collagens especially those from freshwater and marine fishes and molluscs have been gaining considerable attention in recent years as replacement of land animals or mammalian raw materials (Ri et al., 2007).

The first aquatic-derived collagen been characterized was the soluble collagen from the swim bladder of teleosts back in year 1955 (Montero et al., 1995). Following that, the number of fish or marine species studied for collagen extraction is continually growing. Extraction and functional characterization of acid- and/or pepsin soluble collagen has been reported for skins and bones of different fish species, such as, trout (*Salmo irideus*) and hake (*Merluccius merluccis*) (Montero et al., 1990), plaice (*Pleuronectes platessa*) (Montero et al., 1995), squid (*Illex argentines*) (Kołodziejska et al., 1999), cuttlefish *Sepia lycidas* (Nagai et al., 2001) and *Sepiella inermis* (Shanmugam et al., 2012), cod (*Gadus morhua*) (Sadowska et al., 2003; Żelechowska et al., 2010), bigeye snapper (*Priacanthus tayenus*) (Jongjareonrak et al., 2005b; Nalinanon et al., 2007), brownstripe red snapper (*Lutjanus vita*) (Jongjareonrak et al., 2005a), backed toadfish (*Lagocephalus gloveri*) (Senaratne et al., 2006), and skate (*Raja kenojei*) (Hwang et al., 2007).

In recent years, extensive studies have also resulted in reports on collagen extraction from the carp (*Hypophthalmichthys molitrix*)(Rodziewicz-Motowidło et al., 2008), deep-sea redfish (*Sebastes mentella*) (Wang et al., 2008a; Wang et al., 2011),

threadfin bream (*Nemipterus hexodon*) (Nalinanon and Benjakul, 2008), walleye pollack (*Theragra chalcogramma*) (Yan et al., 2008), unicorn leatherjacket (*Aluterus monoceros*) (Nalinanon and Benjakul, 2008), seaweed pipefish (*Syngnathus schlegeli*) (Khan et al., 2009), shark (*Carcharhinus limbatus*) (Kittiphattanabawon et al., 2010a), catfish (*Tachysurus maculates*) (Bama et al., 2010; Singh et al., 2011), balloon fish (*Diodon holocanthus*) (Huang et al., 2011), cobia (*Rachycentron canadum*) (Zeng et al., 2012) and barramundi (*Lates calcarifer*) (Jamilah et al., 2013). Although strict comparisons are difficult since methodologies may differ considerably from one work to another, fish collagens extracted from different species were characterized as generally containing three polypeptide chains, each consisting 1000 amino acid residues and weighing approximately 100 kDa, which was having close resemblance to mammalian and avian type I collagen (Zahrani, 2011).

Fish scales also contribute to the residues of fish processing industry and they contain proteins that are made up of collagen, keratin and mucin (Wang and Regenstein, 2009). Utilization of fish scales for collagen extraction has been reported for sea bream (*Pagrus major*) and red tilapia (*Oreochromis niloticas*) (Ikoma et al., 2003), black drum (*Pogonia cromis*) and sheepshead (*Archosargus probatocephalus*) (Ogawa et al., 2004), sardine (*Sardinops melanostictus*) (Nomura et al., 1996; Harada et al., 2007), deep-sea redfish (*Sebastes mentella*) (Wang et al., 2008a) and lizardfish (*Saurida* spp.) (Wangtueai and Noomhorm, 2009). Besides marine species, scales from freshwater fishes such as grass carp (*Ctenopharyngodon idellus*) (Li et al., 2008), snakehead (*Channa argus*) (Liu et al., 2008), Asian silver carp (*Hypophthalmichthys molitrix*) (Wang and Regenstein, 2009), rohu (*Labeo rohita*) and catla (*Catla catla*) (Pati et al.,

2010a), carp (*Cyprinus carpio*) (Zhang et al., 2011) and spotted golden goatfish (*Parupenes heptacanthus*) (Matmaroh et al., 2011) were also categorized as potential raw materials for collagen extraction. Unlike skins, fish scales are mainly composed of collagen and hydroxyapatite ($Ca_5(PO_4)_3OH$), where hydroxyapatite constitutes about 38 – 46 % with a small amount of calcium carbonate ($CaCO_3$) (Wang and Regenstein, 2009; Zhang et al., 2011). Consequently, pre-treatment to remove Ca component from the fish scales before extraction process is vital in order to obtain final yield and purity of the collagen (Gómez-Guillén et al., 2011). Pati et al., (2010a) reported that collagen from the scales of rohu (*Labeo rohita*) and catla (*Catla catla*) possessed similarity in properties to mammalian counterpart whilst Li et al. (2008) found that collagen from the scales of grass carp fish (*Ctenopharyngodon idellus*) was made up of $\alpha_1\alpha_2\alpha_3$ heterotrimer. On top of that, the abundance of glycine and high contents of alanine, proline, hydroxyproline and glutamic acid in collagens from the scales of sardine (*Sardinops melanostictus*), red sea bream (*Pagrus major*) and Japanese sea bass (*Lateolabrax japonicus*) (Nagai et al., 2004) had aroused the interest among researchers to further exploiting fish scales as one of the promising sources of collagen.

Isolation and characterization of marine invertebrate collagens from the body wall of starfish (*Asterias amurensis*) (Lee et al., 2009), purple sea urchin (*Anthocidaris crassispina*) (Nagai and Suzuki, 2000) and sea cucumber (*Stichopus japonicus*) (Saito et al., 2006) confirmed the existance of fibrillar and non-fibrillar collagen in these invertebrates. It was further supported by the work of Toshiharu (1972) who described that the collagen of echinodermal animals was similar in chemical properties to mammalian skin collagen. In separate work by Ri et al., (2007) and Mizuta et al. (2007),

collagen was also extracted from scallop *Patinopecten yessoensis* mantle and it was found that collagen fiber was densely distributed in the inner connective tissue matrix of the mantle pallial in comparison to the inner fold part which was rich in muscle fibers. As for sea cucumber, about 20.8 % and 24.3 % (dry weight basis) collagens extracted from the skin and connective tissue, respectively, of *Parastichopus californicus* were characterized as type I with three α_1 chains of approximately 138 kDa each (Liu et al., 2010).

These reports indicated that marine sources have potential in supplementing the skin of land vertebrates and invertebrates as a source of collagen. Aquatic-derived collagens are used widely in food and in the manufacture of glues (Wasswa et al., 2007). Fish collagens form the typical fibrillar collagen structures but differ at the primary amino acid sequence level (Wijesundara and Malaweera, 2013). The total imino acid content of aquatic-derived collagen is significantly lower where the amount of hydroxyproline is only about 62 % of that found in calf skin collagen (Olsen et al., 2003). As a result, aquatic-derived collagen has a significant lower melting temperature which results in lower thermal stability.

2.1.4 (d) Recombinant Collagen

The tools of recombinant protein expression are now being applied to provide recombinant sources for collagen. The primary focus is to explore other alternatives to bovine collagen for its applications. Several recombinant systems have been developed for the production of human sequence collagens. With the application of genetic engineering, recombinant collagen have been successfully reported in yeast (Olsen et al.,

2001; Myllyharju, 2009), tobacco (Merle et al., 2002), silkworms (Tomita et al., 2003), *Escherichia coli (E-coli)* (Hua et al., 2006; Xue et al., 2010), and barley (Ritala et al., 2008). Prolyl 4-hydroxylase (P4H), an $\alpha_2\beta_2$ tetramer in vertebrates, plays an important role in the synthesis of all collagens, as 4-hydroxyproline-deficient collagen polypeptide chains cannot form triple helices that are thermally stable (Olsen et al., 2003; Myllyharju, 2009). P4H is also required to form hydroxyproline within the collagen chains via hydroxylation of proline and lysine for the assembly and physiological stability of the final triple helix conformation (Shoseyov et al., 2010). In order to produce recombinant collagen, an active P4H tetramer from its subunits needs to be assembled *in vitro* (Myllyharju, 2009) and it was found that the coexpression of collagen and mammalian-derived prolyl-hydroxylase in yeast, insects, bacteria and plants supports the formation of stable and hydroxylated collagen (Shoseyov et al., 2010).

Recombinant collagens and gelatins have the potential to alleviate many of the issues associated with animal-derived versions. *E-coli* is a widely used bacterial platform for expressing simple heterologous proteins. Due to its low manufacturing costs, production of human-like collagen (HLC) using *E-coli* remains the choice of processing. However, recombinant *E-coli* fermentation possesses the problem of acetic acid production which can inhibit both cell growth and recombinant HLC production (Xue et al., 2010). In addition, costly infrastructure and limited production capacity associated with expression of recombinant molecules via microbial fermentation or mammalian cell expression systems also hinder the development of recombinant collagen (Shoseyov et al., 2010).

2.1.5 Type I Collagen Extraction Methods

Extraction of collagen can be divided into three main parts which include preparation of raw material, extraction and finally the recovery process (Benjakul et al., 2012). Only a very minimal fraction of collagen dissolves in water and the amount depends on the age of the animal and type of tissue extracted (Nair et al., 2010). Kasoju et al., (2010) described that the major obstacle for dissolution of collagen from animal tissues is the presence of covalent cross-links between the collagen molecules. Collagen from tissues of young animals is not as extensively cross-linked as that of mature tissues and thus more amenable to extraction. The nature of cross-links in different tissues therefore determines the particular solvent to be used and the amount of collagen yield extracted (Nair et al., 2010). For aquatic-derived collagen, especially from fish, collagen can be extracted from the skins, bones and scales. These raw materials require cleaning, size reduction and appropriate pre-treatment prior to the extraction process (Benjakul et al., 2012). For most of the studies, collagen extraction is normally done by acid extraction, in which the resulting collagen is termed "acid soluble collagen". Nevertheless, the acid process results in low yield and the application of pepsin in combination with acid extraction is then implemented to increase the yield of collagen. The resultant collagen is referred to as "pepsin soluble collagen" (Whitehurst and Oort, 2009). All procedures are performed at low temperature (approximately 4°C) in order to avoid thermal denaturation and bacterial growth (Benjakul et al., 2012).

2.1.5 (a) Sample/ Raw Material Preparation

Generally, the presence of contaminants in the form of non-collagenous proteins, lipids or colour pigment, are expected in the raw materials for collagen extraction

(Whitehurst and Oort, 2009). As for fish scales and bones, they are also containing calcium and other inorganic matters in which several pre-treatments are necessary to remove the contaminants so as to obtain higher purity of the extracted collagen (Benjakul et al., 2012). The removal of residual meat (flesh) and cleaning steps are performed before they are pre-treated with chemicals. Alkaline pre-treatment to remove non-collagenous proteins and colour pigments is one of the widely used method where NaOH solution at a concentration of 0.1 M is used as the solvent (Jongjareonrak et al., 2005b; Wang et al., 2008a; Kittiphattanabawon et al., 2010a). NaCl and H_2O_2 have also been used for the same purposes (Wijesundara and Malaweera, 2013). In order to eliminate calcium from bones and scales of fishes, ethylenediaminetetraacetic acid (EDTA) is used for the decalcification due to its chelating funtion. Consequently, it leads to the formation of porous and decalcified raw material with higher surface area that can be readily subjected to collagen extraction (Benjakul et al., 2012). Pre-treated fish skins, on the other hand, also require defatting step where by butyl alcohol is used to remove lipids and fats prior to the collagen extraction process (Yan et al., 2008; Singh et al., 2011).

2.1.5 (b) Neutral Salt Extraction

Neutral salt solution (0.15 – 2 M NaCl) is one of the most commonly used solvent to extract freshly synthesized and negligibly (minimal) crosslinked collagen from tissues or raw materials (Nair et al., 2010). As reported by Gross et al. (1955), the electron microscope examination of fibrils after salt extraction showed that most of the collagen fibrils were relatively normal in appearance, in compared to extraction by acid or acid buffers where the structural organization of the fibrils was sometimes badly

disrupted. In another work by Neklyudov et al. (2003) to find the best raw materials among porcine skin, subcutaneous tissue of cattle and various tendon types of cattle to obtain collagen fractions with physicochemical properties required for utilization in food industry, water-salt extraction of collagen from these raw materials had been carried out. Minced samples of collagen-containing raw materials were mixed with 1 – 10 % solution of NaCl at ratio ranging from 1:1 to 1:10 w/w. Collagen fractions were then extracted under continuous stirring and temperature controlled environment. Even though most tissues have little or no salt-extractable collagen, difference in temperature, rate of stirring and solvent to tissue ratio will inevitably alter the composition of the extracted and purified collagen (Kasoju et al., 2010; Nair et al., 2010).

2.1.5 (c) Dilute Acid Extraction

Collagen extraction process via acid solubilization is the most favorable method for collagen preparation. It is more efficient than the neutral salt solution (Kasoju et al., 2010) because under acidic condition, positive charge of the collagen polypeptides becomes dominant, enhancing the repulsion among tropocollagens, thus promoting solubilization in the solvent (Benjakul et al., 2012). Generally, this extraction method does not pose any damage on the triple helical structure of the extracted collagen. It was evidenced by the Fourier transform infrared (FTIR) spectra of the acid soluble collagens from the skin of striped catfish (*Pangasianodon hypophthalmus*) and Nile perch (*Lates niloticus*) where presence of helical structure was found (Muyonga et al., 2004; Singh et al., 2011). Generally, collagen extraction from various sources can be carried out with both organic acids (acetic, chloroacetic, citric and lactic) and inorganic acid (hydrochloric). The yield of extracted collagen depends on the animal species used and

the age as well as the parameters of extraction. A number of studies have referred to collagen from different marine animals extracted by acetic acid (Skierka and Sadowska, 2007). Among all, extraction of collagen in 0.5 M acetic acid was widely carried out and the yields varied from 2 – 90 %, especially those reported in the skin of young and adult Nile perch (*Lates niloticus*) (Muyonga et al., 2004), skin of Baltic cod (*Gadus morhua*) (Sadowska et al., 2003), skin and bone of bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon et al., 2005) as well as skin of grass carp (*Ctenopharyngodon idella*) (Wang et al., 2009).

The solubility of collagen in acid solution plays a key role in determining the extraction efficiency. Increasing the concentration of acetic acid from 0.1 to 0.5 M resulted in increasing yield of minced cod (*Gadus morhua*) skin collagen from 52 to 59 % (Benjakul et al., 2012). Similar observation was reported by Wang et al. (2009) in which the amount of grass carp skin (*Ctenopharyngodon idella*) collagen extracted was increased with the increase of acetic acid concentration to 0.5 M, and thereafter decreased. In order to improve the resulting yield of acid soluble collagen (ASC), re-extraction of the residual materials with 0.5 M acetic acid was also attempted. For instance, re-extraction was performed for bone of carp (*Cyprinus carpio*) (Duan et al., 2009) and also skin and bone of bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon et al., 2005) to increase the yield of ASC. Nevertheless, numerous reports in the literature were associated with the findings that not all raw materials dissolved completely in the acidic extracting solvent, greatly influenced by the cross-links of collagen in the samples (Nagai et al., 2001; Muyonga et al., 2004; Huang et al., 2011).

2.1.5 (d) Enzyme Aided Acid Extraction

In order to solubilize an additional fraction of collagen, digestion of collagen-containing tissues with enzyme particularly pepsin in acid solution was performed. By applying the enzymatic pre-treatment of connective tissues with proteolytic enzymes non-specific for collagen such as pepsin, trypsin, pancreatin, ficin, bromelain or papain, the yield of extracted collagen could be improved (Żelechowska et al., 2010). Under these circumstances, in order to obtain collagen from animal skin for instance, the cleaned skin was hydrolysed or treated with an acid such as acetic acid before could be digested with enzymes such as pepsin to remove telopeptides (Aukkanit and Garnjanagoonchorn, 2010). The enzyme removed only the non-helical ends (telopeptides) of the collagen. Because of the cutting of the non-helical region, they removed inter-molecular crosslinks even when they were the most stable in acid medium. In literature, few reports had been published on the improvement of collagen extraction efficiency by pepsin digestion (Nalinanon et al., 2007; Bama et al., 2010; Singh et al., 2011). Pepsin digestion rendered a higher yield as compared to the yield of solely acid extracted collagen (Benjakul et al., 2012). The difference in extracted ASC and pepsin soluble collagen (PSC) suggested that there were many inter-chain cross-links at the telopeptide region of the collagen which resulted in low solubility in acid. Pepsin cleaved the crosslinked regions at the telopeptide without damaging the integrity of the triple helix and thus increased the solubility of collagen in acid (Bama et al., 2010).

Benjakul et al. (2012) recently provided further description on the difference of yields between ASC and PSC, focusing on the impact of pepsin treatment on the proportion of compositions of the extracted collagen. It was reported that more β- and γ-

chains, dimmer and trimer were noticed in ASC in contrast to PSC. This led to the speculation that cleavage of telopeptide region by pepsin resulted in the conversion of cross-links, such as the β- and γ-chains, into the α-chains, which could be of ease for extraction. This was in agreement with the findings in various sources such as the skin of ocellate puffer fish (*Takifugu rubripes*) (Nagai et al., 2002), skin of brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak et al., 2005a), skin of largefin longbarbel catfish (*Mystus macropterus*) (Zhang et al., 2009), seaweed pipefish (*Syngnathus schlegeli*) (Khan et al., 2009), skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010a) and the skin of blacktip shark (*Carcharhinus limbatus*) (Kittiphattanabawon et al., 2010b), where the extraction efficiency was increased through pepsin digestion in acidic extracting solvent. Flow chart for the preparation of ASC and PSC is shown is Figure 2.4.

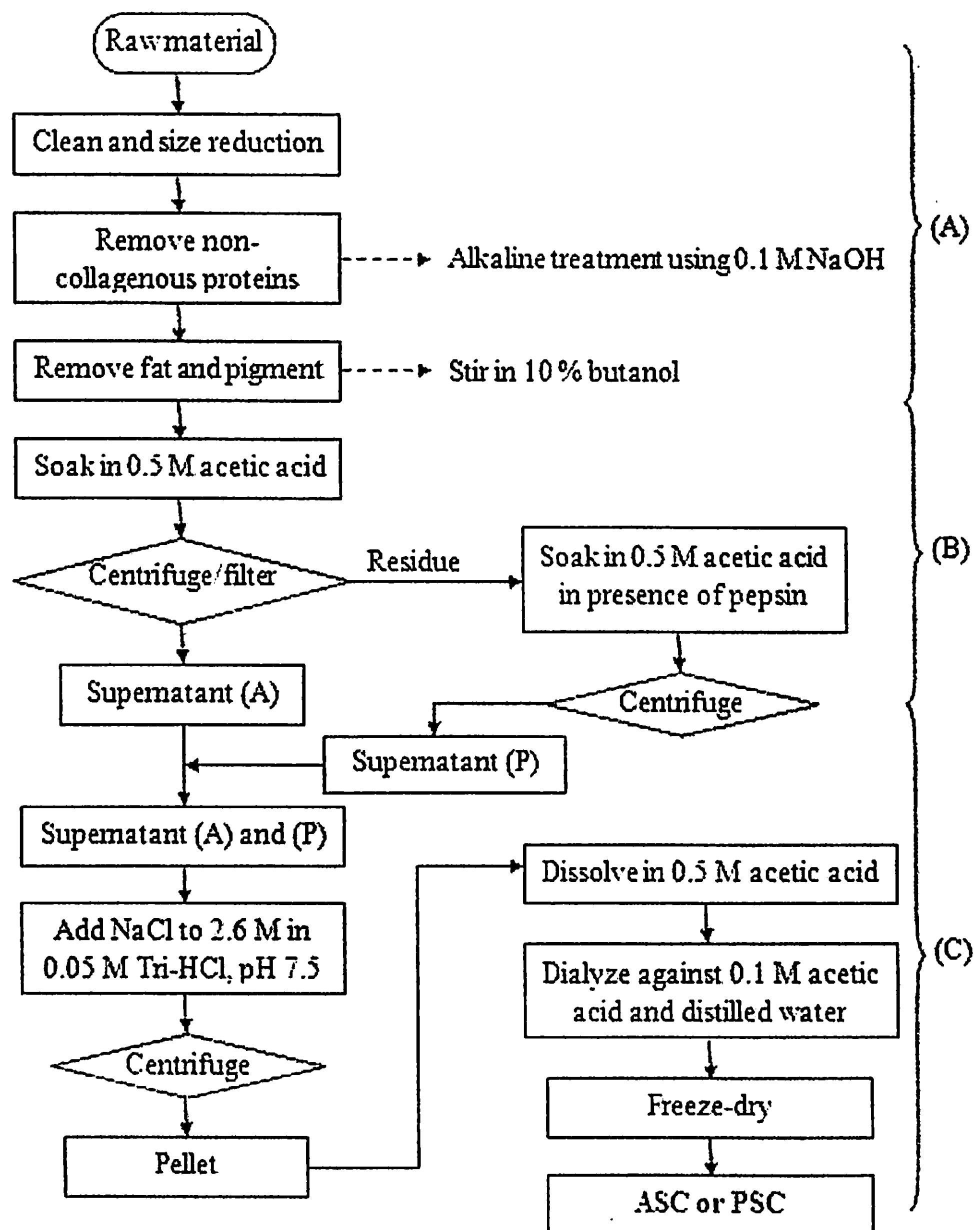

Figure 2.4: Flow chart for the general preparation of acid soluble collagen (ASC) and pepsin soluble collagen (PSC). All procedures are performed at 4°C. (A) Preparation of raw material. (B) Extraction. (C) Recovery (Benjakul et al., 2012).

2.1.5 (e) Recovery of Collagen

After extraction, the collagen was recovered by salt precipitation before subjected to dialysis and freeze-drying. The concentration of NaCl used for the collagen precipitation in different studies varied from 0.8 to 2.6 M (Nagai and Suzuki, 2000; Jongjareonrak et al., 2005a; Nalinanon et al., 2007). Precipitation was aimed to maximize the collagen recovery and remove impurities in the extract (Benjakul et al., 2012). It was then followed by dialysis in very dilute acid solutions and distilled water prior to freeze drying. The freeze-dried product or sample is used as collagen and termed ASC or PSC based on the preparation method.

2.1.6 Process Parameters Affecting Type I Collagen Extraction Efficiency

Influence of specific process parameter towards extraction process is crucial in improving the efficiency and achieving a time saving as well as cost-efficient process. Collagen has a unique structure, size and amino acid sequence. The molecule consists of three polypeptide chains intertwined and resembles a three-stranded rope. Non-helical telopeptides are attached to both ends of the molecule. Telopeptide-poor collagen, which was produced by eliminating the telopeptide by non-specific enzymes or pepsin, was reported as a good example formed under acidic conditions. Severe conditions would damage the integrity of collagen (Lin and Liu, 2006a). Therefore, most studies of collagen extraction had been limited to low temperature and short time exposure to enzyme. Effects of acetic acid concentration, extraction temperature, extraction time, enzyme dosage and solvent to material ratio were among the parameters which had been investigated in literature in collagen extraction processes. It is necessary to understand

the effects of extraction conditions in order to obtain higher recovery of collagen (Wang et al., 2008b).

2.1.6 (a) Acetic Acid Concentration

With great extractability towards collagen, acetic acid is the most popular solvent used in most of the collagen extraction processes. Effect of acetic acid concentration had been studied to investigate its impact on the extraction efficiency of collagen extraction from body parts of grass carp (*Ctenopharyngodon idella*) such as the skin (Wang et al., 2008b; Wang et al., 2009) and the swim bladder (Zhang et al., 2010a). Acid concentration was varied from 0.1 to 0.9 M and the yields were compared. From the results, increment in the yield was observed with the increase of acetic acid concentration to approximately 0.5 M, but thereafter decreased. Verheul et al. (1998) mentioned that pH value was in charge of the density of protein, which would modify the electrostatic interactions between protein molecules and their structures. Difference in acetic acid concentration led to the variations in the pH of the extraction bulk, thus affecting the solubility of collagen in the solvent. With the increase of acid concentration, pH of the extraction bulk was lowered and denaturation of collagen was expected (Carvalho et al., 2003). This explained the finding that lesser yield was achieved in the solvent concentration of 0.7 M and 0.9 M, compared to the one at 0.5 M (Wang et al., 2009).

2.1.6 (b) Extraction Temperature

The ability of collagen molecule to maintain its native 3D structure is a determination factor of whether it can function properly as a biomaterial, e.g. to form

films or porous sheets. Most extraction studies have been conducted at low temperatures (usually 4°C) to avoid damage to the collagen integrity (Aukkanit and Garnjanagoonchorn, 2010). Collagen was a thermal instable protein which could be denatured at room temperature and this sensitivity to temperature was associated with its chemical structure (Wang et al., 2009). Lin and Liu (2006b) in their recent study to investigate the effects of pepsin digestion at different temperatures and times on the properties of telopeptide-poor collagen from bird feet reported that the yield of bird feet treated at 4 °C was significantly lower than those of the treatments at 12, 18 and 24 °C. Nonetheless, the SDS-PAGE electrophoretogram revealed that more low molecular fragments present in the collagen samples treated at 12, 18 and 24 °C compared to the one at 4°C. It proved that temperature was an important parameter influencing the yields and characteristics of extracted collagen. Similarly, pepsin-solubilised collagens were extracted from the fresh skin of silver-line grunt (*Pomadasys kaakan*) at 4, 10, 20 and 28 °C for 6 hours. Aukkanit and Garnjanagoonchorn (2010) reported that the extraction at 10°C gave the highest yield of collagens (439.32 ± 96.43 mg/g fresh skin, dry basis), which were identified as type I. Extraction was not favourable at too low temperature due to the reduced activity of pepsin at lower temperature. However, extraction at higher temperatures of 20 – 28°C resulted in the formation of low-molecular weight peptide fragments, thus reducing the yield of type I collagen (Aukkanit and Garnjanagoonchorn, 2010).

The nature of collagen varies depending on the sources, particularly variation in the amino acid composition especially the levels of imino acids (proline and hydroxyproline) among different sources. Collagen with lower amount of

hydroxyproline showed lower thermal stability than those with relatively higher amount (Muyonga et al., 2004). Presence of hydroxyproline in the inter-chain linkage of the hydrogen bond would stabilize the triple-helix structure of collagen. Consequently, different denaturation temperatures were reported for collagen from different sources. In short, temperature control along collagen extraction was vital to maintain and keep the integrity of collagen. Increase of temperature to a certain extent could improve the solubility of collagen and accelerate the mass transfer. However, high temperature also led to the denaturation of collagen, which reduced the solubility in solvent (Wang et al., 2009).

2.1.6 (c) Extraction Time

The effect of time on the yield and properties of collagen is also important. A positive relationship was found between time and yield of collagen where the yield increased with the extension of time especially in the range of 12 – 24 hours for the extraction of acid soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*) (Wang et al., 2009). Though improvement in yield was observed when the extraction period was longer than 24 hours, it was not significant. Likewise, the optimal extraction period for collagen extraction from yellowfin tuna (*Thunnus albacores*) dorsal skin was found to be approximately 23.5 hours even though it was extended to 36 hours (Woo et al., 2008). Extraction of collagen from Baltic cod (*Gadus morhua*) skin also showed that collagen yield at 72 hours was not significantly different from that of 24 hours (Sadowska et al., 2003).

In classical extraction, the rate of mass transfer was controlled by the diffusion process of analyte from the matrix into the extracting bulk which was time-concerned. As described by Wang et al. (2008b), the recovery of analyte would keep increasing along with the extension of time. However, in the presence of pepsin digestion, different extraction periods would be the main influence affecting the yield and properties of the extracted collagen (Aukkanit and Garnjanagoonchorn, 2010). Extended extraction time was reported to result in the loss of integrity of collagenous materials. At a higher temperature and longer period of pepsin digestion, larger amount of telopeptide of tropocollagen would be digested and formation of collagen with less fibril-forming ability would be promoted. The fibril forming capacity was an important index of collagen molecular integrity and denaturation was the main reason of the reduction in collagen fibril-forming ability (Lin and Liu, 2006b).

2.1.6 (d) Solvent to Material Ratio

Solvent to material ratio is an important variable affecting the extraction efficiency. Larger solvent to material ratio enhanced the extraction process by inducing greater concentration gradient and promoting the diffusion of desired solute from the matrix into extracting solvent. In the case of collagen extraction from grass carp (*Ctenopharyngodon idella*) skin, an increasing solvent to material ratio led to a higher yield of collagen. The ratio was varied from 5 to 25 mL/g and when the ratio was greater than 15 mL/g, improvement in the collagen yield was no longer obvious (Wang et al., 2009). As for the work of Zhang et al. (2010a), the optimal ratio to achieve higher yield of acid soluble collagen from the swim bladders of grass carp (*Ctenopharyngodon idella*) was found to be 17.85 mL/g. Therefore, it was necessary to determine the appropriate or

optimum amount of solvent to be used in the extraction process so as to achieve highest yield without the wastage of solvent.

2.1.6 (e) Enzyme Dosage

Enzymatic pre-treatment of raw material enhanced the yield of collagen extracted by removing intra- and inter-molecular covalent cross-links in the collagen molecules, especially the lysine and hydroxylysine residues, ester bonds and bonds with saccharides (Skierka and Sadowska, 2007). Pepsin, trypsin, pancreatin, ficin, bromelain and papain were among the proteolytic enzymes used in various collagen extraction studies. Besides optimization study on collagen extraction from yellowfin tuna (*Thunnus albacores*) dorsal skin, grass carp (*Ctenopharyngodon idella*) skin and pig hide, not much have been reported for the effects of different enzyme dosage on the extraction yield in literature (Wang et al., 2008b; Woo et al., 2008). A positive correlation was found between the yield and amount of enzyme in all studies. Nevertheless, excessive enzyme dosage could lead to extreme digestion of collagen, resulting in more low-molecular weight peptide fragments thus lower extraction yield.

2.1.7 Applications of Type I Collagen

Collagen is widely known as an excellent biomaterial, especially biopolymer that offers its utilization in diverse applications particularly in industrial and biomedical fields. In the former, collagen has been utilized for quite a long time, whilst the latter have placed collagen as an object of intense research in recent years (Ezquerra-Brauer et al., 2012). In pharmaceutical applications, collagen can be used for production of wound dressings, vitreous implants and as carriers for drug delivery (Senaratne et al., 2006;

Singh et al., 2011). With its moisturizing and anti-aging properties, collagen stands as another popular constituent in cosmetics and skin care application (Senaratne et al., 2006). Industrially, it has been utilized to produce edible casings for the meat processing industries (sausages/ salami/ snack sticks etc.) which are made of dispersed insoluble collagen extruded as a tube and formed or cut into links as it is filled (Meena et al., 1999; Singh et al., 2011). In addition to that, denatured collagen in the form of gelatin is also widely utilized in food processing industry (Senaratne et al., 2006). In short, an increasing number of new applications have been found for collagen in products, in line with the growing trend to replace synthetic agents with more natural ones (Gómez-Guillén et al., 2011).

2.1.7 (a) Biomedical Application

In biomedical field, biomaterials are referring to those natural or artificial materials that are used as a whole or as part of a system which treats or replaces a tissue, organ or function of the human or animal body (Albu et al., 2011). Collagen represents one of the most used biomaterials owing to various unique features and characteristics such as excellent biocompatibility, biodegradability and weak antigenecity, well-established structure, biologic characteristics and to the way it interacts with the body (Lee et al., 2001). Due to these properties, type I fibrillar collagen can be processed into diverse form of biomaterials to be used as burn or wound dressings, osteogenic and bone filling materials, antithrombogenic surfaces, collagen shields in ophthalmology, being also utilized in tissue engineering for applications such as skin replacement, bone substitutes and artificial blood vessels as well as aortic heart valves repair (Albu et al., 2011). The main attraction of collagen exploitation as a biomaterial is probably due to its

low immunogenicity (Ezquerra-Brauer et al., 2012), where the implantation of collagen has long been proven to show good tissue tolerance both *in vivo* and *vitro* since 1970s (Zahrani, 2011).

Collagen is also a common constituent of soaps, shampoos, facial creams, body lotions and other cosmetic products (Zahrani, 2011). It is found that the use of collagen in cosmetic emulsions resulted in safe and stable product. Besides exhibiting advantages such as non-irritating to skin, collagen is also effective in penetrating aging skin to improve moisture content and elasticity of the skin (Meena et al., 1999). At present, the improvement and development in cosmetic surgery has initiated yet another spike to the collagen business where a simple injection of collagen in the form of dermal filler is used to fill the area under the skin (Zahrani, 2011). Since collagen plays an important role in the formation of tissues and organs, and is involved in various functional expressions of cells (Nair et al., 2010), it becomes an essential suture material and haemostatic agent in orthopedics, surgeries and dental treatments (Meena et al., 1999; Pati et al., 2010b; Zahrani, 2011).

Lee et al. (2001) and Nair et al. (2010) described the usage of collagen in drug delivery system as broad and comprehensive as it could be extracted into aqueous solution and molded into various forms of delivery systems. Collagen can be processed into different kinds of presentations such as sheets, tubes, sponges, powders, films, injectable solutions and dispersions, making it a functional component for specific application and system (Ezquerra-Brauer et al., 2012). For instances, collagen acts as drug delivery agents as in shields in ophthalmology, sponges for burns/wounds, mini-

pellets and tablets for protein delivery, gel formulation in combination with liposomes for sustained drug delivery, as controlling material for transdermal delivery, nanoparticles for gene delivery, aqueous injection for local cancer treatment as well as films for the delivery of human growth hormone, immunostimulants, tetracycline and growth factors (Meena et al., 1999; Lee et al., 2001; Nair et al., 2010). Apart from this, another recent discovery about collagen employment in biomedical industry is reported in the formulation of membranes and hydrogels, in which collagen interacts with another material to form a composite (e.g. chitosan), with practical emphasis on medicine, dentistry and pharmacology (Thacharodi and Rao, 1995; Lima et al., 2006).

The usage of collagen in biomedical application has been rapidly growing and widely expanding to bioengineering areas. This is primarily because it is easily absorbable in the body and has very low antigenicity that poses no harm and toxic to the recipients (Lee et al., 2001). Reports of adverse reactions to collagen have only been restricted to localized redness and swelling following plastic surgery using collagen implants and wound breakdown with the use of catgut suture material (Webster et al., 1984; Carroll, 1989). With that, advantages and disadvantages of collagen as a biomaterial in biomedical industry are summarized and presented in Table 2.2.

Table 2.2: Main advantages and disadvantages of collagen as a biomaterial in biomedical applications (Lee et al., 2001; Nair et al., 2010; Ezquerra-Brauer et al., 2012)

Advantages	Disadvantages
1) Available in abundance and easily purified from living organisms	1) High purification cost

2) Non-toxic and non-antigenic	2) Complex handling properties
3) Biodegradable and biocompatible	3) Variability in isolated collagen
4) Synergic with bioactive compounds	4) Hydrophilicity may cause swelling
5) Promotes blood coagulation	5) Possible side effects (mineralization)
6) Can be formulated in different forms	
7) Biodegradability can be regulated by cross-linking	
8) Easily modified to produce materials as desired	
9) Compatible with synthetic polymers	

2.1.7 (b) Non-Biomedical Application

The safety of collagen for human consumption is also evidenced by its diverse general applications in various industry not related to bio-medical field. According to Zahrani (2011), the knowledge that collagen produces gelatin in hydrolyzed state (or denatured and degraded by heat and chemicals) and that gelatin can be used as fine glue dated back to 8000 years when humans would use collagen-based glue as a protective lining on rope baskets, embroidered fabrics, to hold utensils, also in crisscross decorations on human skulls. If collagen is heated and solubilized, the three tropocollagen strands separate into globular and random coils, producing gelatin that is not a good dietary source due to lacking of adequate amounts of essential amino acids (Gómez-Guillén et al., 2011). Following that, the Egyptians used it as an adhesive about 4000 years ago, while the native Americans used it in bows about 1500 years ago by boiling the skin, hooves and sinews of horses and other animals to obtain glue (Zahrani, 2011).

In the food industry, an increasing number of new applications have been found for collagen in products such as emulsifiers, foaming agents, colloid stabilizers, fining agents, biodegradable packaging materials and microencapsulating agents, particularly for meat processing industries (Gómez-Guillén et al., 2011). Up to present, collagen is the most commercially successful edible protein film where film-forming collagen has been traditionally used in the meat industry, for the production of edible sausage casings. It has marked a success in replacing natural gut casings for sausages (Senaratne et al., 2006; Alizadeh and Behfar, 2013). It was reported that collagen films and casings were capable to act as an effective barrier to prevent the penetration of moisture, oxygen, carbon dioxide, fatty and aromatic substances, thus helped to increase the shelf life of food (e. g. fruits, vegetables, candies and nuts) and maintain their quality (Alizadeh and Behfar, 2013). On the other hand, collagen is also another important component in leather industry. The presence of collagen in animal skins which predominates other classes of proteins such as elastin, keratin, glycoproteins, albumins and globulins, is very much desired because it is responsible for the formation of leather by combination with tanning agents (Kanagaraj et al., 2006).

2.2 Malaysian Freshwater Fishes

2.2.1 Aquaculture in Malaysia

Aquaculture in Malaysia began to develop in the 1920's with extensive culture of Chinese carps, mainly the bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*) and grass carp (*Clarias idellus*) in ex-mining pools. In the mid 1930's, marine shrimp trapping ponds were first developed in Johor, the southern state of Peninsular Malaysia. It was then until the early 1940's that the culture of blood cockles *(Anadara*

granosa) began. Later in the mid 1950's, extensive culture of freshwater fish was then started in earthen ponds (FAO, 2012). As for Sabah and Sarawak (East Malaysia), the aquaculture sector had only started to grow in the early 1990's. Currently, there are three practices of Malaysia's aquaculture which include the fresh water, brackish water and marine aquaculture (Hamdan et al., 2012). Consequently, aquaculture has been identified as a potential important export earner after oil palm and rubber in Malaysia.

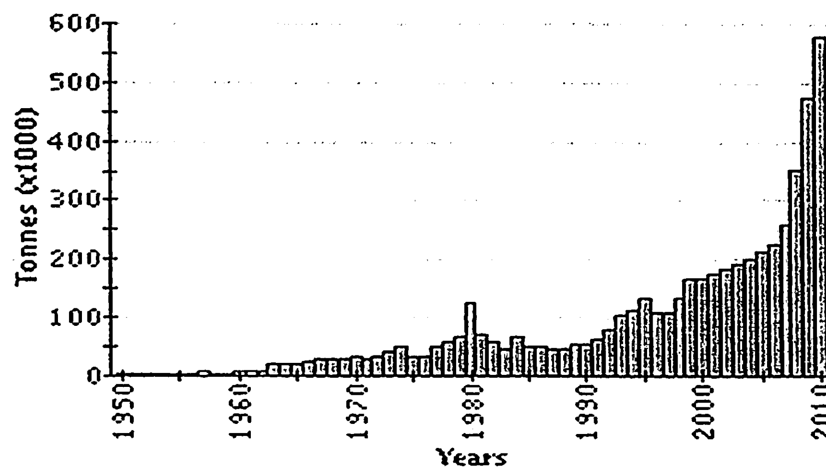

Figure 2.5: Reported aquaculture production in Malaysia (from 1950) (FAO, 2012).

According to the report by Food and Agriculture Organization, FAO (2012), aquaculture is one of the most productive agricultural sectors in terms of income per hectare per annum and return to investment, in comparison to other sectors such as oil palm, rubber, paddy, fruits and vegetables. In the year 2011, a total of 28 599 fish farmers and culturist were involved in the aquaculture industry, marking an increase of approximately 8.78 % as compared to 26 291 persons in year 2010 (DOF, 2011). This has noted a success in the attempt by the Department of Fisheries (DOF) Malaysia to encourage the expansion of freshwater industry (Airina and Jamaludin, 2012). In year

1990, production from aquaculture was approximately 52 302 tonnes and by 1994, production had doubled to 114 114 tonnes. The production of aquaculture has been steadily increased since then. Figure 2.5 shows the total aquaculture production in Malaysia according to FAO statistics from the year 1950 to 2010.

Table 2.3: Production and value of freshwater aquaculture, Malaysia 2011 (DOF, 2011)

Freshwater Aquaculture	Quantity (tonnes)	Value (RM Million)
Keli (Freshwater Catfish)	46 777.80	153.06
Tilapia Merah (Red Tilapia)	33 259.93	247.59
Patin (River Catfish)	10 891.51	95.99
Tilapia Hitam (Black Tilapia)	9 536.30	54.33
Udang Galah (Giant Freshwater Prawn)	334.44	9.34
Others	21 428.75	123.94
Grand Total	122 218.73	684.15

Freshwater aquaculture is generally increasing in Malaysia where the culture of freshwater fishes for food is developed and established in ponds, ex-mining pools and cages (Anon, 2011). According to Ng (2011), freshwater species is defined as the species that spend their adult lives and breed in freshwater beyond tidal influence. The majority species used in aquaculture are of freshwater catfish, river catfish, red tilapia, black tilapia and freshwater prawn. The production and values of these freshwater species are presented in Table 2.3, contributed almost 122 218 tonnes that valued about RM 684 million to the fishery industry in year 2011 (DOF, 2011). Even though this sector is still developing compared to neighboring countries like Thailand and Indonesia,

it is categorized as a strategic industry to fulfill the domestic demand of high protein resources and export demand of fish products (Hamdan et al., 2012).

Investment and allocation of huge funding by the Malaysian government were among the actions taken to improve and provide better facilities to the aquaculture industrial zone area in recent years (FAO, 2012). Aquaculture activities especially freshwater industry contributed to the increasing production of national food as well as resolved the problems of insufficient marine fisheries landings and exploitation of marine fishes (Hamdan et al., 2012). In addition, it was also important to reduce the poverty problem especially in rural areas even though with the traditional aquaculture practices and benefitted the communities by the generation of income and employment. Major freshwater fisheries districts in Peninsular Malaysia are depicted in Figure 2.6.

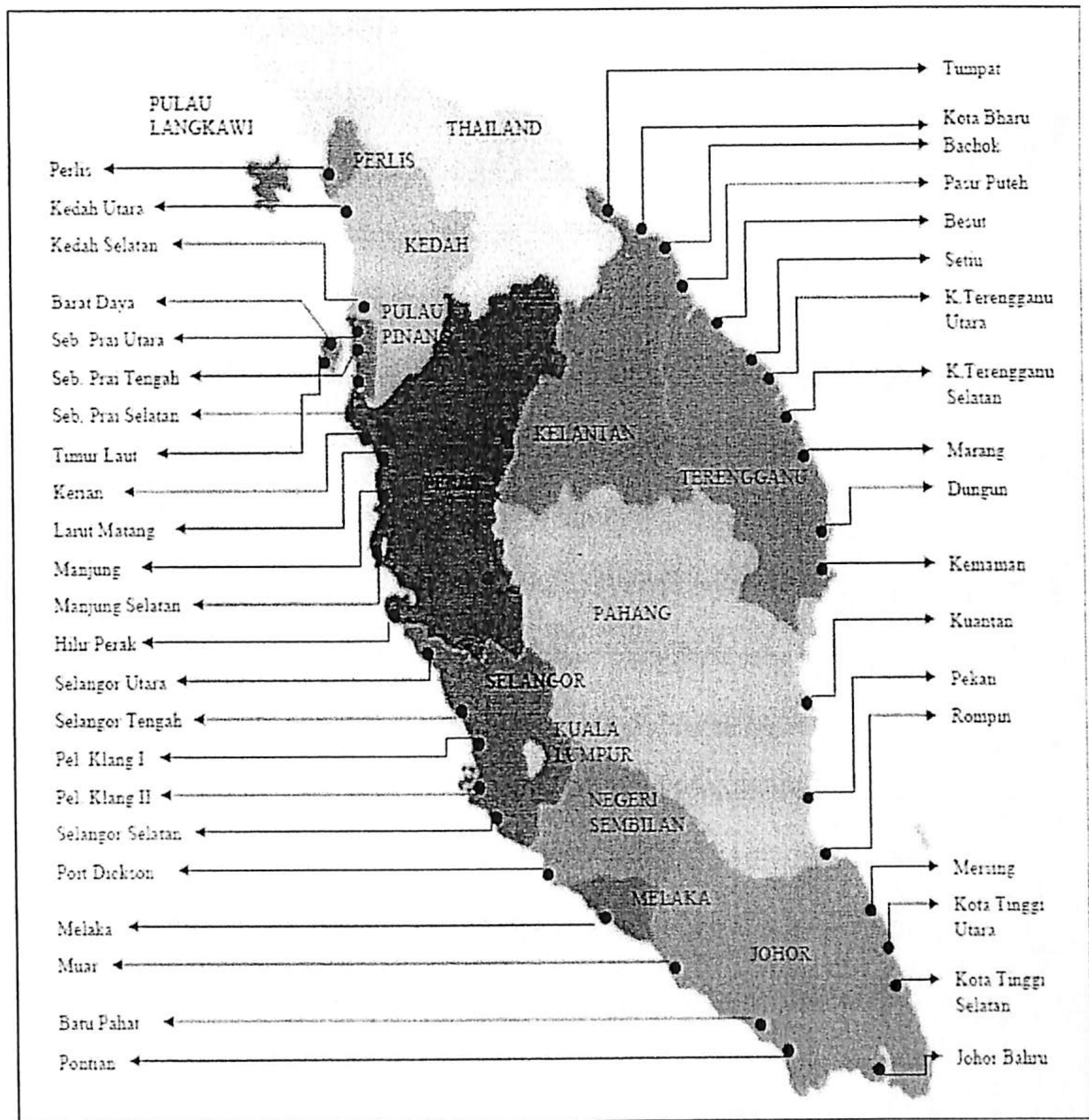

Figure 2.6: Fisheries districts in Peninsular Malaysia (DOF, 2011).

2.2.2 Applications of Malaysian Freshwater Fishes

2.2.2 (a) Food Source

The demand for fish in the world generally and in Malaysia particularly, has increased considerably in recent years because fish is well-known as an excellent and relatively cheaper source of animal protein of high biological value (Mokhtar et al., 2005;

Jabeen and Chaudhry, 2011). According to the report of Marine Institute of Malaysia (MIMA), a dramatic increase in the fish consumption among Malaysians was observed where an average Malaysian consumed approximately 54 kg per annum in year 2010 as compared to 20 kg in year 1970 (Teh, 2012). Freshwater fishes stand as an important source of protein to the communities in Malaysia where marine fishes are limited and hard to obtain, especially in inland areas (Yaakob and Ahyaudin, 1994). Freshwater fishes were also found to be a viable resource to meet the demand in the market when there was inconsistency in the supply of marine fishes (Abbas et al., 2006). Among the Malays, freshwater fishes come in different form of meals such as curried, spiced, fried or roasted one, supplying basic nutrition to daily lives. Following that, the fish-based industry in Malaysia such as surimi and fillet processing industry are developing progressively due to the high demands of fish-based products in the market (See et al., 2010). Not only available in fresh and frozen forms, freshwater fishes such as *Patin* (*P. sutchi*) and red tilapia are among the most common species found in processed food such as fish balls, fish cakes, fish sausages and other buttered or breaded products such as nuggets, burgers and fingers (Abbas et al., 2006). The processing plants for these products are located throughout the country, mainly in the vicinities of urban dwellings. QL Foods Sdn. Bhd. is known as the largest surimi producers in Malaysia with the average production of surimi products of 82 000 MT every year (Pangsorn et al., 2007).

2.2.2 (b) Therapeutic and Nutritive Source

The Malays, the Orang Asli of Peninsular Malaysia and tribal communities in East Malaysia have the habit of utilizing materials that originated from plants, animals and natural resources as the source of traditional medicine. Among the diverse types of

freshwater species in Malaysia, the Malaysian Channidae (including *Channa micropeltes, C. striatus* and *C. gachua*), the mudskipper (*Periophtalmus* spp.), and the freshwater eel (*Monopterus albus*) were widely exploited in traditional Malay medicines (Abdullah et al., 2010; Shafri and Abdul Manan, 2012). The popularity of *C. striatus* or locally known as "Haruan" in Malaysia as a folk medicine also overwhelmed among communities in other Southeast Asia countries such as Thailand, Vietnam, Cambodia as well as China (Shafri and Abdul Manan, 2012). This freshwater species was extensively studied for its excellent wound healing ability and was highly recommended to be consumed by patients in the post-operative period to promote wound healing and relieve pain (Zuraini et al., 2006). Besides that, it was well accepted as an energy booster among the sick or elderly, as well as to mothers who were recuperating from both normal and caesarean delivery (Shafri and Abdul Manan, 2012). This led to the emergence of broth and tonic of *C. striatus* extracts in the market due to the recognized health benefits of this species. Regular consumption of freshwater eels on the other hand, was claimed to be helpful in boosting up the body's immune system, stabilizing blood pressure, and enhancing one's memory power (Abdullah et al., 2010).

There was strong evidence suggesting that consumption of fish containing *n*-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was favorable for human health and beneficial in preventing cardiovascular diseases (Steffens, 1997). Therefore, numerous scientific researches have been carried out to identify the hidden potential of Malaysian freshwater fishes as a functional food. In the recent work of Airina and Jamaludin (2012), DHA and EPA were reported to be in the range of 0.63 – 1.41 % and 0.11 – 0.25 %, respectively

for three Malaysian freshwater fishes *C. striatus* ("Haruan"), *P. hypothalamus* ("Patin"), and *C. macrocephalus* ("Keli"). Similarly, in the previous report by Endinkeau and Tan (1993), it was found that Malaysian freshwater fish consisting of rice-field eel (*M. alba*), sultan fish (*Leptobarbus hoevenii*), grass carp (*Ctenopharyngodan idellus*), walking catfish (*Clarius* spp.), common carp (*Cyprinus carpio*), African bream (*Oreochromis* spp.) and snakehead fish (*C. Striatus*) contained high levels of unsaturated fatty acids compared to the saturated ones. Even though the omega-3 acids were generally low in most species, Endinkeau and Tan (1993) found that the quantity was high in rice-field eel (*M. alba*) which was approximately 9.4/ 100 g of oil, making it comparable to that of salmon, cod and herring. Up to now, most of the Malaysians are not exposed to the detailed nutritive values of these freshwater species, but it warrants consideration for commercial exploitation through these scientific findings. In order to enhance the consumption and utilization of Malaysian freshwater fishes, more researches are needed to further identify the characteristics and potentials of these fishes.

2.2.3 Potential Source of Collagen

Fish collagen has received considerable attention for its potential as an ingredient in processed functional food manufacturing, as well as for cosmetic, biomedical and pharmaceutical applications (Gómez-Guillén et al., 2011). Various extractions and functional characterizations of acid- and/or pepsin-soluble collagen have also been reported for different fish species. Fish offal such as bone, skin and scale has been the prime light for the isolation of collagen in all these studies. Information on the collagen extracted from fish muscle is very scarce in literature. Even though isolations of type I collagen from fish muscle were done by Sato et al. (1988), Kimura et al. (1988) and

Eckhoff et al. (1998), attentions have been given more to its role in the development of meat texture rather than the extraction process and characterization of the extracted collagen. According to Foegeding et al. (1996), about one tenth of mammalian muscle protein was collagen, which contributed to the texture of the muscle or the flesh. The amount of collagen in fish muscle was about one tenth of that in mammalian muscle. As a result, the texture of fish muscle was not as tough as that of meat, due to lesser cross-linked and lower melting temperature of fish collagen (Sikorski et al., 1984). The solubility of fish muscle collagen varied significantly among various species of fishes as presented in Table 2.4. Mathew and Hassan (1996) described this variation as the reflection of the difference in the degree and properties of inter- and intra-molecular cross-linking of the collagen.

Table 2.4: Collagen content of white muscle from different species of fishes (Mathew and Hassan, 1996)

Common Name	Scientific Name	Acid Soluble Collagen (%)
White Pomfret	*Pampus argenteus*	0.19
Sardine	*Sardinella longiceps*	0.36
Mackerel	*Rastrelliger kanagurta*	0.38
Sole	*Cynoglossus semifasciatus*	0.40
Vatta	*Caranx spp.*	0.50
Ribbon fish	*Trichulus savala*	0.12
White bait	*Anchoviella*	0.09
Tilapia	*Oreochromis mossabiscus*	0.47
Common carp	*Cypinus carpio*	0.49
Rohu	*Labes rohita*	0.28
Palankanni	*Megalops cyprinoides*	0.38
Paral	*Farbus spp.*	0.66

Veloori	*Kowala koval*	0.66
Kilimeen	*Nempterus japonicus*	0.09
Mullet	*Mugil cephalus*	0.94
Catla	*Catala catla*	0.71
Tuna	*Euthynnus affinis*	1.06
Whiting	*Sillage sihama*	1.00
Ray	*Himantura*	2.30
Shark	*Scoliodon sorrakowah*	2.13

Muscle makes up the majority percentage of the total body weight of a particular fish. Collagen constitutes 1 – 2% of muscle tissues and accounts for almost 6% of the weight of strong and tendinous muscles (Quereshi et al., 2010). Kimura et al. (1988) discovered that the denaturation temperature of type I collagen extracted from the muscle of freshwater lives such as carp and eel were higher than the skin collagen, respectively. This has brought up the possibility that the collagen extracted from fish muscle might be more suitable as the alternative of mammalian collagen in terms of the thermal stability. Currently, freshwater species in Malaysia, especially cultured fishes are still under-utilized and not much has been done for further exploitation of these natural resources for other applications besides food industry. Therefore, it would be of great interest to investigate the type I muscle and skin collagen contents of Malaysian freshwater fishes in the efforts to discover more promising potentials of these natural resources.

2.3 Optimization of Process Parameters

2.3.1 Statistical Based Optimization

2.3.1 (a) Design of Experiment (DOE)

Experimental design methods are widely applied in different disciplines for various applications. It is an important tool in the scientific and engineering world for the product realization process. The application of experimental design brings advantages such as improvement in process yields, reduction in product variability, reduction in development time as well as reduction of overall costs. Not only useful in the evaluation of basic design configurations and material alternatives, designed experiments also have extensive applications in selecting design parameters and identifying the key product design parameters that influence product performance (Montgomery, 2009). Though classical approach of one-factor-at-one-time (OFAT) method was simple, in which only one process variable or factor was varied at one time while keeping the others fixed, it was associated with few disadvantages such as time consuming and interactions between process factors were not estimatable (Czitrom, 1999). Therefore, statistical experimental designed methods were more appropriate to be applied in research field where screening and optimization of process parameters could be analyzed. Several process parameters involved could be varied simultaneously, somehow with lesser number of experiments and more efficiently (Montgomery, 2009).

In this study of collagen extraction from Malaysian freshwater fishes, the optimization study started with the screening of significant process variables towards the extraction yield via Fractional Factorial Design (FFD). Further optimization was done through Response Surface Methodology (RSM) coupled with Central Composite Design

(CCD) in order to study the interaction effect between the process parameters and influence of individual factor towards the yield of extraction.

2.3.1 (b) Fractional Factorial Design (FFD)

A major application of fractional factorials was in the screening step where many process parameters were considered and the objective was to identify those parameters (if any) that had significant effect on the response (Montgomery, 2009). FFD was popular in two-level multi-factorial designed with each process parameter was prepared in two level, designated as low level (-1) and high level (+1) (Zhu et al., 2010). The assumption in using a FFD was that higher order interactions were likely to be of little consequence and so their aliasing could safely be ignored. The order at which such aliasing appeared in a FFD was termed its resolution (Enda and Daniel, 2007). A 2^{k-p} FFD was of resolution IV if the main effects were clear of two factor interactions and some two-factor interactions were aliased with each other. Therefore, if three factors and higher interactions were suppressed, the main effects could be estimated directly in a 2_{IV}^{k-p} where k was the total number of process factors involved and p referred to the independent generator (Montgomery, 2009). Resolution IV designs were particularly popular because they avoided the confounding of main effects and two-factor interactions found in resolution III designs while avoiding the larger sample size requirements of resolution V designs (Jones and Montgomery, 2010).

The applications of FFD in various optimization studies were widely reported in literature such as production of elastase by *Bacillus* sp. (Chen et al., 2002), avilamycin production by *Streptomyces viridochromogenes* (Zhu et al., 2007), succinic acid

production from biodiesel based glycerol and astaxanthin extraction from *Phaffia rhodozym* (Zhu et al., 2010). There were multiple process variables that were 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, FFD was used in this study to screen the significant variables involving in the collagen extraction process. Insignificant variables were eliminated in order to obtain a smaller and more manageable set of process variables.

2.3.1 (c) Response Surface Methodology (RSM)

The limitations and drawbacks of single factor optimization that was practiced conventionally could be eliminated by applying response surface methodology (RSM). RSM was a mathematical modeling technique that relates independent and dependent variables and subsequently generated regression equations that represented the interrelations between the input parameters and output properties (Yang et al., 2007). In short, the principles and applications of RSM involved three steps: (1) experimental design where the independent variables and the corresponding experimental levels were set using statistical experimental designs such as the central composite design (CCD) or Box-Behnken design (BBD); (2) response surface modeling through regression analysis, and (3) process optimization using the response surface model (Zhang et al., 2010b). The eventual objective of the RSM was to determine the optimum operating conditions for the system and had been widely utilized in food science and technology, microbiology and enzyme applications.

In most of the RSM problems, the relationship between the response and independent variables was unknown. Therefore, the first step in RSM was to find a suitable approximation for the true functional relationship between the respond, y, and the set of independent variables, x_1, x_2, $x_3 ... x_n$ where n was the total number of independent variables. It could be expressed as:

$$y = f(x_n) + \varepsilon \qquad (2.1)$$

with ε represented the noise or error observed in the response y. The true response function f was normally unknown, thus low-order polynomial in some region of the independent variables was preferable for the approximation of f. If the response could be defined by a linear function of the independent variables, then the approximation function was a first-order model expressed by:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 .... + \beta_k x_k + \varepsilon \qquad (2.2)$$

In the case of curvature in the response surface, a higher degree polynomial could be used (e.g. the second order model) as follow:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=2}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (2.3)$$

Almost all RSM problems were associated with one or both of these models (Montgomery, 2009). Few reports that were available in the literature demonstrated that mostly central composite design (CCD) and Box-Behnken design (BBD) were effective in investigating the optimum collagen extraction conditions from the skin of yellowfin tuna (*Thunnus albacares*) (Woo et al., 2008), grass carp (*Ctenopharyngodon idella*) (Wang et al., 2008b), scales of yellowfin tuna (*Thunnus albacares*) (Han et al., 2010), and swim bladder of grass carp (*Ctenopharyngodon idella*) (Zhang et al., 2010a).

According to Wang et al. (2008b), the mathematical model suggested by RSM resulted in high correlation value, R^2 and P value less than 0.0001, which implied good agreement between the predicted values and the actual values of the yield of acid soluble collagen from grass carp (*Ctenopharyngodon idella*). This was also supported by Woo et al. (2008) and Zhang et al. (2010a) who stated that the generalization of the suggested models by RSM in collagen extraction optimization studies was very good. The relationship between the yield of collagen and the process parameters were also well evaluated.

Central Composite Design (CCD) was an efficient technique in process optimization in which it was an experimental strategy to seek the optimum conditions for a multivariable system. It contained an embedded factorial or fractional factorial design with center points that was augmented with a group of 'star point' which allowed the estimation of curvature (Zhang et al., 2007a). As the center of the design space to a factorial point was defined as ±1 unit for each factor, the distance from the center of the design space to a 'star point' was $\pm\alpha$ with $|\alpha|>1$. CCD was commonly used to fit second order model and consisted of three groups of design points which could be divided as follow (Montgomery, 2009):

a) Two-level factorial design which consisted of all possible combination of +1 and -1 levels of the factors. For the case of two factors (variables), there were four design points, which were (-1, -1), (+1, -1), (-1, +1) and (+1, +1).
b) The axial points whereby all the factors were set to the level 0 or the midpoint and only one factor was set to have the value of $\pm\alpha$. Therefore, for a two-factor problem,

the axial points were $(-\alpha, 0)$, $(+\alpha, 0)$, $(0, -\alpha)$ and $(0, +\alpha)$. The value of α was set to the rotatable value which could provide equal precision of estimation of the surface in all directions.

c) Center points whereby the levels were set to 0 or midpoint of each factor range. Center points were repeated 4 – 6 times to obtain good estimation of experimental error.

2.3.2 Artificial Intelligence Based Optimization

2.3.2 (a) Artificial Neural Network (ANN)

Artificial neural network (ANN) had emerged as an attractive tool for non-linear multivariate modeling in the last two decades (Desai et al., 2008; Zainal et al., 2010). It had typically been used as a "black-box" tool, which was, no prior knowledge about the process was assumed but the goal was to develop a process model based only on observations of its input-output behavior (Psichogios and Ungar, 1992). ANN was a mathematical system that simulated biological neural networks and was often described as a massively interconnected network structure consisting of many simple processing elements (neurons) with the ability to perform parallel computation for data processing (Agatonovic-Kustrin et al., 1998; Baş and Boyacı, 2007). ANN was capable of handling multiple independent and dependent variables simultaneously and to do this prior knowledge on the functional relationship did not need to be known. Each neuron received information through input connections, processed the information and produced the output which was distributed through output connections. Each connection was associated with a real number quantity known as weight (Desai et al., 2008). In other words, the neurons summed weighted inputs and then applied a linear or non-linear

function to the resulting sum to determine the output. These neurons were arranged in layers and were combined through excessive connectivity (Shen et al., 2007). A neural network in its basic form was usually composed of several layers of neurons, there being one input layer, one output layer and at least one hidden layer (Figure 2.7).

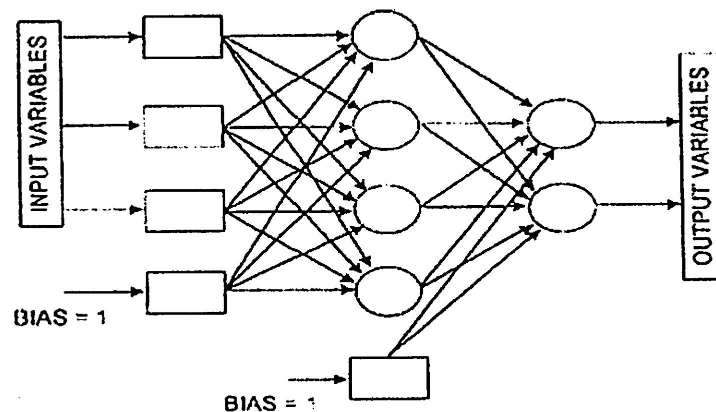

Figure 2.7: Architecture of ANN. The lines connecting the neurons represent the weight. Also shown are the bias neurons used to shift the neuron transfer function and to improve the network performance (Desai et al., 2008).

There were various types of ANNs. Amongst them were the Hopfield, Kohonen, Radial Basis Function and the most common and popular network was the feedforward artificial neural network (FANN). Feedforward back-propagation network was normally applied in analytical applications and was very powerful in function optimization modeling (Lou and Nakai, 2001). Information from various sets of inputs was fed forward through the network to optimize the weight between neurons, or to train it. As described by Agatonovic-Kustrin et al. (1998), the error or bias in prediction was then propagated through the system and the inter-unit connections were changed to minimize the error in prediction. This was a continuous process with multiple training sets until

the minimum error was attained. Training an ANN network was therefore accomplished by adjusting these weights by minimizing a non-linear error function (Desai et al., 2008). However, a major disadvantage of ANN was the difficulty in explaining the relationship between independent and response variables resulting from the ambiguously defined weight, which as mentioned earlier, was a black box (Lou and Nakai, 2001).

As a consequence, neural techniques required some ways of evaluating the performance of the network. Since it was a system which learned to associate the inputs with outputs, network performance evaluation on the training data may not be sufficient. Coit et al. (1998) stated that in order to assure confidence in a black box model, the model must be constructed and validated using data which adequately and accurately reflected the process domain. Thus three types of data sets were used: (1) training data: used to train network; (2) testing data: used to monitor the neural network performance during training; and (3) validation data or unseen data: used to measure the performance of a trained network (Agatonovic-Kustrin et al., 1998). The number of the layers and processing elements in layers varied from one process to another. There was no strict rule available to identify the optimal number of layers and neurons. In fact, it was generally defined by trial and error (Baş and Boyacı, 2007). The procedures of constructing a FANN are summarized in Figure 2.8.

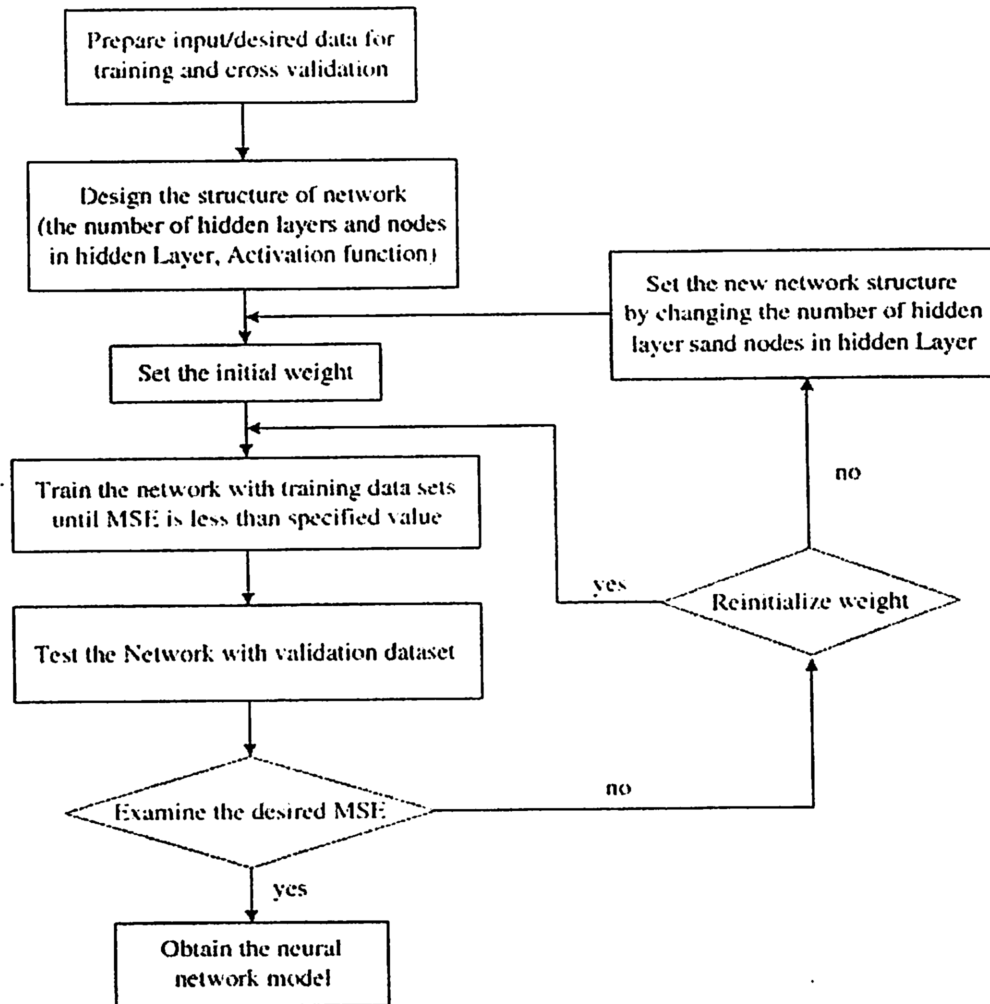

Figure 2.8: Procedures in generating a feedforward ANN model (Kittisupakorn et al., 2009)

2.3.2 (b) Bootstrap Re-sampling Neural Network

According to Baş and Boyacı (2007), an ANN derived its computing power via massively parallel-distributed structure and its ability to learn, and therefore generalized. Generalization on the other hand, referred to the ability of neural network in producing reasonable outputs for inputs that were not encountered during training process (Haykin, 1994). Since a good process control performance was greatly dependent on the accuracy

of model representation of the process, ANN models must be robust or stable when they were applied to unseen data (Zainal et al., 2010). Over-fitting and under-fitting were the common concerns in developing neural network models. Over-fitting happened when the network errors were large even though the training data set was driven to a very small error value while the later was referring to the phenomena that neural network itself failed to capture the relationship within the complex, resulting in poor generalization capability of the networks (Ahmad et al., 2008). Therefore, the search of techniques and alternatives to improve the generalization capability of neural network model has been intensified. To cope with these problems, improvement methods such as bootstrap re-sampling and multi neural network were introduced.

Bootstrap re-sampling was actually a way to create random data sets for training and testing in neural network. The goal of re-sampling was to create equal number of bad and good data sampling, thus improved the generalization ability of the network via the identification of the characteristics of the scarce classes (Dupret and Koda, 2001). Additionally, sampling by bootstrap was also found to be effective in increasing the robustness of the neural network models (Zhang et al., 1998).

2.3.2 (c) Genetic Algorithm (GA)

Genetic Algorithm (GA) was an optimization technique inspired by natural selection and natural genetics (Karegowda et al., 2011) which was widely applied in the search for an optimal value of a complex objective function or fitness function by simulation of biological evolutionary process based, such as in genetics, on crossover and mutation (Izadifar and Jahromi, 2007). It solved linear or non-linear problems by

maintaining and manipulating a population of solutions and searching for better solutions based on 'survival of the fittest' strategy (Desai et al., 2005; Shen et al., 2007). New population generated would then proceed with further selection, crossover and mutation, until the termination criterion was satisfied (Karegowda et al., 2011). The application of GA required the determination of few fundamental aspects which included the chromosome representation, the selection function, genetic operators making up the reproduction function, the creation of the initial population, termination criteria, as well as the evaluation function (Shen et al., 2007). Besides, it was possible to use ANN models in GA as the guiding function in order to solve the problem domains, leading to success in the application of soft computing techniques in food production systems in recent years (Izadifar and Jahromi, 2007).

2.3.2 (d) Artificial Neural Network/Genetic Algorithm (ANN/GA)

The power of ANN was that it was generic in structure and demonstrated the ability to learn from data gathered by users, thus widely known as a reliable modeling tool for process that was experienced lack of information (Zainal et al., 2010). Also, ANN could work well even with relatively less data in which experimental data of RSM was sufficient to build an effective ANN model (Desai et al., 2008). Lately, GA based on ANN model as an objective or fitness function has been applied successfully in optimizing the input space of various bioprocess studies (Singh et al., 2009; Zafar et al., 2012). To solve an optimization problem, GA repeatedly modified a population of individual solutions. Selection of individual from the current population to act as the parents who produced children for the next generation based on the selection, crossover,

and mutation rules was done randomly. Over successive generations, the population would finally evolve towards an optimal solution (Singh et al., 2009).

ANN and GA were two of the most promising soft computing intelligence techniques and were widely applied in many engineering optimization problems. ANN has been recognized as a powerful and practical approach to model extremely complex non-linear process while GA was used in various diverse optimization systems in biochemical engineering. Implementation of ANN/GA has been blooming in recent years for various applications such as in the prediction of bacteria thermal inactivation rate (Lou and Nakai, 2001), optimization study of biomass and yield of β-glucan (Desai et al., 2005), vegetable oil hydrogenation process (Izadifar and Jahromi, 2007), media optimization in fermentative production of scleroglucan (Desai et al., 2008) and optimization of polyhydroxybutyrate production by *Azohydromonas lata* (Zafar et al., 2012). Nevertheless, the application of this technique in the optimization of collagen extraction processes is still scarce in the literature.

2.4 Collagen Extraction Kinetics and Modeling

2.4.1 Empirical Kinetic Model

Numerous researches have been conducted to describe the kinetics and mechanism of extraction process, particularly solid–liquid extraction for various vegetal (plant) tissues (Sturzoiu et al., 2011). However, there is no kinetic model for collagen extraction being reported so far. Kinetic models can be divided into physical and empirical models. According to Kitanović et al. (2008), empirical models described the mathematic variations of the amount of extracted compound in either the raw material or

liquid extract with time. They are normally simpler as compared to the physical models but are appropriate for engineering purposes. Kinetic model is a useful engineering tool, considerably facilitating design, optimization and simulation of extraction processes and contributing to better utilization of energy, solvent and time. Simple illustration of collagen extraction process is presented in Figure 2.9.

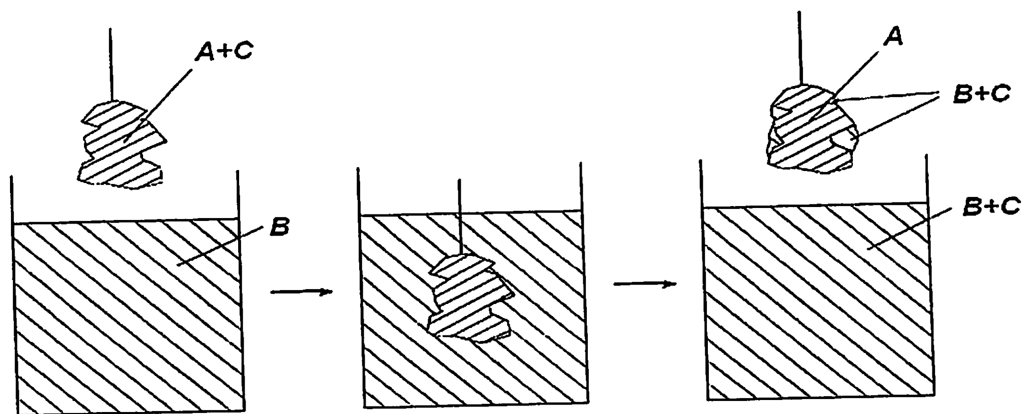

Figure 2.9: Illustration of collagen extraction process where A, B and C represents raw material, solvent and target-collagen respectively.

Four two-parametric kinetic models namely power law, parabolic diffusion, Peleg's and Elovich's equation are commonly being applied in the modeling of solutes recovering from different types of solid materials (plant materials, soil, ores, and wastes). The applications of these empirical kinetic models are based on the following assumptions:

i. The raw materials are isotropic and of equal size;
ii. The distribution of solute within the raw material is uniform and varies only with time;

iii. Net diffusion occurs only towards the external surface of the raw materials; and

iv. The diffusion coefficient of solute is a constant.

2.4.1 (a) Power Law Model

The power law model, which was similar to Freundlich type, was applied widely in the diffusion process of an active agent through non-swelling devices (Sturzoiu et al., 2011). It could be applied as follows:

$$y = Bt^n \tag{2.5}$$

where y was the yield of collagen (g/g), B referred to the constant incorporating the characteristics of the carrier-active agent system, t was the time in minutes, and n was the diffusional exponent, an indicative of transport mechanism. In literature, n was less than 1 for extraction from plant or vegetal materials. The constants for this model must be estimated using a regression analysis. In the linearized form, the equation was transformed into

$$ln\, y = n\, ln\, t + ln\, B \tag{2.6}$$

2.4.1 (b) Parabolic Diffusion Model

Orthogonal polynomial was another useful empirical equation in solid liquid extraction. With the general form:

$$Y(x) = \sum_{i=0}^{n} A_i \emptyset_i\, (x) \tag{2.7}$$

where A_i was the parameter to be determined and Φ_i was the function of x. Therefore, the yield of collagen, y, was given by:

$$y = \sum_{i=0}^{n} A_i t^{1/2} \tag{2.8}$$

Kim et al. (2002) fitted the third-degree polynomial in the form of Eq. 2.8 to the leaching data of the simulated and real paraffin waste.

$$y = A_0 + A_1 t^{1/2} + A_2 t \quad (2.9)$$

The three terms in Eq. 2.9 represented three kinetic behaviours observed during leaching. The first term was accounted for the washing of loosely bound materials which would be leached instantaneously, while the second and third represented diffusive release and chemical reactions (dissolution, corrosion, or solubility control), respectively. In this study of collagen extraction, since there was no chemical reaction involved, Eq. 2.9 could be simplified into:

$$y = A_0 + A_1 t^{1/2} \quad (2.10)$$

This was known as the parabolic diffusion equation which corresponded to two-step extraction mechanisms, consisted of washing and followed by diffusion. A_0 was known as the washing coefficient, indicating amount of collagen extracted instantaneously when the fish skins were submerged into acetic acid (solvent). Likewise, A_1 was known as the diffusion rate constant.

2.4.1 (c) Peleg's Model

The shape of the collagen extraction curves was similar to that of sorption curves (moisture content versus time). Therefore, it was possible to describe these collagen extraction curves using the model proposed by Peleg (Bucić-Kojić et al., 2007) which was also known as the hyperbolic model. In the case of extraction, the model was adapted and used in the form of Eq. 2.11:

$$y = y_o + \frac{t}{K_1 + K_2 t} \quad (2.11)$$

where y_0 was the initial yield of collagen extracted at t=0, K_1 was the Peleg's rate constant (min g skin/g collagen) and K_2 was the Peleg's capacity constant (g skin/g collagen). Since y_0 for most of the extraction processes was zero, Eq. 2.11 was simplified and used in the form:

$$y = \frac{t}{K_1 + K_2 t} \tag{2.12}$$

2.4.1 (d) Elovich's Model

The following logarithmic relation was a modified form of Elovich's equation. It was fitted to leaching curves such as the extraction of polycyclic aromatic hydrocarbons from coal tar-contaminated soil (Paterson et al., 1999). The relationship assumed that the rate of adsoprtion could be replaced by the rate of extraction and leaching rate decreased exponentially with increasing extraction yield. It was expressed as in Eq. 2.13:

$$y = e \ ln t + a \tag{2.13}$$

2.5 Summary

The literatures reveal that aquatic lives are gradually gaining attention as potential alternative sources for collagen extraction. In Malaysia, freshwater fishes particularly those of the cultured species are under-utilized and abundant in the market. It therefore offers an interesting approach to extract this biomaterial from body parts (muscle and skin) of the freshwater fishes as a replacement to mammalian collagen. Also, in regard to engineering point of view, optimization of extraction process parameters via artificial intelligence method deserves a credit owing to its superior performance in terms of accuracy and predictive capability over the conventional statistical optimization method. It would be an interesting attempt to conduct the parameters optimization of collagen extraction process using soft computing intelligence technique since it is scarce in the literature. There is also a need to derive a suitable and proper kinetic model for the collagen extraction process to facilitate the process simulation, design and control. In fact, kinetic and modeling studies, as well the reaction mechanisms concerning collagen extraction process regardless of the raw materials used are very limited in the literature.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Materials and Chemicals

The materials and chemicals used in this research are listed in Table 3.1. Unless stated otherwise, all chemicals are of analytical grade and used without pre-treatment.

Table 3.1: List of materials and chemicals

Materials/Chemicals	Supplier	Purpose
Freshwater fishes 1) Catfish (*Clarias gariepinus* X *Clarias macrocephalus*) 2) Red tilapia (*Oreochromis niloticus*) 3) Black tilapia (*Oreochromis mossambicus*) 4) Pangasius catfish (*Pangasianodon hypophthalmus*) 5) Sultan fish (*Leptobarbus hoevenii*) 6) Labyrinth fish (*Trichogaster trichopterus*)	Wet market in Parit Buntar, Perak, Malaysia	Raw material for the extraction of type I collagen
Brine shrimp (*Artemia salina*) egg	Cheaw Thean Yeang Aquarium Sdn. Bhd., Penang, Malaysia	Toxicology assay
Sodium hydroxide (NaOH)	Merck	Pretreatment process
Butyl alcohol (C_4H_9OH)	Merck	Pretreatment process
Acetic acid (CH_3COOH)	Fisher Scientific	Extraction process

Pepsin (0.7 FIP U/mg)	Merck	Extraction process
Sodium chloride (NaCl)	Merck	Collagen precipitation
Collagen standard	Merck	Standard
Bovine serum albumin (Fraction V)	Merck	Standard
Sodium carbonate (Na_2CO_3)	Merck	Characterization
Copper sulphate pentahydrate ($CuSO_4.H_2O$)	R&M Chemicals	Characterization
Folin-Ciocalteu's phenol reagent (FC)	Merck	Characterization
Potassium sodium tartarate	Fisher Scientific	Characterization
Tris-glycine buffer	Bio-Rad Laboratories	Characterization
Laemmli sample buffer	Bio-Rad Laboratories	Characterization
Protein molecular weight marker (M.W. 30 000 – 200 000)	Sigma-Aldrich	Characterization
Coomassie brilliant blue R-250 staining solution	Bio-Rad Laboratories	Characterization
Coomassie brilliant blue R-250 destaining solution	Bio-Rad Laboratories	Characterization
Potassium bromide (KBr)	Merck	Characterization
Hydrochloric acid (HCl)	R&M Chemicals	Characterization
Potassium dichromate ($K_2Cr_2O_7$)	Fluka Chemika	Toxicology assay

3.2 Equipments

The equipments and facilities used in this research are listed in Table 3.2. The usage of the equipment is also described.

Table 3.2: List of equipment and facilities

Equipment/Facility	Brand/Model	Usage
Hot plate with magnetic stirrer	Heidolph	Stirring and extraction
Oven	Memmert UNB 800	Drying
Chiller	Polyscience	Temperature control
Centrifuge	Eppendorf 5702 R	Separation
UV-Vis spectrophotometer	Thermo Scientific Evolution 201	Analysis
pH meter	Mettler Toledo Delta 320	Analysis and pH adjustment
Weighing balance	Shimadzu AC220	Weight measurement
Vortex mixer	Heidolph Reax Top	Mixing
Freeze dryer	Telstar Cryodos	Drying in low temperature
Overhead stirrer	IKA RW20	Mixing
Image analyzer*	FUJIFILM Luminescent Image Analyzer LAS-3000	SDS-PAGE gel image capture
HPLC (High performance liquid chromatography)*	Agilent Technology	Analysis
Fourier transform infra-red spectrometry (FTIR)	Shimadzu Scientific Instruments' IR-Prestige-21	Analysis
Viscometer	Brookfield DV-III	Analysis

*All equipments were obtained from School of Chemical Engineering, USM, besides image analyzer (Centre of Advanced Analytical Toxicology Services, USM) and HPLC (School of Biological Sciences, USM).

3.3 Research Methodology Flow Chart

The process methodology involved in the extraction of collagen from Malaysian freshwater fishes is presented in Figure 3.1.

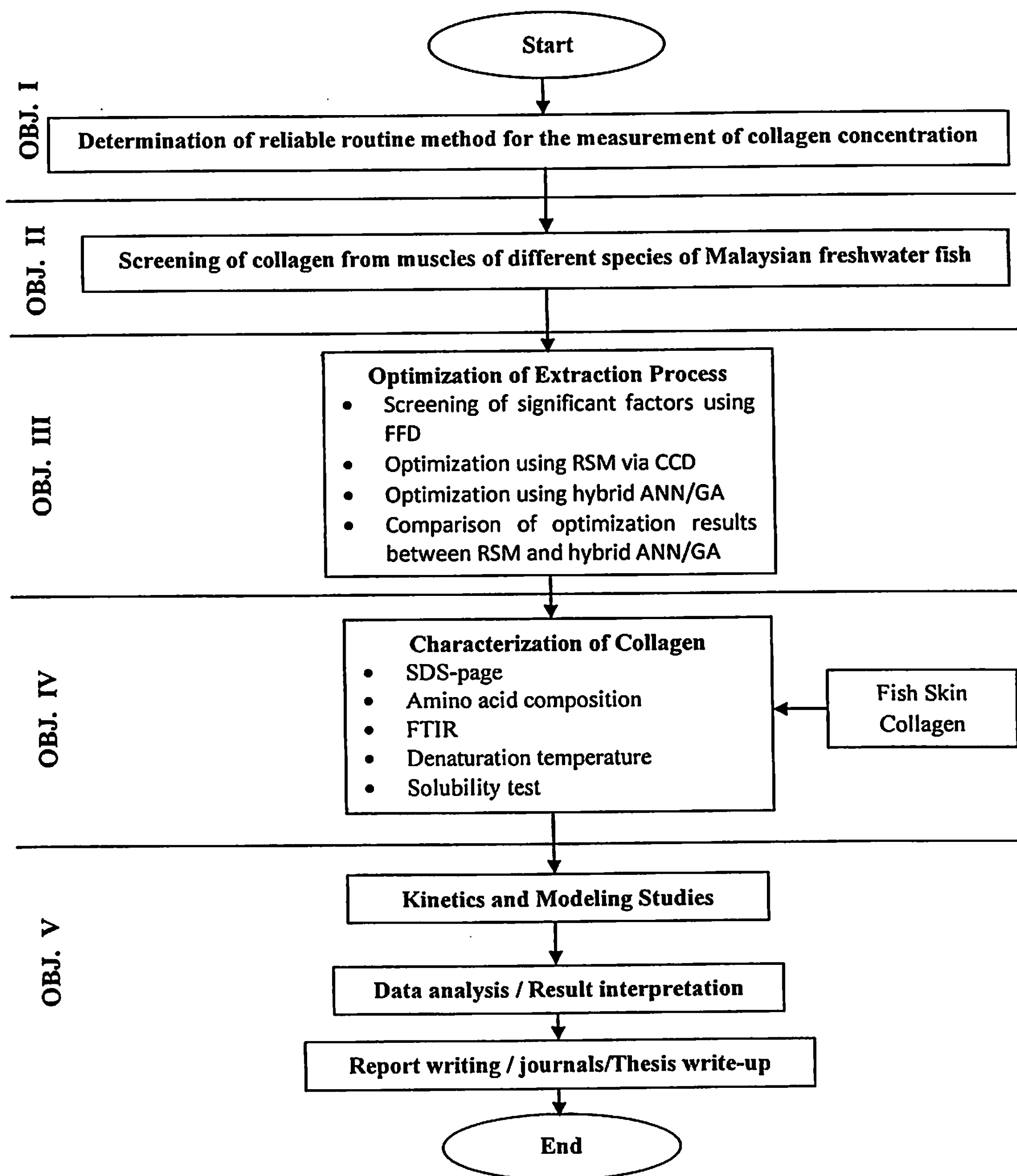

Figure 3.1: Research methodology flow chart of this study

3.4 Screening of Collagen from Malaysian Freshwater Fishes

3.4.1 Preparation of Raw Material

Six different species of Malaysian freshwater fishes: cultured hybrid catfish (*Clarias gariepinus* X *Clarias macrocephalus*), red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasianodon hypophthalmus*), sultan fish (*Leptobarbus hoevenii*), and labyrinth fish (*Trichogaster trichopterus*), were screened for the presence of collagen (Appendix A). The fishes were obtained from the local wet market in Parit Buntar, Perak, Malaysia. Upon arrival at the laboratory, the fishes were killed, dissected, deboned and de-scaled (if required). The skin and muscle were cleaned of adhering tissues before being cut into small pieces (1 cm × 1 cm). The skin was manually removed by using a sharp knife. Both skin and muscle for all fishes were then washed with distilled water and kept frozen at -20 °C prior to collagen extraction.

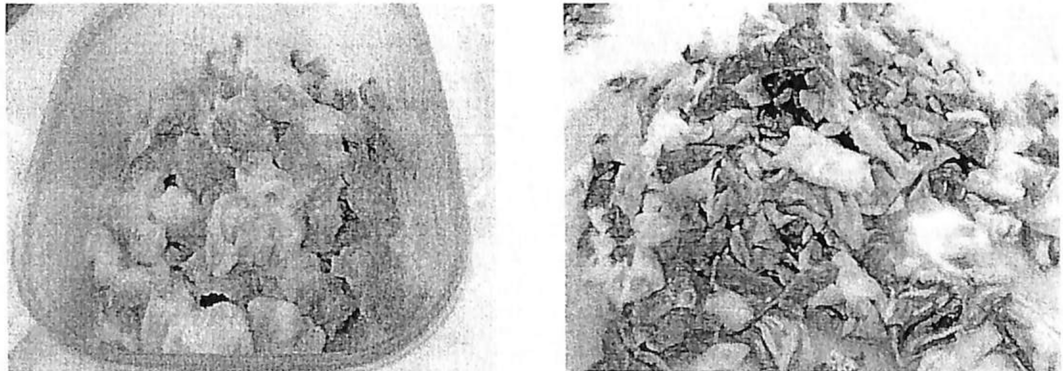

Plate 3.1: Muscle (left) and skin (right) from hybrid *Clarias* sp.

3.4.2 Extraction of Type I Collagen

3.4.2 (a) Extraction of Acid Soluble Collagen (ASC)

Extraction process was performed according to the method as described by a few researchers (Kimura et al., 1988; Kittiphattanabawon et al., 2005; Wang et al., 2009), with slight modifications. The ratios of pretreatment solutions to the raw materials and centrifugation period and speed have been adjusted accordingly in this study. The whole procedure was carried out at 4 °C. To remove non-collagenous proteins, the muscle was mixed with 0.1 M NaOH at a ratio of 1:20 (w/v) (muscle:NaOH). The mixture was stirred for 5 – 6 hr. The NaOH solution was changed every 2 hr. The samples were then washed thoroughly with excessive distilled water until the pH was neutral or slightly basic. Deproteinised muscle was defatted with 10 % butyl alcohol with a sample to alcohol ratio of 1:20 (w/v) for 24 hr. The alcohol solution was changed every 8 hr. Subsequently, defatted muscle was then washed with cold water and subjected to collagen extraction by aqueous acetic acid. The raw material was actively stirred in 30 volumes (v/w) of 0.5 M acetic acid for 20 hr to extract the acid soluble collagen. The viscous collagenous material was separated from the insoluble components by high speed centrifugation at 20,000 × g for 40 mins. The soluble collagen solution was obtained from the supernatant. The collagen was precipitated by adding NaCl to a final concentration of 0.8 M. Resulting sediment was collected by centrifugation at 20,000 × g for 30 mins. To further purify the collagen, it was re-dissolved in minimal amount of acetic acid, dialyzed against 0.1 M acetic acid, followed by distilled water and lyophilized. The freeze-dried product was designated as acid soluble collagen (ASC).

3.4.2 (b) Extraction of Pepsin Soluble Collagen (PSC)

The PSC was obtained using similar method as the ASC (Section 3.4.2 (a)) except that the defatted muscle was continuously stirred in 30 volumes (v/w) of 0.5 M acetic acid containing 1.50 % (w/w) pepsin for 20 hr. Similarly, the freeze-dried product was designated as pepsin soluble collagen (PSC). 1 FIP Unit of pepsin corresponded to amount of pepsin that catalyzed the conversion of 1 micro mole of substrate per minute. Schematic diagram for the collagen extraction process is illustrated in Figure 3.2.

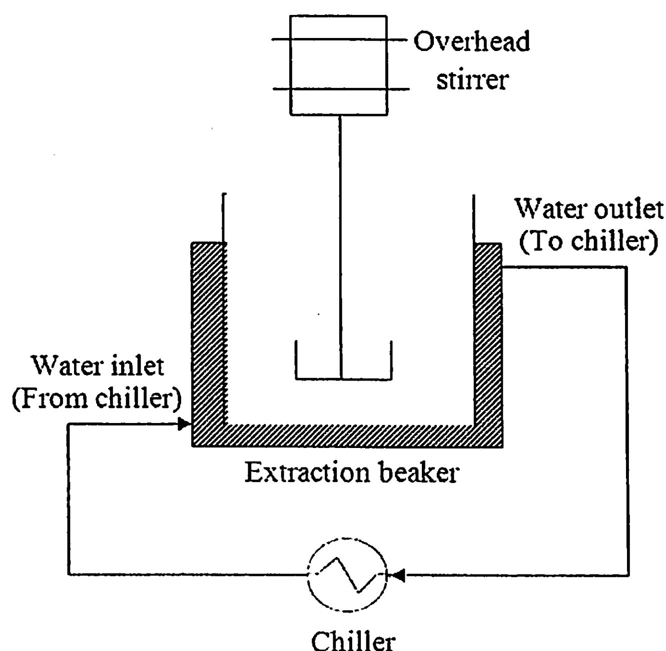

Figure 3.2: A simple schematic illustration of the collagen extraction process

3.4.2 (c) Collagen concentration measurement by Lowry's modification method

Solutions and reagents used included Folin-Ciocalteu reagent, reagent A and reagent B were prepared as described by Komsa-Penkova et al. (1996). Brieftly, reagent A was a solution of 0.4% potassium-sodium tartrate, 10% Na_2CO_3 and 0.5 M NaOH.

Reagent B consisted of 2% potassium-sodium tartrate, 3% $CuSO_4.5H_2O$ and 0.1 M NaOH. The Folin-Ciocalteu reagent was diluted with distilled water at the ratio of 1:15.

Extracting mediums extracted at timed intervals from were centrifuged at 3,840 × g for 15 minutes and the supernatant were collected, known as collagen solutions. 0.80 ml of the collagen solution was incubated with 0.72 ml of reagent A and 0.08 ml of reagent B at 50°C for 20 minutes. This was followed by addition of 2.40 ml of Folin-Ciocalteu reagent to the samples after cooling to room temperature. The solution was shaken vigorously immediately after the addition and incubated at 50°C for another 10 minutes. After cooling the sample to room temperature, absorbance was read at 650 nm against the reference. A calibration curve was established using different dilutions of calf skin Type I collagen in 0.05 M acetic acid prior to the measurement to obtain linear correlation between concentration and absorbance value of the collagen solutions. Proper dilutions (with dilution factor 5 and 8) were done to samples to obtain absorbance readings within linear range of the calibration curve. The testing for each set of dilution was performed in quintuplicate.

Higher dilutions (e.g. 15, 20 and 30) were done to the pepsin soluble collagen samples due to much higher absorbance were recorded as compared to acid soluble collagen. The yields of collagen from the fishes muscle were calculated as proposed by Li et al. (2009):

$$Y = \frac{V \times C}{W} \times 100\% \qquad (3.1)$$

where Y is the yield of collagen in %, 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 lyophilized weight of fish muscle in g.

3.5 Optimization of Pepsin Soluble Collagen (PSC) Extraction Process

3.5.1 Screening of Significant Process Parameters using Fractional Factorial Design (FFD)

In this study, 8 independent factors (Table 3.3) were tested at both high (+1) and low (-1) levels. The range of the process parameters was set based on the reported values in literature (Wang et al., 2008b; Woo et al., 2008; Wang et al., 2009; Zhang et al., 2010a). The yield of extracted PSC was listed as the response variable. In order to evaluate the effect of process parameters, 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 of the mean values. Data analyses were also performed using Design Expert software for the selection of most influential factor(s) among the proposed process parameters.

Table 3.3: Process parameters and levels for the fractional factorial design (FFD)

Process Parameters	PSC	
	Coded 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 (°C)	5	15
X_4 : Acetic Acid to Material Ratio (ml/g)	5	20
X_5 : NaOH concentration (M)	0.1	0.9
X_6 : NaOH to Material Ratio (ml/g)	5	10
X_7 : NaOH Treatment Time (hr)	2	6
X_8 : Extraction Stirring Speed (rpm)	100	250

3.5.2 Optimization of Process Parameters Using One-Factor-At-One-Time (OFAT) Method

OFAT approach was a conventional method for many industrial processes in optimizing process parameters (Nei et al., 2009). It sequentially tuned each process parameter individually while holding all others fixed, assuming the various treatment parameters did not interact and that the response variable was 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 parameters from the results of FFD. Effects in variation of each significant parameter towards the extraction efficiency, and the influences reflected on the pattern of yields' profiles were also studied and investigated. For the extraction of PSC, the process was carried out as described in

Section 3.4.2 (b) with the exception that the defatted muscle was actively stirred in acetic acid with varying concentrations (0.1 M, 0.5 M, 0.7 M, and 0.9 M) and acetic acid to muscle ratios (10 ml/g, 20 ml/g, 25 ml/g, and 30 ml/g) containing pepsin at 1.5 % (w/w) for 24 hr under different stirring speeds (200 rpm, 300 rpm, 400 rpm, and 500 rpm) in order to extract pepsin soluble collagen.

3.5.3 Optimization of Muscle Pepsin Soluble Collagen (M-PSC) Extraction Process Parameters using Response Surface Methodology (RSM)

Response surface methodology was employed for experimental design, data analysis, and model building with the aid of the software Design Expert (Version 6.0.6, Stat-Ease Inc., Minneapolis, Minnesota USA). Central composite design (CCD) with three variables was used to determine the response pattern and then to establish a model. According to the CCD, the total number of experimental combinations is $2^k + 2k + n_0$, where k was the number of independent variables and n_0 was the number of repetitions of the experiments at the centre point. For statistical calculation, the experimental variables X_i have been coded as x_i according to the following transformation equation:

$$x_i = \frac{X_i - X_0}{\partial X} \tag{3.2}$$

where x_i was the dimensionless coded value of the variable X_i, X_0 was the value of X_i at the center point, and ∂X was the step change (Zhang et al., 2010b). In this study, the central composite design with three factors and three levels, including six replicates at the center point, was used for fitting a second order response surface. Three independent variables used in this work were acetic acid concentration (X_1), acetic acid to muscle ratio (X_2), and stirring speed (X_3), while the dependent variable was the yield of PSC.

The ranges and center point values of all independent variables were based on the results of preliminary experiments as described in Section 3.5.2 (Table 3.4). The yield of PSC was analyzed by multiple regressions to fit into the following polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (3.3)$$

where *Y* was the dependent variable (yield of M-PSC), β_0 was intercept, β_i , β_{ii}, β_{ij} were regression coefficients and X_i indicated the linear terms, X_i^2 for the quadratic terms for a single variable, X_iX_j for the interaction terms (Zeng et al., 2012). Six replicates at the central point of the designed model were used to estimate the pure error sum of squares. In order to evaluate the effect of the process variables, 20 experiments were performed in random order to cover all combinations of the factor levels in the experimental design. All experiments were done in triplicate and data presented were mean values.

Table 3.4: Experimental range and values of the independent variables used in CCD

Independent Variables	Symbol	Coded Levels		
		-1	0	1
Acetic Acid Concentration (M)	X_1	0.1	0.5	0.9
Acetic Acid to Muscle Ratio (ml/g)	X_2	20	25	30
Stirring speed (rpm)	X_3	300	400	500

3.5.4 Optimization of Process Parameters using Artificial Neural Network (ANN) and Genetic Algorithm (GA)

3.5.4 (a) Development of Feedforward Artificial Neural Network (FANN)

The input parameters chosen in the current work were based on the results from Section 3.5.1 in which for the extraction of muscle collagen, the independent variables

involved were acetic acid concentration (M), acetic acid to muscle ratio (ml/g), and the stirring speed (rpm). The yield of the muscle pepsin soluble collagen (M-PSC) was evaluated as the output. The FANN model was developed based on the experimental data of RSM in Section 3.5.3. Prior to the model development, bootstrap re-sampling was applied to resample the collected data. In short, the M-PSC extraction process was conducted in 20 batches contributed to 20 samples data. Therefore, in order to create the model, these data were divided into two sections where half of them were taken as a validation data or unseen data, whilst the remaining half were re-sampled using bootstrap method into 300 data for training and testing. The general idea on the network development is illustrated in Figure 3.3.

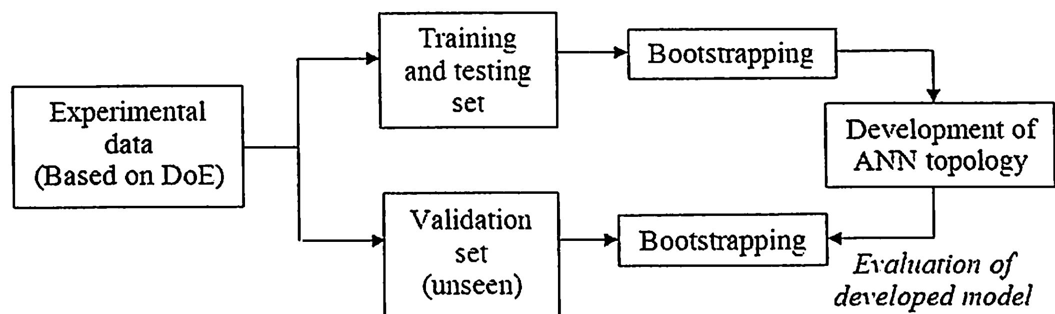

Figure 3.3: Development of artificial neural network

Using MATLAB (version 7.8.0.347, Mathworks, Natick, MA), a FANN trained with an error-back-propagation algorithm was designed. Then the neural networks were trained by the Levenberg-Marquardt optimization algorithm. Cross validation technique was applied on the testing set to overcome the overfitting and underfitting behavior of the trained network. In cross validation techniques, neural network building data were

divided into testing and training data sets and the mean squared error (MSE) for both were plotted against training epoch and when the testing MSE started to rise, the training was terminated and the value of weight and number of neurons that the network had in that previous step was chosen as the result of the training run.

Hidden neurons were associated with the logarithmic sigmoid activation function whereas output layer neurons used the linear activation function. To cope with different magnitudes in the input and output data, all the data were scaled to zero mean and unit standard deviation. The data for neural network model building need to be divided into: (1) Training data (for network training); (2) Testing data (for cross-validation based network structure selection); and (3) Unseen validation data (for evaluation of the final selected model). Single hidden layer neural networks with different numbers of hidden neurons were trained on the training data and tested on the testing data. The network with the lowest mean squared errors (MSE) on the testing data was considered as having the best network topology. In assessing the developed models, MSE and correlation coefficient (R^2) on the unseen validation data were used as the performance criterion.

3.5.4 (b) Evaluation of Model Predictability

The performance of the FANNs was statistically measured by mean squared error (MSE) and correlation coefficient (R^2) obtained as follows (Wang et al., 2008b):

$$MSE = \frac{1}{N}\sum_{i=1}^{N}(y_i - y_{di})^2 \tag{3.4}$$

$$R^2 = 1 - \frac{\sum_{i=1}^{N}(y_i - y_{di})^2}{\sum_{i=1}^{N}(y_{di} - y_m)^2} \tag{3.5}$$

where N was the number of data, y_i was the predicted value by ANN model, y_{di} the actual or experimental value, and y_m was the average of actual values. The network having minimum MSE and maximum R^2 was selected as the best FANN model.

3.5.4 (c) Genetic Algorithm (GA) Based Optimization

Once a generalized ANN model has been developed, its input space was optimized using GA. The input vectors were comprised of process parameters of the model and became the decision variable for GA. The optimization process was carried out through a simple cycle of four stages as such: (1) initialization of solution populations known as chromosomes, (2) fitness computation based on objective function (in this study referring to FANN model), (3) selection of best chromosome, and (4) genetic propagation of selected parent chromosomes using genetic operators such as crossover and mutation to create the new populations of chromosomes. It was a continuous process until a suitable result was obtained. The best string that evolved after repeating the loop until a convergence would be selected as the solution to the optimization problem. The general procedures opted in the optimization process for the extraction of both M-PSC and S-PSC were shown in Figure 3.4.

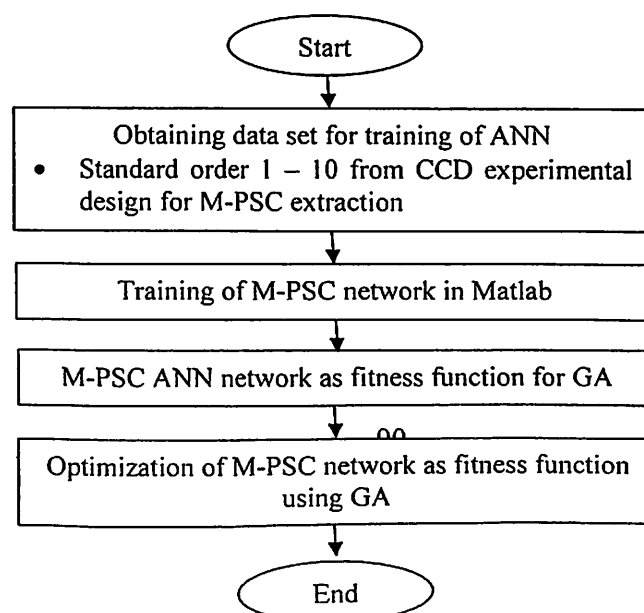

Figure 3.4: Solution methodology for optimization using ANN/GA

3.6 Characterization of Extracted Collagen

3.6.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the methods of Laemmli (1970) as described by Zeng et al. (2012) using discontinuous Tris-glycine buffer system with 4 % stacking gel and 12 % resolving gel. The collagen samples were dissolved in 0.05 % (v/v) acetic acid. Then, the dissolved collagen samples were added to Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 5 % of 2-mercaptoethanol at the ratio of 1:1. The mixtures were kept at boiling water for about 5 mins before loading to the SDS-PAGE. A total of 20 μg of each collagen sample was loaded on the SDS-PAGE. After electrophoresis, the gel was stained with 0.10 % (w/v) Coomassie blue R-250 in 30 % (v/v) methanol and 10 % (v/v) acetic acid and destained with 40 % (v/v) methanol and 10 % (v/v) acetic acid. Pre-stained SDS-PAGE broad range standards were used to estimate the molecular weight of both of the acid and pepsin soluble collagens from the fish muscle. The image of the gel was captured using FUJIFILM Luminescent Image Analyzer LAS-3000 (Fujifilm, Tokyo, Japan). The electrophoresis pattern of the collagen samples were analyzed using Multi Gauge v3.0 software (Fujifilm, Tokyo, Japan).

3.6.2 Amino Acid Analysis

The freeze dried collagen samples were hydrolyzed in inert atmosphere with 6 M HCl containing 1 % phenol at 110 °C for 24 hr. The hydrolysates were then dried under vacuum. This was followed by derivatization, drying, and dilution with sample diluents. The amino acids derivative samples were analyzed using high performance liquid chromatography (HPLC) (1260 Infinity, Agilent Technologies, Malaysia) and compared against the standard amino acids which were analyzed prior to these. The peak area of each amino acid in the chromatogram was calculated and compared with that of the standard and reported as number of residue per thousand amino acids content.

3.6.3 Functional Group Analysis

Fourier Transform Infrared (FTIR) spectra were determined from discs containing 2 mg collagen in approximately 100 mg potassium bromide (KBr). Infrared spectra were observed in the range between 4000 and 500 cm^{-1} using an infrared spectrophotometer (Shimadzu Scientific Instruments' IR-Prestige-21, Thermo Fisher Scientific, Malaysia).

3.6.4 Determination of Collagen Denaturation Temperature

The denaturation of collagen in solution was performed according to the method as described by Pati et al. (2010a). A Brookfield viscometer (Brookfield DV-III, Mecomb Malaysia Sdn Bhd, Malaysia) was filled with 0.30 % (m/v) collagen solution in

0.05 M acetic acid. The viscometer was then immersed in a water bath held at 4 °C and left to stand for 15 min, in order to allow the collagen solution to equilibrate to the water bath temperature. The temperature was raised stepwise up to 50 °C and maintained at each temperature for 10 min. Collagen solution viscosities were then measured at temperature intervals of 2 °C from 4 °C up to 50 °C. Fractional viscosities were computed for each temperature as follows:

$$Fractional\ viscosity = \frac{measured\ viscosity - minimum\ viscosity}{maximum\ viscosity - minimum\ viscosity} \quad (3.6)$$

Thermal denaturation curves were then obtained by plotting the fractional viscosities against temperature for the collagen. The denaturation temperature was taken to be the temperature at which fractional viscosity was 0.5.

3.6.5 Collagen Solubility Study

The solubility of collagens was determined by the method of Singh et al. (2011). The collagens were dissolved in 0.05 M acetic acid to obtain a final concentration of 6 mg/ml, and the mixtures were stirred at 4 °C until collagens were completely solubilized.

3.6.5 (a) Effect of pH

Collagen solution (3 mg/ml; 8 ml) was transferred to a 50 ml centrifuge tube and the pH was adjusted with either 6 M NaOH or 6 M HCl to obtain the final pH ranging from 1 to 10. The volume of solution was made up to 10 ml by distilled water which previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20, 000 × g at 4 °C for 30 min. Protein content in the supernatant was determined by the method of Matmaroh et al. (2011) using bovine serum albumin as the

standard. Relative solubility was computed in comparison with that obtained at the pH rendering the highest solubility.

3.6.5 (b) Effect of NaCl

5 ml of collagen (6 mg/ml) was mixed with 5 ml of NaCl in 0.05 M acetic acid at various concentrations of 0 %, 2 %, 4 %, 6 %, 8 %, 10 %, and 12 % (v/v). The mixture was stirred continuously at 4°C for 30 min, followed by centrifugation at 20, 000 × *g* for 30 min at 4 °C. Protein content in the supernatant was measured and the relative solubility was calculated as described previously (Section 3.6.5 (a)).

3.7 Kinetics and Modeling of Collagen Extraction

In this study, four two-parametric kinetic models namely power law, parabolic diffusion, Peleg's and Elovich's equations, which were commonly being applied in modeling of recovering of solutes from different types of solid materials (plant materials, soil, ores, and wastes) were validated based on the following assumptions:

I. Fish muscle was isotropic and of equal size;

II. Distribution of collagen within the fish muscle was uniform and varied only with time;

III. Net diffusion occurred only towards the external surface of fish muscle; and

IV. Diffusion coefficient of collagen was a constant.

Experimental results of collagen extraction were analyzed using the linearized equations of the selected empirical kinetic models as summarized in Table 3.6. Model parameters were calculated by linear regression using Microsoft Excel software.

Table 3.6: Proposed empirical kinetic models in linearized forms

Model	Model Equation	Linearized Form
Power Law	$y = Bt^n$	$\ln y = n \ln t + \ln B$
Parabolic Diffusion	$y = A_0 + A_1 t^{1/2}$	-
Peleg's Equation	$y = \frac{t}{K_1 + K_2 t}$	$\frac{t}{y} = K_1 + K_2 t$
Elovich's Equation	$y = e \ \ln t + a$	-

Selection of the best empirical kinetic model in representing the collagen extraction data was based on the process flow as presented in Figure 3.5.

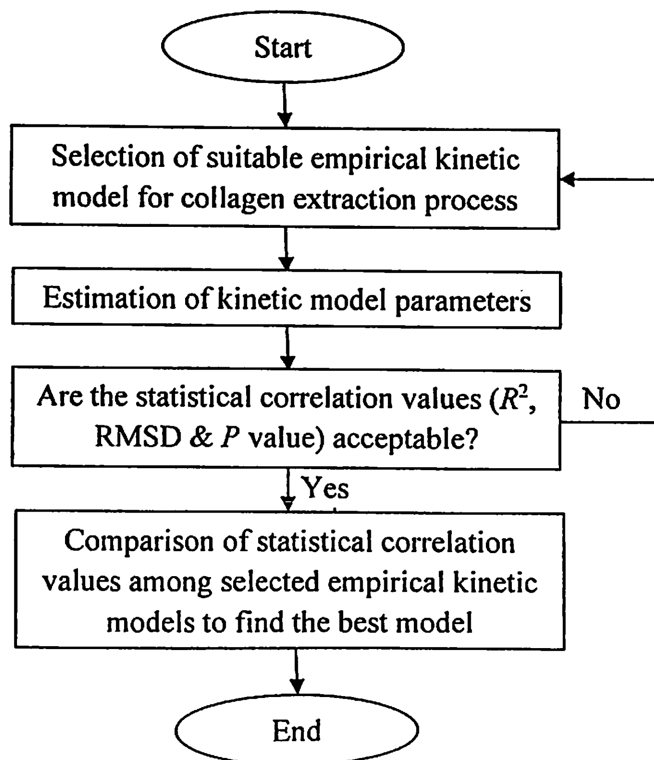

Figure 3.5: Flow chart for the kinetic study of collagen extraction process

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Determination of Collagen Concentration Using Modified Lowry's Method

The inset in Figure 4.1 shows the relationship between absorbance and concentration of calf skin Type I collagen from 0.01 – 3.00 g/ml. The basic law of light absorption or Beer-Lambert law was obeyed over the collagen concentration range 0.01 – 1.00 g/ml in the photometrically measured solution. A linear relationship between absorbance and collagen concentration with a high coefficient of determination (R^2 =

0.99) was found and is shown in Figure 4.1. This was in accordance with the findings by Komsa-Penkova et al. (1996) and Li et al. (2009) that good linear relationship could be achieved only within certain range of collagen concentrations. The calibration curve could be used to monitor concentration of extracted collagen at any time along the extraction process. It was described by the straight line equation:

$$A = 1.0876c + 0.0561 \tag{4.1}$$

where A was the absorbance and c was the concentration of collagen in mg/ml in the photometrically measured solution.

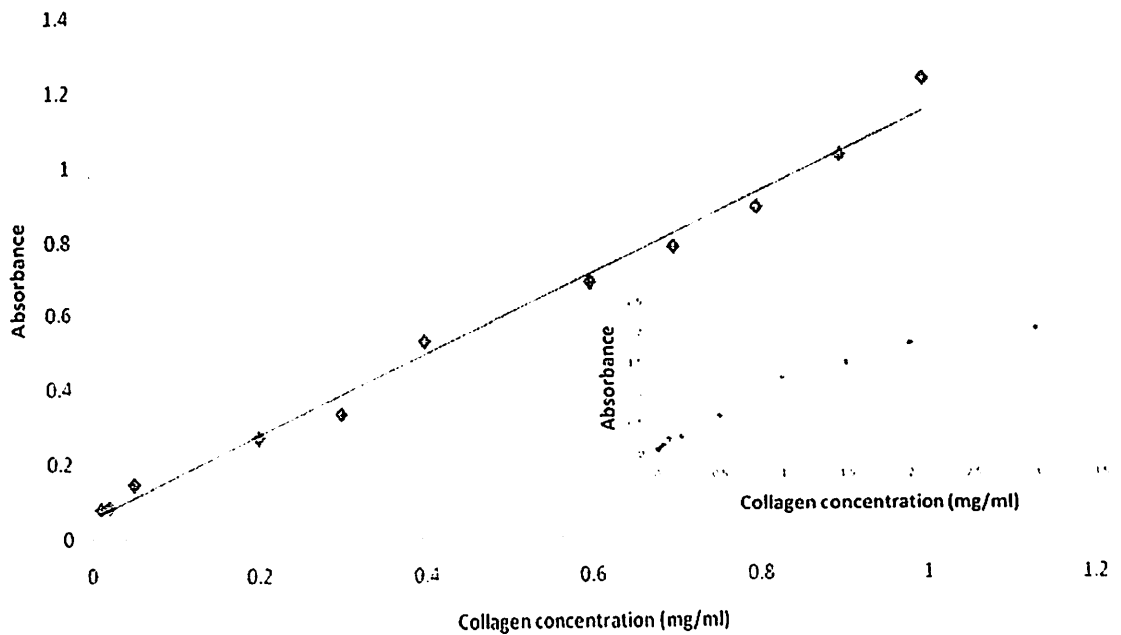

Figure 4.1: The concentration dependence of the absorbance of calf skin Type I collagen solution in 0.05 M acetic acid at 650 nm. The inset shows the relationship between absorbance and wider collagen concentrations from 0.01 – 3.00 mg/ml.

The Lowry's method was a well-known and widely used protein estimation procedure which the sensitivity was claimed to be moderately constant from protein to protein (Waterborg and Matthews, 2002), relying on two different reactions. It started with the formation of copper ion complex with peptide bonds, being stabilized by tartrate under alkaline conditions, known as Biuret chromophore. Colour developed in the second reaction when reduction of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by the reduced copper-peptide bond complex as well as by tyrosine and tryptophan residues took place (Lowry et al., 1951). The reduced Folin-Ciocalteu reagent was blue in colour and the intensity of colour produced was proportional to protein concentration.

Modification of the conventional Lowry's method had successfully adapted this method in determining collagen concentration as well. According to Komsa-Penkova et al. (1996), the presence of imino acids in collagen which ensured the rigidity of collagen polypeptide backbones, resulted in inaccessibility of peptides to Cu^{2+} ions and finally interfered with the formation of copper complexes, causing conventional Lowry's method to be inappropriate to measure collagen concentration. Therefore, incubation of collagen samples at high temperature (50 °C) beyond the denature temperature (40 – 43 °C) was suggested to unfold the triple helix, leading to destruction of rigidity of the polypeptide chains, thus increased the ability to form complexes with Cu^{2+} ions (Komsa-Penkova et al., 1996). Reaction schemes for the Lowry's assay are illustrated in Figure 2

which makes it measurable at the wavelength of 500 – 750 nm using a spectrophotometer (Wrolstad and Decker, 2005).

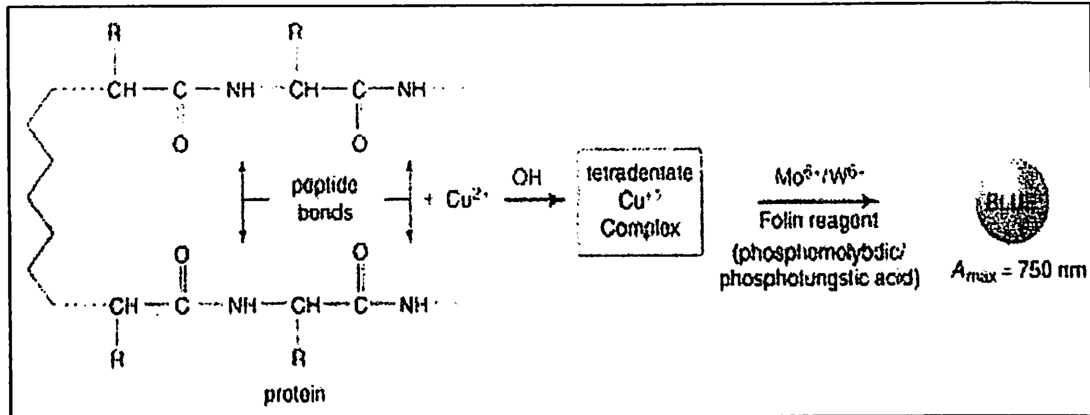

Figure 4.2: Reactions involved in Lowry's assay (Wrolstad and Decker, 2005).

4.2 Screening of Collagen from Selected Malaysian Freshwater Fishes

Both acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the muscle of six selected species of Malaysian freshwater fishes: cultured hybrid catfish (*C. gariepinus* X *C. macrocephalus*), red tilapia (*O. niloticus*), black tilapia (*O. mossambicus*), pangasius catfish (*P. hypophthalmus*), sultan fish (*L. hoevenii*), and labyrinth fish (*T. trichopterus*). They were locally known as "Keli", "Tilapia Merah", "Tilapia Hitam", "Patin", "Jelawat", and "Sepat", respectively (Appendix A). The collagen yields based on wet weight basis, varied in the range of 2.02 ± 0.81 – 8.54 ± 0.98 % whilst in the range of 9.75 ± 1.12 – 36.84 ± 1.82 % for the dry basis. The yields of ASC and PSC (dry basis) from the fish muscle of all selected

fishes are presented in Figure 4.3. For ease of comparison, the yields reported in the subsequent sections referred to the wet yields (unless specified).

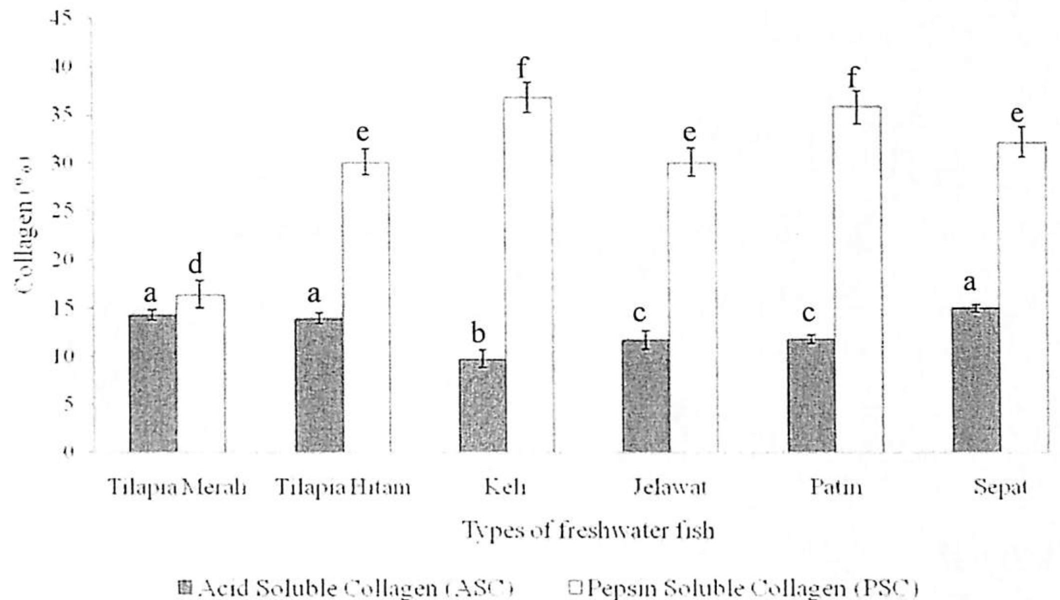

Figure 4.3: Yields (dry) of ASC and PSC from muscle of different Malaysian freshwater fishes. The column with the same alphabet letter was not significantly different (*P*>0.05).

It was found that the collagen yields varied from one fish species to the other. In fact, differences in the yields of extracted ASC and PSC from the muscle were observed among all selected freshwater species. Similar observation was also reported by See et al. (2010) who discovered that the yields of gelatin, the denatured form of collagen varied among the skin of catfish, snakehead, pangasius catfish and red tilapia. Conventionally, quantification of collagen extracted from various sources was done by determining the content of one of the imino acids (Muyonga et al., 2004) known as hydroxyproline (Sadowska et al., 2003; Nalinanon et al., 2007). The amount of hydroxyproline was directly correlated to the amount of collagen present in the raw materials. Kittiphattanabawon et al. (2005) in their studies stated that difference in hydroxyproline

contents between fish species was greatly influenced by the species itself, living environment and body temperature of the fish. Fish species living in colder environment had lower contents of hydroxyproline and the collagen exhibited lower thermal stability than those fishes living in warmer environments (Muyonga et al., 2004; Bae et al., 2008). This was also supported by Pati et al. (2010a) who inferred that higher contents of imino acids in collagen from freshwater fishes were related to their living environments.

Consequently, in the present study, different amount of ASC and PSC were extracted from these six different species of freshwater fish muscle as they were living in different habitats. The hybrid catfish, red tilapia, pangasius catfish and sultan fish selected were the cultured fishes, while the black tilapia and labyrinth fish were wildly harvested in freshwater streams. At the end of the 20-hr-extraction process, for ASC, the yield from red tilapia was found to be the highest (14.29 ± 0.83 %) among all (Figure 4.3). The difference in collagen yields obtained in this study, according to See et al. (2010) depended not only on the variation of the proximate composition of fish muscle, but also the amount of soluble components, as these properties varied with the species of fishes. Nevertheless, Zeng et al. (2012) stated that discrepancies in the construction of collagens among different fish species and the variation in the extraction method performed could be the factors which resulted in the difference of the collagen yields.

The influence of pepsin digestion in the extraction process was obvious in the present study as obvious increment in the yield was observed with the addition of pepsin in the extracting medium. Such increase in the yield of collagen regardless of the sources after pepsin treatment had also been reported by many authors. Among them, Ogawa et

al. (2004), in their research reported that the yield of collagen based on dry weight of black drum skin were 15.80 % for PSC, and 2.30 % for ASC, respectively. Liu et al. (2008) later stated that for channel catfish, 38.40 % of PSC and 25.80 % of ASC were obtained from the skin. Besides, a few researchers also described similar findings for deep sea-redfish (ASC: 47.50 % and PSC: 92.20 %) (Wang et al., 2008a), largefin longbarbel fish (ASC: 16.80 % and PSC: 28.00 %) (Zhang et al., 2009) and the skin of balloon fish (PSC was 5-fold higher than the ASC) (Huang et al., 2011). They were consistent with the observations in this study where the yields of PSC were higher as compared to ASC for all fishes.

It was also found that collagens in the muscle of all selected fishes were not completely extracted with 0.5 M acetic acid, as shown by the lower yield of ASC obtained. This was in agreement with the findings of a few researchers in literature (Kittiphattanabawon et al., 2010a; Huang et al., 2011; Singh et al., 2011; Zeng et al., 2012), who reported that the skin of cobia, striped catfish, balloon fish, and brownbanded bamboo shark were not entirely soluble in 0.5 M acetic acid, instead further solubilisation of the remaining residues was achieved by limited pepsin digestion, thus making the extracting medium very viscous. Collagen molecule was made up of three polypeptide chains intertwined and resembled a three-stranded rope, where non-helical telopeptides were attached to both ends of the molecule (Lin and Liu, 2006a). Enzymatic treatment of fish muscle with pepsin assisted in cleaving only the non-helical ends (telopeptides) of the collagen which act as the inter-molecular crosslink (Bama et al., 2010; Singh et al., 2011), without damaging the integrity of the triple helix. With partial cleavage at the telopeptide region, collagen localized in the loosened molecule

structure could be further extracted, led to higher solubility of collagen in acid (Nalinanon et al., 2007). Therefore, difference in the yields of ASC and PSC from muscle of catfish, black tilapia, red tilapia, pangasius catfish, sultan fish, and labyrinth fish might be contributed by the existence of inter-molecular crosslink at telopeptides region of the collagens that resulted in lower solubility in acid.

In this study, smallest variance between the yield of ASC and PSC was observed in red tilapia. On the contrary, the variation between the yield of ASC and PSC was most pronounced in the case of hybrid catfish. With the aid of pepsin digestion, the yield of muscle collagen increased from 9.75 ± 1.12 % to 36.84 ± 1.82 % at the end of 20-hour-extraction period. Therefore, it could be deduced that the muscle of catfish, particularly of this selected hybrid *Clarias* sp. contained the most inter-molecular cross-links at the telopeptides region and served as the most potential species to be studied in further recovery and isolation of muscle collagen in subsequent stages.

4.3 Optimization of Collagen Extraction Process

4.3.1 Screening of Process Parameters Using Fractional Factorial Design (FFD)

The assumption in using a FFD was that higher order interactions were likely to be of little consequence, thus their corresponding aliasing could be safely ignored. Resolution was termed as the order at which such aliasing appeared 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 the muscle of hybrid *Clarias* sp., FFD was arranged with such factors at different levels (Table 3.3). The corresponding results of the experiments are presented in Table 4.1.

Table 4.1: FFD with corresponding response (using coded variables) for extraction of muscle PSC from hybrid *Clarias* sp.

No.	X_1 (M)	X_2 (hr)	X_3 (°C)	X_4 (mg/l)	X_5 (M)	X_6 (mg/l)	X_7 (hr)	X_8 (rpm)	Yield of PSC (%)
1	-1	-1	1	1	-1	-1	1	1	3.38
2	1	-1	-1	1	1	-1	1	-1	3.70
3	1	1	1	-1	-1	-1	1	-1	4.17
4	1	-1	1	1	-1	1	-1	-1	3.69
5	-1	1	1	-1	-1	1	-1	1	3.27
6	0	0	0	0	0	0	0	0	6.94
7	-1	-1	1	-1	1	1	1	-1	1.99
8	1	1	1	1	1	1	1	1	6.56
9	0	0	0	0	0	0	0	0	6.62
10	-1	1	-1	1	-1	1	1	-1	4.19
11	1	1	-1	1	-1	-1	-1	1	6.97
12	1	-1	-1	-1	-1	1	1	1	5.11
13	-1	-1	-1	-1	-1	-1	-1	-1	1.82
14	1	-1	1	-1	1	-1	-1	1	2.57
15	-1	1	-1	-1	1	-1	1	1	3.25
16	1	1	-1	-1	1	1	-1	-1	3.73
17	0	0	0	0	0	0	0	0	6.46
18	0	0	0	0	0	0	0	0	6.51
19	-1	1	1	1	1	-1	-1	-1	3.03
20	-1	-1	-1	1	1	1	-1	1	3.96

Analysis of variance (ANOVA) was performed on the main effects and the results are summarized in Table 4.2. The F value and the probabilities of $Pr > F$ are also shown. ANOVA for this experiment (F=14.59 > $F_{(8,19,0.01)}$ = 6.75) indicated that the chosen variables significantly affected the yield of PSC extracted from the muscle of hybrid *Clarias* sp. In addition, regressive analysis of the variables shown in Table 4.3 revealed that X_1 ($Pr > F = 0.01\%$), X_2 ($Pr > F = 0.12\%$), X_4 ($Pr > F = 0.05\%$), and X_8 ($Pr > F = 0.14\%$) influenced the extraction efficiency significantly, where as effects of other remaining variables with $Pr > F > 5$ % could be neglected. Also, the deduced first-order multiple regressions as shown in Eq. 4.2:

$$Y = 38.53 + 7.44X_1 + 5.42X_2 - 2.38X_3 + 6.15X_4 - 2.55X_5 + 2.42X_6 + 1.89X_7 + 5.13X_8 \qquad (4.2)$$

including all the above mentioned variables resulted in linear correlation coefficient (R^2) values at 0.92 and Adj. R^2 at 0.86.

Table 4.2: Analysis of variance of FFD for muscle PSC from hybrid *Clarias* sp.

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

According to Annuar et al. (2008), the R^2 value was frequently used to judge whether the model correctly represented the data, implying that, if R^2 was close to one, then the regression model was correct This indicated that the predicted data were well fitted by the model which variations caused by the variables accounted for 92 % for the

extraction of PSC in this study. The experimental results and the model values of Eq. 4.2 are compared in Figure 4.4.

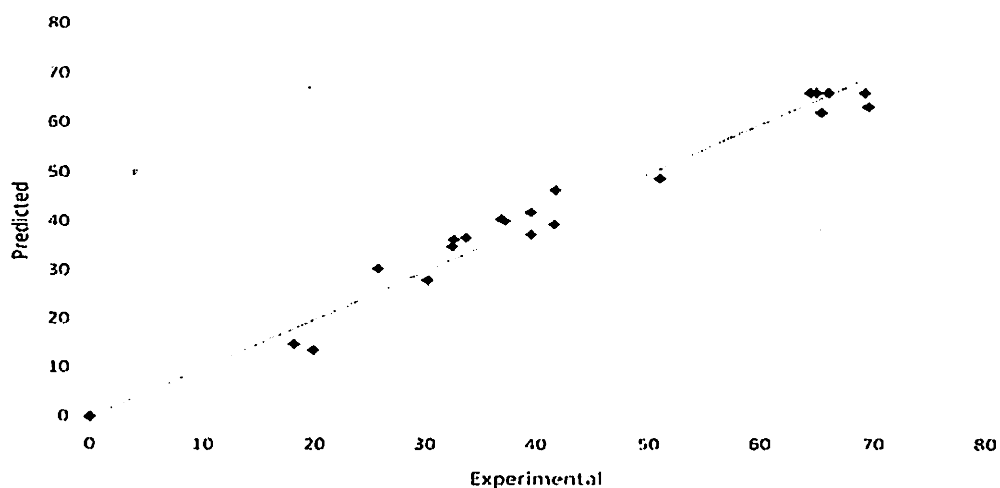

Figure 4.4: Comparison of experimental results and values calculated by the statistical model for extraction of muscle PSC

Table 4.3: Regressive analysis of FFD for muscle PSC from hybrid *Clarias* sp.

Variable	Coefficient Estimate	Standard error	*F* value	*Prob* > *F*	Significance
Intercept	38.53	1.22	14.59	0.0001	Significant
X_1	7.44	1.22	37.43	0.0001	Significant
X_2	5.42	1.22	19.92	0.0012	Significant
X_3	-2.38	1.22	3.83	0.0790	Not significant
X_4	6.15	1.22	25.58	0.0005	Significant
X_5	-2.55	1.22	4.42	0.0619	Not significant
X_6	2.42	1.22	3.98	0.0740	Not significant
X_7	1.89	1.22	2.42	0.1506	Not significant
X_8	5.31	1.22	19.11	0.0014	Significant

From the results in Table 4.3, it can be found that the amount of extracted muscle PSC was greatly influenced by the concentration of acetic acid, extraction time, acetic acid to muscle ratio, and the extraction stirring speed. They were the significant parameters which affecting the yield of muscle collagen extracted in this study. In other words, parameters involving in the stage of dilute acid extraction were found to be predominant over those affecting the alkaline pre-treatment step. This finding was consistent with the work of Wang et al. (2009) who found out that the acetic acid concentration, time, and solvent to material ratio were factors that imposed significant effects on the extraction efficiency of acid soluble collagen (ASC) from grass carp skin. In fact, these factors showed a significant role in the extraction process.

4.3.2 Optimization of Process Parameters Using One-Factor-At-One-Time (OFAT) Method for Extraction of Muscle Collagen

4.3.2 (a) Effect of Acetic Acid Concentration

Skierka and Sadowska (2007) and Cheng et al. (2009) 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. Acetic acid was the most favourable extracting solvent used in collagen extraction studies from marine and land animals. 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., 2008; Aukkanit and Garnjanagoonchorn, 2010;

Figure 4.5 shows the effect of different concentrations of acetic acid on the yield of PSC from the muscle of hybrid *Clarias* sp.

The yield of muscle PSC increased with the increase of acetic acid concentration to 0.5 M. However, a reverse trend was observed beyond this level. The highest yield was achieved when 0.5 M acetic acid was used as the extracting medium and PSC as much as 14.90 ± 0.33 % was extracted. For higher concentration of acetic acid particularly at 0.9 M, the yield of muscle PSC was found to be 13.09 ± 0.34 %, which was significantly (P<0.05) lower than that of 0.5 M. These results were in agreement with the work of Wang et al. (2009) in which the yields of acid soluble collagen (ASC) extracted from grass carp skin were found to be in an increasing manner with the increment of acetic acid concentration to 0.5 M, and thereafter decreased.

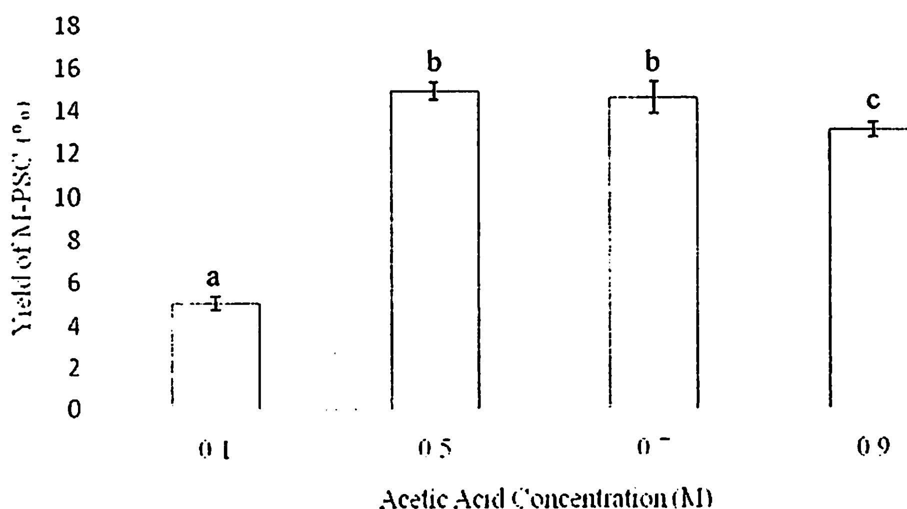

Figure 4.5: Effect of different concentrations of acetic acid on the yield of muscle pepsin soluble collagen (M-PSC) from muscle of hybrid *Clarias* sp. with the conditions: The extraction time of 24 hr, acid to muscle ratio at 10 ml/g, and

stirring speed at 400rpm. The column with the same alphabet letter was not significantly different ($P>0.05$).

Difference in the yields obtained through different concentrations of acetic acid employed was probably due to different solubility of collagen in the acidic extracting medium. Among the concentrations of acid tested, 0.1 M was the least effective solvent for the extraction of collagen. During the 24 hr of extraction, about 5 % of muscle collagen was dissolved. The amount of dissolved collagen was increased when the muscle was incubated in higher concentration of acetic acid. Incomplete solubility of the muscle suggested that inter-molecular cross-links were still present in collagen molecules. According to Skierka and Sadowska (2007), the initial stage of collagen solubilisation was the hydration of fibrous collagen which proceeded by exposure to acids. The modification of electrostatic interaction and structure of this protein might occur along with the changes in acid concentration since the pH value was in charge of the protein charge density (Verheul et al., 1998).

As in the present study, extracting medium of very low pH (0.9 M, pH 2.39) would reduce the collagen water absorption ability. Skierka and Sadowska (2007) in their studies mentioned that the positively charged amine groups of proteins would form bonding with anions at very low pH (refers to CH_3COO^- for acetic acid aqueous solution), hence leading to weaker electrostatic repulsive forces between the one-nominal charged group. This resulted in tightening of the structure of collagen fibres and reduced the ability to form bonding with water, thus decreasing the solubility of collagen in the medium. Besides, Wang et al. (2009) later stipulated that collagen was denatured

at extremely low pH value as collagenous fibres started to shrink at pH around or below 2.0, making protein hydration impossible. These explained the observations in this study that beyond the acetic acid concentration of 0.5 M, a significant decrease in the yield of PSC was observed.

4.3.2 (b) Effect of Acetic Acid to Material Ratio

Solvent to material ratio was an important variable affecting the efficiency of extraction. In this study, the effect of solvent amount (acetic acid) to material (muscle of hybrid *Clarias* sp.) ratio on the extractability is shown in Figure 4.6. An increasing acetic acid to muscle ratio could lead to a higher yield of muscle PSC. The ratio was varied from 10 – 30 ml/g. Nevertheless, when the ratio was raised to more than 25 ml/g, improvement in the yield of the extracted collagen was no longer significant.

Undoubtedly, higher solvent to material ratio was able to increase the concentration gradient and diffusion rate of collagen particles from the fish muscle into the extracting acetic acid, subsequently enhanced the efficiency of the extraction processes (Wang et al., 2009). Using a large amount of solvent however was 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 muscle at 25 ml/g was already sufficient for PSC extraction carried out in this study.

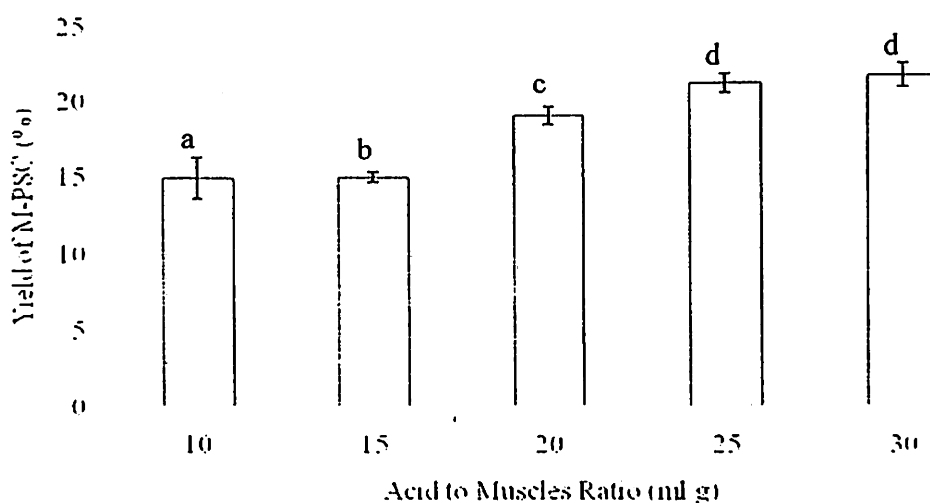

Figure 4.6: Effect of different acetic acid to muscle ratio on the yield of muscle pepsin soluble collagen (M-PSC) with the conditions: The acetic acid concentration of 0.5 M, extraction time of 24 hr, and stirring speed at 400 rpm. The column with the same alphabet letter was not significantly different (P>0.05)).

4.3.2 (c) Effect of Extraction Time

The effect of extraction time on the yield of collagen extracted was also another notable factor that required proper investigation. Positive relationship was found between the extraction time and yield of muscle PSC attained. The yield increased with the extension of time especially when the extraction time ranged from 4 – 16 hr at 4 °C (Figure 4.7). Though the extraction process was prolonged until 24 hr, no further significant improvement in the yield was observed. Similar remark was 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 muscle and skin matrix played a key role in the efficiency of extraction in the present study. The mass transfer rate was controlled by the diffusion process which was time-concerned. Therefore the recovery of analyte (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 period 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 was an important index of collagen molecular integrity and the denaturation of collagen caused a reduction in fibril-forming capacity (Lin and Liu, 2006a). 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.

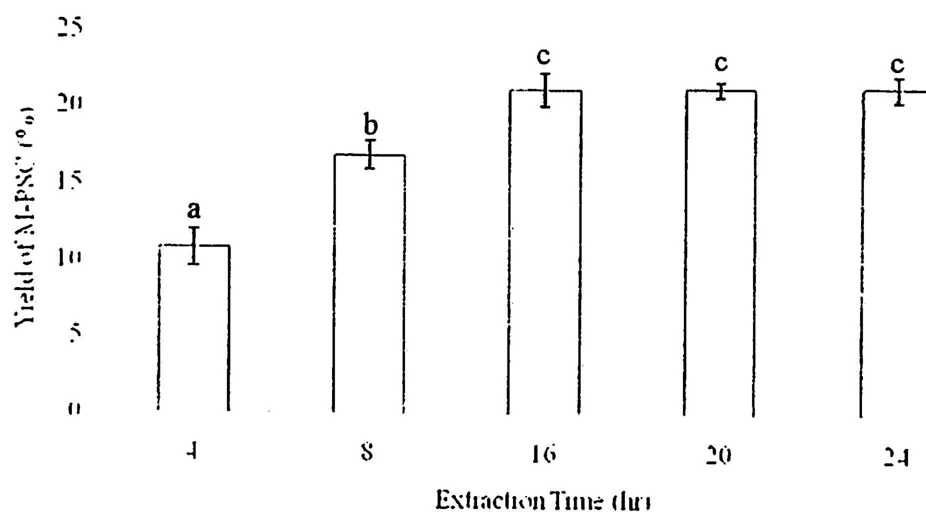

Figure 4.7: Effect of different extraction time on the yield of muscle pepsin soluble collagen (M-PSC) with the conditions: The acetic acid concentration of 0.5 M, acid to muscle ratio at 25 ml/g, and stirring speed at 400 rpm. The column with the same alphabet letter was not significantly different (P>0.05)).

4.3.2 (d) Effect of Stirring Speed

Based on the results of FFD in Section 4.2.1, stirring speed also contributed significant effect towards the extraction efficiency of muscle PSC. Mass transfer was a common phenomenon in any extraction processes, especially in the diffusion-controlled extraction. Stirring speed affected equilibrium time and the amount of analyte (collagen) extracted in the extracting medium. Since mass transfer was limited by diffusion, the more efficient the stirring was, the better would be the mixing between solvent and raw material. Consequently, shorter equilibrium time would be achieved and thus higher amount of analyte would be extracted in the pre-equilibrium conditions (Sanja et al., 2010). However, information on the variation of stirring speed in collagen extraction studies was still scarce in literature. Though collagen extraction was conventionally

suggested to be carried out under vigorous stirring (Nagai et al., 2004), too high stirring speed could possibly lead to generation of excessive heat to the extraction process.

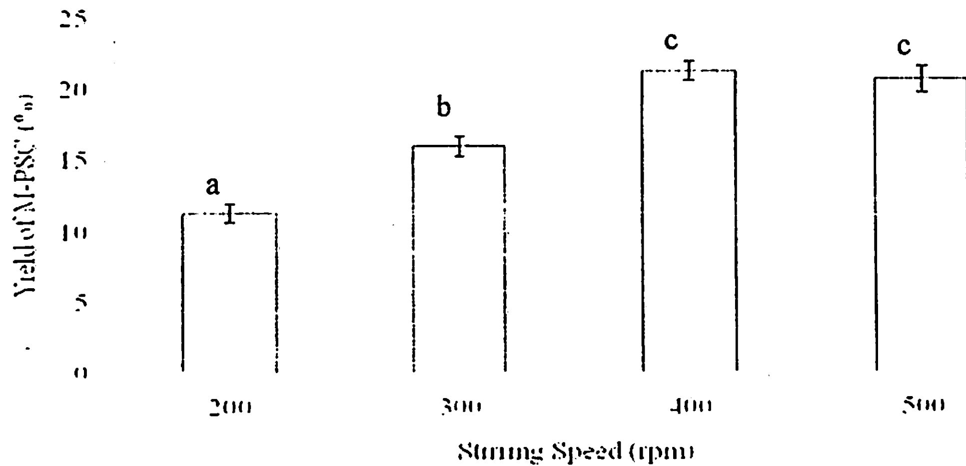

Figure 4.8: Effect of stirring speed on the yield of muscle pepsin soluble collagen (M-PSC) with the conditions: The extraction time of 24 hr, concentration of acetic acid at 0.5 M, and acid to muscle ratio at 25 ml/g. The column containing the same letter was not significantly different ($P>0.05$).

Collagen could be easily denatured easily under this circumstance (Wang et al., 2008b). 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 M-PSC yield at 21.24 ± 0.68 % under the employed extraction conditions (Figure 4.8). A positive relationship was found with the increase of stirring speed on the yield of M-PSC. The amount of M-PSC extracted increased significantly ($P<0.05$) with the elevation of stirring speed from 200 to 400 rpm. This could be due to

the mass transfer between the fish muscle and acetic acid which was greatly enhanced with increasing stirring speed. Greater solubility of muscle in acetic acid was achieved, resulting in greater driving force for collagen particles to diffuse from the fish muscle into the medium. Further increase of the speed after 400 rpm however did not result in significant improvement of the yield.

4.3.3 Optimization of Process Parameters Using Response Surface Methodology (RSM) for Extraction of Muscle Collagen

All 20 experimental points were evaluated and the results of the corresponding yield of muscle pepsin soluble collagen (M-PSC) based on the experimental design are shown in Table 4.4. Response surface optimization was favorable over the conventional single parameter optimization in that it saved time, space, and raw materials (Zhang et al., 2010b). A maximum M-PSC extraction yield of 22.76 ± 0.94 % was obtained under the experimental conditions of acetic acid concentration 0.5 M, acetic acid to muscle ratio 25 ml/g, and stirring speed of 400 rpm. By applying multiple regression analysis on the experimental data, the predicted response variable and the independent variables were related in terms of coded values by the following quadratic equation (Eq. 4.3):

$$Y = 21.09 + 4.41X_1 + 2.01X_2 + 2.16X_3 - 5.032X_1^2 - 0.09X_2^2 - 2.63X_3^2 + 0.66X_1X_2 + 0.22X_1X_3 - 1.22X_2X_3 \quad (4.3)$$

where Y, yield of extracted M-PSC (%); X_1, acetic acid concentration (M); X_2, acetic acid to muscle ratio (ml/g); and X_3, stirring speed (rpm). The coefficients with one factor represented the effect of that particular factor, while the coefficients with two factors and those with second-order terms represented the interaction between the two factors and the quadratic effect, respectively (Rodrigues et al., 2006). ANOVA was used to

evaluate the significance of the coefficients of the model. The regression coefficient values of equation are listed in Table 4.5.

Table 4.4: Response surface CCD and results for the yield of M-PSC from hybrid *Clarias* sp.

No.	X_1, Acetic acid concentration (M)	X_2, Acid to muscle ratio (ml/g)	X_3, Stirring speed (rpm)	Y, Yield of M-PSC (%)
1	-1 (0.1)	-1 (20)	-1 (300)	5.10 ± 0.35
2	1 (0.9)	-1 (20)	-1 (300)	11.65 ± 0.65
3	-1 (0.1)	1 (30)	-1 (300)	5.09 ± 0.23
4	1 (0.9)	1 (30)	-1 (300)	15.17 ± 1.30
5	-1 (0.1)	-1 (20)	1 (500)	10.05 ± 0.18
6	1 (0.9)	-1 (20)	1 (500)	18.35 ± 0.83
7	-1 (0.1)	1 (30)	1 (500)	6.02 ± 1.18
8	1 (0.9)	1 (30)	1 (500)	16.08 ± 0.87
9	-1 (0.1)	0 (25)	0 (400)	11.05 ± 0.55
10	1 (0.9)	0 (25)	0 (400)	20.18 ± 0.78
11	0 (0.5)	-1 (20)	0 (400)	15.09 ± 0.83
12	0 (0.5)	1 (30)	0 (400)	20.02 ±0.66
13	0 (0.5)	0 (25)	-1 (300)	13.96 ± 0.54
14	0 (0.5)	0 (25)	1 (500)	21.65 ± 0.98
15	0 (0.5)	0 (25)	0 (400)	22.56 ± 1.20
16	0 (0.5)	0 (25)	0 (400)	20.38 ± 0.73
17	0 (0.5)	0 (25)	0 (400)	21.25 ± 0.51
18	0 (0.5)	0 (25)	0 (400)	20.65 ± 0.55
19	0 (0.5)	0 (25)	0 (400)	22.76 ± 0.94
20	0 (0.5)	0 (25)	0 (400)	21.98 ± 0.85

The *P*-values were used as a tool to check the significance of each coefficient, subsequently indicating the pattern of the interactions between the variables. Zhang et al. (2010b) stated that for any of the terms in a particular model, a large regression coefficient and a small *P*-value were implying a more significant effect on the respective response variables. This indicated that the smaller the values of *P*, the more significant the corresponding coefficient. From Table 4.5, it could be seen that the linear coefficients (X_1, X_2, X_3) and the quadratic term coefficients (X_1, X_3) were significant, with very small *P*-values ($P<0.05$). Interaction between the coefficient X_2 and X_3, namely the acetic acid to muscle ratio and stirring speed was also another significant effect in the proposed model with the *P*-value at 0.0175.

Table 4.5: Estimated coefficients of the fitted quadratic polynomial equation for different responses of M-PSC from hybrid *Clarias* sp.

Parameter	Regression Coefficient	Standard Error	*F*-value	*P*-value	Indication
Linear					
X_1	4.41	0.39	130.85	<0.0001	Significant
X_2	2.01	0.39	27.27	0.0004	Significant
X_3	2.16	0.39	31.38	0.0002	Significant
Quadratic					
X_1^2	-5.03	0.74	46.82	<0.0001	Significant
X_2^2	-0.09	0.74	0.016	0.9034	Not Significant
X_3^3	-2.63	0.74	12.81	0.0050	Significant
Interaction					
$X_1 X_2$	0.66	0.43	2.35	0.1560	Not Significant
$X_1 X_3$	0.22	0.43	0.25	0.6266	Not Significant
$X_2 X_3$	-1.22	0.43	8.07	0.0175	Significant

According to Wang et al. (2008b) and Zhang et al. (2010a), exploration and optimization of a fitted response surface might produce poor or misleading results unless the model exhibited a good fit, which made the checking of the model adequacy essential. The *P*-value of the model was less than 0.0001 (Table 4.6). Meanwhile, the lack of fit value of the model was 0.0722 which was not significant. These two values confirmed that the model fitness was good. In addition, a lower value of coefficient of variation (CV) at 7.09 % which was lower than 10 % indicated a better precision and reliability of the experiments (Nath and Chattopadhyay, 2007). CV was frequently used as an indication of the degree of precision with which the treatments were compared. The higher the value of CV, thc lowcr thc rcliability of the experiment was (Zhang et al., 2010a). As for the adequate precision value, which was a measure of the signal (response) to noise (deviation) ratio, a ratio greater than four was desirable (Zhang et al., 2010b). In this study, the ratio was found to be 21.58, indicating an adequate signal and therefore the model was significant for the extraction process. Another important analysis by ANOVA was the checking of the precision of a particular model by the determination of correlation coefficient (R^2) (Nath and Chattopadhyay, 2007). In the present study, the value of R^2 (0.9733) for Eq. 4.3 indicated a close agreement between the experimental results and the predicted values. Thus it was proven that the proposed regression model was able to define the true behavior of the collagen extraction system well.

Table 4.6: Analysis of variance (ANOVA) for the response surface quadratic model of the yield of M-PSC from hybrid *Clarias* sp.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Model	9	542.67	60.30	40.54	<0.0001
Residual	10	14.87	1.49		
Lack of fit	5	11.98	2.40	4.15	0.0722
Pure error	5	28.89	0.58		
Total	19	557.54			

R^2= 0.9733; Dependent mean=17.21; Adj. R^2= 0.9493; CV= 7.09

Graphical representations of the regression model, known as the response surfaces and contour plots were obtained with the aid of Design Expert software version 6.0.6. The results of the M-PSC extraction yield as influenced by acetic acid concentration, acetic acid to muscle ratio, and stirring speed are illustrated in Figure 4.9 (a), (b) and (c), respectively. Response surface methodology played a key role in identifying the optimum values of the independent variables efficiently, under which response variable could achieve a maximum response (Zhang et al., 2010b). In the response surface and contour plot, the extraction yield of M-PSC was obtained along with two continuous variables, whilst the remaining variable was held constant at the respective zero level (center value of the testing ranges) (Woo et al., 2008). Shapes of the corresponding contour plots indicated whether mutual interactions between the independent variables were significant or not (Zhang et al., 2010b). Elliptical contours were obtained when there was a perfect interaction between the independent variables (Muralidhar et al., 2001; Chang et al., 2007), and the maximum predicted value indicated by the surface was confined to the smallest ellipse in the contour diagram

(Zhang et al., 2010b). From the 3D response surface plots, optimal values of the independent variables could be identified, and the interaction between each independent variable's pair could be easily understood.

The effect of acetic acid concentration and acetic acid to muscle ratio on the yield of M-PSC from muscle of hybrid *Clarias* sp. are shown in Figure 4.9 (a). The yield of M-PSC increased with the increase of acetic acid concentration to a certain value (approximately 0.5M), and thereafter decreased. A similar trend was observed in Figure 4.9 (b) where interaction between acetic acid concentration and the stirring speed is shown. The yield of M-PSC increased with the increase of acetic acid concentration to 0.5 M. However, a reverse trend was observed beyond this concentration. The result was consistent with the work of Wang et al. (2009) in which for the optimization of the ASC extraction conditions from grass carp (*Ctenopharyngodon idella*) skin, they pointed out that more positively charged amine groups of collagen were resulted when the concentration of acetic acid used was at 0.5 M, thus leading to the highest yield among the studied concentrations. Also, the pattern of the yield profile observed in Figure 4.9 (a) and (b) was similar to that of OFAT study in Section 4.3.2. Denaturation of collagen at extremely low pH value ($pH<2$) was another possibility that lower yield was obtained when acetic acid with concentration beyond 0.5 M was utilized.

A positive relationship was found between acetic acid to muscle ratio and the yield of M-PSC (Figure 4.9 (a) and (c)). An increasing acetic acid to muscle ratio could lead to a higher yield of M-PSC. The ratio was varied from 20 – 30 ml/g, but when it was raised to more than 25 ml/g, improvement in the yield of M-PSC was no longer

significant. Similar trend was also observed for the effect of stirring speed on the amount of M-PSC extracted along the process. The amount of M-PSC extracted increased drastically with the elevation of stirring speed from 300 rpm to 400 rpm (Figure 4.9 (b) and (c)). Further increase of the speed after 400 rpm however did not result in significant improvement of the yield. These observations were also consistent with the results of OFAT study in Section 4.3.2. Among the three extraction parameters studied, acetic acid concentration was the most significant factor affecting the yield of M-PSC in this extraction process, followed by stirring speed, and the acetic acid to muscle ratio. This was proven by the regression coefficients significance of the quadratic model (Table 4.5) and gradient of slope in the 3-D response surface plot (Figure 4.9 (a) – (c)).

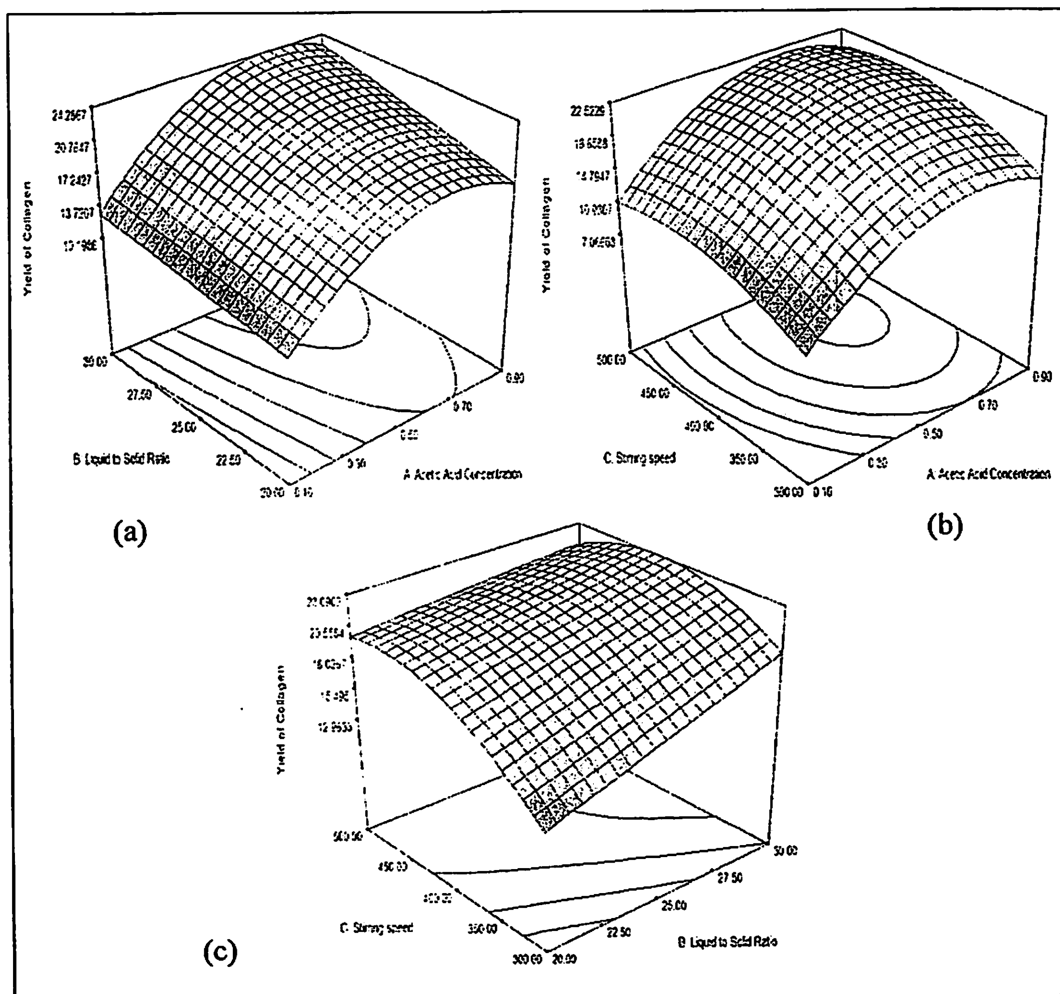

Figure 4.9: Response surface plot showing the effects of: (a) Acetic acid concentration and acetic acid to muscle ratio on the yield of M-PSC. The stirring speed was constant at 400 rpm. (b) Acetic acid concentration and stirring speed on the yield of M-PSC. The acetic acid to muscle ratio was constant at 25 ml/g. (c) Acetic acid to muscle ratio and stirring speed on the yield of M-PSC. The acetic acid concentration was constant at 0.5 M.

By prediction with computing program, the optimal conditions to obtain the highest yield of M-PSC were identified as follows: an acetic acid concentration of 0.67 M, the acetic acid to muscle ratio of 24.65 ml/g, and the stirring speed of 423.84 rpm (Appendix B). In order to check on the adequacy of the predictive model, 5 sets of

experiments were repeated at the suggested optimum conditions in order to experimentally obtain the maximum yield of M-PSC from the muscle of hybrid *Clarias* sp. As shown in Table 4.7, the percentage error differences between the experimental and predicted values were in the range of 0.67 – 4.72 %. Teoh et al. (2012) described that when the differences between the experimental and predicted response were less than 10 %, the validity of the model was verified. Therefore, the developed model for M-PSC extraction process was then validated.

Table 4.7: Validation of the data and constructed model for extraction of M-PSC from hybrid *Clarias* sp.

	Experimental Yield (%)	Predicted Yield (%)	Error (%)
Trial 1	22.87 ± 0.64	22.46 ± 0.00	1.83
Trial 2	22.61 ± 0.42	22.46 ± 0.00	0.67
Trial 3	21.47 ± 0.33	22.46 ± 0.00	4.41
Trial 4	21.40 ± 0.91	22.46 ± 0.00	4.72
Trial 5	22.03 ± 0.87	22.46 ± 0.00	1.91
Average	22.08 ± 0.56	22.46 ± 0.00	2.71

4.3.4 Optimization of Process Parameters Using Artificial Neural Network (ANN) and Genetic Algorithm (GA) for Extraction of Muscle Collagen

4.3.4 (a) Artificial Neural Network Topology

Based on the results of FFD in Section 4.3.1, the input parameters chosen in ANN modeling for the extraction of M-PSC were acetic acid concentration (M), acetic acid to muscle ratio (ml/g), and the stirring speed (rpm). The design of experiment, which was used for the network training and their respective experimental yields were similar to the design of experiments generated by RSM. This was because Desai et al.

(2008) previously mentioned that the experimental data of RSM was sufficient to build an effective ANN model.

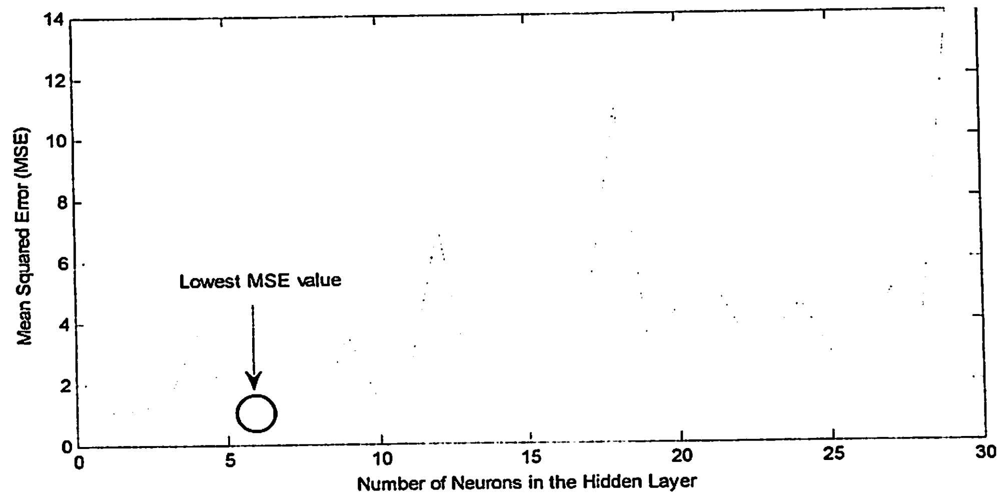

Figure 4.10: Selection of number of neurons in the hidden layer for the extraction of M-PSC

Since there was no theoretical principle in choosing the proper network topology, several structures were tested to obtain the best one. The number of neurons in the hidden layer were varied from 1 – 30 and the network was trained and tested after each addition of neuron. The lowest MSE obtained for M-PSC model was 0.9038 with 6 neurons in the hidden layer (Figure 4.10). The configuration of the ANN for M-PSC was therefore defined as 3-6-1.

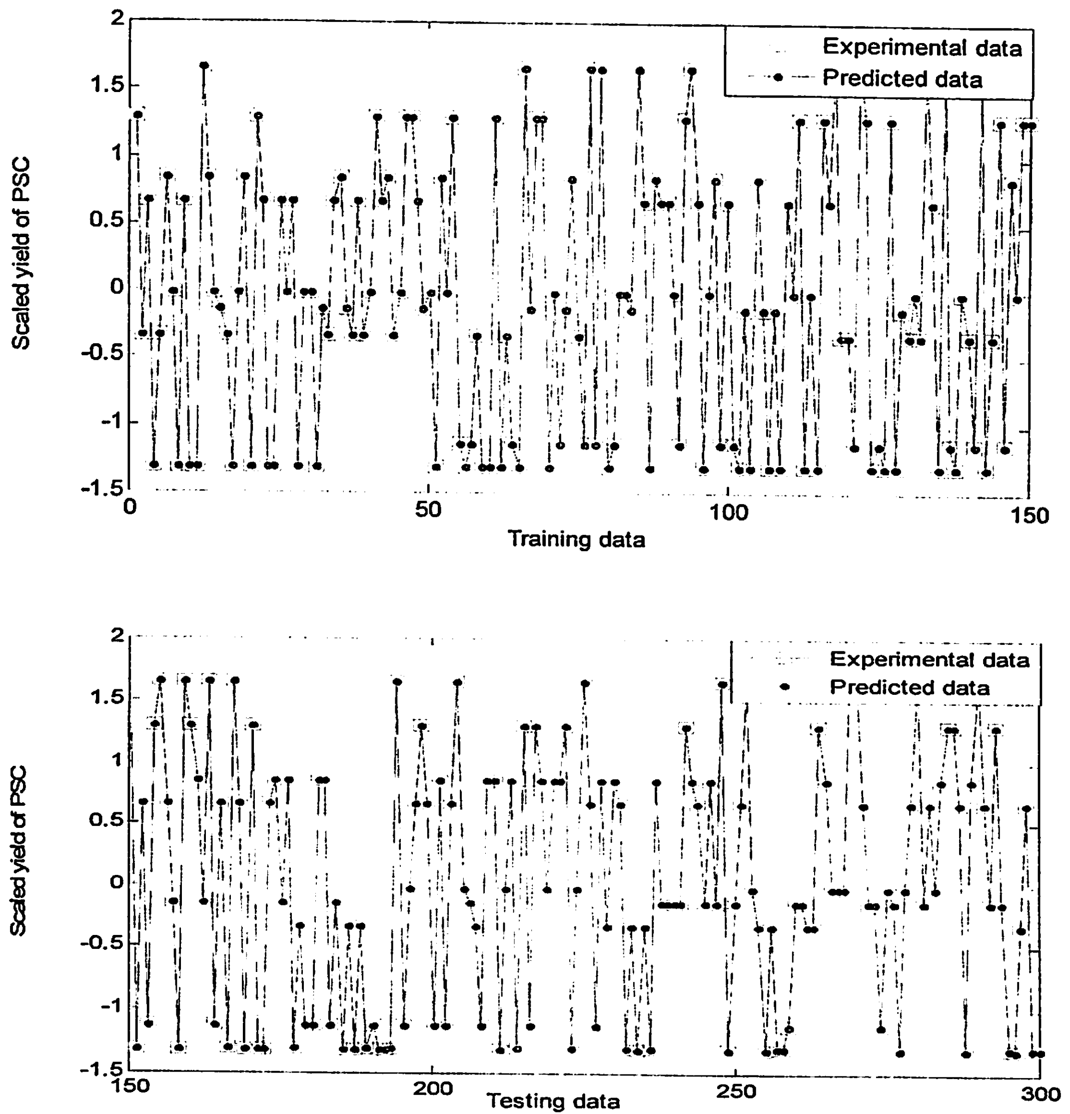

Figure 4.11: Training and testing data for ANN model of M-PSC

The tan-sigmoid transfer function was used as the activation function for hidden layer, and linear transfer function was applied for the output layer. The predicted data for training and testing sets are illustrated in Figure 4.11 for ANN model of M-PSC. It was obvious that the actual and predicted values were more or less the same and in

agreement with each other for the model. Matlab coding or M-file for the development of ANN model is shown in Appendix C.1.

4.3.4 (b) Prediction Accuracy of Trained Network

The MSE between experimental and model predicted M-PSC yield for the training and testing set was 2.0273×10^{-9} and 2.4191×10^{-9}, respectively; while the correlation coefficient value, R^2, between the predicted model and experimental yield pertaining to the training and testing set on the other hand was both maximum ($R^2 = 1$). In fact, evaluation of the established ANN model with the validation data (previously unseen), indicated that the model has a very good accuracy with an R^2 value of 0.9527 and MSE value of 0.1672 (Table 4.8), implying an excellent generalization capacity of the network.

Table 4.8: Correlation coefficient and sum squared error for training, testing and validation sets of M-PSC ANN model

	M-PSC ANN Model	
Data set	Correlation coefficient (R^2)	Mean squared error (MSE)
Training	1.000	2.0273×10^{-9}
Testing	1.000	2.4191×10^{-9}
Validation	0.9527	0.1672

The predicted and actual values for the unseen data are presented in Figure 4.12. Close agreement between the experimental results and theoretical values predicted by ANN indicated that the trained ANN model was capable in defining the true behavior of

the collagen extraction system adequately. This was also supported by the relatively low values of MSE for all data sets.

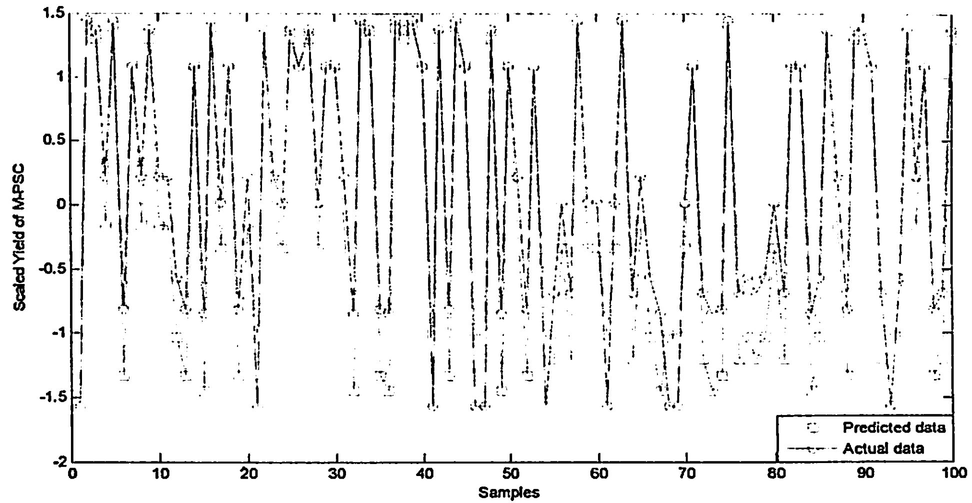

Figure 4.12: Actual and predicted output for unseen data of M-PSC

4.3.4 (c) Genetic Algorithm Based Optimization

The GA-based technique was applied to optimize the input space of ANN model with the objective of maximization of collagen yields (M-PSC) in the extraction process. The objective function could be defined as follows:

$$Maximize\ F(x) = \lambda f(x_1, x_2, \dots \dots x_m) \tag{4.4}$$

Subject to $x_{min} \leq x \leq x_{max}$

where F(x) denoted the optimization function of the process optimization (ANN model); f represented the functional relationship between objective function and the key process parameters; λ denoted the weight of the objective function; $x = [x_1, x_2, \dots, x_m]$ was the matrix consists of collagen extraction process parameters, $m \geq 1$; x_{max} and x_{min} were the upper and lower bounds of the process parameters, respectively. During GA-

implementation, the search for the optimal solutions was restricted between the bounds specified in the design of experiments (Table 3.4).

The GA-based optimization procedure was repeated several times for different randomly initialized population of the candidate solutions (chromosomes) and also for different GA specific parameters. This was to ensure that entire search space was search thoroughly to find the global optimum (refer Appendix C.2 for the M-file coding). The ANN predicted yield of M-PSC at GA optimized condition was 23.81 % with the extraction process conditions as follows: an acetic acid concentration of 0.70 M, the acetic acid to muscle ratio of 25.40 ml/g, and the stirring speed of 443.75 rpm (Appendix C.3).

4.3.4 (d) Validation of Predictive Model

In order to check on the adequacy of the predictive model and accuracy of the GA optimized conditions, 5 sets of experiments were repeated at the suggested optimum conditions for the extraction of M-PSC in order to experimentally obtain the maximum yield of PSC from the muscle of hybrid *Clarias* sp. As shown in Table 4.9, the percentage error differences between the experimental and predicted values for the extraction processes were in the range of 0.24 – 3.43 %. The validity of the models was confirmed with very small differences resulted between the experimental and predicted response (always less than 5 %) (Teoh et al., 2012). The average PSC yields extracted in the verification experiments was 23.41 ± 0.61 % for the muscle collagen, which was in close agreement with the solutions predicted by the ANN-GA models.

Table 4.9: Validation of the predicted model generated by ANN/GA algorithm for extraction of M-PSC

		Experimental Yield (%)	Predicted Yield (%)	Error (%)
M-PSC	Trial 1	23.77 ± 0.67	23.81 ± 0.00	0.24
	Trial 2	23.00 ± 0.23	23.81 ± 0.00	3.43
	Trial 3	23.13 ± 0.98	23.81 ± 0.00	2.90
	Trial 4	23.52 ± 0.55	23.81 ± 0.00	1.22
	Trial 5	23.62 ± 0.79	23.81 ± 0.00	0.85
	Average	23.41 ± 0.61	23.81 ± 0.00	1.73

Based on the validation results, it was proven that the optimization using ANN/GA successfully provided an adequate model to represent the non-linear nature between different extraction conditions and the resulting collagen yields in an efficient manner. Consequently, apart from the conventional RSM optimization method, the approach of process optimization using the ANN/GA technique was sufficiently general and thus could also be employed in modeling and optimization of collagen extraction studies and other bioprocess researches.

4.3.5 Comparison of Collagen Yield between Response Surface Methodology (RSM) and Artificial Neural Network/Genetic Algorithm (ANN/GA)

The estimation capabilities between the statistical- and artificial intelligence-based optimization techniques namely RSM and ANN/GA, respectively, were compared and examined. The comparison was made on the basis of various parameters such as the R^2 for both training and validation sets, predicted yields by both techniques and the average error (%) of the experimental yields based on the optimum conditions suggested.

The generalization ability could best be judged with the unseen (validation) dataset (Desai et al., 2008). The comparative values are tabulated in Table 4.10.

Table 4.10: Comparison of predictive capability between RSM and ANN/GA model

	Parameters	RSM	ANN/GA
M-PSC Model	Training R^2	0.9733	1.000
	Validation R^2	0.8270	0.9527
	Predicted Yield (%)	22.46 ± 0.00	23.83 ± 0.00
	Average Experimental Yield (%)	22.08 ± 0.56	23.41 ± 0.61
	Average Yield Error (%)	2.71	1.73

RSM was one of the most popular and widely used techniques in optimization studies of chemical and biochemical processes. Nonetheless, it had some limitations that RSM employed the linear and second-order equations for modeling of the processes (Baş and Boyacı, 2007). It was uncertain that all changes in the process could be sufficiently explained with only a second-order equation. Therefore, in the present study, ANN/GA model had shown significant higher generalization and predictive capability as compared to RSM based on the R^2 values of training and validation datasets for the extraction of M-PSC. In addition, higher yield of M-SPC was predicted by ANN/GA model with the same design of experiments data. Validation of the predictive results given by both optimization techniques revealed that the prediction made by RSM had a greater deviation (error) than the ANN/GA model prediction (Table 4.10). This higher predictive accuracy of ANN/GA model could be attributed to its universal ability to approximate non-linearity of the systems, whilst RSM was only restricted to the second-order polynomial (Desai et al., 2008). In this study, the ANN/GA methodology showed a

clear superiority over RSM as a modeling technique for datasets showing non-linear relationships. It exhibited better data fitting and estimation capabilities than the latter. Therefore, ANN/GA could be an alternative to RSM as a modeling method for collagen extraction processes.

4.4 Characterization of Extracted Collagen from Hybrid *Clarias* sp.

4.4.1 Characterization of Fish Muscle Collagen

4.4.1 (a) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoresis patterns of muscle acid soluble collagen (M-ASC) and muscle pepsin soluble collagen (M-PSC) from the muscle of hybrid *Clarias* sp. are shown in Plate 4.1. Results showed that both M-ASC and M-PSC consisted of β (dimers) and only 1 α chain ($\alpha 1$ band). Besides the absence of $\alpha 2$ band, they were different from the patterns of S-ASC and S-PSC as there was no γ (trimers) band in these muscle collagens.

Based on the SDS-PAGE analysis on the extracted M-ASC and M-PSC, it was obvious that the electrophoretic patterns were not comparable to that of the typical type I calf skin collagen (standard). Not to mention that only fade β and $\alpha 1$ bands were obtained, there was also no band for high molecular weight component (i.e. γ band) which was the main characteristic of type I collagen. In addition, presence of tropomyosin at the molecular weight of approximately 35 kDA in the electrophoresis pattern of M-ASC confirmed the dissimilarity between the extracted muscle collagens of hybrid *Clarias* sp. with the desired type I collagen (Okano and Tetsuya, 1999; Werner et

al., 2007). Tropomyosin was an important protein compound which played a pivotal role in regulating the muscle contraction (Okano and Tetsuya, 1999). Nevertheless, the existence of lower molecular weight components was not expected in the SDS-PAGE characterization study of type I collagen.

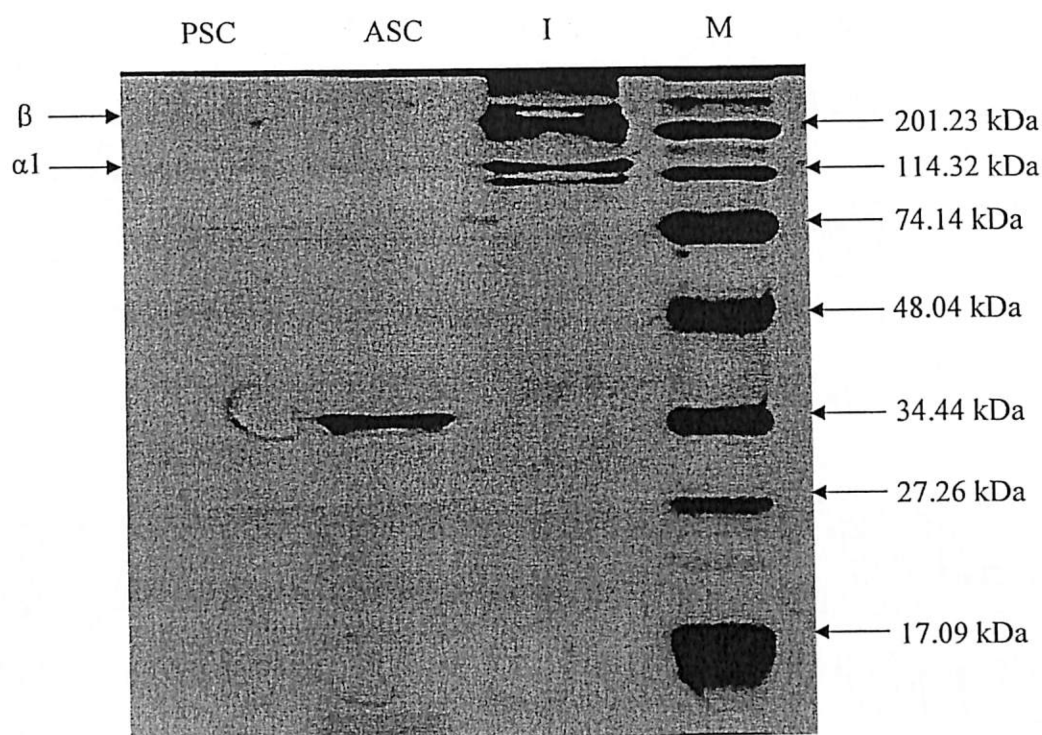

Plate 4.1: SDS-PAGE of acid soluble collagen (M-ASC) and pepsin soluble collagen (M-PSC) from the muscle of hybrid *Clarias* sp. under reducing condition. M and I denoted high molecular weight protein markers and type I collagen from calf skin, respectively

4.4.1 (b) Amino Acid Analysis

The amino acid compositions of the M-ASC and M-PSC extracted from the muscle of hybrid *Clarias* sp. are presented in Table 4.11. The composition was expressed as amino acid residues per 1000 total amino acid residues. It was found that

the most abundant amino acid for both M-ASC and M-PSC was the glutamic acid (Glu) which accounted for 146 and 134 units of the total amino acids, respectively. This had deviated from the characteristic of type I collagen in which glycine (Gly) content was supposed to be the highest among all amino acids (Kittiphattanabawon et al., 2005; Huang et al., 2011). Even though absence of cysteine was in accordance with the characteristics of skin collagens, yet relatively low content of alanine, proline and hydroxyproline was observed in both M-ASC and M-PSC. Also, these muscle collagens from hybrid *Clarias* sp. were rich in histidine and both contained relatively high amount of lysine and isoleucine. Such results were significantly different from those reported in literature for the amino acid composition of type I collagen from various sources (Muyonga et al., 2004; Zhang et al., 2009; Kittiphattanabawon et al., 2010a). According to Berillis et al. (2011), the chemical and physical properties of collagen proteins in fishes were different in tissues such as skin, swim bladder, and the myocommata in muscle. Therefore, this explained the discrepancies of amino acid compositions between the expected and muscle collagens obtained from the hybrid *Clarias* sp. in the present study.

On the other hand, the amino acid compositions of the extracted M-ASC and M-PSC in this study also differed from the muscle collagens extracted from the eel (*Anguilla japonica*), common mackerel (*Scomber japonicus*), saury (*Cololabis saira*), chum salmon (*Onchorhynchus beta*) and carp (*Cyptinus carpio*) (Kimura et al., 1988). It was reported that the compositions of muscle and typical type I skin collagens were identical to each other in eel (*Anguilla japonica*), common mackerel (*Scomber japonicus*), saury (*Cololabis saira*) and carp (*Cyptinus carpio*) (Kimura et al., 1988).

Table 4.11: Amino acid composition of fish muscle collagen of hybrid *Clarias* sp. (amino acid residues per 1000 total amino acid residues)

Amino acid		Acid soluble collagen (M-ASC)	Pepsin soluble collagen (M-PSC)
Aspartic acid	Asp	73	70
Serine	Ser	35	39
Glutamic acid	Glu	146	134
Glycine	**Gly**	49	**51**
Histidine	his	113	146
Arginine	Arg	86	64
Threonine	Thr	68	65
Alanine	Ala	54	59
Hydroxyproline	**Hyp**	**18**	**22**
Proline	**Pro**	**35**	**29**
Cystine	Cys	0	0
Tyrosine	Tyr	43	30
Valine	Val	39	41
Methionine	Met	19	23
Lysine	Lys	77	78
Isoleucine	Ile	45	39
Leucine	leu	72	76
Phenylalanine	Phe	28	34
Total		1000	1000

Berillis et al. (2011) described that diet supplementation with vitamin A and C promoted a significant increase in collagen biosynthesis, especially of type I. They found that fishes fed on organic feed which had a higher content of vitamins (A, C, and E) and metals (zinc, manganese, and iron), resulted in a bigger collagen fibril diameter than fishes that fed on the conventional feed. The total lipid content, fatty acid

proportions, and amino acid compositions between white muscle tissues of fishes also differed with the types of feed used in the culture of the fishes. Consequently, these differences between the results of the present study with the literature may be attributed to the constituents of the diet of the fishes where in the case of hybrid *Clarias* sp., commercial feed in pellet form was used for its culture instead of organic feed.

4.4.2 Characterization of Fish Skin Collagen

4.4.2 (a) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoresis patterns of skin acid soluble collagen (S-ASC) and skin pepsin soluble collagen (S-PSC) from the skin of hybrid *Clarias* sp. are shown in Plate 4.2. Results showed that both S-ASC and S-PSC consisted of at least two α chains (α1 and α2). Both collagens contained inter- and intra- molecular cross-linked components of β (dimers) and γ (trimers) similar to species previously reported such as Nile perch (*Lates niloticus*) (Muyonga et al., 2004), bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon et al., 2005), brown backed toadfish (Senaratne et al., 2006), largefin longbarbel catfish (*Mystus macropterus*) (Zhang et al., 2009), brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010a), and striped catfish (*Pangasianodon hypophthalmus*) (Singh et al., 2011). The electrophoretic pattern of hybrid *Clarias sp.* skin was also similar to that of calf skin collagen. Apart from that, α1 and α2 chains of both S-ASC and S-PSC were found at a ratio of approximately 2:1. This was a typical feature of type I collagen, which was the major collagen in dermal tissue (Muyonga et al., 2004). Nevertheless, it was found that the proportion of high MW components was greater in S-ASC as compared to S-PSC, as evidenced by higher

band intensity of β and γ chains as well as more cross-linked components than the latter. Therefore, the results of the present study implied that the intra- and inter-molecular crosslinks of collagen were richer in S-ASC than in S-PSC. As suggested by Singh et al. (2011) and Matmaroh et al. (2011), pepsin was able to cleave peptides at the telopeptide region, in which β and γ chains were cleaved into α components, resulting in increased band intensity of the α-chains in S-PSC. Based on the electrophoretic mobility and subunit composition, it was suggested that collagens from the skin of hybrid *Clarias* sp. were type I collagen.

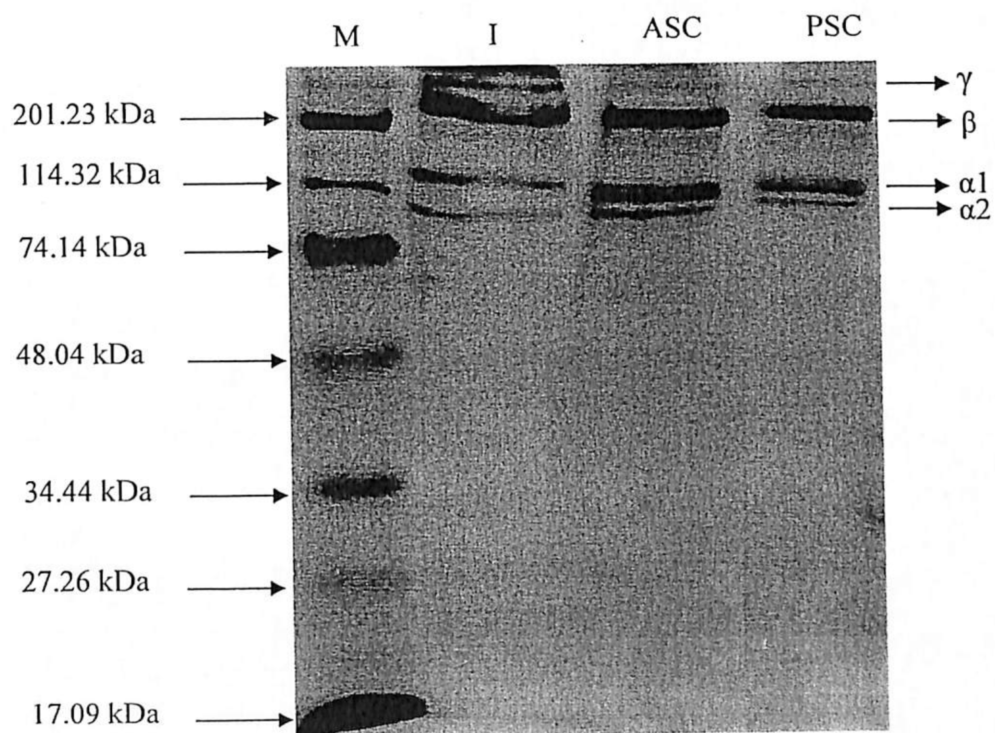

Plate 4.2: SDS-PAGE of acid soluble collagen (S-ASC) and pepsin soluble collagen (S-PSC) from the skin of hybrid *Clarias* sp. under reducing condition. M and I denoted high molecular weight protein markers and type I collagen from calf skin, respectively.

4.4.2 (b) Amino Acid Analysis

Table 4.12 shows the amino acid composition of the S-ASC and S-PSC extracted from the skin of hybrid *Clarias* sp.

Table 4.12: Amino acid composition of fish skin collagen of hybrid *Clarias* sp. (amino acid residues per 1000 total amino acid residues)

Amino acid		Acid soluble collagen (S-ASC)	Pepsin soluble collagen (S-PSC)
Aspartic acid	Asp	60	57
Serine	Ser	46	50
Glutamic acid	Glu	105	95
Glycine	**Gly**	**207**	**223**
Histidine	his	2	2
Arginine	Arg	86	102
Threonine	Thr	21	23
Alanine	Ala	90	95
Hydroxyproline	**Hyp**	**55**	**59**
Proline	**Pro**	**130**	**126**
Cystine	Cys	0	0
Tyrosine	Tyr	13	6
Valine	Val	36	33
Methionine	Met	19	20
Lysine	Lys	41	40
Isoleucine	Ile	28	15
Leucine	leu	31	30
Phenylalanine	Phe	30	24
Total		1000	1000

The composition was expressed as amino acid residues per 1000 total amino acid residues. Since collagen was triple helical in nature with the characteristic amino acid of $(Gly\text{-}Pro\text{-}Hyp)_n$ (Singh et al., 2011), in this study, glycine (Gly) was the most abundant compound with the amount of 207 and 223 units of the total amino acids present in S-ASC and S-PSC, respectively (Table 4.12). Even though most of the characterization studies of collagen reported glycine content of approximately 30% of the total amino acids (Kittiphattanabawon et al., 2005; Senaratne et al., 2006; Huang et al., 2011), result of the present study was almost similar to the characteristics of collagen isolated from the skin of Nile perch (*Lates niloticus*) as reported by Muyonga et al. (2004) where the glycine content was in the range of 21 – 22 %, which was slightly lower than 1/3 of total amino acids. In general, glycine occurred uniformly, at every third residue throughout most of the collagen molecules (Zeng et al., 2012), except for the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the molecules (Senaratne et al., 2006). Both collagens were also rich in proline, glutamic acid, alanine, and hydroxyproline (Table 4.12). In addition, relatively low content of methionine, isoleucine, tyrosine, and histidine was consistent with the amino acid compositions of other aquatic collagens reported in literature (Huang et al., 2011; Singh et al., 2011; Zeng et al., 2012). Absence of cysteine in collagens obtained from this hybrid species also emphasized the presence of type I collagen.

The amount of imino acid (proline + hydroxyproline) of S-ASC and S-PSC was found to be 185/1000 residues for both collagens. It was observed that the imino acid residues in S-ASC from hybrid *Clarias* sp. skin was higher than that in balloon fish skin (*Diodon holocanthus*) (179/1000 residues) (Huang et al., 2011) and cod skin (*Cyprinus*

carpio) (179/1000 residues) (Duan et al., 2009) but was slightly lower than carp (*Cyprinus carpio*) skin (190/1000 residues) (Duan et al., 2009), brownbanded bamboo shark skin (*Chiloscyllium punctatum*) (204/1000 residues) (Kittiphattanabawon et al., 2010a), and striped catfish skin (*Pangasianodon hypophthalmus*) (206/1000 residues) (Singh et al., 2011). As for the S-PSC, the imino acid content was higher than that from balloon fish skin (*Diodon holocanthus*) (174) (Huang et al., 2011) but lower than that from brownstripe red snapper (*Lutjanus vitta*) skin (221) (Jongjareonrak et al., 2005a), striped catfish (*Pangasianodon hypophthalmus*), cobia (*Rachycentron canadum*), tilapia (*Oreochromis niloticus*), and yellowfin tuna (*Thunnus albacares*), which contained imino acids ranging from 190 to 216/1000 residues (Wrolstad and Decker, 2005; Huang et al., 2011; Singh et al., 2011; Zeng et al., 2012). Fish collagens were normally made up of lower imino acid contents as compared to mammalian collagens (Kittiphattanabawon et al., 2005) where porcine dermis collagen and calf skin collagen were reported to contain 220/1000 residues and 215/1000 residues of imino acid, respectively (Kittiphattanabawon et al., 2005; Kittiphattanabawon et al., 2010a). Variation in the imino acid content, especially hydroxyproline, between animals was correlated with the difference in their habitats, particularly temperature of the living environments (Kittiphattanabawon et al., 2010a; Huang et al., 2011). Bae et al. (2008) reported that the collagens isolated from fish species living in warm environment had higher amount of hydroxyproline and exhibited higher thermal stability than those living in cold environment. This was due to the fact that hydroxyproline stabilized the triple helix structure of collagen molecules (Senaratne et al., 2006).

4.4.2 (c) **Functional Group Analysis**

The infrared spectra of S-ASC and S-PSC obtained by using Fourier Transform Infrared Spectroscopy (FTIR) as well as the major peaks are shown in Figure 4.13 and Table 4.13. FTIR spectra obtained in the present study were similar to those of collagens from other fish species (Muyonga et al., 2004; Singh et al., 2011). The amide A bands of S-ASC and S-PSC were found at wavenumber of 3348 and 3336 cm^{-1}, respectively. According to Abe and Krimm (1972), amide A band was associated with the N-H stretching frequency. A free N-H stretching vibration was expected to occur in the range of 3400 – 3440 cm^{-1}, but Doyle et al. (1975) mentioned that when the NH group of a peptide was involved in hydrogen bond, the position might be shifting to a lower frequency, usually around 3300 cm^{-1}. Therefore, a shift towards lower wavenumbers of amide A as observed in the present study could be described as the indication of water-mediated hydrogen bonding in these collagens, probably with the carbonyl group of the peptide chain. Similar results were also reported by Yakimets et al. (2007) and Zeng et al. (2012).

Amide B band of both collagens was observed at 2943 cm^{-1} and 2951 cm^{-1}, in agreement with that reported by other researchers (Kittiphattanabawon et al., 2010a; Pati et al., 2010a; Singh et al., 2011). The amide I band, with characteristic frequencies in the range of 1600 – 1700 cm^{-1}, was mainly associated with the stretching vibrations of carbonyl groups along polypeptide backbone (Singh et al., 2011). In addition, it was also a sensitive marker of peptide secondary structure (Pati et al., 2010a). Amide I of S-PSC was found at lower wavenumber (1654 cm^{-1}) compared to S-ASC (1655 cm^{-1}). Similar pattern was observed for Amide II bands.

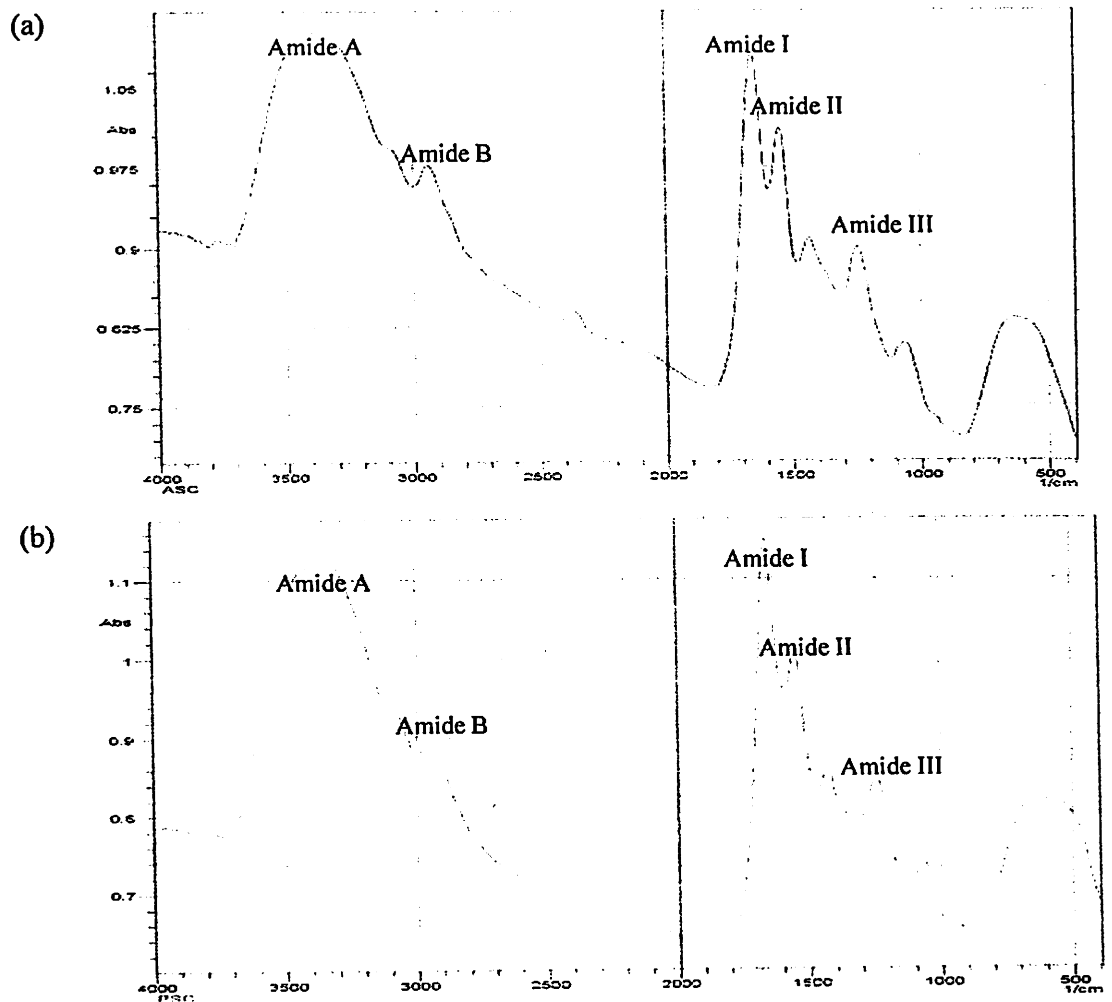

Figure 4.13: Fourier transform infrared spectra of type I collagen from skin of hybrid *Clarias* sp. (a) Acid soluble collagen (S-ASC) and (b) Pepsin soluble collagen (S-PSC)

Amide I, II and III bands were known to be correlated to the degree of molecular order and associated with the triple helical structure of collagen, resulting from the C=O stretching, N-H bending, and C-H stretching (Muyonga et al., 2004; Zeng et al., 2012). Amide I and II bands of S-PSC shifted to lower wavenumber than the S-ASC, suggested that the latter had more or stronger hydrogen bonds. This was in accordance with the peak location of Amide A band for both collagens. Based on the location of Amide I and

II peaks, it appeared that S-PSC from hybrid *Clarias* sp. skin had a lower degree of molecular order, since a shift of these peaks to lower wavenumbers was associated with a decrease in the molecular order (Zeng et al., 2012). Thus, it could be deduced that pepsin disrupted non helical region of the telopeptide, resulted in lower intermolecular cross-links in S-PSC. This was in agreement with the electrophoresis analysis of the S-ASC and S-PSC (Section 4.4.2 (a)).

Apart from that, the intensity ratio between Amide III band and 1454 cm^{-1} band had been used to indicate the stability of the triple helical structure of collagen (Plepis et al., 2004; Matmaroh et al., 2011). In the present study, the ratio was found to be 1.17 for both S-ASC and S-PSC. A ratio of approximately 1.0 indicated the presence of helical structure (Plepis et al., 2004; Singh et al., 2011). Due to the same intensity ratio obtained for both collagens, pepsin hydrolysis obviously had no pronounced effect on the triple-helical structure of S-PSC and it was confirmed that the structure was maintained for both S-ASC and S-PSC isolated from the skin of hybrid *Clarias* sp.

Table 4.13: Fourier transform infrared spectra peak locations and assignment for type I collagen from hybrid *Clarias* sp. skin

Region	Peak wavenumber (cm^{-1})		Assignment	References
	S-ASC	S-PSC		
Amide A	3348	3336	NH stretching	(Sai and Babu, 2001)
Amide B	2943	2951	CH_2 asymmetrical stretching	(Abe and Krimm, 1972)
-	2364	2380	CH_2 symmetrical stretching	(Abe and Krimm, 1972)

Amide I	1655	1654	C=O stretching, hydrogen bonding coupled with COO-	(Payne and Veis, 1988)
Amide II	1547	1547	NH bending coupled with CN stretching	(Jackson et al., 1995)
-	1315	-	CH_2 wag of proline	(Jackson et al., 1995)
Amide III	1246	1246	NH bending coupled with CN stretching	(Jackson et al., 1995)
-	1065	1068	C-O stretching	(Jackson et al., 1995)
-	-	953	C-O stretching	(Jackson et al., 1995)
-	632	640	Skeletal stretching	(Muyonga et al., 2004)

4.4.2 (d) Determination of Collagen Denaturation Temperature

Figure 4.14 shows the changes in fractional viscosity, with increasing temperature, for both S-ASC and S-PSC from the skin of hybrid *Clarias* sp. Both collagens exhibited a rapid loss of viscosity with heating. This could be attributed to the denaturation of collagen (Muyonga et al., 2004). Collagen denatured at temperatures above 40 °C, forming a mixture of random-coil single, double and triple strands (Kittiphattanabawon et al., 2005).

The thermal denaturation temperature (T_d) was determined to be approximately 32 °C and 31 °C for S-ASC and S-PSC, respectively. This suggested that denaturation temperatures of collagen from hybrid *Claris* sp. skin were in agreement with those collagens isolated from tropical and sub-tropical species (living in warm environment),

such as black drum (*Pogonias cromis*) (ASC 34.20 °C, PSC 35.80 °C), sheepshead (*Archosargus probatocephalus*) (ASC 34 °C, PSC 34.30 °C), balloon fish (*Diodon holocanthus*) (ASC 29.01 °C, PSC 30.01 °C), and carp (*Ctenopharyngodon idella*) (ASC 28 °C) (Zeng et al., 2012). The T_d observed in this study was however found to be higher than those from temperate (cold water) fish species. It was previously reported that collagen denaturation temperatures for cod (*Gadus morhua*) was 15 °C, Alaska Pollack (*Theragra chalcogramma*) was 16.80 °C, Japanese seabass (*Lateolabrax japonicus*) was 30 °C, skip jack tuna (*Katsuwonus pelamis*) was 29.70 °C, and chum salmon (*Oncorhynchus keta*) was 19.40 °C (Muyonga et al., 2004). Variation of denaturation temperature of collagen from different species with their imino acid contents and habitats are presented in Table 4.14.

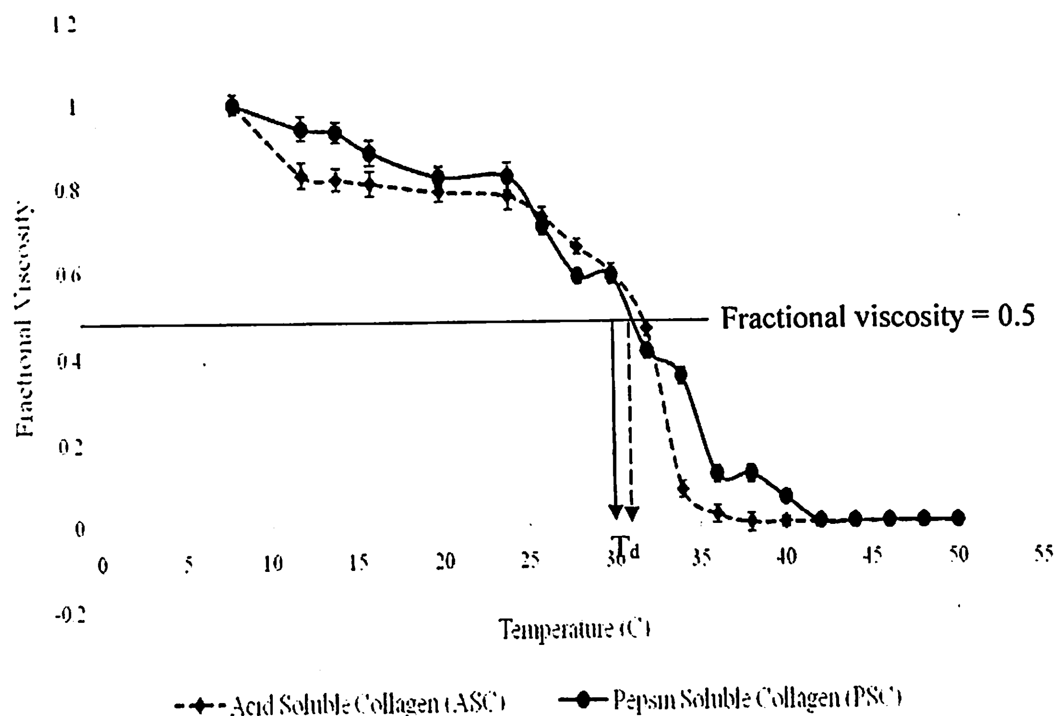

Figure 4.14: Change in fractional viscosity with temperature of skin collagen from the hybrid *Clarias* sp.

Table 4.14: Variation of denaturation temperature of collagen from different sources with their imino acid contents and habitats

Habitat	Species	Source	Imino acid content	Denaturation temperature (°C)	References
Land animals	Calf	Skin	215	40.8	(Kittiphattanabawon et al., 2010a)
	Pig	Skin	220	37	(Kittiphattanabawon et al., 2005)
Sea	Ornate threadfin bream (*Nemipterus hexodon*)	Skin	188	33.35	(Nalinanon et al., 2011)
	Deep-sea redfish (*Sebastes mentella*)	Skin	160	15.7	(Wang et al., 2008a)
	Brown backed toadfish (*Lagocephalus gloveri*)	Skin	170	28	(Senaratne et al., 2006)
	Walleye pollock (*Theragra chalcogramma*)	Skin	184	24.6	(Yan et al., 2008)
Freshwater	Rohu (*Labeo rohita*)	Scale	201	36.5	(Pati et al., 2010a)
	Catla (*Catla catla*)	Scale	214	36.5	(Pati et al., 2010a)
	Silver carp (*Hypophthalmichthys molitrix*)	Skin	192	29	(Zhang et al., 2009a)
	Nile tilapia (*Oreochromis niloticus*)	Skin	210	32	(Zeng et al., 2009)
	Carp (*Cyprinus carpio*)	Skin Scale Bone	190 192 192	28 28 28	(Duan et al., 2009)
	Catfish (*Clarias gariepinus* X *Clarias macrocephalus*)	Skin	185	32	Present study

As mentioned in most studies in the literature, the amount of imino acid (proline and hydroxyproline) was directly associated with the thermal stability of collagen

through hydrogen bonds (Muyonga et al., 2004; Singh et al., 2011). Senaratne et al. (2006) explained that the higher the imino acid content, the helical structure of the collagen was more stable, resulting in higher denaturation temperature. This was then supported by Zeng et al. (2012) who stated that proline and hydroxyproline imposed restrictions on the conformation of a polypeptide chain and helped to strengthen the triple helical structure of collagen.

In addition, the difference in T_d among collagens from different animals and fishes was also contributed by both the body and habitat temperatures (Pati et al., 2010a; Singh et al., 2011). It was stated earlier that species living in colder environments had lower amount of imino acid. As in the present study, the water temperature from where the hybrid *Clarias* sp. was caught ranged 28 – 34°C. Therefore, this explained why the T_d values of S-ASC and S-PSC were higher than those from cold water and more or less similar to those from warm water environment.

4.4.2 (e) Collagen Solubility Study

The solubility of S-ASC and S-PSC from the skin of hybrid *Clarias* sp. was influenced by different pH (Figure 4.15). Both collagens showed high solubility in very acidic pH ranging from 1 – 5, with the relative solubility higher than 80 %. At pH above 5, a significant decrease in the solubility was observed. At pH 6 and 7, the solubility of S-ASC and S-PSC reached the minimum. Low solubility was also found in the neutral and slightly alkaline pH range. Similar solubility patterns had also been reported by Nalinanon et al. (2007), Matmaroh et al. (2011) and Zeng et al. (2012). According to

Huang et al. (2011), collagen had isoelectric points ranging from pH 6 to 9, and this supported the results in the present study.

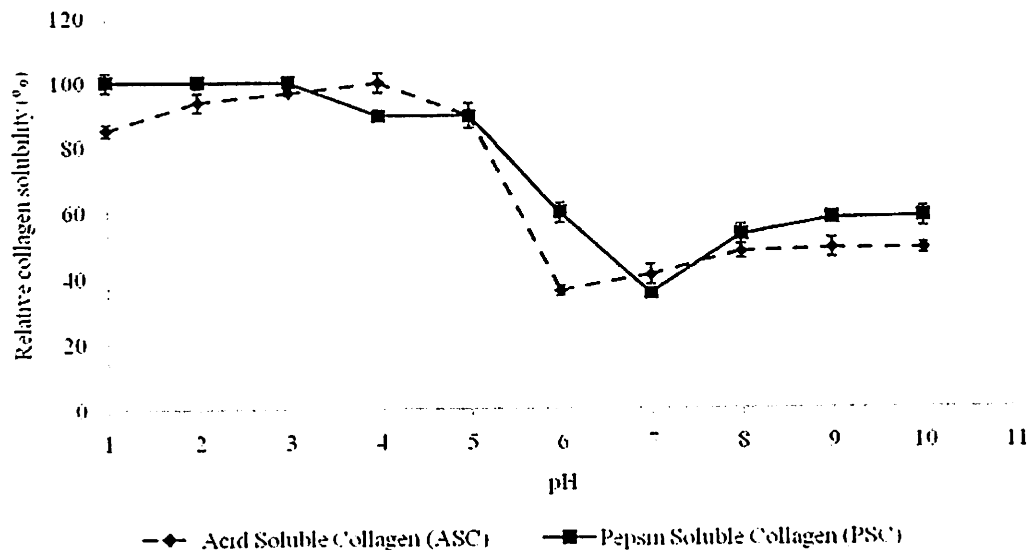

Figure 4.15: Relative solubility of S-ASC and S-PSC at different pH. The vertical bars represent the standard deviations (n=3)

It was known that when a protein dissolved in buffer at its isoeletric point, total net charges of the protein molecules were zero. Subsequently, the hydrophobic-hydrophobic interaction increased, leading to precipitation and aggregation of the protein, resulting in low solubility in solution (Huang et al., 2011; Singh et al., 2011). On the other hand, when pH was lower or higher than the isoelectric point, the net positive or negative charge residues of proteins increased, resulting in increased solubility by the repulsive forces between the chains (Kittiphattanabawon et al., 2005; Huang et al., 2011; Zeng et al., 2012). Similar result was also reported for collagen from bigeye snapper (*Priacanthus tayenus*) (Nalinanon et al., 2007), tiger puffer (*Takifugu rubripes*), red stingray (*Dasyatis akajeri*), sea chub (*Kyphosus bigibbus*) (Bae et al.,

2008), and balloon fish (*Diodon holocanthus*) (Huang et al., 2011). Besides, the S-PSC in this study showed higher solubility than the S-ASC for all of the tested pHs except at pH 4 and 7. This led to the deduction that the S-PSC may contain a lower degree of crosslink or weaker bonds compared to the S-ASC. Huang et al. (2011), on the other hand described that part of peptide bonds were hydrolyzed in addition to the peptide bonds hydrolysis in telopeptide region of the collagen molecules, leading to higher solubility of S-PSC than S-ASC. This was in accordance with the results obtained in the present study.

On the other hand, the solubility of S-ASC and S-PSC at various NaCl concentrations was measured as depicted in Figure 4.16. The solubility of hybrid *Clarias* sp. skin collagens gradually decreased with increasing NaCl concentrations until reaching 4 % (w/v), after which a drastic decrease was observed for both collagens at 6 % (w/v) NaCl and above. Generally, it had been reported in literature that the solubility of collagen from striped catfish (*Pangasianodon hypophthalmus*), cobia (*Rachycentron canadum*), bigeye snapper (*Priacanthus tayenus*), yellowfin tuna (*Thunnus albacares*), balloon fish (*Didion holocanthus*), tiger puffer (*Takifugu rubripes*), dusky spinefoot (*Siganus fuscescens*), and red stingray (*Dasyatis laevigata*) in acetic acid solution decreased with increasing NaCl concentration (Kittiphattanabawon et al., 2005; Bae et al., 2008; Woo et al., 2008; Huang et al., 2011; Singh et al., 2011; Zeng et al., 2012). This could be related to the 'salting out' phenomena, which occurred at relatively high NaCl concentration (Singh et al., 2011). According to Matmaroh et al. (2011), at low concentrations of NaCl, salt ions bound weakly to the charged groups on protein surface,

The predicted results gave a relatively good agreement with the experimental data, with the linear correlation coefficient, R^2 values above 0.9. This showed that the proposed empirical models were sufficient to describe both the fast washing action and slow-diffusion of extraction process for the selected species of freshwater fishes. Since there were many examples existed where the R^2 was closed enough to one but the model was still not appropriate, hence the root mean square deviation (RMSD) was used with the R^2 for the comparison of various empirical extraction models. A model with small RMSD represents the data more accurately than the models with larger RMSD (Kitanović et al., 2008).

Table 4.15 shows the calculated parameters for the proposed empirical models, and the statistical correlation values. It showed that regardless of which model was applied, individual average value of the RMSD were lower than 10 % for all the models, except that of Peleg's model, which was slightly higher at 11.65 %. Therefore, the power law model, parabolic diffusion model and Elovich's equation were identified as being appropriate and suitable to model and govern the collagen extraction by pepsin digestion from fish muscle. In addition, it was also found that the correlation coefficient, R^2, values were high for all the empirical models, ranging $0.973 < R^2 < 0.993$. In fact, the order of the models arranged in increased R^2 was:
Elovich's model > Peleg's model > Parabolic diffusion model > Power law model.

This showed that the Power law model, having the lowest value of RMSD (at 0.023) and the highest value of the linear correlation coefficient (at 0.993), was selected as the best empirical model for the muscle collagen extraction from freshwater fishes.

Figure 4.18 presents the averaged values of the RMSD and R^2 in the form of histogram and line chart for each model.

Table 4.15: Summary of model parameters and statistical correlation values for each empirical kinetic model

	Type of fish	Power Law		Parabolic Diffusion		Peleg		Elovich	
		n	B	Ao	A1	K_1	K_2	E	a
Model Parameters	Catfish	0.26	59.51	127.08	7.56	0.31	0.0025	70.10	-125.18
	Black Tilapia	0.32	30.57	67.31	6.83	0.56	0.0030	62.40	-155.33
	Pangasius Catfish	0.30	42.21	91.93	7.82	0.43	0.0025	71.58	-163.72
	Sultan Fish	0.29	38.79	81.81	6.43	0.50	0.0030	58.55	-126.70
	Labyrinth Fish	0.24	59.72	120.03	6.17	0.34	0.0029	56.72	-83.01
Statistical Correlation Values	R^2	0.994		0.990		0.988		0.973	
	RMSD (%)	2.255		2.728		11.650		5.187	

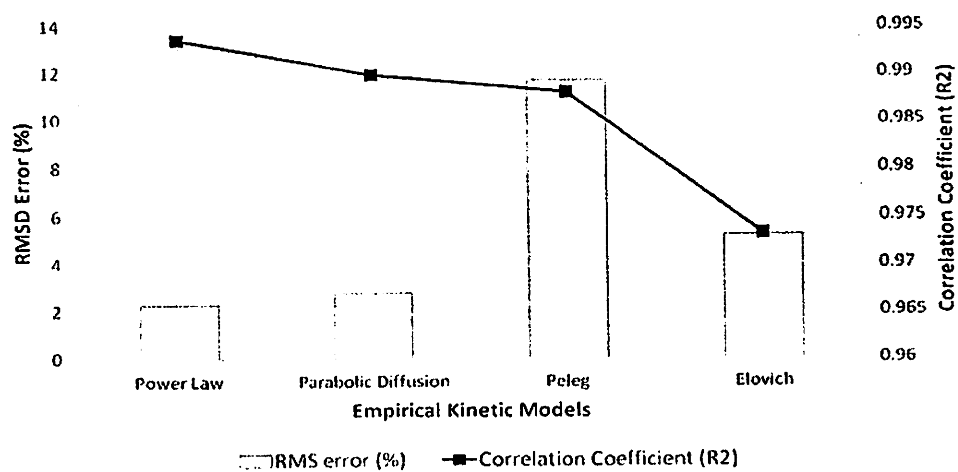

Figure 4.18: Averaged values of root mean square deviation and correlation coefficient for all empirical models.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This chapter presents the conclusion of the whole experimental work achieved according to the research objectives and several recommendations for future work.

5.1 Conclusion

The present work attempted to extract type I collagen from Malaysian freshwater fishes in the effort to boost up the commercial values of these cheaply available natural resources as well to find alternatives for mammalian derived type I collagen, which associated with some religious constraints and have spurred concerns among health conscious consumers over its utilization in various applications. Collagens were extracted from the skins and muscles of few selected Malaysian freshwater fishes and characterized. Kinetic and modeling study of the extraction process was also carried out. The conclusions obtained in this study can therefore be summarized as follow:

i. Modified Lowry's method had been proven to be a simple way that was suitable for routine measurement of collagen concentration in any collagen extraction processes.

ii. Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the muscle of selected cultured catfish (hybrid of *Clarias gariepinus* X *C. macrocephalus*), red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mossambicus*), pangasius catfish (*Pangasianodon hypophthalmus*), Sultan fish

(*Leptobarbus hoevenii*), and labyrinth fish (*Trichogaster trichopterus*), freshwater fishes that were widely consumed in Malaysia. The extracted collagen yields were higher for PSC as compared to ASC for all tested species. Existence of inter-molecular cross-links between collagen molecules of the extracted collagens were proven for all the fishes due to differences between the yield of ASC and PSC, with higher yield obtained for the later. Presence of collagen in fish muscles revealed that these few species of Malaysian freshwater fishes were potential to be utilized as alternative sources of mammalian collagens. Nevertheless, variation between the yield of ASC and PSC was most pronounced in the case of catfish (hybrid *Clarias* sp.). With the aid of pepsin digestion, the yield increased from 9.75 ± 1.12 % to 36.84 ± 1.82 % at the end of 20-hour-extraction period, serving it as the most potential species to be studied in further recovery and isolation of muscle and skin collagens in subsequent stages.

iii. With the aid of fractional factorial design (FFD) and analysis of variance, the effective main parameters which significantly influenced the extraction efficiency of muscle pepsin soluble collagen (M-PSC) from the muscle of hybrid catfish (*Clarias* sp.) were obtained by conducting the least number of experimental runs. Result of the first order factorial design showed that acetic acid concentration, acid extraction time, acetic acid to muscles ratio, and stirring speed had significant effect ($P<0.05$) on the yield of M-PSC.

iv. In the optimization study of process variables to obtain the highest yield of M-PSC, both statistical- and artificial intelligence optimization approaches were applied. Response surface methodology (RSM) namely central composite design (CCD) was employed to optimize the extraction process conditions of M-PSC.

The optimization results were then compared with the prediction of hybrid artificial neural network/genetic algorithm (ANN/GA) model for the extraction of the collagen. The hybrid ANN/GA model had shown significant higher generalization and predictive capability as compared to RSM based on the R^2 values of training and validation datasets. Also, higher yield of M-SPC was predicted by ANN/GA model with the same design of experiments data. Validation of the predictive results given by both optimization techniques revealed that the prediction made by RSM had a greater deviation (error) than the ANN/GA model prediction. In this study, the ANN/GA methodology showed a clear superiority over RSM as a modeling technique for datasets showing non-linear relationships. Therefore, ANN/GA could be an alternative to RSM as a modeling method for collagen extraction processes.

v. The ANN predicted yield of M-PSC at GA optimized condition was 23.81 % with the extraction process conditions as follows: an acetic acid concentration of 0.70 M, the acetic acid to muscles ratio of 25.40 ml/g, and the stirring speed of 443.75 rpm. The adequacy and precision of hybrid ANN/GA to model and predict the collagen extraction processes were evidenced in the validation of the predictive models where the percentage error differences between the experimental and predicted values for both extraction processes were always less than 5 %.

vi. ASC and PSC extracted from the skins and muscles of *Clarias* sp. were characterized. It was found that the properties and characteristics of M-ASC and M-PSC from the muscles of *Clarias* sp. was not promising to be comparable with the typical type I collagen. This was proven with the SDS-PAGE analysis as

well as the amino acid composition analysis. Both skin collagens, namely S-ASC and S-PSC however were classified as type I and they were quite similar in terms of structures and characteristics. Both collagens contained α1 and α2 chains with the presence of high molecular weight crosslinks (γ and β) observed in the gel electrophoresis. Fourier transform infrared (FTIR) spectra of both ASC and PSC were almost similar, suggesting that pepsin hydrolysis did not disrupt the triple helical structure of collagen. It was noticed that Amide A, I and II bands of PSC were obtained at lower wavenumber as compared to ASC. Based on the amino acid analysis, glycine was the most abundant amino acid found with 207/1000 residues and 223/1000 residues present in ASC and PSC, respectively. Their amounts of imino acids were 185/1000 residues for both, which was relatively high. The thermal denaturation temperatures (T_d) were determined to be 31.5°C and 31°C, respectively. Both collagens exhibited high solubility in acidic pH (1 – 5) and below 4% (w/v) NaCl concentration. From the characterization results, it could be concluded that the skins of hybrid *Clarias* sp. served as a better alternative source of type I collagen for further applications, in contrast to the muscles.

vii. The empirical kinetic models for the collagen extraction from the muscle of cultured hybrid catfish (*Claris* sp.) were studied using four two-parametric models, namely the power law, parabolic diffusion, Peleg's and Elovich's models. The power law model provided the best fit in describing the muscle collagen extraction kinetics. This model (with the lowest RMSD of ±2.25% and highest R^2 of 0.993) fitted to the experimental data somewhat better the rest of the empirical models.

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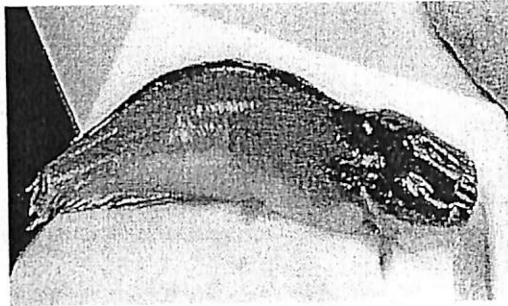
APPENDICES

Appendix A: Selected Freshwater Fishes for Collagen Extraction



Common Name: Hybrid Catfish

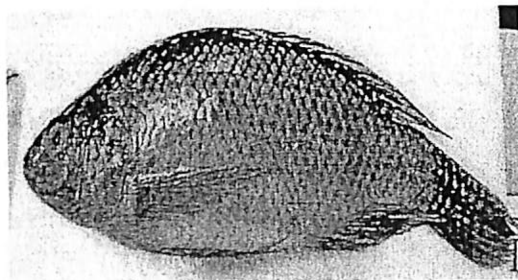
Scientific Name: *C. gariepinus* X *C. macrocephalus*

Local Name: "Keli"

Common Name: Red Tilapia

Scientific Name: *O. niloticus*

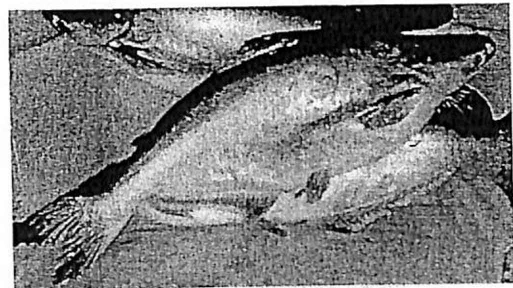
Local Name: "Tilapia Merah"



Common Name: Black Tilapia

Scientific Name: *O. mossambicus*

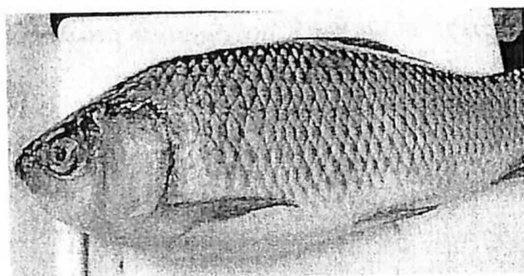
Local Name: "Tilapia Hitam"



Common Name: Pangasius Catfish

Scientific Name: *P. hypophthalmus*

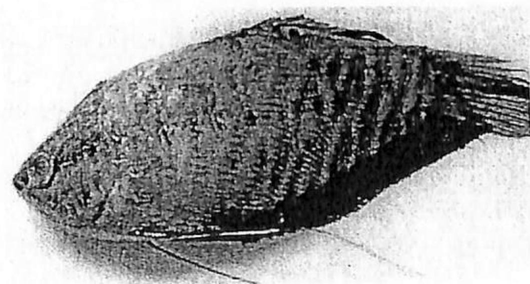
Local Name: "Patin"



Common Name: Sultan Fish

Scientific Name: *L. hoevenii*

Local Name: "Jelawat"



Common Name: Labyrinth Fish

Scientific Name: *T. trichopterus*

Local Name: "Sepat"

Appendix B: Optimization by Response Surface Methodology (Suggested optimum process conditions for the extraction of M-PSC by CCD)

File Edit View Display Options Design Tools Help

Notes for CCD design don
Design
Status
Evaluation
Analysis
Yield of Collagen(Ar
Optimization
Graphical
Point Prediction

Solutions Tool
Report
Ramps
Histogram

Criteria Solutions Graphs

Solutions 1 2 3 4 5 6 7 8 9 10

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Acetic Acid Con	is in range	0.1	0.9	1	1	3
Liquid to Solid Ra	is in range	20	30	1	1	3
Stirring speed	is in range	300	500	1	1	3
Yield of Collager	maximize	50.933	222.76	1	1	3

Solutions

Number	Acetic Acid Co	Liquid to Solid	Stirring speed	Yield of Collag	Desirability	
1	0.67	24.66	423.84	224.619	1.000	Selected
2	0.74	24.23	450.46	224.637	1.000	
3	0.70	23.57	466.59	224.464	1.000	
4	0.65	24.29	445.06	225.69	1.000	
5	0.74	24.75	436.85	224.232	1.000	
6	0.67	25.65	441.07	223.967	1.000	
7	0.67	25.54	458.97	223.338	1.000	
8	0.63	23.36	438.95	224.308	1.000	
9	0.73	25.63	452.40	223.084	1.000	
10	0.67	23.52	466.18	224.631	1.000	

10 Solutions found

Therefore, validation the CCD model was carried out the following process conditions:

- ✓ Acetic acid concentration = 0.67 M
- ✓ Acetic acid to muscles ratio = 24.65 ml/g
- ✓ Stirring speed = 423.84 rpm

Appendix C: Optimization by ANN/GA

Appendix C.1: Development of ANN model for extraction of M-PSC

```
clc
clear all

%Original set of data
data=[51.0353 0.1 20 300;116.54 0.9 20 300;50.933 0.1 30
300;151.673 0.9 30 300;100.514 0.1 20 500;183.45 0.9 20
500;60.2 0.1 30 500;160.82 0.9 30 500;110.498 0.1 25
400;201.823 0.9 25 400;150.91 0.5 20 400;200.22 0.5 30
400;139.551 0.5 25 300;216.465 0.5 25 500;220.564 0.5 25
400;50.2009 0.1 10 400;100.0056 0.5 10 400;92.9438 0.7 10
400;90.9176 0.9 10 400;106.7770 0.5 25 200];

%Split the data into 2
data1= data(1:10,:);
data2= data(11:20,:);

% divide data into train + test set
x = data1(:,[2:4]);
y = data1(:,1);

xx =[];
yy=[];

for i = 1:30; % Defined the neural networks combination
( Layer )
% We need to apply bootstrap for every single nets to get
a different input and output
% Bootstrap for training data
    b = i;
    bxy =bootsr([x y],i) ;
    bx = bxy(:,1:3);
    by = bxy(:,4);
    xx=[xx;bx];
    yy=[yy;by];

end

%validation set
xv=data2(:,[2:4]);
yv=data2(:,1);

xvv=[];
yvv=[];
```

```
for i = 1:30; % Defined the neural networks combination
( Layer )
% We need to apply bootstrap for every single nets to get
a different input and output
% Bootstrap for training data
   b = i;
   bxvyv =bootsr([xv yv],i) ;
   bxv = bxvyv(:,1:3);
   byv = bxvyv(:,4);
   xvv=[xvv;bxv];
   yvv=[yvv;byv];

end

%CREATE A FEEDFORWARD NETWORK
%to normalize data with mean 0 and variance 1 for
training set
a=mean (yy);
b=std(yy);
CV=(yy-mean(yy))/std(yy);

%to normalize data with mean 0 and variance 1 for
validation set
g=mean (yvv);
h=std(yvv);
CVv=(yvv-mean(yvv))/std(yvv);

MV=xx';
CV=CV';
%DIVIDE DATA INTO TRAINING, VALIDATION AND TESTING SETS
%divide data by index
trainInd=1:100;
valInd=101:200;
testInd=201:300;
[trainMV,valMV,testMV]=divideind(MV,trainInd,valInd,testI
nd);
[trainCV,valCV,testCV]=divideind(CV,trainInd,valInd,testI
nd);

%CREATE NETWORK
%define the criteria of the training network
[N M]=size(CV);
sse=[];
yvp=[];
for i=1:30;
net=newff(MV,CV,i,{},'trainrp');
net.divideFCn='divideind';
```

```
net.divideParam.trainInd = trainInd;
net.divideParam.valInd = valInd;
net.divideParam.testInd = testInd;
net.trainParam.goal=1e-5;
net.trainParam.epochs=1000;
net.trainParam.lr=0.01;

s=(RandStream('mcg16807','Seed',0));
RandStream.setDefaultStream(s);


%TRAIN THE NETWORK
%Initializing the weights
net=init(net);

%Train the network
[net,tr]=train(net,MV,CV);

%Calculate mse from validation set
y=sim(net,xvv');
yvp=[yvp y'];
sse1= sum((y-CVv').^2)/300;
sse=[sse sse1]

end

[j,k]=min(sse)
net=newff(MV,CV,k,{},'trainlm');
net.divideFCn='divideind';
net.divideParam.trainInd = trainInd;
net.divideParam.valInd = valInd;
net.divideParam.testInd = testInd;
net.trainParam.goal=1e-5;
net.trainParam.epochs=1000;
net.trainParam.lr=0.01;

s=(RandStream('mcg16807','Seed',0));
RandStream.setDefaultStream(s);


%TRAIN THE NETWORK
%Initializing the weights
net=init(net);

%Train the network
[net,tr]=train(net,MV,CV);
```

Appendix C.2: Application of GA to find optimum extraction conditions and yields in Matlab

```
%Train the network
[net,tr]=train(net,MV,CV);

%Calculate mse
yvp_net=sim(net,xvv');
sse2= sum((yvp_net-CVv').^2)/M

rr= [CVv yvp_net'];
r=corrcoef(rr)

%to optimize trained NN using GA
objFcn = @(x)-sim(net,x');  % Function that simulates NN
and returns output
LB=[0.1 20 300];
UB=[0.7 30 500];
[xOpt,fVal] = ga(objFcn, 3,[],[],[],[],LB,UB)  % Find the
minimum of objFcn with 3 inputs

%optimum yield of collagen
yield=abs(fVal)*h+g
```

LIST OF PUBLICATIONS

Journals

1) Kiew, P. L. and Mashitah, M. D. (2012). Screening and empirical kinetic models of collagen extraction from selected Malaysian freshwater fish. Journal of Food Process Engineering, doi:10.1111/j.1745-4530.2012.00683.x.

2) Kiew, P. L. and Mashitah, M. D. (2012). Collagen extraction from Malaysian cultured catfish (hybrid *Clarias* sp.): Kinetics and optimization of extraction conditions using response surface methodology. ISRN Chemical Engineering, doi:10.5402/2012/835391.

3) Kiew, P. L. and Mashitah, M. D. (2013). Screening of significant factors in collagen extraction from hybrid *Clarias* sp. using a statistical tool. International Food Research Journal, 20(4), 1913 – 1920.

4) Kiew, P. L., Ahmad, Z. and Mashitah, M. D. (2013). A hybrid of back propagation neural network and genetic algorithm for optimization of collagen extraction from Malaysian cultured catfish (hybrid *Clarias* sp.). Biotechnology and Bioprocess Engineering, 18, 257 – 265.

5) Kiew, P. L. and Mashitah, M. D. (2013). Extraction of acid and pepsin soluble collagen from selected Malaysian freshwater fish muscles: Modified Lowry's measurement method. Journal of Agrobiotechnology, 4, 16 – 31.

6) Kiew, P. L. and Mashitah, M. D. (2013). Isolation and characterization of collagen from the skin of Malaysian catfish (hybrid *Clarias* sp.). Journal of the Korean Society for Applied Biological Chemistry, 56, 441-450.

Appendix C.3: Optimization solutions for extraction of M-PSC as generated by hybrid ANN/GA model

Solution generated by Matlab is as follow:

```
Window   Help
Current Directory: C:\Users\Kiew Peck Loo\Documents\MATLAB
Command Window
New to MATLAB? Watch this Video, see Demos, or read Getting Started.

j =

    0.5038

k =

     6

sse2 =

    0.1672

r =

    1.0000    0.9527
    0.9527    1.0000

Optimization terminated: average change in the fitness value less than

xOpt =

    0.7000   25.4017  443.7529
```

Lowest mean squared error (MSE) attained with 6 neurons in the hidden layer of ANN model

R^2 of the trained ANN model

Values of X_1, X_2 and X_3, respectively

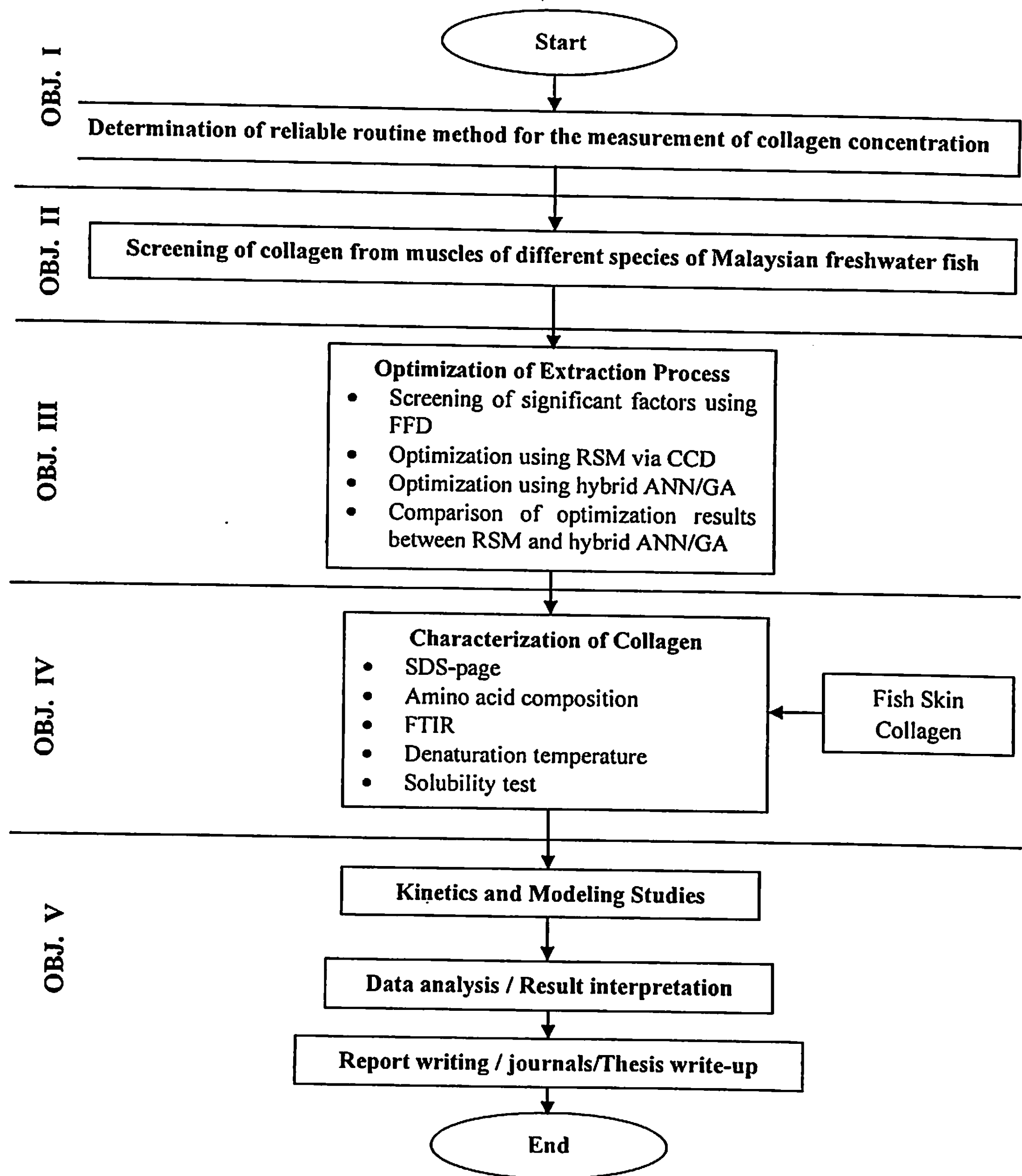
```
Command Window
New to MATLAB? Watch this Video, see Demos, or read Getting Started.
Optimization terminated: average change in the fitness value less than

xOpt =

    0.7000   25.4017  443.7529

fVal =

   -1.7570

yield =

   23.8096
```

M-PSC yield predicted by hybrid ANN/GA

Proceedings

1) Kiew, P. L. and Mashitah, M. D. (2012). *Ikan keli* (*Clarias gariepinus* X *C. macrocephalus*): A potential source of type I collagen. In: Proceedings of International Conference on Science, Technology & Social Sciences, Pahang, Malaysia, 20 – 22 November 2012.

2) Kiew, P. L. and Mashitah, M. D. (2013). Isolation of collagen from the skin of *Keli*: Potential alternative for mammalian type I collagen. In: Proceedings of International Conference of Environment (ICENV), Penang, Malaysia, 11 – 13 December 2012.



Start
OBJ. I
Determination of reliable routine method for the measurement of collagen concentration
OBJ. II
Screening of collagen from muscles of different species of Malaysian freshwater fish
OBJ. III
Optimization of Extraction Process
Screening of significant factors using FFD
Optimization using RSM via CCD
Optimization using hybrid ANN/GA
Comparison of optimization results between RSM and hybrid ANN/GA
OBJ. IV
Characterization of Collagen
SDS-page
Amino acid composition
FTIR
Denaturation temperature
Solubility test
Fish Skin Collagen
OBJ. V
Kinetics and Modeling Studies
Data analysis / Result interpretation
Report writing / journals/Thesis write-up
End

USM UNIVERSITI SAINS MALAYSIA

BORANG PENYERAHAN ASET / INVENTORI

==

A. BUTIR PENYELIDIK

1. NAMA PENYELIDIK : PROF. DR. MASHITAH BT. MAT DON
2. NO STAF : AE 50109
3. PTJ : KEJURUTERAAN KIMIA.
4. KOD PROJEK : ....
5. NO. AKAUN GERAN : 203/PJKIMIA/6730068
6. TARIKH TAMAT PROJEK : 31/7/15

B. MAKLUMAT ASET / INVENTORI

BIL	KETERANGAN ASET	NO HARTA	NO. SIRI	HARGA (RM)
		Tiada —		
		vote 35 telah dipindahkan ke vote 29 (rujuk lampiran).		

C. PERAKUAN PENYERAHAN

Saya dengan ini menyerahkan aset/ inventori seperti butiran (B) di atas kepada pihak universiti:

....

PROFESSOR DR. MASHITAH MAT DON
SCHOOL OF CHEMICAL ENGINEERING
ENGINEERING CAMPUS
UNIVERSITI SAINS MALAYSIA, SERI AMPANGAN,
14300 NIBONG TEBAL, SEBERANG PERAI SELATAN.
PULAU PINANG, MALAYSIA.

Tarikh: 11/8/15

D. PERAKUAN PENERIMAAN

Saya telah memeriksa dan menyemak setiap alatan dan didapati :

- ☐ Lengkap
- ☐ Rosak
- ☐ Hilang : Nyatakan..........
- ☐ Lain-lain : Nyatakan ....

Diperakukan Oleh :

(..........)
Tandatangan
Pegawai Aset PTJ

Tarikh: ....

*Nota -
Satu salinan borang yang telah diperakukan perlu dimajukan oleh Pegawai Aset untuk tujuan rekod kepada :

a) Unit PengurusanHarta, Jabatan Bendahari
b) Pejabat RCMO

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Memorandum

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Ruj. Kami : X0068
Tarikh : 26 Ogos 2014

Profesor Madya Dr. Mashitah Bt Mat Don
Pusat Pengajian Kejuruteraan Kimia
Universiti Sains Malaysia
Kampus Kejuruteraan

Puan,

KEPUTUSAN BORANG HAL EHWAL PENGURUSAN GERAN (HEPG)

TAJUK PROJEK : *COLLAGEN EXTRACTION FROM FRESHWATER FISH: KINETICS, MECHANISM AND CHARACTERIZATION STUDIES*
NO. AKAUN : 203 / PJKIMIA / 6730068 (GERAN ERGS)

Saya dengan hormatnya merujuk kepada perkara di atas dan Borang HEPG puan yang telah diterima pada 22 Ogos 2014 adalah berkaitan.

2. Sukacita dimaklumkan bahawa pihak universiti telah meluluskan permohonan puan untuk pindaan vot seperti berikut:-

Bil.	Vot Asal	Vot Baru	Jumlah Pindaan (RM)
1.	Vot35000	Vot29000	10,500.00

3. Semoga pindaan vot ini membantu melancarkan perjalanan penyelidikan pihak puan.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"
'Memastikan Kelestarian Hari Esok'

Yang menjalankan tugas,

(ADRIL ELLMI BIN MOHD ADNAN)
Pegawai Sains
Pejabat Pengurusan & Kreativiti Penyelidikan

PUSAT PENGAJIAN KEJURUTERAAN KIMIA
29/8/14
2/9/14

s.k. Pengarah
Pejabat Pengurusan dan Kreativiti Penyelidikan
Universiti Sains Malaysia

Dekan
Pusat Pengajian Kampus Kejuruteraan Kimia
Universiti Sains Malaysia
Kampus Kejuruteraan

Ketua Penolong Bendahari
Jabatan Bendahari
Universiti Sains Malaysia
Kampus Kejuruteraan