



# Application of Intelligence Computing to Optimizing Enzymatic Bioprocessing in Cartilage Hydrolysis

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This study uses the Taguchi orthogonal method and artificial neural network to optimize enzymatic bioprocessing of animal waste cartilage (chicken, mini pig and hog). Specifically, the artificial neural network is used in parallel with the Taguchi orthogonal array process for enzymatic hydrolysis of the cartilage tissue to optimize the best quality of bioactive peptides. The experiment was designed using Taguchi orthogonal array optimal level L25 physical parameters and key media components, namely temperature, pH, enzyme/substrate ratio, substrate concentration, and reaction time. The experimental results were used to train the artificial neural network (ANN) to predict the optimizing enzymatic bioprocessing in animal cartilage hydrolysis. The analysis was performed on a personal computer using NeuroSolutions 6.0 software. The experiment of an enzymatic hydrolysate of three animal cartilages followed the Taguchi orthogonal design, and we discovered that  $60 \pm 1^\circ\text{C}$  is the most effective temperature to hydrolyze cartilage. These peptides of molecular size smaller than 10kDa (with 95% values between 10.7kDa and 2.5kDa) were capable of stimulating the porcine chondrocytes to produce glycosaminoglycan (GAG) and type II collagen in vitro. NeuroSolutions 6.0 back-propagation analysis achieved a convergence value of  $R^2=0.9762$ , indicating that the enzymatic bioprocessing has good performance. Therefore, this study suggests that integrating artificial neural network and Taguchi method when constructing an optimal enzymatic bioprocessing model could significantly increase and improve the quality of final bioactive peptide products. It also suggests that integrating artificial neural network and Taguchi method in the construction of an optimal enzymatic bioprocessing in cartilage hydrolysis could be used as nutraceutical component in bone and joint health

*Keywords: Intelligence computing, Taguchi orthogonal array, Neural network, Cartilage hydrolysis, Enzymatic bioprocessing*

Cartilage is a flexible connective tissue found in various parts of humans and other animals, including the

joints between bones, the rib cage, the ears, the nose, the bronchial tubes and the intervertebral discs [1]. It is chemically abundant in collagen, proteoglycans, acidic polysaccharides and water. Conceivably, cartilage can be hydrolyzed to be a potential bioactive material of collagen extraction. In fact, hydrolyzed collagen has been applied in the leather and film industries, pharmaceuticals, cosmetics, biomedical materials and food manufacturing [2]. In particular, some clinical studies report that oral ingestion of hydrolyzed collagen ease the joint pain of osteoarthritis or rheumatoid arthritis, with those having the most severe symptoms showing the most benefit [3, 4 and 5]. Normally, hydrolyzed collagen is isolated from animal (chicken, bovine, porcine, rabbit, duck and antler) cartilages [6, 7 and 8], and fish (skate and shark) cartilages [2, 9 and 10].

A review of methods for extracting hydrolyzed collagen from animal cartilage shows the following. A study conducted in 2005 found that hydrolyzed collagen absorbed small peptides in blood, indicating that the process of hydrolysis involves breaking down the molecular bonds between individual collagen strands using combinations of heat, acids, alkalis, or enzymes, and then reducing collagen proteins of about 300,000 Da into small peptides with an average molecular weight between 2000 and 5000 Da [11]. Specifically, the process is characterized by the following steps: (1) obtaining raw materials from fresh animal cartilage, removing the adipose tissue, cutting adipose tissue into small pieces and soaking a 3% sodium hydroxid for 10-30 hours; (2) using phosphate-buffered saline (abbreviated PBS) to adjust pH value to 3.5-9.5, in proportion to the concentration of animal cartilage particle in g substrate; (3) Adding a plant extract of papain, bromelain or alkaline protease enzymatic protecting agent based on the ratio of animal cartilage particle to g substrate and then adding the composite (enzyme/substrate ratio), incubating with stirring hydrolysis for 4-8 hours; (4) transferring the reaction contents to 45-100°C water-bath for 8-15 minutes off the enzyme while awaiting completion of incubation; and (5) after cooling to room-temperature, centrifuging the reaction mixture at 3500-5000rpm for 30 minutes at 4°C. Finally, the supernatant fluid and precipitate are separated and freeze-dried [12-17].

Resorting to the above methods [12-17], whether enzymatic hydrolysis, acid hydrolysis or alkaline hydrolysis is used for extracting animal cartilage collagen, the products need to be assured of safety, efficiency, and quality testing before being marketed. Thus, to optimize bioprocessing of cartilage hydrolysis for recovery and production, a combination of various microbial, chemical, enzymatic technologies is

necessary for a chondroprotective effect [2]. It is believed that intelligence computing technology can bring about a significant advancement in product development (as archived pharmaceutical industry) as well as in future business execution and prediction.

#### **-Specific Aims**

The primary aim of this study is to use intelligence computing technologies (such as Taguchi orthogonal method and artificial neural network) to optimize enzymatic bioprocessing of animal waste cartilage (chicken sternal cartilage, mini pig laryngeal and tracheal, and hog laryngeal and tracheal) and generate hydrolysis conditions for preparation of hydrolyzate collagen. Specifically, the artificial neural network is used in parallel with the Taguchi orthogonal array process for enzymatic hydrolysis of the cartilage tissue to produce the best yield and quality of bioactive peptides with clinical efficacy for functional ingredients of nutraceuticals. Ultimately, the goal is to develop a valuable and market-potential nutraceutical product for bone and articular joint health care.

A systematic review of the scientific evidence put forth between 1979~2010 [18, 19] shows that a new era in the management of osteoarthritis and nutrition, from nutraceuticals to functional foods, is possible. According to a report published in 2007, nutraceuticals sales were projected to reach \$74.7 billion at an AAGR (Average Annual Growth Rate) of 9.9%. In fact, the global market for nutraceuticals is growing day by day and was expected to reach \$176.7 billion in 2013 [20]. Particularly, chondroprotective agents including collagen hydrolysate have been used widely as nutraceuticals in osteoarthritis treatments [21]. As such, identification and selection of bioactive factors would be important for engineering mimetic biomaterials/ biological biomaterials to provide not only mechanical support but also biological cues in the induction of a synthesized special cartilage extracellular matrix by chondrocytes. Motivated by such a trend, this study was conducted to advance both academic and industrial interests.

## **MATERIALS AND METHODS**

#### **-Materials**

##### ***Enzymatic Bioprocessing Materials***

This study obtained chicken sternal cartilage from an industrial poultry meat processor (Kai Shing Trading Co., Ltd., Yunlin, Taiwan). The mini pig laryngeal and tracheal and hog laryngeal and tracheal were obtained from PigModel Animal Technology Co., Ltd. The three animal cartilage tissues were stored and frozen before they were processed and prepared as cartilage hydrolysates. Fresh porcine knee joints were purchased from a local meat market and used for isolation of chondrocytes in less than 6hrs. Papain was obtained from Merck (No.107147 Papain 6000USP-U/mg). DMEM/Ham's F12 medium, Fetal Bovine Serum (FBS), Penicillin and Streptomycin were from Invitrogen Corporation, (Carlsbad, CA, USA). Peopidium iodide, Alcian blue, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), 1,9-dimethyl-methylene blue (DMB), chondroitin sulfate A, calf thymus DNA and Hoechst 33324 and reagent graded chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

#### ***Taguchi Orthogonal Working Model***

This study obtained the Qualitek-4 from a local agent in Taiwan.

#### ***Artificial Neural Network Software***

NeuroSolutions 6.0 software was obtained from the local agent that provided the Qualitek-4.

#### ***-Methods***

##### ***Enzymatic Bioprocessing Materials***

Optimal levels L2<sup>5</sup> of physical parameters and key media components, namely temperature, pH, enzyme/substrate, substrate concentration, and reaction time were determined.

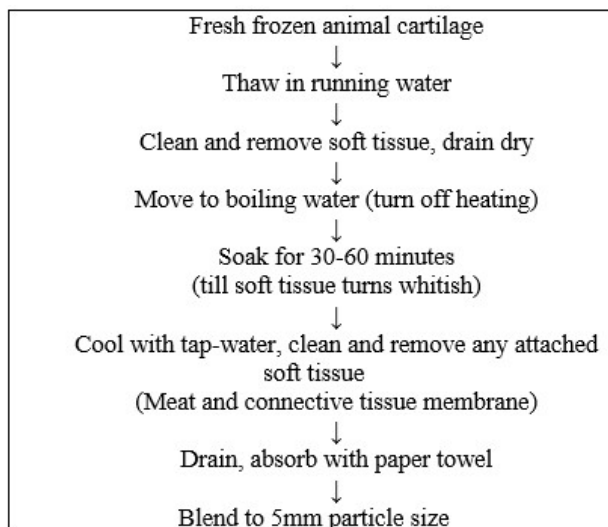
##### ***Getting Sartetd with Qualitek-4 [31]***

##### ***Preparation of Cartilage Hydrolysate (see Table 1)***

##### ***Papain Hydrolysis of Animal Cartilage***

Based on the hydrolyzing procedure of avian cartilage used in the study by Vouland *et al.* [14, 15], efforts for the Taguchi orthogonal working model (Qualitek-4 Windows software) were made to reach optimal levels of physical parameters, namely temperature, pH, enzyme/substrate ratio, substrate concentration and duration time [22, 31]. From published information [12-17], this study established the papain hydrolysis of animal cartilage to achieve the following conditions: 60-70±1°C, pH4.5, pH5.5 and pH6.5, enzyme/substrate ratio 0.5%, 0.75%, and 1.0%, substrate concentration 30%, 40% and 50%, duration time 2hrs and 3hrs. At the

end of incubation time, the reaction contents were transferred to 100°C water bath for 10 minutes. After cooling to room temperature, the reaction mixture was centrifuged at 3500rpm (2200×g) for 30minutes at 4°C. The supernatant fluid and precipitate were separated and freeze-dried. The dried products were stored at room temperature in tide container vials.



*Table 1. Blending Animal Cartilage to 5mm Particle Size*

### ***Isolation, Cultivation and Identification of Porcine Articular Chondrocytes***

Full-thickness cartilage slices were harvested from porcine knee joints. Then the slices were cut into 1mm<sup>3</sup> pieces and placed in dishes containing 0.2% type I collagenase and transferred to a 37°C incubator for 16 hours. The chondrocytes liberated were filtered through 10μ m filter to remove undigested cartilage fragments. The isolated cells were washed and re-suspended in completed medium (DMEM/Ham' s F12 medium containing 10% FBS and 100 U/ml Penicillin and 100 μ g/mL Streptomycin) and their viability was determined using trypan blue dye exclusion.

Cells were expanded by monolayer culture in completed medium. At 80 to 90% confluences, the cells were trypsinized and re-suspended in completed medium at a concentration of 1×10<sup>5</sup> cells/ ml. The 5ml cell suspension was placed into culture flask and at 37°C in a humidified 5% CO<sub>2</sub> incubator. This was the P1 passage of the primary culture of chondrocytes.

### ***Cell Morphology***

A cell climbing sheet of P3 chondrocytes was washed with PBS, fixed with 4% paraformaldehyde for 15 minutes, stained with 1% alcian blue for 30 minutes and washed with ddH<sub>2</sub>O. Cell morphology was observed using a microscope and photography was taken at a magnification of 100× for further examination.

#### ***Evaluation of Cell Cytotoxicity and Proliferative Effects***

The cartilage hydrolysate on the chondrocyte cultures by MTT assay. The chondrocytes were cultured in completed medium and cultured overnight. For cytotoxicity assay, chondrocytes were treated with cartilage hydrolysate in serial dilutions of concentrations 200mg/ ml to 0.0002mg/ml for 24hours. For proliferation analysis, the cells were treated with various concentrations of cartilage hydrolysate (2mg/ml, 0. 2mg/ml and 0.02mg/ml) for 2, 7 and 14 days. After treatment, 10μ M MTT (5mg/ml in PBS) were added to each culture well, and the cultures were incubated at 37°C for 4hours. The purple-blue MTT formazan precipitate was dissolved in 100μ l DMSO and cells were shaken for 5 minutes. Absorbance was measured at 570 nm using ELISA reader.

#### ***Biochemical Analysis***

The P3 chondrocyte culture were treated with cartilage hydrolysate and assessed for Glycosaminoglycans (GAG) type II collagen and DNA content. Briefly, cells were washed with PBS and digested by incubation for 16 hours at 60°C in papain digest buffer. The measurement of total sulphated GAG in the chondrocyte culture was performed using the 1,9-dimethyl-methylene blue (DMB) dye method. Chondroitin sulfate A from bone cartilage was diluted with distilled water to prepare a standard curve (0 to 80μ g/ml). A 96-well plate was used and 40 μ l of GAG standards or the papain-digested samples were piped into each well, then 250 μ l of DMB solution was added to each well. Absorbance at 525 nm was determined using a spectrophotometer (Biorad Plate-Reader, BioRad Laboratories, Hemel Hempstead, UK) at a wavelength of 595 nm.

#### ***Determination of DNA Content Using Hoechst 33324 Fluoresce-Metric Assay***

DNA from calf thymus was used to prepare a standard dilution curve ranging from 0 to 2.5μ g/ml. 100μ l aliquots of standards or sample and Hoechst 33324 (1μ g/ml) were added into a 96-well plate. The samples were read on a fluorimeter with a 365nm excitation filter emission set at 460nm, and the GAGs were normalized to the DNA content.

### ***Quantification of Type II Collagen***

Porcine type II collagen was determined by means of the Collagen type II ELISA kit (MD Bioscience, Inc, Switzerland). To solubilize the newly synthesized collagen, the cell culture medium was removed and pepsin solution was added to the cell layer and digested the collagen at 2-8°C overnight. Subsequently, TXB (0.1M Tris, 0.2M NaCl, 5mM CaCl<sub>2</sub>, pH 7.8-8.0) was added to stop the reaction. To monomerize the remaining polymeric collagen, pancreas elastase solution was added to the samples and incubated at 2-8°C overnight. After centrifugation (10,000rpm for 5 minutes), the supernatant was assayed for type II collagen by ELISA.

### ***Molecular Size Distribution***

The cartilage hydrolysate was characterized by HPLC analysis using an LC system consisting of a Waters model 600E solvent delivery system (a Waters model 996 Photodiode Array Detector and a Waters 717 Plus auto-sampler using a 200  $\mu$ l sample loop). Empower software was used to control the system and to perform the data analysis. The size exclusion experiments were performed at room temperature using a 10 $\times$  300nm Superdex™ peptide column (GE Healthcare) eluted at 0.4ml/min with 10mM phosphate buffer, pH7.4 and containing 0.15M NaCl as the mobile phase. Sample volumes of 40 $\mu$ l were used. The molecular mass of the peptides was estimated with molecular weight markers of range 2,512-16,949 (Amersham Biosciences, Code No. 80-1129-83). Above data obtained were analyzed using SPSS software. Results are expressed as mean  $\pm$  SD.

### ***Application Supervised ANN for Optimizing Enzymatic Bioprocessing***

NeuroSolutions is a neural network development environment by NeuroDimension, Inc. It combines a modular, icon-based (component-based) network design interface with an implementation of advanced learning procedures, such as conjugate gradients, Levenberg-Marquardt and backpropagation through time. The software is used to design, train and deploy neural network (supervised learning and unsupervised learning) models to perform a wide variety of tasks, such as data mining, classification, function approximation, multivariate regression and time-series prediction.

care service training, service redesign, and Standard Operating Procedures (SOP). However, these actions are still insufficient to close the gaps between the provided services and seniors' needs (Chen et al., 2005,

2006; Chen *et al.*, 2004; Chuang, 2007; Keleher *et al.*, 2007).

## RESULTS

### Biological and Enzymatically Experimental Results

#### -Hydrolysis of Three Animal Cartilages

This biochemical structure of cartilage has a high water content of 60-97%. Our hydrolyzed rate was in good agreement with published results. The high water content was also in the range of reported data [12-15].

#### -Molecular Size Distribution

The molecular weight distribution of the cartilage papain hydrolyzed product is shown in Figure 1 (SEC-HPLC chromatograms). Based on the area under cover the molecular weight distribution, more than 97% of cartilage hydrolysate' s molecular weight was less than 10.7kDa. We also have observed that all of the molecular weight of cartilage lysate was less than Ribonuclease A (Mwt. 13700) as analyzed by Superdex 75 (data not shown)]. Additionally, 58% of cartilage hydrolysate has a molecular weight of between 10.7 and 6.2kDa, while 40% of cartilage lysate has a molecular weight of less than 6.2kDa.

Figure 2 shows the heat-hydrolyzed (not enzyme-hydrolyzed) cartilage hydrolysate. More than 83% of molecules in the heat-hydrolyzed cartilage product appeared to be above the upper separation limit of SEC-HPLC (before retention time of 20 minutes). The result indicated that the majority of heat-hydrolyzed cartilage products still retained their high molecular weight structures. As shown in Figure 3, about 14% of small molecular weight product appeared after a retention time of 42 minutes. Its identity and properties require further study.

#### -Cytotoxic and Mitogenic Effects of Cartilage Hydrolysate

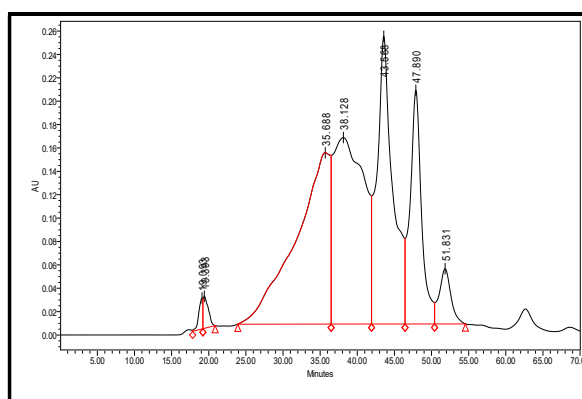
Figure 4 shows the cartilage papain-hydrolysate is not cytotoxic to the primary porcine chondrocytes at a concentration below 20mg/ml and lower. At a concentration of 200mg/ml, it displayed a significantly lowered viability that could be due to other physical properties or its having very weak toxicity.

Figure 5 shows the cartilage papain-hydrolysate had no mitogenic effects on porcine chondrocyte culture. Cartilage papain-hydrolysate stimulating GAG expression

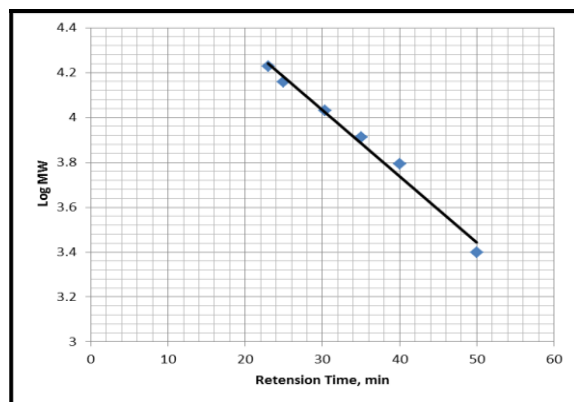


Figure 6 shows the cartilage papain-hydrolysate induced GAG production by the primary porcine chondrocyte cultures at day 14. Figure 7 shows the treatment of cultured chondrocytes with 0.2 and 0.02mg/ml sample induced a marked increase in type II collagen synthesis. At the end of the culture period (18 days), type II collagen synthesis was almost 11.9-fold higher in sample stimulated cultures in comparison with the control cells.

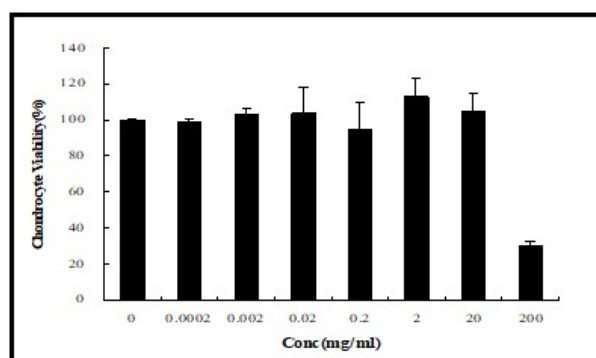
Our cartilage hydrolysate was presented in molecular size smaller than 10kDa (Fig.1, 2 and 3) with the capability of stimulating chondrocytes to express GAG (Fig. 6) and type II collagen (Fig. 7) , thus fulfilling the requirement of being dietary peptides. Dietary peptides are known to have biological importance beyond their nutritive value of intact protein and individual amino acids [12-17, 46]. It is well recognized that apart from their basic nutritional role many food proteins contain encrypted within their primary structures peptide sequences capable of modulating specific physiological functions [43-46]. In this study, the cartilage hydrolysate was prepared by papain hydrolysis and resulted in various sizes of a fragment including peptides and saccharide-aggregates, with stimulating/enhancing of the anabolic activity of chondrocyte. This bioactive property was similar to reports of bioactive peptides having been found in enzymatic protein hydrolysates [12-17].



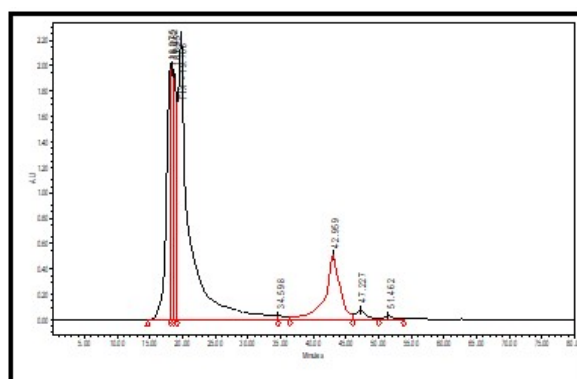
**Figure 1. HPLC Chromatogram of Cartilage Hydrolysate Products: Cartilage Hydrolysate Product was Separated by the Superdex™ Column**



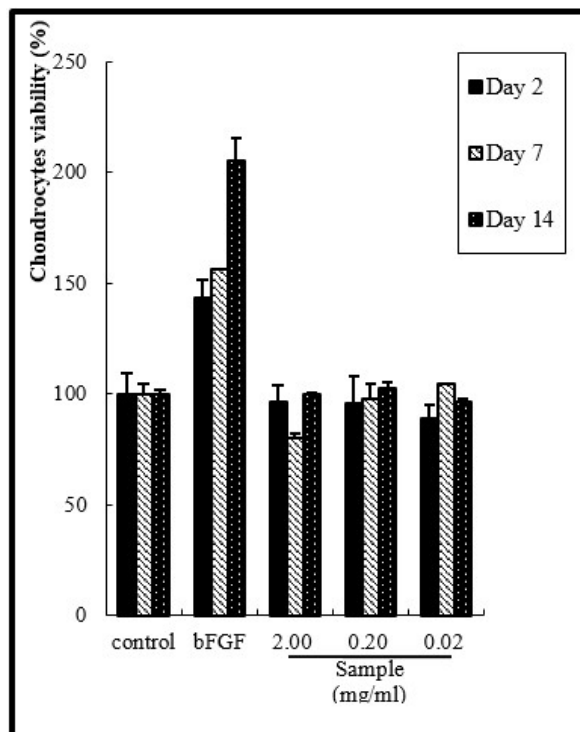
*Figure 2. HPLC Chromatogram of Amersham Standards (80-1129-83)*



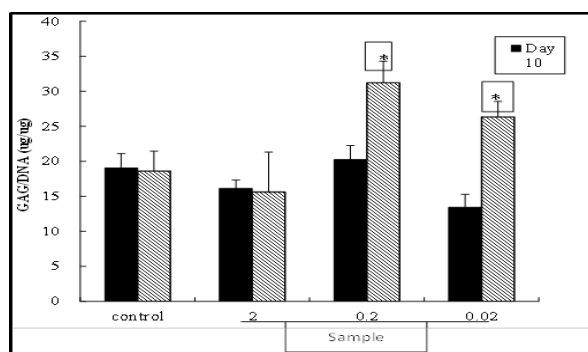
*Figure 3. HPLC Chromatogram of Heat-Hydrolyzed Cartilage Products*



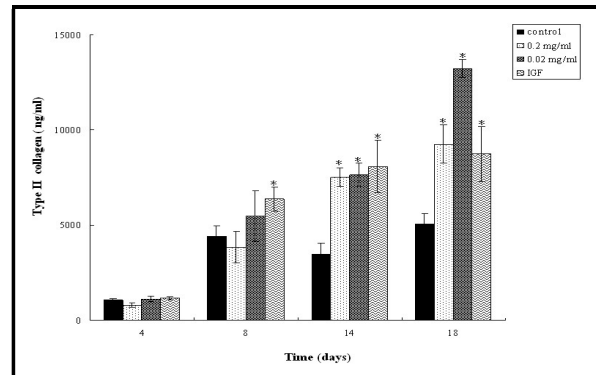
*Figure 4. Cytotoxicity Evaluation of the Sample on Chondrocytes. Chondrocytes were treated with various concentrations of the sample for 24hrs. Chondrocytes Cytotoxicity was determined by MTT assay*



**Figure 5. Effects of the Sample on Chondrocytes Proliferation** Chondrocytes were treated with various concentrations of the sample for 2, 7 and 14 days. Chondrocytes viability was determined by MTT assay



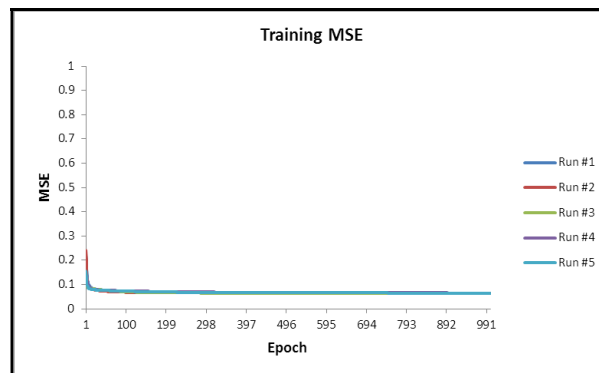
**Figure 6. Effects of the Sample on GAG Content of Chondrocytes.** Chondrocytes were treated with various concentrations of the sample for 10 and 14 days. GAG and DNA content were determined by DMMA assay and Hoechst 33324. \* $p < 0.05$  compared with untreated controls



**Figure 7. Effects of the Sample on Type II Collagen Expression of Chondrocytes.** Chondrocytes were treated with three concentrations of cartilage hydrolysate product for indicated durations. \* $p < 0.05$  compared with untreated controls

### Neural Network Assessment and Statistical Analysis

NeuroSolutions 6.0 neural network software (Back-propagation Network) was into the production process to parallel the orthogonal array in the construction of the prediction model for the 89 experiments. The biological enzymatically results (target value: supernatant recovery rate) demonstrated good consistency in the sample duplications and experimental groups, and the artificial intelligence computing convergence value ( $R^2=0.9762$ ) indicated this enzymatic bioprocessing had good performance (Figure 8-24).



**Figure 8. Training MSE: Papain Hydrolysis of Chicken Sternal Cartilage**

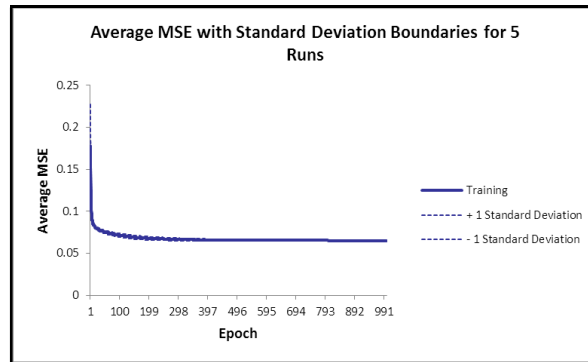


Figure 9. Training Report: Papain Hydrolysis of Chicken Sternal Cartilage

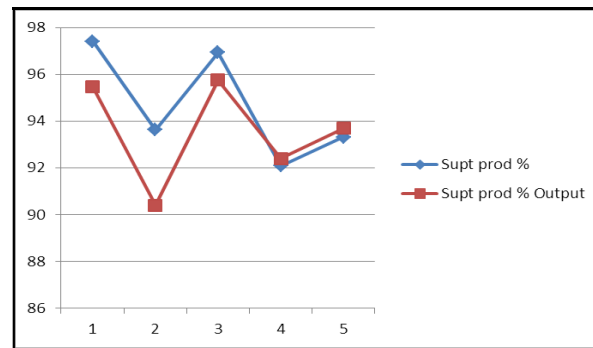


Figure10. Testing Trend: Papain Hydrolysis of Chicken Sternal Cartilage

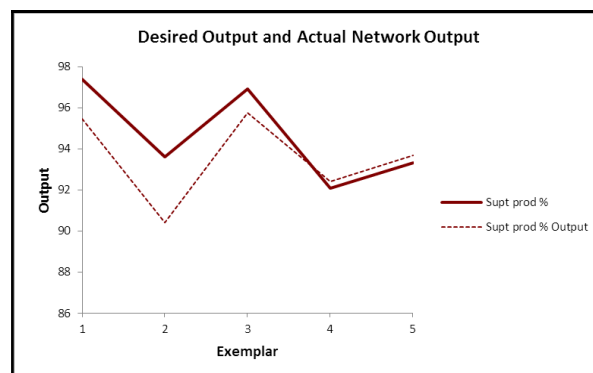
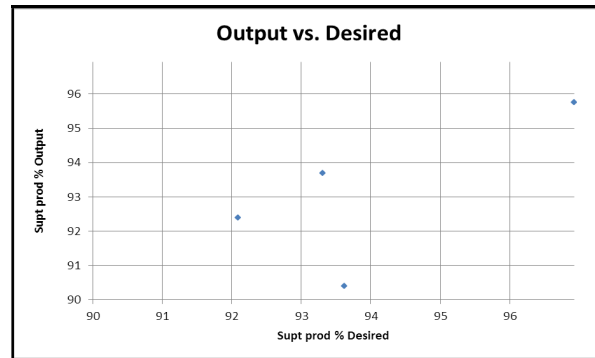
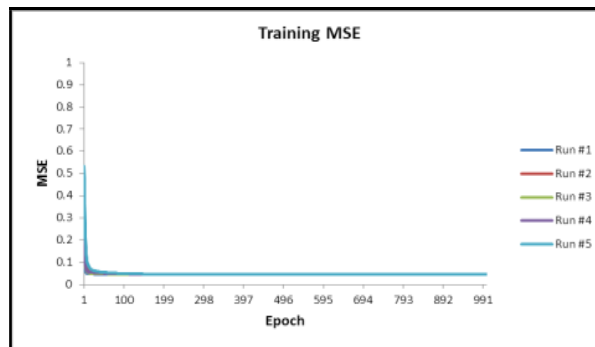


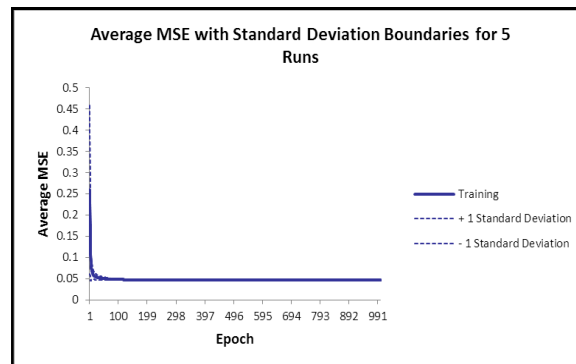
Figure11. Testing Trend: Papain Hydrolysis of Chicken Sternal Cartilage



*Figure 12. Testing Report: Papain Hydrolysis of Chicken Sternal Cartilage*



*Figure 13. Training MSE: Papain Hydrolysis of Mini Pig Laryngeal and Tracheal Cartilage*



*Figure 14. Training Report: Papain Hydrolysis of Mini Pig Laryngeal and Tracheal Cartilage.*

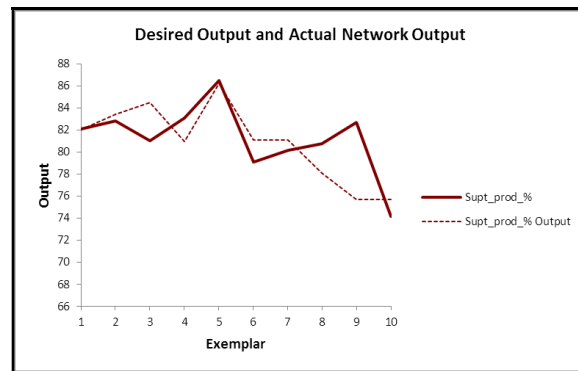


Figure 15. Testing Trend: Papain Hydrolysis of Mini Pig Laryngeal and Tracheal Cartilage

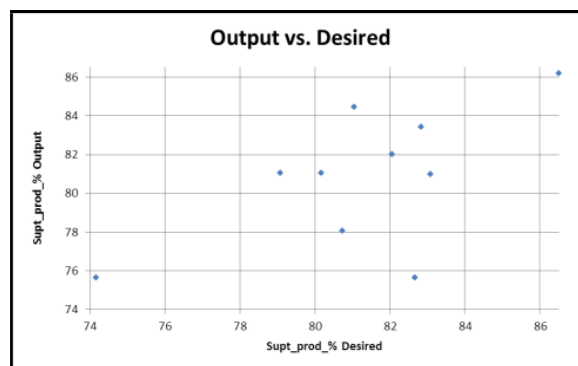


Figure 16. Testing Report: Papain Hydrolysis of Mini Pig Laryngeal and Tracheal Cartilage

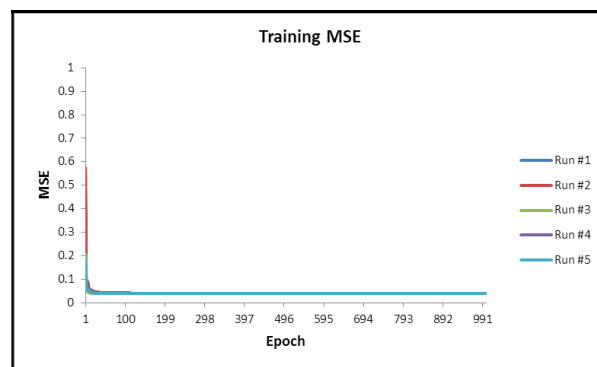


Figure 17. Training MSE: Papain Hydrolysis of Hog Laryngeal and Tracheal Cartilage

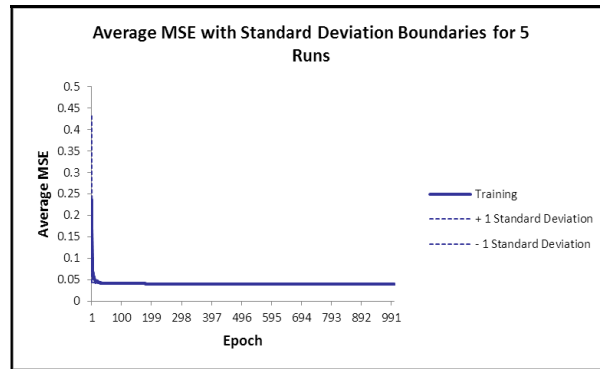


Figure 18. Training Report: Papain Hydrolysis of Hog Laryngeal and Tracheal Cartilage

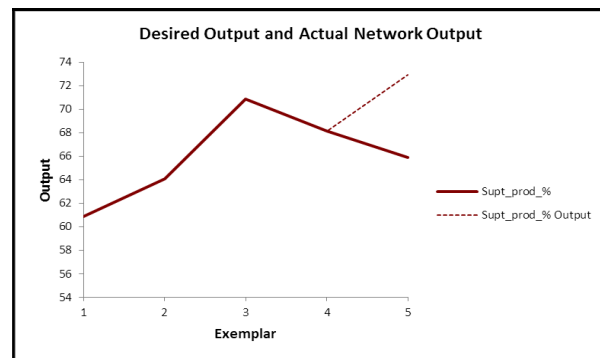


Figure 19. Testing Trend: Papain Hydrolysis of Hog Laryngeal and Tracheal Cartilage

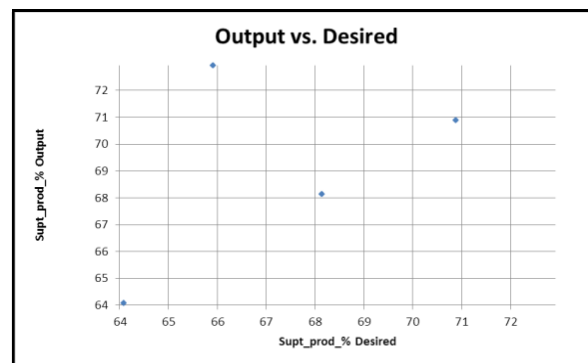


Figure 20. Testing Report: Papain Hydrolysis of Hog Laryngeal and Tracheal Cartilage



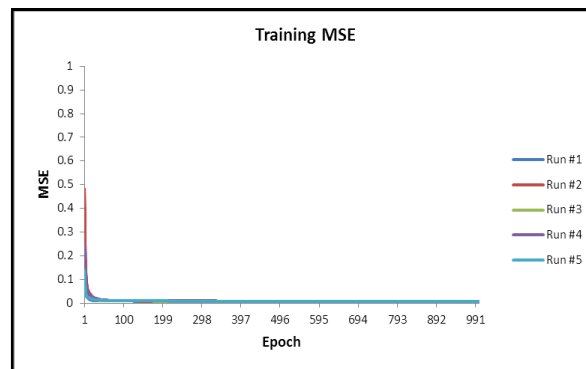


Figure 21. Training MSE: Papain Hydrolysis of Mixture of Three Animal Cartilages

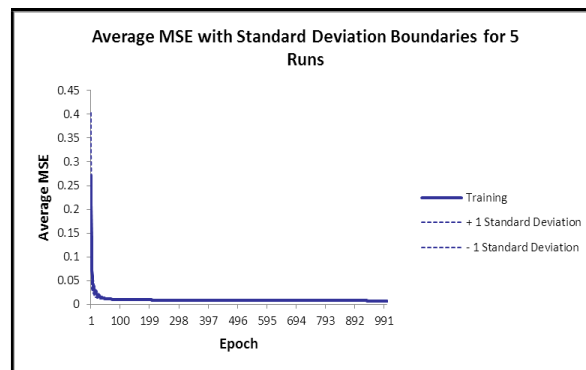


Figure 22. Training Report: Papain Hydrolysis of Mixture of Three Animal Cartilages

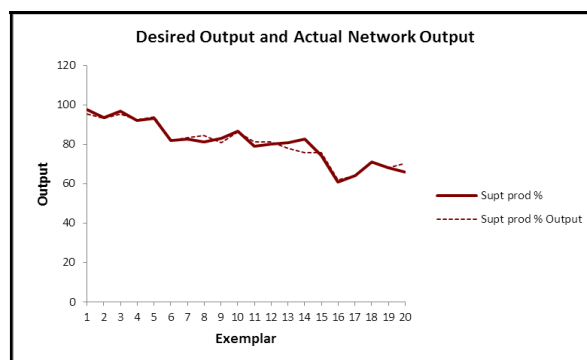
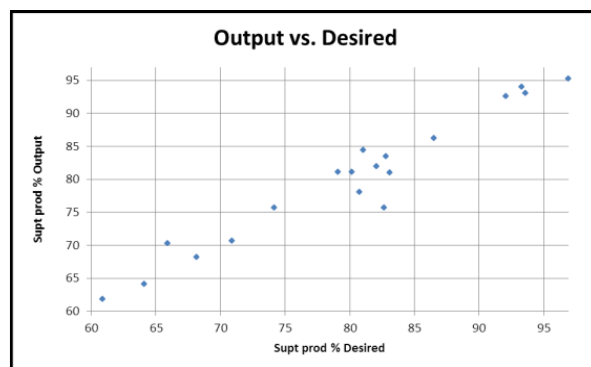


Figure 23. Testing Trend: Papain Hydrolysis of Mixture of Three Animal Cartilages



*Figure 24. Testing Report: Papain Hydrolysis of Mixture of Three Animal Cartilages*

## DISCUSSION

### Neural Network Performance

NeuroSolutions 6.0 neural network software (Back-propagation Network) was incorporated into the production process to parallel the Taguchi orthogonal array in the construction of the prediction model for the 89 experiments. The biological enzymatically results were 92.09-93.03% (target value: supernatant recovery rate) appeared to have good consistency in the sample duplications and experimental groups, and the artificial intelligence computing convergence value was  $R^2=0.9762$ , indicating this enzymatic bioprocessing had good performance. However, the analysis indicated the papain enzyme/substrate ratio as the major impact factor in this enzymatic hydrolysis to produce high yields of final bioactive peptide products (Figure 8-24).

### Optimizing Enzymatic Bioprocessing

In 1999, collagen hydrolysate (prepared from gelatin) was shown to be absorbed in its high-molecular weight by containing peptides of 2.5kDa to 15kDa [13-18]. Recently, a report showed hydrolyzed chicken sternal cartilage extract of novel low molecular weight (from 0.05kDa to 10kDa with average 5.5 kDa), indicating an improved osteoarthritis-related system in a Randomized, Double-Blind, Placebo-Controlled Trail [13-16]. The molecular size of our cartilage hydrolysate was smaller than 10.7 kDa, which is comparable to the published result [14-17].

Significantly, this study provides a simple, easy and economical way of preparing 10 to 100 grams of cartilage hydrolysate required for further investigations.

Hydrolyzed chicken cartilage powder is a natural source of BSE free type II collagen and chondroitin sulfate. Extensive research has shown the benefit of these two substances for both healthy and unhealthy joints. Furthermore, ongoing research and studies continue to uncover their mechanisms of action and to strengthen the evidence of their efficiency [14-17].

**-Hydrolyzed collagen type II having an average molecular weight**

Animal cartilage-derived material comprising hydrolyzed collagen type II, said hydrolyzed collagen type II having an average molecular weight of between about 1,500 and 2,500 Daltons [14-17].

**-Comprising hydrolyzed collagen type II having an average molecular weight**

A method for treating an individual with a connective tissue disorder, comprising orally administering to said individual an effective daily amount of chicken sternal cartilage-derived material comprising hydrolyzed collagen type II having an average molecular weight of between 1,500 and 2,500 Daltons [14-17].

**-Intelligence computing technology can optimize enzymatic bioprocessing in animal waste cartilage**

It is believed that intelligence computing technology can bring about significant advancement in product development (as archived pharmaceutical industrial) as well as in future **business** execution and prediction (such as Taguchi orthogonal method and artificial neural network) by optimizing enzymatic bioprocessing in animal waste cartilage (chicken sternal cartilage, mini pig laryngeal and tracheal, and hog laryngeal and tracheal) and generating hydrolysis conditions for preparation of hydrolyzate collagen. Specifically, the artificial neural network is used in parallel with the Taguchi orthogonal array process for enzymatic hydrolysis of the cartilage tissue to produce the best yield and quality of bioactive peptides with clinical efficacy for functional ingredients of nutraceuticals.

## CONCLUSION

This study comprises two parts: one is the biological experiment of papain hydrolysis of animal (chicken, mini pig and hog pig) cartilage and the other is the neural network computing and analysis in an attempt to locate a predicting model for the formulation of a better enzymatical production procedure for bioactive-functional peptides.

The enzymatic hydrolysate of three animal cartilages was processed following the Taguchi orthogonal design. It was discovered that a hydrolyzing temperature of  $60\pm 1^{\circ}\text{C}$  is the most effective to hydrolyze the cartilage. The chicken sternal cartilage could be hydrolyzed by papain to produce a molecular size smaller than 10kDa (with 95% in 10.7kDa and 2.5kDa). These peptides were demonstrated to be capable of stimulating the porcine chondrocytes to produce glycosaminoglycan (GAG) and type II collagen *in vitro*. Thus, it is concluded that animal cartilage papain-hydrolysate contain bioactive peptides or factors and could be a good agent for chondrogenesis and regeneration of cartilage tissue. The product of this research can be applied in nutraceuticals since it has been granted “GRAS” status by the US FDA.

In the second part of this study, through NeuroSolutions 6.0 analysis, it is concluded that the good conversion value ( $R^2=0.9762$ ) is an indication that based on this study experiments can perform neural network analysis. The training MSE-papain hydrolysis of a mixture of three animal cartilages also indicated the best final MSE =0.00779.

However, the testing input/output data: papain hydrolysis of a mixture of three animal cartilages indicate the papain enzyme/substrate ratio to be the major impact factor in this enzymatic hydrolysis to produce high yields of the final bioactive peptide products.

Indeed, the chicken sternal cartilage can be efficiently hydrolyzed by papain to produce a molecular weight smaller than 10kDa with 95% in 10.7kDa, 6kDa and 2.5kDa fragments or peptide with biological activity capable of stimulating the primary porcine chondrocyte to express GAG and type II collagen in culture. The cartilage hydrolysate could be used as ingredients in food supplements and nutraceuticals. Also importantly, it should be considered as safe as collagen hydrolysate which has been granted GRAS status by the US FDA in 2003. Finally, the cartilage hydrolysate could also be a biomimetic biomaterial for bone and connective tissue regeneration studies.

#### **FUTURE DIRECTIONS**

This study results suggest that intelligence computing manipulation of bioprocessing engineering could improve the goods of final bioactive peptide products. Nevertheless, waste animal cartilages can indeed become the green resources for biotechnology to convert low valued by-products of the meat industry for eventual use as functional food ingredients and nutraceutical components for bone and joint health.

Bioprocess engineering is a conglomerate of mathematics, biology and industrial design. It also consists of various biotechnological processes used in industries for large-scale production of biological products for optimization of yield and quality [48]. It is believed that the intelligence computing technology can bring about significant advancements in product development (as archived pharmaceutical industrial) as well as in future business execution and prediction.

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