Research Article

Enzyme-Assisted Extraction of Fucosylated Chondroitin Sulfate from Sea Cucumber *Holothuria scabra* and *Bohadschia argus* and their Potential in Pharmaceutical Applications

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Abstract
Due to the health benefit of fucosylated chondroitin sulfate (FuCS), the efficient method for extraction of FuCS from raw materials is a crucial issue in reducing the production cost. In this study, enzymatic extraction of FuCS from two species of sea cucumber, *Holothuria scabra* and *Bohadschia argus* was undertaken using two protease enzymes, alcalase and papain. Response surface methodology (RSM) was employed in determining the optimal extraction conditions with the highest yield of FuCS concentration. The predicted optimal papain-assisted extraction conditions of *Holothuria scabra* and *Bohadschia argus* obtained a predicted FuCS yield of 1609.73 mg/100 g and 444.51 mg/100 g, respectively. To compare extraction efficiencies of two protease enzymes, employment of the RSM optimal conditions to *Holothuria scabra* resulted in 1538.76 ± 20.26 mg/100 g and 1295.50 ± 14.28 mg/100 g of purified FuCS for papain and alcalase, respectively. Whereas *Bohadschia argus* produced 412.39 ± 10.12 mg/100 g and 461.11 ± 8.45 mg/100 g purified FuCS for papain and alcalase, respectively. The acquired FTIR and NMR spectrums of extracted FuCS showed typical bands of sulfation patterns and were compared to commercial FuCS. The extracted FuCS showed enzyme type dependent antioxidant activity, and significant tyrosinase inhibitory activity than commercial FuCS. It also exhibited similar anti-glucosidase activity as commercial FuCS. Thus, this study reveals potential applications of enzyme-assisted FuCS from sea cucumber in food and pharmaceutical industries.

Keywords: Bioactivity, Enzymatic extraction, Fucosylated chondroitin sulfate, Functional ingredient, Sea cucumber

1 Introduction

Sea cucumbers have been utilized as an ingredient in traditional meals to serve as a health supplement in many Asian countries. Sea cucumber meats are enriched with protein content (5.45–5.78%) but low in fat and cholesterol (0.17–0.37%), therefore they are suitable sources for the preparation of healthy foods [1], [2]. The body walls of sea cucumbers are composed of collagen and acidic polysaccharides,

making them flexible and water-enriched structures. Acidic polysaccharides, for example, glycosaminoglycans (GAGs) such as hyaluronic acid (HA), keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS)/heparin [3]–[5], have been demonstrated for their various biological activities and further applied in medical, cosmetic, and food supplement uses [6]–[8]. Currently, CSs are commercially available in a high purity form or as a crude extract that is obtained from different types of feedstocks, including shark cartilage, cattle, pigs, and chickens. However, there are concerns about the risks of spreading plagues from these raw materials, such as bovine-borne mad cow disease and the H1N1 virus found in poultry. Shark cartilage is also restricted in some countries due to the Shark Conservation Act. Additionally, religious restrictions on some types of raw materials, e.g., pigs and cattle, also limit the use of commercial CS products.

Among these acidic polysaccharides, fucosylated chondroitin sulfates (FuCS) [9]–[11] and sulfated fucans are enriched in sea cucumber body walls, and their wide spectrum of biological activity has been investigated [12]. As an alternative option to the current CS commercial products, sea cucumbers are potential feedstocks as they are consumed in regular and traditional meals throughout the world. Anti-coagulant [13], anti-thrombotic [14]–[16], and anti-inflammatory [17] are biological characteristics described for FuCS isolated from a variety of sea cucumbers [18]. FuCS is a member of glycosaminoglycan, and its chemical structure has the same hyaline backbone compositions as those found in mammalian CS, which are made of 4-linked glucuronic acid and 3-linked N-acetyl galactosamine residues within disaccharide repeating units. On the other hand, FuCS is composed of a sulfated fucosyl branch chain with β-1,3-linkages to the glucuronic acid residues. The fucosylation pattern is an important factor in determining anticoagulant properties and bioactivity [18]. The positions of the sulfates in the two primary chondroitin sulfates, A and C, are different. Sulfation occurs at position 4 in CS A and at position 6 in CS C. As a result, these two polysaccharides are referred to as chondroitin 4-sulfate and chondroitin 6-sulfate, respectively. Depending on the source, the chondroitin chain has a different number of repeating uronic acids. The number of repeating units may vary even within the same tissue [19].

From the previous reports on CS extraction, there are different extraction methods according to the source of raw materials, such as CS extraction using acid or alkali extraction under high pressure and enzymatic extraction [20]. Enzymatic extraction gives a different pattern of end products, making it the preferred method of extracting chondroitin sulfate. After enzymatic digestion with various enzymes with different specificities and targets [21], [22], various types of single-chain CS products are obtained. For example, with papain extraction, the molecular size of CS is approximately 23–40 kDa, which is similar to commercial products [23]. The digested single-chain CS has the benefit of promoting its absorption in the digestive system. In this study, two commercially available protease enzymes, papain and alcalase, were selected to be applied in the extraction process of two types of sea cucumbers to maximize the extraction efficiency of CS. The distinct characteristics of papain and alcalase result in different patterns of hydrolyzed CS products due to enzyme specificity. Papain specifically cleaves peptide bonds of basic amino acids, such as leucine or glycine [24]. On the other hand, alcalase has a high affinity for aromatic amino acids, including glutamic acid, methionine, leucine, alanine, serine, and lysine [25]. Therefore, the resulting hydrolysis patterns of CS produced by these enzyme-assisted extraction are expected to be diverse profiles, which result in different biological activities. Thus, this study also aimed to characterize the hydrolyzed CS obtained from enzymatic extractions by Fourier Transform Infrared Spectrometer (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy and analyze their biological activities, e.g. anti-oxidant, anti-tyrosinase, anti-inflammation and anti-amylase, to be used as a guideline for their applications in food supplement, medical and cosmetic uses.

2 Materials and Methods

2.1 *Sample preparation*

Dried tigerfish sea cucumber (*Bohadschia argus*) and white sea cucumber (*Holothuria scabra*) samples were brought to the laboratory from Thailand and Papua New Guinea (courtesy provided by Wonnapob Co., Ltd., Bangkok, Thailand). The sea cucumbers were rehydrated for their wet weights by immersing

them in deionized water for 96 h at 4 °C. Every 24 h, deionized water was replaced. The moisture content was measured, and the weight was recorded. Sea cucumbers were manually chopped into 1-cm-thick slices and kept frozen at –20 °C until used. Food-grade proteases (papain and alcalase supplied by Value Industrial Products Co. Ltd., Bangkok, Thailand) and chondroitin-4-sulfate from the bovine trachea (Fluka, Singapore) were employed as standards for quantitative and quantitative analysis. The rest of the chemicals in the experiment were of analytical grade.

2.2 *Enzyme assisted extraction and purification of chondroitin sulfate*

The extraction of proteins is dependent on two critical factors: the extraction condition and the enzyme specificity. Protein and glycan sulfate found in marine connective tissue are broken down during enzyme assisted extraction to liberate CS. For the extraction process, a method adapted from Garnjanagoonchorn *et al.* [23] was used (Figure 1). 2.0 g of sliced sea cucumber was homogenized in 20 mL of 0.1 M sodium phosphate buffer containing 5 mM ethylenediamine tetraacetic acid, 5 mM cysteine hydrochloride, and 0.02 percent sodium azide. The papain enzyme (at a concentration range of 0.005–0.5% w/v) was added to the homogenized sample. The parameters of enzyme hydrolysis, time (15–180 min) and temperature (55–65 °C), were varied according to the Box-Behnken Design (BBD). The sample was taken when the enzymatic hydrolysis reaction was stopped by heating at 95 °C in a waterbath, yielding a clear solution. The unhydrolyzed fraction of sample was removed as a pellet by centrifugation at 8,000 rpm for 15 min at 4 °C. To determine the crude CS yield, an aliquot of the hydrolysate fraction was freeze dried, until constant dried weight was achieved. Then, the dried weight of purified CS was measured to calculate the extraction yield as follows.

$$Extraction\ \ yield\,(\%) = \frac{D_{W1}}{D_{W2}} \times 100$$

Where D_{W1} and D_{W2} are dried weight of freeze dried CS powder and dried weight of sea cucumber sample, respectively.

To further purify CS, NaCl was added to get a final concentration of 10% (w/v) (Figure 1). The mixture was stored at 4 °C overnight to allow precipitate to form. The precipitated sample was collected by centrifugation for 30 min at 10,000 rpm at 4 °C, then the supernatant fraction was harvested for further purification step. The supernatant was dialyzed for 24 h in cold distilled water at 4 °C. Subsequently, 1.5 g of cetylpyridinium chloride was added to precipitate CS, and the pellet was collected by centrifugation at 15,000 rpm for 15 min at 4 °C, and the pellets were washed with 40 mL of 0.4 M NaCl. To remove cetylpyridinium thiocynate, the precipitate was dispersed in 2.1 M NaCl and 100 mL of 1 M potassium thiocyanate was added, and the washed precipitate was harvested by filtering through filter paper (Whatman No. 4). Finally, the solution was dialyzed with cold distilled water for 4 h and freeze dried to obtain dried CS to be used in bioactivity testing. To confirm repeatability, at least 3 repeats of experiments were done.

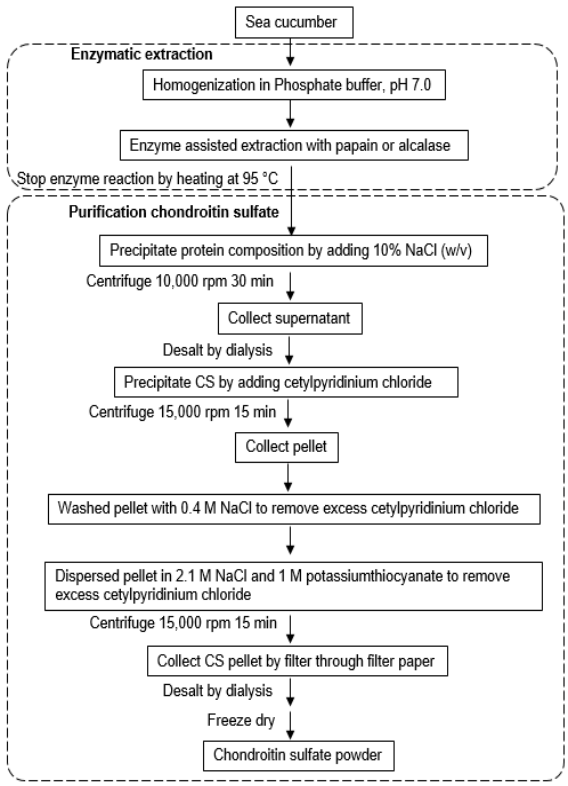


Figure 1: Chondroitin sulfate extraction and purification procedure.

2.3 Optimization of CS extraction

The Design-Expert program was used to optimize parameters for the extraction of CS from *Holothuria Scabra* and *Bohadschia argus* based on RSM with Box-Behnken design (BBD) (V.7.0.0, Stat-Ease, Inc, Minneapolis, USA). This approach is commonly used to assess the relationship between response values and variables [26]. This strategy also has the advantage of minimizing the numbers of experiments, which helps to reduce the investment cost and process time. In this study, the enzyme concentration (X_1: 0.005–0.5%), extraction time (X_2: 15–180 min), and extraction temperature (X_3: 55–65 °C) were used as extraction parameters. The ranges of the three independent variables were coded as +1, 0 and 1 for high, intermediate, and low values, and varied at three levels. A total of 17 experimental runs were carried out in accordance with this design for the optimization of the extraction process (Table 1). The CS extraction yield was chosen as the response (Y).

Based on our preliminary study, we chose these ranges for independent parameters. For temperature, although the enzyme is active at room temperature (+/–20 °C), papain activity was shown to be the best between 55 and 65 °C, and after 65 °C, enzyme inactivation was observed. For hydrolysis time, the amount of time takes to extract a sample depends on the raw material used, for example, 48 h for bovine cartilage. However, in the case of sea cucumber with relatively softer tissue, the extraction time was reduced to 15–180 min. The enzyme concentration was set between 0.005–0.5% based on a prior study by Nguyen *et al.* [27]. Each of the 17 experimental runs in the BBD was performed in triplicate. Then, the obtained experimental results were fit in different mathematical models and the model with the highest correlation coefficient value (R^2) with the significance fit (p-value < 0.05) was selected to forecast the optimal condition for enzymatic extraction.

2.4 *Quantitative analysis of chondroitin sulfate*

The content of CS was measured using the Dimethylmethylene Blue Assay (DMMB) as described by Thomas-Coulson *et al.* [28]. 20 µL of standard chondroitin-4-sulfate solutions with different concentrations (0, 2, 4, 6, and 8 µg/mL) and 200 µL of DMMB dye were mixed and added in a 96 well microplate. The absorbance at 525 nanometer of each standard chondroitin-4-sulfate solution was determined by spectrophotometry and the standard curve was

Table 1: Experimental design and responses (CS yield) of the dependent variables to extraction conditions by using papain enzyme

Run Order	Extraction Condition			CS Yield (mg/100 g dry weight)	
	Enzyme Concentration (X_1) (%)	Extraction Times (X_2) (min)	Extraction Temperature (X_3) (°C)	*Holothuria Scabra*	*Bohadschia argus*
1	0.005	99	55	1595.24	426.98
2	0.005	15	60	478.98	252.28
3	0.500	180	60	655.23	343.69
4	0.252	15	55	671.76	395.28
5	0.252	99	60	1465.80	392.24
6	0.252	180	65	688.28	394.58
7	0.252	99	60	1531.90	410.03
8	0.005	99	55	1019.67	279.92
9	0.252	99	60	1468.56	393.07
10	0.252	99	65	1523.64	407.82
11	0.252	99	60	1606.25	429.93
12	0.005	99	60	1658.58	443.93
13	0.252	180	55	677.26	432.88
14	0.005	180	60	601.99	362.85
15	0.252	15	65	627.69	409.29
16	0.500	99	65	1633.79	437.30
17	0.500	15	60	539.97	404.13

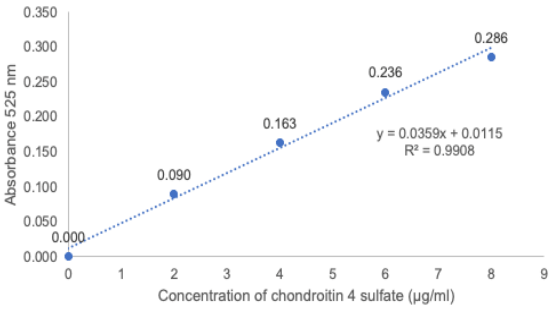


Figure 2: Standard curve obtained from DMMB assay to measure chondroitin-4-sulfate.

prepared for further calculation of CS amount. To determine the CS concentration in the extracted sample, a volume of 20 µL from each sample was pipetted into a microplate well containing 200 µL of DMMB dye. After mixing, the absorbance at 525 nanometer was read and the CS concentration in the sample was calculated based on the standard curve (Figure 2). The measurements of CS concentration were conducted with three repeats.

2.5 *Determination of total phenolic contents*

Folin-Ciocalteu reagent (FC) was used to determine the total phenolic content of CS powder. 250 µL of the sample were added to 626 µL of FC reagent (0.2 mg/mL) and adequately mixed. After 5 min, 1 mL of a 7.5% Na_2CO_3 solution and 1 mL of distilled water were added. The mixture was then incubated in the dark for 40 min at room temperature (25 °C). The mixture's absorbance was measured at 727 nm compared to a blank (60% methanol). The total phenolic content of CS samples was measured in mg of gallic acid equivalents per g of dry sample weight (mg GAE/g).

2.6 *Antioxidant DPPH scavenging activity assay*

The DPPH assay was used to determine the free radical scavenging activity in CS powder [29]. In a 96-well microplate, 50 µL of extracts at various concentrations and 50 µL of DPPH (Sigma-Aldrich, USA) solution (0.5 mg/mL in ethanol) were placed in each well. After 30 min of processing at 25 °C, the absorbances were measured with a microplate reader at 515 nm. As a control, ascorbic acid (Sigma-Aldrich, USA) was employed. The following percentages of DPPH radical scavenging activity were calculated:

$$Scavenging\ activity\ (\%) = [(A_0 - A_1)/A_0] \times 100$$

The absorbance of the control sample was A_0, and the absorbance of the treatment sample was A_1. From the graph displayed between the free radical scavenging percentages and the sample concentrations, the 50% scavenging (IC_{50}) concentrations were computed.

2.7 *Tyrosinase inhibition activity by the modified dopachrome method*

The modified dopachrome method measured tyrosinase inhibitory activity using tyrosine as a substrate [30]. In 96-well microplates, 50 µL of various concentrations of samples, 50 µL of 0.1 mg/mL L-tyrosine (Sigma-Aldrich, USA), 50 µL of 0.1 mg/mL mushroom tyrosinase (Sigma-Aldrich, USA), and 50 µL of 0.1 mM phosphate buffer were added. As a control, ascorbic acid was employed. The mixture was incubated at 37 °C for 60 min. A microplate reader was used to measure absorbances at 450 nm before and after incubations. The following equation was used to compute the percentages of tyrosinase inhibition:

$$Inhibition\ activity\ (\%) = [(A - B) - (C - D)]/(A - B) \times 100$$

Where A represents the blank's absorbance after incubation, B represents the blank's absorbance before incubation, C represents the samples' absorbance after incubation, and D represents the samples' absorbance before incubation. The concentrations giving 50% inhibition (IC_{50}) were computed.

2.8 *Anti-inflammatory*

According to the protocol previously reported [31], the reaction mixture contained 0.2 mL of fresh hen's egg albumin, 2.8 mL of phosphate buffer saline (pH 6.4), and 2 mL of different concentrations of the tested CS extract, resulting in concentrations of 400, 800, 2000, 4000, 8000, and 16,000 µg/mL. As a control, a similar volume of double-distilled water was used. The mixtures were then heated at 70 °C for 5 min, then cooled down to 37 °C for 15 min. Their absorbance was measured at 660 nm after cooling. Diclofenac was employed as a reference at final concentrations of 50,

100, 250, 500, 1000, and 2000 µg/mL, respectively, and was handled similarly for absorbance determination. Using the following formula, the percentage inhibition of protein denaturation was calculated:

$$\% \, Inhibition = ([V_t/V_c] - 1) \times 100$$

Where V_t denotes the absorbance of the test sample and V_c denotes the absorbance of the control,

2.9 *Alpha-amylase inhibition*

Alpha-amylase inhibitory activity based on starch iodine color assay was carried out to assess the alpha-amylase inhibition by CS powder extracts with a slight modification based on the starch-iodine test [32]. CS powder of varied concentrations (100 µL) was added to 100 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.04 units of alpha-amylase solution and incubated at 37 °C for 10 min. Then 100 µL of soluble starch (1%, w/v) was added to each reaction and incubated at 37 °C for 15 min. 1 M HCl (20 µL) was added to stop the enzymatic reaction, followed by the addition of 100 µL of iodine reagent (5 mM I2 and 5 mM KI). The color change was noted, and the absorbance was read at 620 nm on a microplate reader. The control reaction, representing 100% enzyme activity, did not contain any CS extract. Inhibition of enzyme activity was calculated as:

$$Enzyme\ activity\ inhibition\ (\%) = (Abs2 - Abs1) / (Abs4 - Abs3) \times 100$$

where *Abs1* is the absorbance of the incubated solution containing the sample, starch, and amylase, *Abs2* is the absorbance of the incubated solution containing the sample and starch; *Abs3* is the absorbance of the incubated solution containing starch and amylase; and *Abs4* is the absorbance of the incubated solution containing starch substrate.

A dark-blue color indicates the presence of starch; a yellow color indicates the absence of starch; and a brownish color indicates partially degraded starch in the mixture. In the presence of inhibitors from the CS extracts, the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex, whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by alpha-amylase.

2.10 *FTIR analysis of extracted CS*

Qualitative determination of extracted CS powder samples was performed by the procedure described by Sundaresan *et al.* [33]. The CS component in sea cucumber was identified using FTIR (Bruker AVANCE III, 500 MHz) by comparing sample spectra (between 400–4000 cm^{-1} with 4 nm window interval) to the standard CS (chondroitin-4-sulfate from the bovine trachea (Fluka)) spectrum.

2.11 *NMR spectroscopy of extracted CS*

Thirty milligrams of extracted CS were solubilized in 99.9% D_2O and freeze-dried. The sample was re-dissolved again in 99.96% D_2O and transferred into a Shigemi tube. 1H spectra of each sample were recorded by using a Bruker AV-600 spectrometer equipped with a probe with proton frequencies of 600.13 MHz at 333 K with HOD suppression by pre-saturation. Chemical shifts are relative to trimethylsilylpropionic acid at 0.0 ppm for 1H spectra.

2.12 *Statistical analysis*

Statistical analysis was performed to analyze the amounts of total phenolics and activities of different assays (including DPPH, anti-tyrosinase, anti-inflammatory, and anti-amylase) in CS extracts of sea cucumbers. All tests were carried out in triplicate, and the data were calculated for mean and standard deviation of three replicates. The data were processed by using a one-way analysis of variance (ANOVA), and mean separations were analyzed with Duncan using the IBM SPSS.

3 Results and Discussion

3.1 *Optimization of enzymatic extraction of CS*

To optimize the enzymatic extraction of CS from sea cucumber samples, *Holothuria scabra* and *Bohadschia argus*, the extraction parameters, including papain concentration, extraction time and extraction temperature were varied based on RSM with BBD. The CS yields obtained from each tested experiment, a

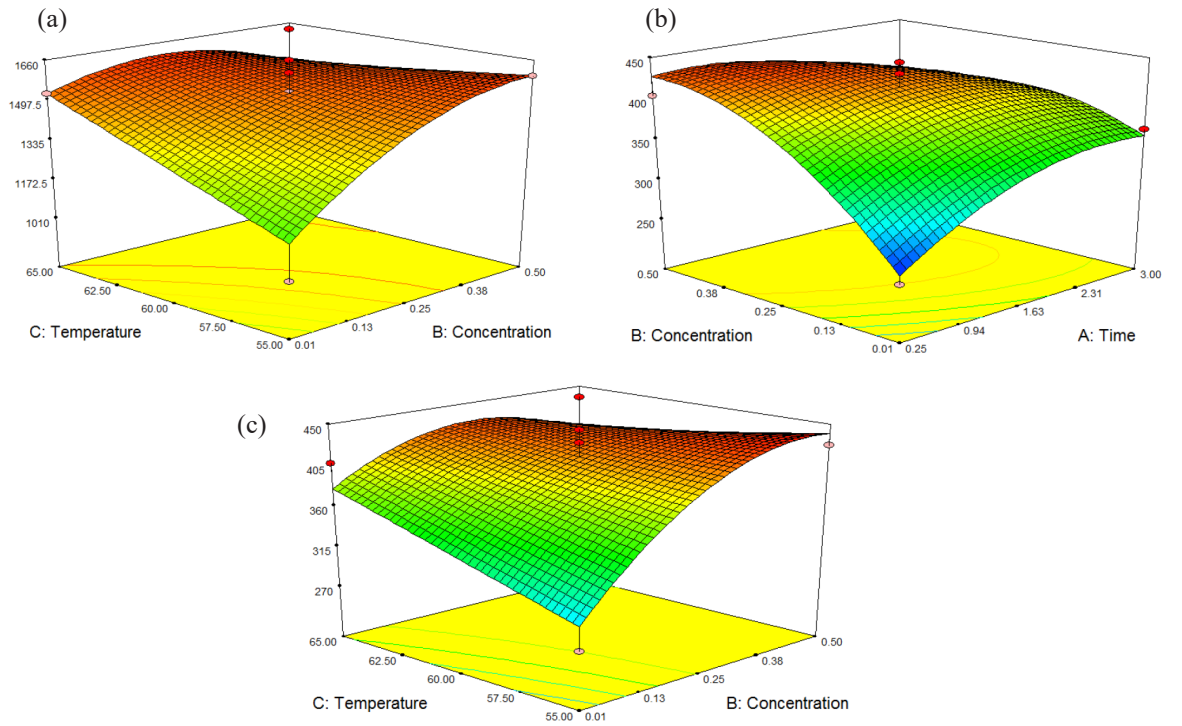


Figure 3: The contour plots representing the influence of papain extraction parameters and CS yields of (a) *Holothuria scabra* and (b, c) *Bohadschia argus*. (a) The effect of papain concentration and extraction temperature on CS yield of *Holothuria scabra*. (b) The effect of papain concentration and extraction time, and (c) enzyme concentration and extraction temperature on CS yields of *Bohadschia argus*.

total of 17 runs, were measured and analyzed (Table 1). Based on regression analysis of the experimental data, the second-order mathematical models were suggested to represent the relationships between extraction parameters and CS yields. Using ANOVA analysis, the appropriateness of the predicted model was evaluated and the p-value obtained to determine the significance of the model. The reliability of the predicted model was determined based on *p*-value and correlation coefficient (R^2) value and the *p*-value less than 0.05 and R^2 more than 0.85 advocated the significance of the predicted model. The model *p*-values of extractions of *Holothuria scabra* and *Bohadschia argus* are < 0.0001 and 0.317, respectively, whereas model R^2 values are 0.9682 and 0.8708, respectively. These statistical analysis results suggested the high reliability of the predicted model for enzymatic extraction. The influence of extraction parameters on CS yield was explained in the second-order mathematical model using regression analysis on the experimental data (Table 2). The contour plots were generated based on the mathematical models, which could help to visualize the relationship between two extraction parameters as well as the interacting effects of two extraction parameters on CS yield at a time (Figure 3) [34].

Various enzymatic extraction methods by using different types of enzymes, such as alcalase, trypsin, pepsin and proteinase are applied in the process, especially papain, the most common used enzyme [22]. The obtained contour plots help to visualize and observe the effects of extraction factors on CS yields. In the case of *Holothuria scabra*, at lower level of extraction temperature, the extraction yields of CS increased when the higher papain concentration was used. However, at higher temperatures, the optimal enzyme concentration was identified to be at the middle level [Figure 3(a)]. For *Bohadschia argus*, the interacting effect of papain concentration vs. extraction time and papain concentration vs. extraction temperature were analyzed to be the significant factors

Table 2: Predicted optimal conditions and mathematic models of papain extractions of CS from *Holothuria scabra* and *Bohadschia argus*

Parameters	Predicted Optimal Condition	
	Holothuria scabra	***Bohadschia argus***
Enzyme concentration (%)	0.23	0.48
Time (min)	99	60.60
Temperature (°C)	65	56.53
Chondroitin sulfate yield (mg/100g dry)	1609.73	444.51
Predicted mathematical models*		
Holothuria scabra	$Y_{HS} = -256.78301 + 86.80478 \times X_2 + 2212.35692 \times X_1 + 8.25309 \times X_3 - 125.62718 \times X_3 \times X_1 - 25.16768 \times X_1 \times X_3 - 16.16515 \times X_2^2 - 670.37628 \times X_1^2$	
Bohadschia argus	$Y_{BA} = -2120.22564 + 1535.51868 \times X_2 + 6872.55469 \times X_1 + 36.47787 \times X_3 - 94.02424 \times X_3 \times X_1 - 463.95372 \times X_2^2 - 1637.79206 \times X_1^2$	

Note: **Y* represents the CS yield (mg/100g dry). X_1, X_2 and X_3 represent papain concentration (%), extraction time (min) and extraction temperature (°C), respectively.

[Figure 3(b) and (c)]. At the shorter extraction time, the more papain concentration was used the higher CS yield was obtained. When the longer extraction time was used, the optimal enzyme concentration was found to be the middle level of concentration [Figure 3(b)]. Interestingly, a similar trend of extraction effects, papain concentration vs. extraction temperature, on CS yield in *Bohadschia argus* [Figure 3(c)] and *Holothuria scabra* [Figure 3(a)] was observed. However, the predicted optimal temperatures from the mathematical models of *Holothuria scabra* and *Bohadschia argus* were 65 °C and 56.53 °C, respectively (Table 2). The observed interacting factors could be used to explain the mechanism of extraction yield. The results showed that the highest concentration of enzyme, papain, did not guarantee the maximum CS yield due to the interacting effects of extraction temperature and time, in which the enzyme concentration at 0.23% and 0.48% were the predicted optimal concentration for *Holothuria scabra* and *Bohadschia argus*, respectively (Table 2). Similar observations about extraction effects on CS yields were observed. For example, CS was extracted from sturgeon cartilage by enzyme-assisted process [35]. The optimal condition for extraction was found to be at 0.2% enzyme loading, 55 °C and 7.5 h. The enzymatic extraction by alcalase enzyme was applied for CS extraction from Prionace glauca, shark, and head wastes. The optimization experiment was conducted to identify the condition to be at 55.7 °C and pH 8.2, with alcalase loading at 7.2 AU/kg of cartilage (0.3% v/w), and the maximum CS yield was approximately 11 g/L [25]. The analysis of the contour plot could help to identify the optimal condition for extraction to achieve the highest CS yield (red color zone in the plot) by compromising the cost, in terms of enzyme price, and energy use for temperature and time.

To validate the predicted optimal condition, the extraction experiments were conducted by using the predicted optimal condition obtained from RSM (Table 2). The predicted CS yields obtained from *Holothuria scabra* was 1609.73 mg/100 g when using the condition at 0.23 % papain concentration, 65 °C extraction temperature, and 99 min extraction time. The CS yield was determined to be 1538.67 mg/100 g dry in this validation experiment, with a model error of 4.41 %. In the case of *Bohadschia argus*, the predicted CS yield at 444.51 mg/100 g was obtained at 0.48 % papain concentration, 56.53 °C extraction temperature, and 60.60 min extraction time. In the validation run, the CS yield was determined to be 412.39 mg/100g, with a model error of 6.51 %. This result demonstrated that the actual experimental value was in good agreement with the mathematic model values, therefore it suggested the high reliability of the optimization experiment (Table 2).

After obtaining the optimal condition for enzymatic extraction of CS by using papain enzyme, the CS extract was done by using alcalase enzyme to compare with papain at the same extraction parameters in Table 2. Then, the yields of crude CS and purified CS of *Holothuria scabra* and *Bohadschia argus* were determined (Table 3). The results showed that the CS yields, both crude and purified fractions, obtained from papain-assisted extraction were higher than alcalase-assisted extraction, and this trend is well agreed in both 2 species of sea cucumbers. The difference in

extraction yields from papain and alcalase could be due to several reasons. Firstly, the preferred working condition for papain is in the range of pH 5.0–7.0 and temperature in the range of 60–70 °C. Whereas, the suitable condition of alcalase is pH range of 8.0–10.0 and temperature range at 50–70 °C. Secondly, their hydrolysis specificities to substrate are different. Papain has a broad specificity by cleaving peptide bonds where aromatic and bulky hydrophobic amino acids are available. While, alcalase has more specific activity by targeting the peptide bonds that are adjacent to hydrophobic amino acids, especially phenylalanine, tyrosine, and leucine [22]. Several studies were conducted to compare and select the suitable enzyme for extraction of CS and suggested that the best enzyme varied depending on the type of biomass and extraction condition, as well as its compatibility with the combined method [36].

Table 3: CS yields extracted from *Holothuria scabra* and *Bohadschia argus* by using the optimal extraction condition.

Samples	Enzyme for Extraction	Crude CS Yield (%wt)	Purified CS Yield (mg/100 g)
H. scabra	Papain	16.35 ± 0.31^{a}	1538.67 ± 20.26^{A}
	Alcalase	10.95 ± 0.22^{d}	1295.50 ± 14.28^{B}
B. argus	Papain	13.78 ± 0.34^{b}	412.39 ± 10.12^{D}
	Alcalase	12.60 ± 0.18^{c}	461.11 ± 8.45^{C}

Note: *a,b,c,d represent the statistical differences of each sample in crude CS yield (p-value < 0.05). A,B,C,D represent the statistical differences of each sample in purified CS yield (p-value < 0.05).

3.2 *Phenolic compounds and antioxidants activities*

The majority of biological compounds have many functions. Many compounds, in particular, can directly or indirectly scavenge free radicals and act as antioxidants in living organisms. Increased amounts of these molecules during oxidative stress appear to be a biological response that, when combined with other antioxidant defense systems, may protect cells from oxidation. Among these structures, CS is a biomolecule that has piqued the curiosity of many scientists due to its antioxidant properties. The activity of CS in decreasing molecular damage induced by free radicals and related oxygen reactants has been demonstrated previously [37].

Table 4 shows that the total phenolic compounds in the tested extracts varied significantly depending on the species and the type of enzyme used during extraction. the aqueous extract of *Holothuria scabra* assisted with papain showed the highest level of total phenols, with 99 ± 0.05 mg GAE/100g extract, while the same extract assisted with alcalase had the lowest amount of phenolic compounds, with 63 ± 0.02 mg GAE/100g extract. In contrast, the aqueous extract of *Bohadschia argus* assisted with papain had a lower amount of phenolic compounds, with 73 ± 0.03 mg GAE/100g extract, compared to when it was extracted with alcalase, which showed a higher amount of phenolic compounds, with 86 ± 0.06 mg GAE/100g extract. These findings suggest that the choice of enzyme can have a significant impact on the extraction of phenolic compounds from these species. Papain-assisted extraction shows a higher efficiency in extracting phenolic compounds in *Holothuria scabra*, while alcalase was more effective in *Bohadschia argus*. The phenolic content of several sea cucumber species has been investigated in numerous research, especially centering on the dried body walls. According to these investigations, sea cucumbers contain a significant amount of phenolics. Althunibat *et al.* discovered that total phenolic compounds found in Stichopus horrens and Holothuria edulis were significantly higher, having 5.24 ± 0.01 mg/g of GAE and 7.33 ± 0.02 mg/g of GAE, respectively. [38] However, Cucumaria frondosa was found to have a greater TPC, measuring 232.67 mg GAE/100 g, according to Mamelona *et al.* Additionally, they discovered that internal organs (including the gonads, digestive system, and respiratory system) subjected to a high pressure extraction process had a higher TPC at 302.82 mg GAE/100g in comparison to untreated samples [39].

Table 4: Total phenolic compound and antioxidant activity of chondroitin sulfate

Sample	Enzyme Assisted	Total Phenolic Compounds (mg GAE/100g extract)	Antioxidant Activity
Commercial CS	-	86 ± 0.00^{b}	90.41 ± 5.20^{C}
H. scabra	Papain	99 ± 0.05^{a}	6.97 ± 1.55^{B}
H. scabra	Alcalase	63 ± 0.02^{d}	7.51 ± 0.60^{B}
B. argus	Papain	73 ± 0.03^{c}	8.06 ± 0.99^{B}
B. argus	Alcalase	86 ± 0.06^{b}	6.87 ± 0.54^{B}
Ascorbic acid	-	-	0.00 ± 4.00^{A}

Note: *a,b,c,d represent the statistical differences of each sample in total phenolic compounds (p-value < 0.05). A,B,C,D represent the statistical differences of each sample in antioxidant activity (p-value < 0.05).

The quantity of extracts needed to scavenge 50% of the DPPH radical is shown in Table 4. With IC_{50} values of 8.060.99 vs. 6.870.54, respectively, our findings show that *Bohadschia argus* assisted with papain demonstrated a stronger radical scavenging activity than when assisted with alcalase. With IC_{50} values of 6.971.55 vs. 7.510.60, respectively, *Holothuria scabra* assisted with papain demonstrated less radical scavenging activity than when assisted with alcalase [38]. Increased enzyme concentration and hydrolysis time improved protein recovery, degree of hydrolysis values, and antioxidant characteristics of hydrolyzed proteins in a prior study on defatted Lemna minor [27]. These findings imply that sea cucumber extracts may be an important source of natural antioxidants, making them potential cytotoxic agents for the treatment of cancer.

3.3 *Tyrosinase inhibition activity*

Tyrosinase is a copper-containing oxidase that contributes to skin pigmentation by generating dopachrome, which enhances the production of melanin [40]. Even though melanin serves to protect the skin, its overproduction has been associated with health problems. Tyrosinase also contributes to the browning reaction that decreases the nutritious content of fruits and vegetables [41]. Using L-tyrosine as a substrate, the inhibitory activity of mushroom tyrosinase was examined in this study. When compared to sea cucumber CS, phenolic extracts from sea cucumber were found to considerably increase the enzyme inhibitory activity. This result was caused phenolic concentrations in sea cucumber extracts.

When compared to sea cucumber CS, phenolic extracts significantly improved tyrosinase enzyme inhibitory activity (Table 5). The free phenolic content of alcalase extracted *Bohadschia argus* samples had a high activity IC_{50} (54.52 ± 1.72), most likely due to the higher phenolic contents in sea cucumber extracts. Furthermore, ascorbic acid, a well-known tyrosinase inhibitor, was utilized as a positive control, demonstrating a low IC_{50} (0.43 ± 0.003). The new information supports the conclusions of prior studies in the literature investigated the tyrosinase inhibitory activity of Ornithogalum narbonne and discovered that the phenolic-rich ethyl acetate extracts had a significant inhibitory effect on the tyrosinase enzymes [41]. Stichopus japonicus, possesses tyrosinase inhibitory activity of 50.84%, as reported by Kim and colleagues [42]. Meanwhile, the viscera extracts of Stichopus japonicus, when detected with saponin, exhibited a tyrosinase inhibitory activity ranging from 25–30% [43]. Tyrosinase inhibitors have been linked to several different inhibitory mechanisms. The copper chelating activity of phenolic compounds is thought to play a critical role. The TPC and tyrosinase inhibitory activities were shown to be highly correlated, implying that the phenolics in the sea cucumbers internal organs play a crucial role in inhibiting tyrosinase. The tyrosinase inhibitory activity of the phenolics in Holothuria scarba and *Bohadschia argus* has not been previously reported.

Table 5: Tyrosinase inhibition activity by the modified dopachrome method of chondroitin sulfate

Sample	Enzyme Assisted	Tyrosinase Inhibition Activity
Commercial CS	-	N/A
H. scabra	Papain	83.76 ± 4.13^{c}
H. scabra	Alcalase	73.50 ± 4.95^{c}
B. argus	Papain	113.24 ± 11.22^{d}
B. argus	Alcalase	54.52 ± 1.72^{b}
Ascorbic acid	-	0.43 ± 0.00^{a}

Note: *a,b,c,d represent the statistical differences of each sample in tyrosinase inhibition activity (p-value < 0.05).

3.4 *Anti-glucosidase and Anti-inflammatory*

According to the trial results, the *Holothuria scabra* extract with alcalase enzyme had the best inhibitory activity of glucosidase when compared to the same enzymatic extraction procedure for *Bohadschia argus*, and it was equivalent to commercial chondroitin sulfate isolated from bovine cartilage (Table 6). On the other hand, the method of extracting CS with the papain enzyme showed the greater glucosidase inhibitory activity for *Bohadschia argus* than that of *Holothuria scabra*. The inhibitory effects of sea cucumbers on glucosidase activity vary depending on the species, as different types of sea cucumbers have unique bioactive compounds. Diverse substances, including chondroitin sulfate from various sources and unsaturated fatty acids from sea cucumber, have been studied for their ability to inhibit the glucosidase enzyme. In comparison to larger molecular weight and other forms of chondroitin sulfate, it has been discovered that type D chondroitin

sulfate is more effective at inhibiting glucosidase activity [44], [45]. The effectiveness of chondroitin sulfate derived from sea cucumber body walls as a glucosidase inhibitor, however, is still undetermined because research on this topic is limited. To completely comprehend how sea cucumber chondroitin sulfate affects glucosidase activity, more studies may be required.

Table 6: Total phenolic compound and antioxidant activity of chondroitin sulfate

Sample	Enzyme Assisted	IC_{50} (µg/mL)	
		Anti-glucosidase	Anti-inflammatory
Commercial CS	-	17.53 ± 0.06^{e}	7.63 ± 0.25^{B}
H. scabra	Papain	2.62 ± 0.05^{a}	17.86 ± 0.00^{C}
H. scabra	Alcalase	16.60 ± 0.11^{d}	27.18 ± 0.14^{E}
B. argus	Papain	7.29 ± 0.00^{c}	26.34 ± 0.38^{D}
B. argus	Alcalase	6.63 ± 0.20^{b}	27.59 ± 0.21^{E}
Diclofenac	-	N.A.*	0.43 ± 0.71^{A}

Note: *N.A. Not applicable

*a,b,c,d represent the statistical differences of each sample in anti-glucosidase (p-value < 0.05). A,B,C,D represent the statistical differences of each sample in anti-inflammatory (p-value < 0.05).

The anti-inflammatory activity of chondroitin sulfate (CS) extracted from different sources has been studied (Table 6). CS extracted from bovine cartilage has been found to exhibit better anti-inflammatory activity than CS from both species of sea cucumber. Among the sea cucumber species, CS extracted from *Bohadschia argus* using papain enzyme exhibited the better anti-inflammatory activity than *Holothuria scabra* using the same enzyme during extraction. However, when extracted with alcalase enzyme, the anti-inflammatory activity of the CS from both species was found to be the same. A study by Mou *et al.* demonstrated that CS from Acaudina molpadioidea showed significant dose-dependent anti-inflammatory activity in the carrageenan-induced inflammation model, with considerable inhibition of paw edema observed in a range of dosages (5–100 mg/kg) and maximum inhibition of 44.8% at a dosage of 100 mg/kg [46]. Soft tissue repair has reportedly been impacted by sea cucumber extracts. Low-dose (1 mg/kg) Stichopus sp extract was discovered to have healing capabilities in comparison to high-dose (10 mg/kg) extract in a research on fractured rabbits [47]. These findings suggest that some structural differences in CS could result in varying anti-inflammatory activities. The sulfate group in the structure of CS is negatively charged and has good water holding capacity, which can cause slower destruction of egg white protein when heated. Further studies are needed to better understand the structural differences in CS and their effects on anti-inflammatory activity.

3.5 *FTIR Spectrum*

FTIR spectroscopy at 400–4000 cm^{-1} revealed CS isolated from sea cucumber using different enzyme-assisted extraction methods (Figure 4). By comparing the acquired spectrum to that of standard CS, the resulting spectrum showed essentially the same absorption bands that are typical and commonly described in the literature for sulfated polysaccharides [33], [48]–[50] (Table 7). The FTIR spectra of CS, as shown in Figure 4, revealed the characteristic peaks of amide group coupling of C-O stretching vibrations, S-O stretching vibrations, -C-O-S, -COO, C-C, C-O-S, and R-SO2-R; R-SO2-R. The typical peaks of –CONH were found at 1605.90 cm^{-1} in commercial CS standard and at 1621.95, 1621.97, 1621.71, and 1621.31 cm^{-1} in extracted samples of *Holothuria scabra*-papain, *Holothuria scabra*-alcalase, *Bohadschia argus*-papain, and *Bohadschia argus*-alcalase, respectively, and the results were consistent with [51]. The FTIR spectrum of chondroitin isolated from shark cartilage in the back part of the cartilage showed a broad and strong tape characteristic at the wavelength of 3000 cm^{-1} and strong absorption at the wave numbers of 1668.31, 1627.81, 1456.16, and 1415.65 cm^{-1}, respectively, while the FTIR spectrum of the side part showed a sharp and strong tape at the wavelengths of 1672.17, 1627.81, and 1454.23 cm^{-1}. The FTIR spectra of CS displayed a significant absorption peak at wave numbers 1637.63 and 1420.03 cm^{-1}, indicating the presence of carboxyl groups, amine, and sulfate, according to the study [52]. Strong peaks in chondroitin separated from the shark backbone were identical to the peaks of 1627 and 1413.72 cm^{-1} in the side part of cartilage, indicating the presence of carboxyl group with amine and sulfate [53]. FTIR analysis of chondroitin sulphate from pig laryngeal cartilage revealed a strong absorption at 1650 and 1556 cm^{-1}, corresponding to the stretching vibration of the carbonyl bond of the amide group and the bending vibration of the N–H bond,

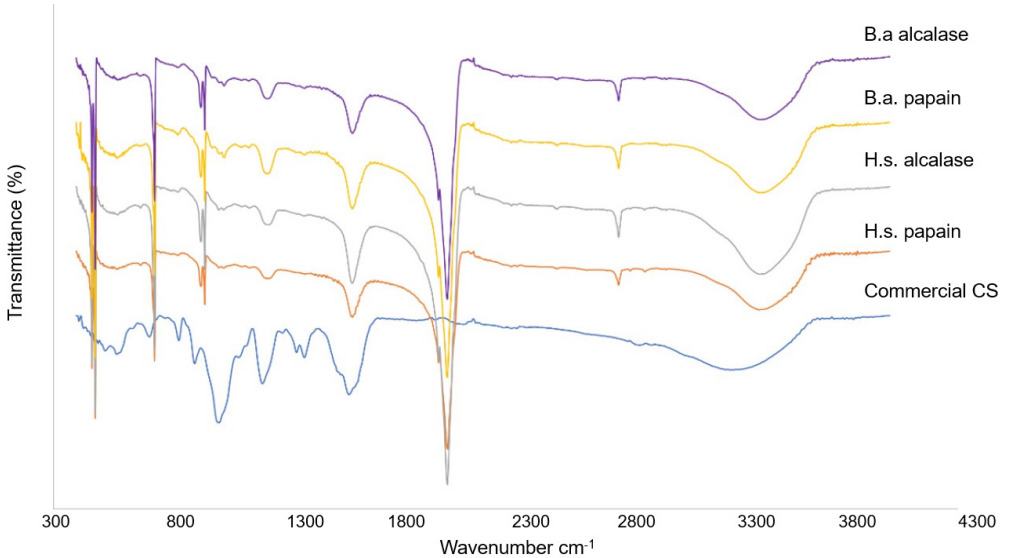
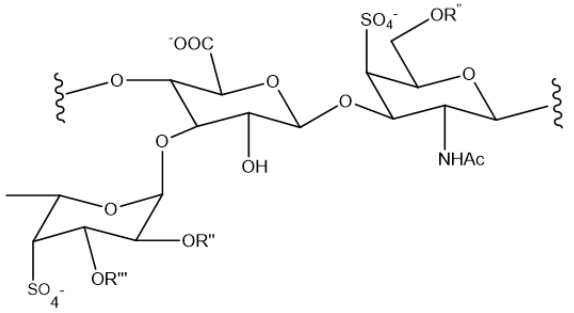


Figure 4: FTIR spectrum of commercial CS, standard bovine sodium chondroitin-4-sulphate; WSC papain, chondroitin sulfate from *Holothuria scabra* extraction by papain; WSC alcalase, chondroitin sulfate from *Holothuria scabra* extraction by alcalase TSC papain, chondroitin sulfate from *Bohadschia argus* extraction by papain; TSC alcalase, chondroitin sulfate form *Bohadschia argus* extraction by papain. T%, Transmittance.

Table 7: Absorption bands observed in the IR spectral region of 400–1800 cm^{-1}

Species	*H. scabra*	*B. argus*	*S. japonicus*	*T. ananas*	*P. graeffei*	*H. atra*	*H. arenicola*	Assignment
Reference	This work	This work	(43)	(44)	(45)	(46)	(46)	
	1621	1621	1623	1635	1643,1646	1633	1640	Amide I vibration
	1539	1445	1556		1545	1559	1554	Amide II vibration
						1451	1454	CH_3, δas
	1401	1407		1419	1423			Acetyl C-N, C-O,υs-COO-
					1372	1380	1384	-CH_3, δas
	1242	1249	1240	1253	1255,1240	1256	1259	S=O, ,υs
			1220			1210	1211	C-H, δ
					1166	1166	1166	Glycosidic C-O,υs
						1128	1128	C-O,C-C-C, C-C-C,υs
	1053	1053			1053		1029	C-O-C, υ
	1025	1025	1033	1031		1029	1001	C-C, υ
			1011			1001	964	C-O, υ
	968	968			960	964		C-C, υs ; C-C-H, δs
	924	921			924	928	927	Ring, ,υs
								b-anomeric C-H,
				853		843	837	C-O-S, υs
	847	849	840		850	851	851	C_4-O-S
						837	837	C_2-O-S
								C_3-O-S
	823	821	820	821		818	818	C_6-O-S
								Ring breathing
	689				693	684	682	O-H, δ
		580		584	583			S-O, υs



G R"=SO_3^-, R"'=H
H R"=H, R"'=SO_3^-
I R"=R"'=H

Figure 5: Structure of fucosylated chondroitin sulfate

respectively, indicating the presence of the acetamido group in chondroitin sulphate. The distinctive peaks of C–O–S were found at 854 cm^{-1} for commercial CS standard, and at 853, 850, 851, and 851 cm^{-1} for *Holothuria scabra*-papain, *Holothuria scabra*-alcalase, *Bohadschia argus*-papain, and *Bohadschia argus*-alcalase, respectively, and results were in agreement with the commercial CS standard. Garnjanagoonchorn *et al*. reported that using the potassium bromide pellet technique and C4S and C6S as standards, FTIR spectroscopy revealed specific absorption bands at 825.78 and 885.25 cm^{-1} attributable to the C-O-S axial and equatorial bending vibrations, which are characteristics of 4-sulphate CS [23]. The characteristic S=O peaks of commercial CS standard were observed at 1224.11 cm^{-1}, and the same were recorded in extracted samples of *Holothuria scabra*-papain, *Holothuria scabra*-alcalase, *Bohadschia argus*-papain, and *Bohadschia argus*-alcalase at 1242.32, 1252.34, 1249.52, and 1251 cm^{-1}, respectively, and the results were in agreement with the characteristic S=O peaks of CS extracted from chicken keel cartilage [54].

3.6 *1H NMR Analysis of fucosylated chondroitin sulfate*

The signals in the 1H NMR have been assigned and selected chemical shift values (δ, ppm) are provided in (Table 8). The chemical shift in range from 2.05 to 1.92 δ, confirms the presence of an acetyl group [10], [55]. The NH residue in fucosyl group shows a shift around 8.46 to 8.59 δ. The downfield shift signals of the respective protons indicate the possible presence of a sulfate group [56]. The 1H signals of the three fucosyl branches G, H, and I are significantly distinguished at 5.69, 5.34, and 5.41 ppm, respectively (Figure 5) [57]. The Fuc3S4S(H) peaks are identified in sample *Holothuria scabra*-alcalase, *Bohadschia argus*-papain, and *Bohadschia argus*-alcalase by the downfield shift of protons, 3S at 4.73, 4.79, and 4.79 and 4S at 4.79, 4.70, and 4.73, respectively [55], [58], [56]. This can be used to determine these fragments in the FCS structure. By integrating these signals, the ratio of monofucosyl branches can be revealed [59].

Table 8: Total phenolic compound and antioxidant activity of chondroitin sulfate

Sample	H1	H2	H3	H4	H5	H6
Standard	4.91	**4.47***	4.23	**4.80**	3.78	3.59
Holothuria scabra - Papain	4.60	3.92	4.27	4.40	3.80	4.08
Holothuria scabra - Alcalase	4.85	3.80	**4.73**	**4.79**	3.68	3.60
Bohadschia argus - Papain	4.85	3.80	**4.79**	**4.70**	3.68	3.60
Bohadschia argus - Alcalase	4.67	3.85	**4.79**	**4.73**	3.73	3.60

Note; *The bold numerals indicate the positions of sulfate

4 Conclusions

The extraction of fucosylated chondroitin sulfate (FuCS) from the two species of sea cucumbers, *Holothuria scabra* and *Bohadschia argus* presents a good prospect for substituting commercial chondroitin sulfate of limited utilization. The consumption of sea cucumbers throughout the world in traditional meals and rich composition of FuCS in body walls makes them promising candidates for FuCS extraction. The use of enzyme-assisted approach yielded a maximum of 1538.76 ± 20.26 mg/100 g of FuCS from *Holothuria scabra* using papain and 461.11 ± 8.45 mg/100 g FuCS using *Bohadschia argus* with alcalase. The enzyme-assisted produced FuCS from both sea cucumbers and commercial FuCS showed similar physical characteristics when subjected to FTIR analysis. Furthermore, extracted FuCS displayed essential enzyme-dependent phenolic compounds concentration, antioxidant activity, tyrosinase inhibition capacity,

anti-glucosidase, and anti-inflammatory activities. With the demonstration of significant concentration of extracted FuCS, intriguing biological properties of FuCS and the absence of health and religious restriction challenges, the production of FuCS from sea cucumbers is bright for the pharmaceutical industry.

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