Media Pendidikan Gizi dan Kuliner

Journal homepage: https://ejournal.upi.edu/index.php/Boga/index

Review: Synthesis of Hyaluronic Acid as Food Supplement and Drugs

Brigitta S Maharani[1], Intan Yustia [1], Sheren Hana Elia[1], Gabriela Chelvina S. Girsang[1], Asep Bayu Dani Nandiyanto[1], Tedi Kurniawan[2]*

[1]Departemen Pendidikan Kimia, Universitas Pendidikan Indonesia, Bandung, Indonesia
[2]Engineering Technology Departement,Community college of Qatar, Qatar
Correspondence: E-mail: nandiyanto@upi.edu

ABSTRACTS

Hyaluronic acid (HA), as a food supplement and drug, is a glycosaminoglycan which is a linear polysaccharide formed from covalently bonded disaccharide units. HA is a polysaccharide formed from repeating units of a disaccharide composed of N-acetyl-D-glucosamine (GalNAc) and D-glucuronic acid (GlcA) with a high molecular weight of between 105 and 108 Da, which are linked alternately by glycosidic bonds (1→ 3) and link(1→4). This structure allows hyaluronic acid to retain large amounts of water and its pseudoplastic fluid properties make hyaluronic acid widely used in the pharmaceutical and cosmetic industries. Sources of hyaluronic acid material can be found from various types of bacteria, land, or marine animals. The purpose of this paper is to present various reviews of hyaluronic acid synthesis methods and determine which method is the most efficient one to do. This review paper contains several research papers from 1934 to 2019 which discuss several methods of hyaluronic acid synthesis including extraction, separation, purification, fermentation, and isolation. Comparison of results shows that the best methods are the repeated batch fermentation and separation because the materials used are quite easy to find and the process is novel yet easy with the high pure products and does not require a long time to obtain HA nanoparticles.

© 2021 Prodi Pendidikan Tata Boga UPI

ARTICLE INFO

Article History:
Received 30 Mar 2021
Revised 10 Apr 2021
Accepted 11 Apr 2021
Available online 11 Apr 2021

Keyword:
Synthesis,
Hyaluronic acid

1. INTRODUCTION

Hyaluronic acid (HA), as a food supplement and drug, is a glycosaminoglycan which is a linear polysaccharide formed from covalently bonded disaccharide units. HA is a polysaccharide formed from repeating units of a disaccharide composed of N-acetyl-D-glucosamine (GalNAc) and D-glucuronic acid (GlcA) with a high molecular weight between 105 and 108 Da, which is linked alternately by glycosidic bonds (1→ 3) and link(1→4) (Weissmann, *et al.*, 1954; Lamberg & Stoolmiller, 1974; Laurent & Fraser, 1992) which were discovered by Meyer and Palmer in 1934 in the vitreous humor of the bovine eye and also isolated from certain bacterial strains, namely hemolytic streptococcus (Meyer, *et al., 1934, 1954;* Kendall, *et al.,* 1937)

Due to its different hydrodynamic properties, HA has been widely applied in the food, drugs, and pharmaceutical and cosmetic industries (Radaeva, *et al.,* 1997; LapcıkJr,*et al.,* 1998; Chong, *et al.,* 2005). These polysaccharides are most commonly referred to as "hyaluronan" because HA exists as a polyanion form and not as a free acid form. However, the name "hyaluronic acid" is often used in the pharmaceutical field (Opdensteinen, *et al.*, 2019). HA can be extracted from various sources namely mammals, marine animals, microbes, and chemical synthesis (Zakerie, *et al.*, 2017). Many methods have been developed for the synthesis of hyaluronic acid. However, each method has advantages and disadvantages. Therefore, a review is needed to find out which method is more effective.

The purpose of this paper is to compare several methods used to produce hyaluronic acid. This review consists of 56 papers from 1934 to 2019. The methods discussed include separation, purification, isolation (in vitro), extraction, and fermentation. It is hoped that this review can help in choosing the best hyaluronic acid synthesis method. The comparison of the synthesis method is presented in Table 1.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Tabel 1. Comparison of the Methods Used in the Synthesis of Hyaluronic Acid

Method	**Type Method**	**Material**	**Results**	**Strengths**	**Weaknesses**	**Reference**
Extraction	Alkaline Process	Swordfish eyeball	0.055 g/L vitreous humor	The materials used are quite easy to find.	It is easy for precipitation to occur if the sediment agitation is disturbed.	Murado, *et al*., (2012)
	Papain	Eyeball Mollusc Bivalve	4.2 mg HA/g dry weight The	Time for the test is quite short	Requires other enzymes for processing.	Kanchana, et al., (2013)
Purification	Ultrafiltration-diafiltration and Electrodeposisi protein	Eyeball shark	0.3 g / L of vitreous humor	Used tool easy to assemble, clean and maintain. And the resulting product is quite a lot.	Using a membrane plate that needs to be cleaned every 5 hours, so it does not get clogged due to dirt buildup.	Murado, *et al*., (2012)
Isolation		Marine Stingray *A. narinari*	HA purified from *A. narinari* liver has a higher molecular mass of 13, 65,863 Da	The materials used in this method are quite easy to find.	Has a longer operating time	Sadhasivam, *et al.*, (2013)

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

Fermentation	*Repeated batch* Fermentation	*Streptococcus zooepidemicus* ATCC 39 920	Increasing the volumetric production rate to be (0.59 g HA L-1 h-1) 2.5-fold of the batch culture (0.24 g HA L-1 h-1)	1. Skipping settlement and lag phase 2. Low production costs 3. Risein dissolved oxygen (DO) level 1. The presence of	1. Inhibitors in the broth reduce the benefits of using large seed volumes 2. Equipping a filtration medium (NWF) in the fermenter to maintain cells that function as seeds will cause more serious problems such as sticky, non-HA-producing strains will grow on solid surfaces and soon become the dominant species.	Huang, *et al*, (2008)

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Separation	Two-stage tangential flow microfiltration (MF) and ultrafiltration (UF)	Fermentation broth	Both schemes effectively separate and purify HA with values above 77% overall yield and a purification factor of around 1000, and scheme 2 is more effective purification overall yield (89%) and water-saving	1. Effectively able to separate HA from the fermentation broth by a factor of 1000. 2. The tangential flow filtration system used in the experiment can be easily scaled, using a properly designed membrane system Saves 3. Water	Has a longer operating time Lama	Zhou, *et al.*, (2006)
	Ultrahigh-voltage capillary gel electrophoresis	*Bovine vitreous humor*	Separation efficiency and resolution of 95 kV was much better than that of 15 kV separation.	For low molecular weight oligomers, the separation efficiency was found to increase by about tenfold, and about three times the resolution.	The increase in resolution decreases as the molecular weight of the polymer increases	Hutterer, *et al.,* (2005)

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

2. METHODS

Based on the reviewed studies, there are several types of synthesis of hyaluronic acid nanomaterials such as extraction consisting of an alkaline process and the use of actinase and papain enzymes, then purification consisting of ultrafiltration-diafiltration, protein electrodeposition, and anion exchange chromatography, then fermentation consisting of repeated batches. fermentation and separation consisting of Two-stage tangential flow microfiltration (MF) and ultrafiltration (UF) and Ultrahigh-voltage capillary gel electrophoresis, then the last method is isolation. This method will be discussed in more detail in the following explanation.

2.1. Extraction Method

2.1.1. Alkaline Process

In this experimental extraction process, two solutions were used, namely, NaOH: ethanol with a concentration of [0.45; 0.85 M] for each experiment, then this test was given fisheye extract and given slowly with vigorous stirring at 5°C for 1-5 hours. After that, centrifugation was carried out when the mixture had settled. If the stirring is disturbed and mass precipitation occurs, it can be centrifuged as much as 6000 g for 15 minutes.

The results of the centrifugation process are divided into 2, namely supernatant and sediment. The supernatant produced from the mixture will be removed from the sediment. Then the alkaline sediment containing HA is redissolved in a small volume of water:ethanol (1:0.75), then an aqueous solution of 0.5 M NaH2PO4 solution:ethanol (1:0.75) is added to neutralize or to take the pH at a set value (Tømmeraas & Melander, 2008).

2.1.2. Enzymatic Process

Various techniques have been developed and optimized to extract HA using detergents, enzymes, and/or solvents to break down the structure and isolate GAG from other polysaccharide complexes so that they are present in the HA tissue (Sadhasivam & Muthuvel, 2014). Extraction using enzymes is the most commonly used technique, such as papain enzymes listed in Table 1. This enzyme has been applied for tissue degradation and protein fraction breakdown to isolate HA molecules from being damaged. Papain is one of the most commonly used enzymes to isolate HA.

In general, initial tissue from marine sources is defatted using acetone. After that, centrifugation was carried out at 10,000 g for 10 minutes and drying at 60°C for 24 hours, the purpose of this heating was so that the enzymes were not degraded. After the sediment was produced (2.4 g), the sediment was dissolved (1 g/20 ml) in 100 mM sodium acetate buffer pH 5.5 containing 5 mM EDTA and 5 mM cysteine. One hundred milligrams of papain were added per gram of tissue and solution and incubated for 24 hours at 60 °C in a stirring vessel. After boiling for 10 min, the mixture was centrifuged at 5000 g for 15 min, and three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000 g for 15 minutes and dried at 60 °C for 6 hours. The dry precipitate (about 715 mg) was dissolved in 10 ml of 0.05 M NaCl. After centrifugation at 10,000 g for 10 min, the supernatant was applied to a column (1 cm × 20 cm) packed with anion exchange resin equilibrated with the same NaCl solution.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Glycosaminoglycans were eluted with a linear gradient of NaCl from 0.05 to 1.2 M from 0 to 150 min using low-pressure liquid chromatography at a flow of 1 ml/min. Then the 2 mL fraction was collected (Volpi & Maccari, 2003)

In addition, papain is also used to extract HA from mollusk shells, (Nakano, *et al*., 1994; Rosa, *et al*., 2012; Volpi & Maccari, 2003) sulfate compounds from sea snakes (Bai, *et al*., 2018) and in hydrolysis. proteoglycans from hammerhead shark fins (Michelacci & Horton, 1989)

2.2. Purification Method

2.2.1. Ultrafiltration and Diafiltration and Electrodeposition Protein

Various purification methods have been used in the final stage of extraction to ensure higher HA purity. Ultrafiltration-diafiltration is the purification method applied and it is a separation measure-based method to remove impurities and concentrate HA in solution (Choi, *et al*., 2014; Lignot, *et al*., 2003; Opdensteinen, *et al.,* 2019). For example, purification of HA isolated from the vitreous humor of swordfish and shark (Murado, *et al*., 2012) was carried out using polysulfone membrane plates with molecular weight limits at 100, 300, and 675 kDa.

Protein electrodeposition was carried out at a current between two platinum electrodes of 10-40 mA and HA was obtained with a purity higher than 99.5%. (Lignot, *et al*., 2003) and HA obtained from fermentation (Choi *et al*., 2014). In one study, different activated carbons were tested (Darco KB-B, Norit CN1, Norit C Extra USP, Norit A SupraEUR…) to remove high molecular weight protein from HA obtained by fermentation, which was derived from the bacterium Streptococcus zooepidemicus(Zakeri, *et al.,* 2017). The results showed that Norit CN1 had the highest protein removal percentage with 97% and 90% endotoxin removal (Choi, *et al*.,2014)

2.3. Fermentation Method

2.3.1. Repeated Batch Fermentation

In this method, the end of each cycle is determined when there is an increase in dissolved oxygen levels. The concentration of the medium is adjusted according to the number of cells in the seed so that repeated cycles are expected to achieve the same cell concentration as the batch rate. The nonwoven fabric (NWF) was used to maintain the cells consisting of a polypropylene core and a polyethylene surface. To complement the nonwoven fabric (NWF) in the fermenter, three pieces of NWF (4 cm × 17 cm) were attached to the three baffles, of which NWF was used as the filter medium(Huang, *et al.,* 2008).

After the broth was drained, the retained cells were released by re-washing using new media for seeding the fermenter. Cell concentration was measured from the optical density of thebroth at 660 nm using a spectrophotometer. Due to changes in cell morphology after entering the stationary phase(Huang, *et al.*, 2007) the correlation of optical density with dry cell weight (DCW) is DCW (g/L) = 0.399 × OD 0.003 for the exponential growth phase, and DCW (g/L) = 0.456 × OD 0.012 for the stationary phase. The concentration of HA was determined by the carbazole method (Bitter, *et al.*, 1962), where optical density was measured at 525 nm and d-glucuronic acid was used as standard.

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

2.4. Separation Method

2.4.1. Two-Stage Tangential Flow Microfiltration (MF) and Ultrafiltration (UF)

In the first step, the membrane performance of the HA fermentation broth filtration was investigated to determine the optimal operating conditions for good HA separation, based on the High-Performance Tangential Flow Filtration (HPTFF) concept. All four types of membranes were used. **Figure 1** shows a schematic diagram of a membrane filtration system. A magnetic gear pump (MG 213, Italy) was used to pump the broth from the 1.5-L feed tank to circulate (cross-flow filtration) along the flat plate membrane surface in the module. Then the applied pressure is controlled by ball valves 1, 3, and monitored with pressure gauges 4 and 5. The cross-flow rate is calculated from the measurement of the circulating retentate flow rate with 6rotor flowmeter. Pressure (membrane inlet and outlet), retentate flow rate are monitored and adjusted to stay closer to the selected value when needed. In the system, the permeate side of the membrane is open to the atmosphere, and thus the pressure on the permeate side is zero. Therefore, the transmembrane pressure (TMP) is defined as the average of the inlet and outlet pressures. In this study, the temperature used was 25 ± 1°C and controlled by a heat exchanger 8. The permeate was collected for 30 seconds at different times and measured with the BS223S electronic balance (Sartorius, Germany; Accuracy 0.001 g) to calculate the permeation flux. Samples are taken periodically for analysis. The system is cleaned by recirculating the 0.1 mol/l NaOH solution twice (for 15 minutes each) after each batch filtration, then the system is rinsed with deionized water to ensure the system is restored to its original performance. The peristaltic pump 13 in **Figure 1** is turned off, and the system is running in total recirculation mode. Both permeate and retentate were recycled back to feed tank 9 to maintain a constant HA concentration (Zhou, *et al.,* 2006).

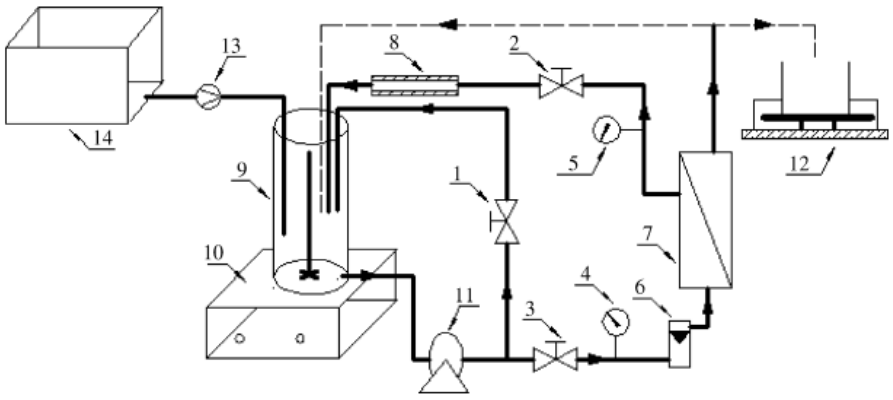


Figure 1. Schematic diagram of the membrane filtration system (1, 2, 3: ball valve; 4, 5: pressure gauge; 6: rotor flow meter; 7: membrane module; 8: heat exchanger; 9: feed tank; 10: agitator; 11: magnetic gear pump; 12: electronic balance; 13: peristaltic pump; 14: diafiltrate reservoir) (Zhou, *et al.,* 2006).

In the second step, a two-stage membrane process for the separation of hyaluronic acid from the fermentation broth was carried out with a 0.45 m membrane and PVDF100 in continuous diafiltration mode. , using optimal

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

conditions. Feed tank 9 is equipped with 300 ml broth. The system initially ran in total recirculation mode for about 90 minutes to stabilize. Then, the peristaltic pump 13 is turned on, and the diafiltrate is added continuously to the feed tank 9 at the same rate as the permeate flow rate to keep the solution volume constant. Two separation schemes were tested. The first scheme is Microfiltration (MF) followed by Ultrafiltration (UF). Pure water is used as the diafiltrate in two stages. The total permeate collected from the MF stage was used as the second diafiltration feed after pre-concentration at a certain volume concentration ratio (VCR). The final HA product was recovered from the UF retentate. The second scheme is similar to the first scheme in that it is expected that the permeate collected from the previous UF stage is used as the diafiltrate for the MF stage (Zhou, *et al.*, 2006).

2.4.2. Ultrahigh-voltage capillary gel electrophoresis

2.4.2.1. Capillary gel electrophoresis (CGE) system

Melted silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 50 mm ID, 360 mm OD, 450 cm long, 40 cm long to the detector for experiments at 95 kV, and 71 cm by 59 cm to the detector for experiments at 15 kV. The inner wall of each capillary was lowered with linear polyacrylamide as described by Hong *et al* in 1998 (Hong, *et al.*, 1998). This method is an adaptation of the method developed by Hjertén(Hjertén, 1985). For the experiment at 15 kV, the gel was forced into the capillary with a nitrogen pressure of about 3.5 bar. For the experiment at 95 kV, the capillaries were filled using an HPLC pump (Shimadzu LC-600; Columbia, MD, USA) to pump the fluoride per fluid (Fluorinert 5320, 3M, St. Paul, MN, USA) into a pressure-tight chamber filled with gels (Hutterer & Jorgenson, 2005).

The fluorine, which is immiscible with the gel, then acts as a liquid “piston”, applying pressure to the gel, and forcing it into a 450 cm long capillary. The typical filling pressure is 250 bar. The sieving matrix used to fill the capillary reservoir and buffer in all experiments was linear polyacrylamide 5%, citric acid 25 mM, Tris 12.5 mM, and urea 4 M adjusted to pH 3.2. A commercial 30 kV power supply (Spellman, Yonkers, NY, USA) was used for the experiment at 15 kV, while a homemade system was used for the experiment at 95 kV. The ultra-high voltage capillary electrophoresis (UHVCE) system used for this work is a slightly modified version of the previously described (Luo & Prestwich, 1999).

A high-voltage power amplifier (TREK, Medina, NY, USA) with a 2.5 kHz square wave input signal from a Stanford Research Systems waveform generator (Sunnyvale, CA, USA) was used to drive the rectification/multiply stack, producing a negative high voltage. The square wave amplitude of the signal generator determines the direct current (DC) output voltage of the multiplier stack. The capillaries are protected from dielectric damage through a metal shielding system consisting of a set of aluminum cylinders. The injection is carried out in an airtight area at the end of the high-voltage equipment. Pile of multiplier, shield, and capillary, immersed in transformer oil (Diala AX,

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

Shell, Houston, TX, USA; dielectric strength, 280 kV/cm) (Hutterer & Jorgenson, 2005).

.

2.5. Isolation Method

Isolation of hyaluronic acid from the liver of marine stingray Aetobatusnarinari Hyaluronic acid isolation method consists of several steps. The first step is the isolation of glycosaminoglycans. Isolation of GAGs was done according to the procedure that Volpi reported (Volpi & Maccari, 2003). The tissue was taken and defatted with acetone and dried. The pellet was solubilized in the sodium acetate buffer pH 5.5 containing EDTA and cysteine. Papain was added per gram of tissue, and the solution was incubated in a stirrer. After boiling, the mixture was centrifuged and three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at 4 ◦C for 24 h. The precipitate was recovered by centrifugation at 5000 × g for 15 min and dried at 60 ◦C for 6 h. After the GAGs are isolated,the next step is the purification of GAGs. After purification, the hyaluronic acid can be determined such as written on Cessaretti paper (Cessaretti, *et al.,* 2003). After determining, the hyaluronic acid can be characterized by several instruments.

3. RESULTS AND DISCUSSION

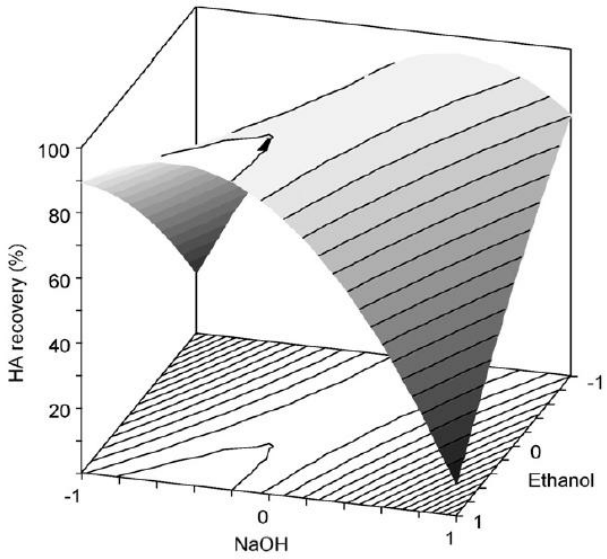
3.1. Extraction

3.1.1. Alkaline Process

In **Figure 2** it is explained that HA recovery occurs when treated with a temperature of 5°C and for 10 hours using an alkaline process, namely 0.7 M NaOH in water: ethanol (1: 0.75). Recovery of HA satisfactorily according to the equation

$$HA = 93.93 - 4.46\,S - 35.25SE - 39.12\,S^2 - 5.25\,E^2 \quad (1)$$

Where S is the molar concentration of NaOH [0.450 : 0.850] and E is the volume of ethanol per volume of water [0.600: 0.900], both of which are significant coefficients with = 0.05 and the consistency is shown in **Figure 2.**



DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Figure 2. The joint effect of NaOH concentration and ethanol proportion on HA recovery (%) in alkaline treatments at 5 ◦C. Independent variables are codified according to the criteria specified in the text. Response surfacecorresponding to equation 1.(Murado, *et al*., 2012)

As for **Figure 3**, it is the percentage of HA recovery shown at different times, with the maximum value in equation 1. The result is up to 97% which is in accordance with equation 2.

$$HA = 100\ e^{-0.00317\,.t} \quad (2)$$

From equations 1 and 2, it can be determined that the stable condition in the alkaline process is at a temperature of 5°C in a volume of 0.9 M of ethanol and 0.56 M of NaOH for 1-5 hours. Then the resulting extract can be continued by diafiltration using a membrane and dilution using phosphate to achieve a purer HA concentration.

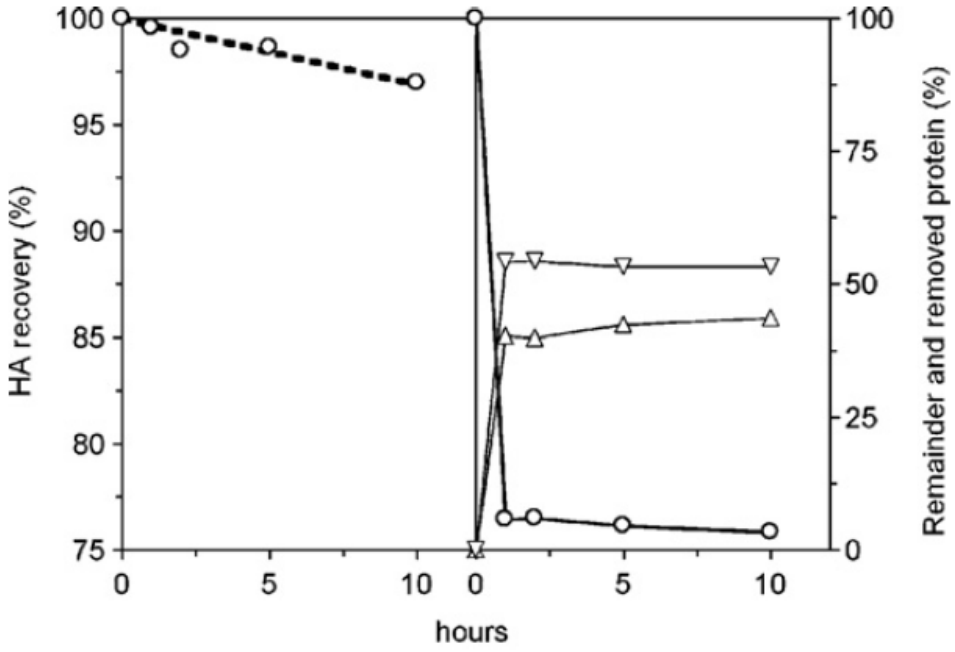


Figure 3. HA recovery (left) and remain and removed protein (right) by alkaline treatment in the maximum of Equation 2, with different times of incubation. Keys: O (left), HA; O (right), remain protein in the extract; removed protein in the sediment; Δ, removed protein in the supernatant.The dotted line to the left shows the fits of the HA data to Equation 2. (Murado, *et al*., 2012)

3.1.2. Enzymatic Process

Using the carbazole assay, the amount of uronic acid-containing polysaccharides was calculated as approximately 6.1 mg/g mollusk dry weight (0.6% w/w), where HA represents a percentage of about 97%. The extraction and purification methods adopted are usually used to purifying glycosaminoglycans from various tissues and sources. After repeated boiling or heating can reduce the possibility that the HA being purified from

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

mollusks is degraded during the purification process. Furthermore, the analysis showed that the GAG produced was not a product of chondroitin sulfate or others but HA.

In **Figure 4** Fractions from 8 to 19, named peak I, from 37 to 58, named peak II, and from 59 to 61, named peak III, then collected to obtain 3 fractions for further analysis. Agarose gel electrophoresis of the three peaks confirmed the presence of unique bands for peaks I and II stained with stains, indicating that metachromasia and a strong blue color are typical of nonsulfated polysaccharides, and two bands for peak III (Fig. stained with toluidine blue (not shown). This polysaccharide is a sign of the presence of GAG, namely Hyaluronic Acid.

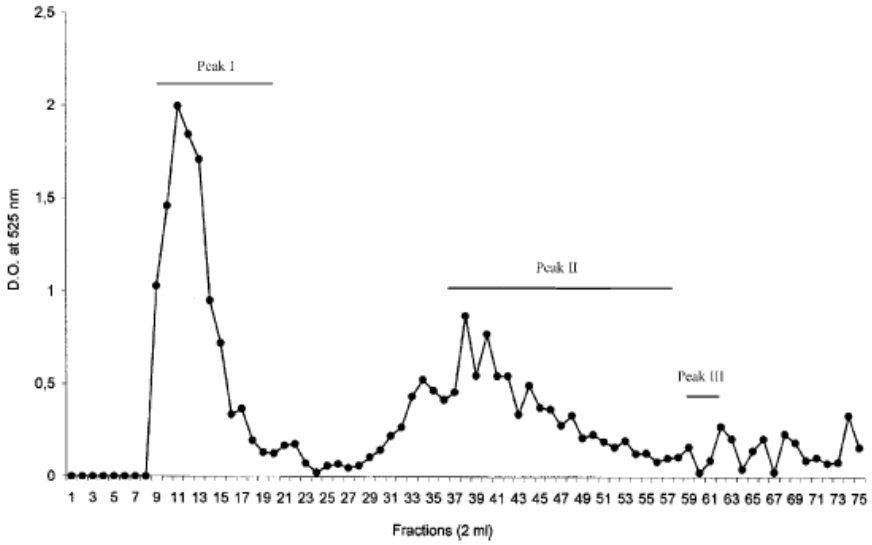


Figure 4. Uronic acid assay of fractions recovered from low-pressure liquid chromatography. The test was performed on 100 µl of each fraction. Peaks named I, II, and III are composed of polysaccharides tested by agarosegel electrophoresis. (Volpi & Maccari, 2003)

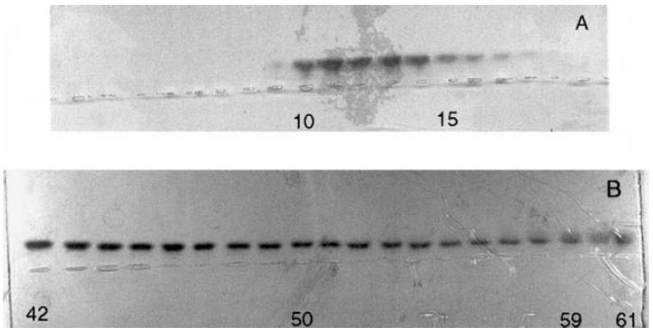


Figure 5. Agarose-gel electrophoresis of fractions recovered from low-pressure liquid chromatography. (A) Fractions from 1 to 21. (B) Fractions from 42 to 61. The gel was dried and stained with Stains-All, and the bands appeared with a strong blue color. (Volpi & Maccari, 2003)

3.2. Purification

In Table 2. It is explained that purification is carried out using an adsorbent such as activated carbon to obtain purer HA. The carbon used is Norit C Gran. When purification was carried out with 2% Norit C Gran with 0.3% HA diafiltration solution

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

and stirred for 5 hours, it would produce particles of < 2µm as much as 43,100 ea/mL which was 66% of the initial yield before being purified (Choi, *et al*., 2014).

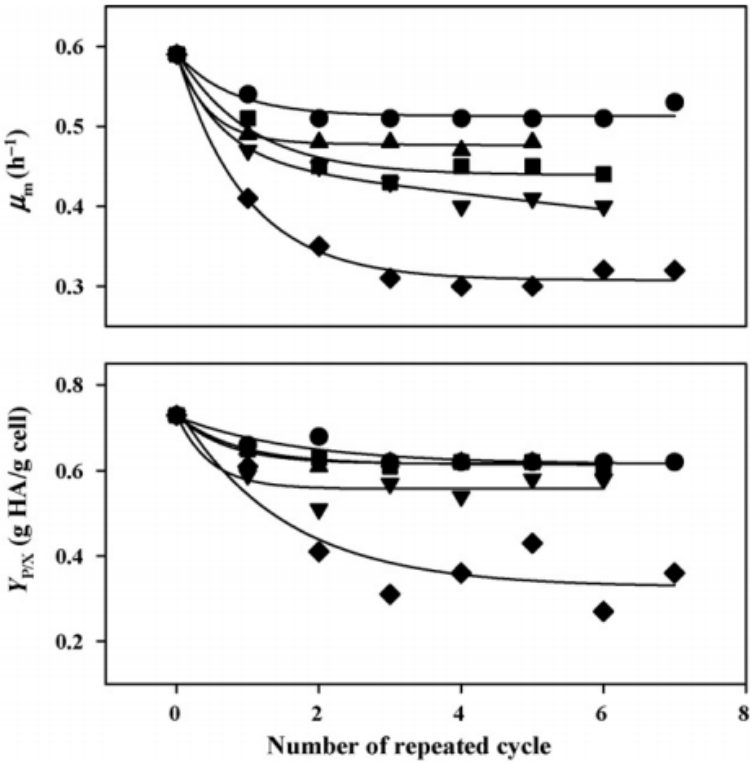
Table 2. Particle Absorbance and Size Distribution Based on Amount of HA and Mixing Time with Activated Carbon Norit C Gran (Choi, *et al*., 2014).

	Norit C Gran. (%)	HA contents (%)	Stirring (hr)	Filtration	Particle (ea/mL, 1/100 dilution)					
					< 2 µm	2 ~ 5 µm	5 ~ 10 µm	10 ~ 15 µm	12 ~ 25 µm	Total
1	0	0	0	O	90 (83%)	13 (12%)	4 (4%)	1 (1%)	0	108
2	0	0.3	0	O	925 (77%)	178 (15%)	69 (6%)	0	3 (0.3%)	1,196
3	2	0	0	O	11,513 (87%)	1,301 (10%)	333 (3%)	3 (0.3%)	24 (0.2%)	13,262
4	2	0.3	0	O	2,251 (85%)	292 (11%)	67 (3%)	24 (1%)	8 (0.3%)	2,642
5	2	0.3	5	X	43,100 (66%)	14,748 (23%)	5,407 (8%)	1,702 (3%)	554 (1%)	65,151
6	2	0.3	13	X	18,898 (22%)	23,986 (29%)	18,685 (22%)	14,451 (17%)	7,990 (10%)	84,010
7	2	0	5	O	37,932 (43%)	28,703 (33%)	13,234 (15%)	5,576 (6%)	2,268 (3%)	87,713

3.3. Fermentation

3.3.1. Repeated Batch Fermentation

Results showed that, with the conventional operation, the maximum specific growth rate (m) and HA-specific productivity (YP / X) decreases with the increasing volume of seeds **(Figure 6).** This is assumed to be due to the presence of inhibitors in the broth, as the seeds contain a liquid which may reduce the benefits of using large seed volumes.



DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

Figure 6. Variations of m and YP/X during the repeated batch culture, here showing m and YP/X decrease with the increasing volume of seed. The repeated cycle of 0 denotes the initial batch culture. Seed volume: (●) 5%, (▲) 10%, (■) 20%, (▼) 30%, and (♦) 40% (v/v) (Huang, *et al.*, 2008).

Therefore, installed *the nonwoven fabric* (NWF) in the fermenter to retain some of the cells while draining the broth. The retained cells were then released and seeded for the next cycle, in which the seed was estimated to be equivalent to 3% inoculum. It can be seen that the cell concentration (from the broth) can be maintained for two repeated cycles. Thereafter, the concentration decreased gradually, as shown by **Figure 7**.

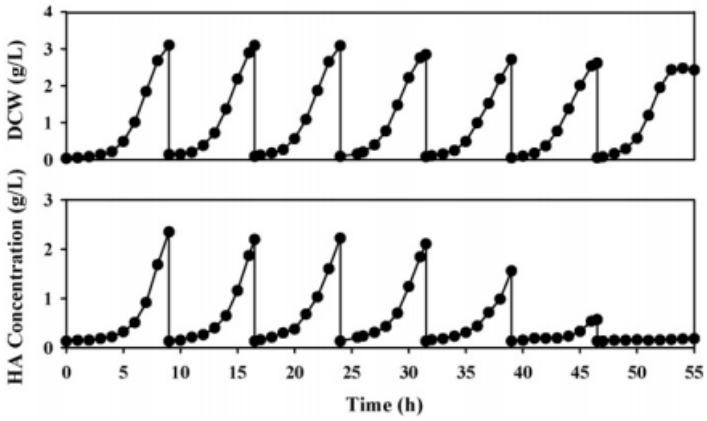


Figure 7. HA production by repeated batch culture with NWF equipped in the fermentor. Symbols: (●) cell density and (○) HA concentration (Huang, *et al.*, 2008).

The decrease in cell concentration was found to be associated with more cells growing in the *nonwoven fabric* (NWF). However, this results in a loss of HA productivity and makes *the nonwoven fabric* (NWF) sticky. Therefore, use an external cartridge filter to retain the current cell broth drain, repeated batch fermentation can be used successfully for the production of HA, thereby increasing the volumetric production rate becomes (0.59 g HA L^{-1} $h^{-}1$) 2.5 times that of batch culture (0.24 g HA L^{1} h^{1}) (See **Figure 8**).

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

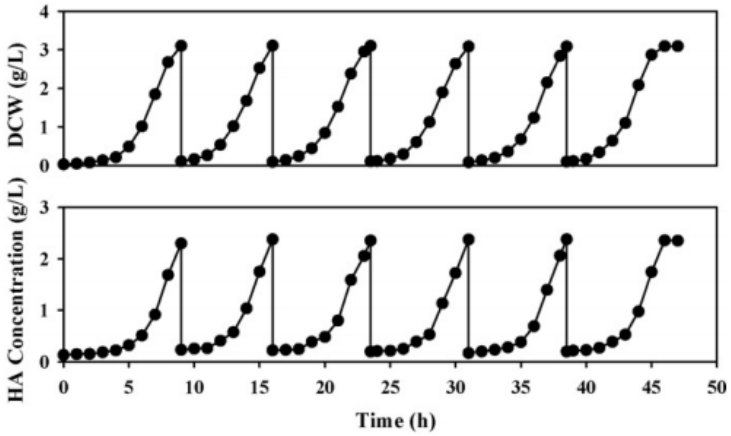
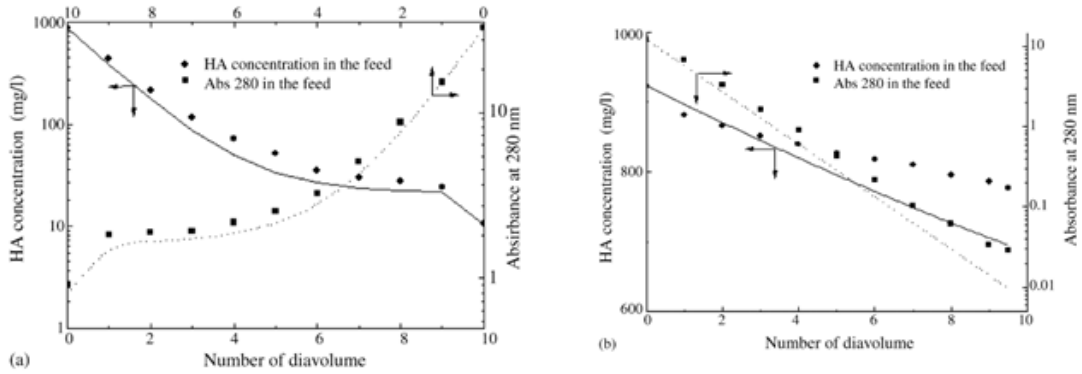


Figure 8. HA production by repeated batch culture with a cartridge filter, where the amount of cell-seeded was 3%. Symbols: (●) cell density and (○) HA concentration(Huang,*et al.*, 2008).

3.4. Separation

3.4.1. *Two-Stage Tangential Flow Microfiltration* (MF) and Ultrafiltration (UF)

Figure 9 shows the variation of HA and protein content in retentate and HA yield at both stages with the amount of diavolume. HA content and Absorbance 280 in the retentate decreased rather rapidly when the diafiltrated volume was less than six times the initial feed volume in the first diafiltration (**Figure 9a**). Then slumped slowly on VD. 19, the concentration of HA is about 20mg/l. One volume of purified water is added at this time because the HA concentration is unlikely to be reduced to the concentration in the diafiltration from the previous UF stage. Then, the concentration of HA degenerates very slow when the protein content in the retentate falls quickly in the last period of the second diafiltration, which signifies an increase in the $S_{pro\,/\,HA}$ is caused by a reduction of $P_{HA}$for UF membrane (See **Figure 9b**).



DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

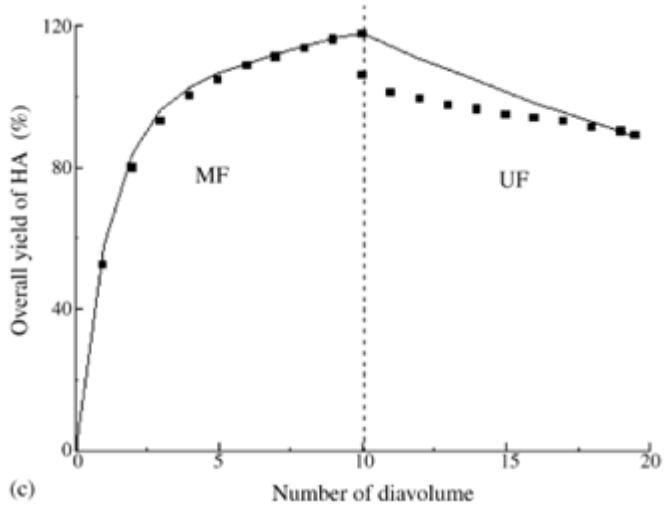


Figure 9. Dependencies of contents of HA and proteins in the retentate in (a) MF stage, (b) UF stage, and (c) HA yield on the number of diavolume for scheme 2 (Zhou, *et al.*, 2006).

Table 3. Comparison of the two schemes tested (Zhou, *et al.*, 2006).

Scheme	*T* (h)	V_D	*Y* (%)	B
1	56.0	16	77.5	973.0
2	73.8	10.5	89.1	1004.6

Note: VD is the number of diavolume, T is the sum of the times needed in two stages, β is the purification factors of HA.

The result of this study is that both schemes can effectively separate HA from the fermentation broth, and the purification factor is around 1000, indicating the product has high purity. Scheme 2 greatly increases the yield of hyaluronic acid (up to 89%) and saves much of the purified water required as the diafiltrate (VD = 10.5). The disadvantage of this method is that it has a longer total processing time. However, much larger membrane filtration areas are usually used in practical (industrial scale) applications of membrane processes, which will shorten the processing time to a large degree.

3.4.2. *Ultrahigh-voltage capillary gel electrophoresis*

3.4.2.1. *Capillary gel electrophoresis (CGE) system*

Figure 10 compared the separation of APTS-derived HA from *bovine vitreous humor* at 95 kV in the UHVCE system with the same sample analyzed at 15 kV in the conventional system. The results show that the efficiency and resolution of the 95 kV separation are much better than that of the 15 kV separation. For low molecular weight oligomers, the separation efficiency was found to increase ten-fold, and approximately three times the resolution (Hutterrer & Jogerson, 2005).

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

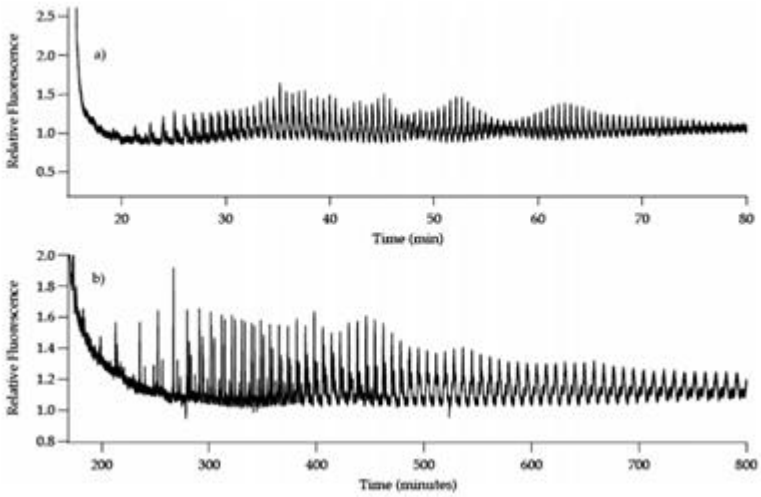


Figure 10. Comparison of separation of hyaluronic acid from bovine vitreous humor at (a) 15 and (b) 95 kV (Hutterer & Jogerson, 2005).

3.5. Isolation

In a study conducted by Sadhasivam*et al,* 2012 , HA was obtained from the liver of stingray *A. narinari* which is a waste from the seafood production industry. The absorbance fraction of 2 ml at 210 nm detects three peaks **(**See **Figure 11).** The amount of hyaluronic acid in the liver *A.narinari* was calculated as 0.81 mg/g of the dry weight of tissue. It has been reported that 6.1 mg/g of HA extracted from the Mollusc species *Mytilusgalloprovincialis* described in a study conducted by Volpi&Maccari, (Volpi & Maccari, 2003) was relatively higher than our results. Therefore based on their OD values, the peak fractions were collected separately to obtain three fractions for further analysis.

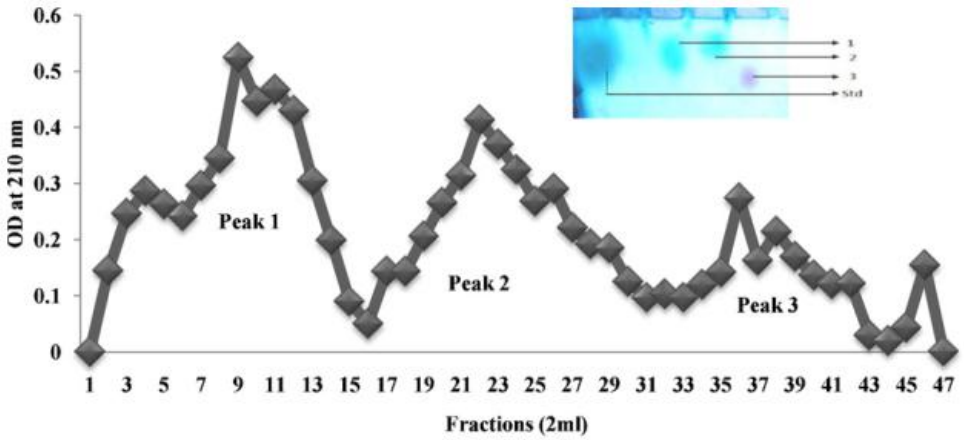


Figure 11. The absorbance of 2 ml fractions detected at 210 nm. The agarose gel (right corner) shows blue bands (peaks 1 and 2) responsible for the presence of HA and one band (peak 3) showing purple color indicating the presence of sulfatedpolysaccharides (Sadhasivam, *et al.*, 2013)

HA is a high molecular weight polysaccharide. HA purified from *A. narinari* liver has a higher molecular mass of 13, 65,863 Da. The molecular weight of HA strongly depends on its biological origin (Zainudin, *et al*., 2014). The molecular weight of HA

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

produced by microbial fermentation ranges from 1×10^6 to 4×10^6 g/mol, Whereas the molecular mass of HA prepared by extraction ranges up to 6×10^6 (Armstrong, *et al*., 1997) which is higher than that produced by microbial fermentation. Earlier low molecular weight hyaluronic acid (200 kDa) was isolated from mollusk *M. Galloprovincialis* (Volpi & Maccari, 2003*)*. Consequently, HA of higher molecular weight was isolated for the first time from marine sources.

HA was analyzed using high-performance thin-layer chromatography. The absorption area of the standard was 16265.8 AU (49.38%), and the sample was 114975.9 AU (52.19%). Different compositions of the mobile phase for HPTLC analysis were tested to obtain high resolution and reproducible peaks. The spectrum of hyaluronic acid sample and standard were shown in **Figure 12A and B.** Peaks with Rf values of 0.02 and 0.03 were detected for standard and sample between 200 and 400 nm with maximum signal generating at 390 and 388.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

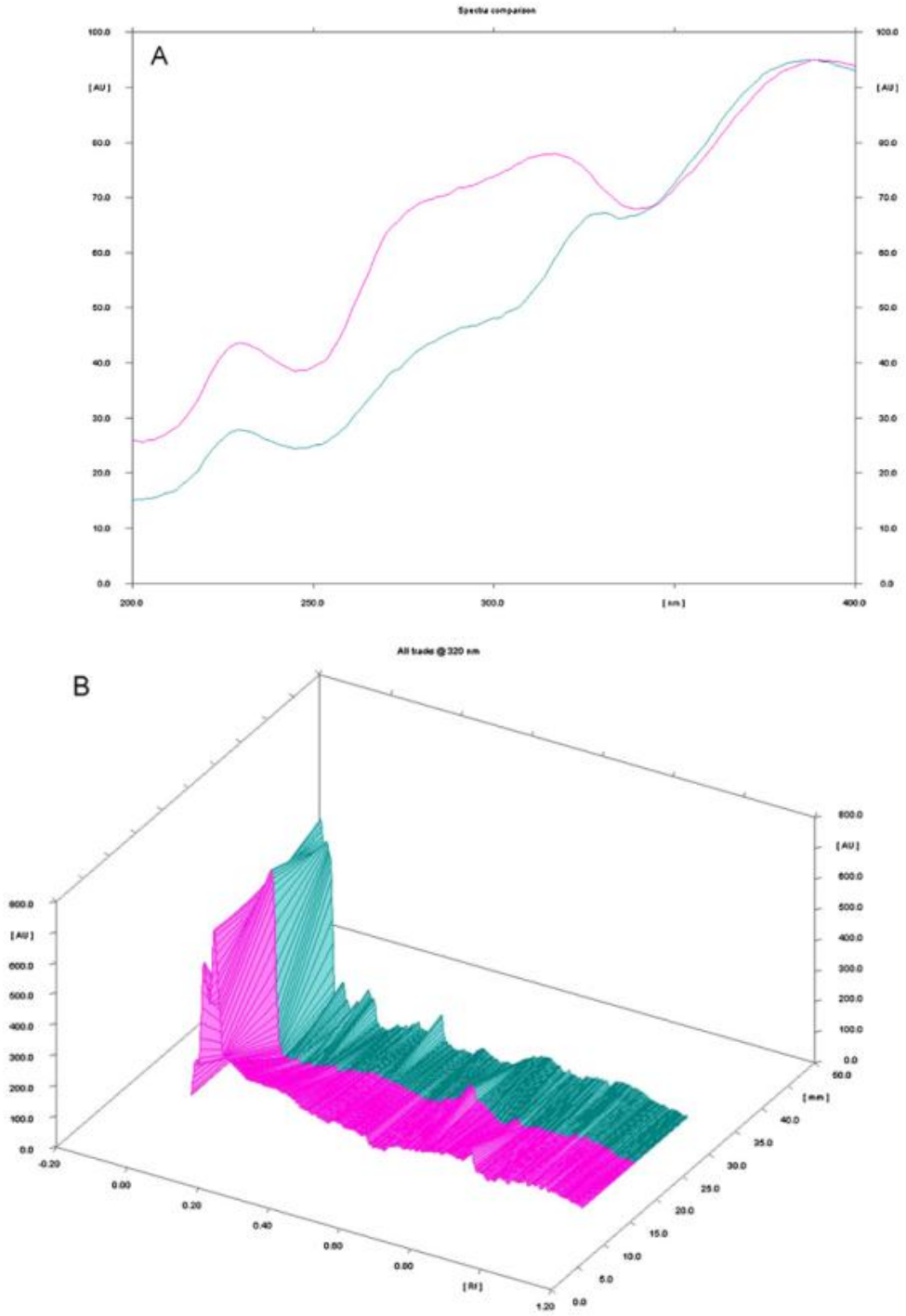


Figure 12. (A) Spectral view for standard and sample (HA) between 200 and 400 nm respectively. (B) Peaks responsible for standard (R_f 0.03) and sample (R_f 0.02) detected at 320 nm(Sadhasivam, *et al*., 2013)

4. CONCLUSION

Hyaluronic acid synthesis can be done by several methods including extraction, purification, fermentation, separation, and isolation. The most efficient for the synthesis of hyaluronic acid is repeated batch fermentation and separation because the materials used are quite easy to find and the process is novel yet easy with the high pure product.

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

5. ACKNOWLEDGEMENTS

We acknowledged Bangdos Universitas Pendidikan Indonesia.

6. REFERENCES

Abdel-Mohsen, A. M., Hrdina, R., Burgert, L., Abdel-Rahman, R. M., Hašová, M., Šmejkalová, D., Michal Kolář, D., M.Pekar, M., Aly, A. S. (2013). Antibacterial activity and cell viability of hyaluronan fiber with silver nanoparticles. *Carbohydrate polymers*, *92*(2), 1177-1187.

Abdel-Mohsen, A. M., Pavliňák, D., Čileková, M., Lepcio, P., Abdel-Rahman, R. M., and Jančář, J. (2019). Electrospinning of hyaluronan/polyvinyl alcohol in presence of in-situ silver nanoparticles: preparation and characterization. *International Journal of Biological Macromolecules, 139*, 730–739.

Abdel-Mohsen, A.M., Hrdina, R., Burgert, L., Krylová, G., Abdel-Rahman, R.M., Krejčová, A., Steinhart, M. and Beneš, L. (2012). Green synthesis of hyaluronan fibers with silver nanoparticles. *Carbohydrate Polymers, 89*(2), 411–422.

Abdel-Mohsen, A.M., Jancar, J., Abdel-Rahman, R.M., Vojtek, L., Hyršl, P., Dušková, M. and Nejezchlebová, H. (2017). A novel in situ silver/hyaluronan bio-nanocomposite fabrics for wound and chronic ulcer dressing: in vitro and in vivo evaluations. *International Journal of Pharmaceutics, 520*(1–2), 241–253.

Almond, A., Brass, A., and Sheehan, J. K. (1998). Deducing polymeric structure from aqueous molecular dynamics simulations of oligosaccharides: predictions from simulations of hyaluronan tetrasaccharides compared with hydrodynamic and X-ray fibre diffraction data. *Journal of Molecular Biology, 284*(5), 1425-1437.

Amagai, I., Tashiro, Y., and Ogawa, H. (2009). Improvement of the extraction procedure for hyaluronan from fish eyeball and the molecular characterization. *Fisheries Science, 75*(3), 805–810.

Armstrong, D. C., and Johns, M. R. (1997). Culture conditions affect the molecular weight properties of hyaluronic acid produced by Streptococcus zooepidemicus. *Applied and Environmental Microbiology*, *63*(7), 2759-2764.

Bai, M., Han, W., Zhao, X., Wang, Q., Gao, Y., and Deng, S. (2018). Glycosaminoglycans from a sea snake (Lapemis curtus): extraction, structural characterization and antioxidant activity. *Marine Drugs, 16*(5), 170.

Bitter, T. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry, 4*, 330-334.

Boas, N. F. (1949). Isolation of hyaluronic acid from the cock's comb. *Journal of Biological Chemistry*, *181*(2), 573-575.

Cesaretti, M., Luppi, E., Maccari, F., and Volpi, N. (2003). A 96-well assay for uronic acid carbazole reaction. *Carbohydrate Polymers, 54*(1), 59-61.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Chen, S. J., Chen, J. L., Huang, W. C., and Chen, H. L. (2009). Fermentation process development for hyaluronic acid production by Streptococcus zooepidemicus ATCC 39920. *Korean Journal of Chemical Engineering, 26*(2), 428-432.

Choi, S., Choi, W., Kim, S., Lee, S.-Y., Noh, I., and Kim, C.-W. (2014). Purification and biocompatibility of fermented hyaluronic acid for its applications to biomaterials. *Biomaterials Research, 18*, 6.

Chong, B. F., Blank, L. M., Mclaughlin, R., and Nielsen, L. K. (2005). Microbial hyaluronic acid production. *Applied Microbiology and Biotechnology, 66*(4), 341-351.

Collins, M. N., and Birkinshaw, C. (2013). Hyaluronic acid based scaffolds for tissue engineering—A review. *Carbohydrate Polymers, 92*(2), 1262–1279.

Cramer, J. A., and Bailey, L. C. (1991). A reversed-phase ion-pair high-performance liquid chromatography method for bovine testicular hyaluronidase digests using postcolumn derivatization with 2-cyanoacetamide and ultraviolet detection. *Analytical Biochemistry, 196*(1), 183-191.

Fakhari, A., and Berkland, C. (2013). Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. *Acta Biomaterialia, 9*(7), 7081–7092.

Garnjanagoonchorn, W., Wongekalak, L., and Engkagul, A. (2007). Determination of chondroitin sulfate from different sources of cartilage. *Chemical Engineering and Processing Process Intensification, 46(*5), 465–471.

Hjertén, S. (1985). High-performance electrophoresis: Elimination of electroendosmosis and solute adsorption. *Journal of Chromatography A, 347*, 191-198.

Holmbeck, S., and Lerner, L. (1993).). Separation of hyaluronan oligosaccharides by the use of anion-exchange HPLC. *Carbohydrate Research, 239*, 239-244.

Holmström, B., and Řičica, J. (1967). Production of hyaluronic acid by a streptococcal strain in batch culture. *Applied microbiology, 15*(6), 1409-1413.

Hong, M., Sudor, J., Stefansson, M., and Novotny, M. V. (1998). High-resolution studies of hyaluronic acid mixtures through capillary gel electrophoresis. *Analytical Chemistry, 70*(3), 568-573.

Huang, W. C., Chen, S. J., and Chen, T. L. (2007). Modeling the microbial production of hyaluronic acid. *Journal of the Chinese Institute of Chemical Engineers*, *38(*3-4), 355-359.

Huang, W. C., Chen, S. J., and Chen, T. L. (2008). Production of hyaluronic acid by repeated batch fermentation. *Biochemical Engineering Journal*, *40*(3), 460-464.

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

Hutterer, K. M., and Jorgenson, J. W. (2005). Separation of hyaluronic acid by ultrahigh-voltage capillary gel electrophoresis. *Electrophoresis, 26*(10), 2027–2033.

Kanchana, S., Arumugam, M., Giji, S., and Balasubramanian, T. (2013). Isolation, characterization and antioxidant activity of hyaluronic acid from marine bivalve mollusc Amussium pleuronectus (Linnaeus, 1758). *Bioactive Carbohydrates and Dietary Fibre, 2*(1), 1–7.

Kendall, F. E., Heidelberger, M., and Dawson, M. H. (1937). A serologically inactive polysaccharide elaborated by mucoid strains of group A hemolytic streptococcus. *Journal of Biological Chemistry, 118*(1), 61-69.

Lamberg, S. I., and Stoolmiller, A. C. (1974). Glycosaminoglycans. A biochemical and clinical review. *The Journal of Investigative Dermatology, 63*(6), 433–449.

LapcıkJr, L., Lapcık, L., De Smedt, S., Demeester, J., and Chabrecek, P. (1998). Hyaluronan: preparation, structure, properties, and applications. *Chemical Reviews, 98*(8), 2663-2684.

Laurent, T. C., and Fraser, J. R. (1992). Hyaluronan. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 6*(7), 2397–2404.

Lesley, J., Hascall, V. C., Tammi, M., and Hyman, R. (2000). Hyaluronan binding by cell surface CD44. *Journal of Biological Chemistry, 275*(35), 26967-26975.

Lignot, B., Lahogue, V., and Bourseau, P. (2003). Enzymatic extraction of chondroitin sulfate from skate cartilage and concentration-desalting by ultrafiltration. *Journal of Biotechnology, 103*(3), 281–284.

Liu, L., Du, G., Chen, J., Wang, M., and Sun, J. (2008). Enhanced hyaluronic acid production by a two-stage culture strategy based on the modeling of batch and fed-batch cultivation of Streptococcus zooepidemicus. *Bioresource Technology, 99*(17), 8532-8536.

Liu, L., Du, G., Chen, J., Zhu, Y., Wang, M., and Sun, J. (2009). Microbial production of low molecular weight hyaluronic acid by adding hydrogen peroxide and ascorbate in batch culture of Streptococcus zooepidemicus. *Bioresource technology, 100*(1), 362-367.

Luo, Y., and Prestwich, G. D. (1999). Synthesis and selective cytotoxicity of a hyaluronic acid–antitumor bioconjugate. *Bioconjugate Chemistry, 10*(5), 755-763.

Maurer, P. H., and Hudack, S. S. (1952). The isolation of hyaluronic acid from callus tissue of early healing. *Archives of Biochemistry and Biophysics, 38*(1), 49-53.

Meyer, K., and Palmer, J. W. (1934). The polysaccharide of the vitreous humor. *Journal of Biological Chemistry, 107*(3), 629-634.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123

Michelacci, Y. M., and Horton, D. S. P. Q. (1989). Proteoglycans from the cartilage of young hammerhead shark Sphyrna lewini. *Comparative Biochemistry and Physiology Part B : Comparative Biochemistry, 92*(4), 651–658.

Murado, M. A., Montemayor, M. I., Cabo, M. L., Vázquez, J. A., and González, M. P. (2012). Optimization of extraction and purification process of hyaluronic acid from fish eyeball. *Food and Bioproducts Processing, 90*(3), 491–498.

Nakano, T., Nakano, K., and Sim, J. S. (1994). A simple rapid method to estimate hyaluronic acid concentrations in rooster comb and wattle using cellulose acetate electrophoresis. *Journal of Agricultural and Food Chemistry, 42*(12), 2766–2768.

Opdensteinen, P., Clodt, J. I., Müschen, C. R., Filiz, V., and Buyel, J. F. (2019). A combined ultrafiltration/diafiltration step facilitates the purification of cyanovirin-n from transgenic tobacco extracts. *Frontiers in Bioengineering and Biotechnology, 6*, 206

Radaeva, I. F., Kostina, G. A., and Zmievskii, A. V. (1997). Hyaluronic acid: Biological role, structure, synthesis, isolation, purification, and applications. *Applied Biochemistry and Microbiology, 33*(2), 111-115.

Rosa, C. S., Tovar, A. F., Mourão, P., Pereira, R., Barreto, P., and Beirão, L. H. (2012). Purification and characterization of hyaluronic acid from chicken combs. *Ciência Rural, 42*(9), 1682–1687.

Sadhasivam, G., and Muthuvel, A. (2014). Isolation and characterization of hyaluronic acid from marine organisms. *Advances in Food and Nutrition Research, 72*, 61–77.

Sadhasivam, G., Muthuvel, A., Pachaiyappan, A., and Thangavel, B. (2013). Isolation and characterization of hyaluronic acid from the liver of marine stingray aetobatus narinari. *International Journal of Biological Macromolecules, 54*, 84-89.

Sumi, T., Ohba, H., Ikegami, T., Shibata, M., Sakaki, T., Sallay, I., and Park, S. S. (2002). Method for the Preparation of chondroitin sulfate compounds. *United States Patent No. 6*, 342,367.

Tammi, R., MacCallum, D., Hascall, V. C., Pienimäki, J. P., Hyttinen, M., and Tammi, M. (1998). Hyaluronan bound to cd44 on keratinocytes is displaced by hyaluronan decasaccharides and not hexasaccharides. *Journal of Biological Chemistry, 273*(44), 28878-28888.

Toffanin, R., Kvam, B. J., Flaibani, A., Atzori, M., Biviano, F., and Paoletti, S. (1993). NMR studies of oligosaccharides derived from hyaluronate: complete assignment of 1h and 13c nmr spectra of aqueous di- and tetra-saccharides, and comparison of chemical shifts for oligosaccharides of increasing degree of polymerisation. *Carbohydrate Research, 245*(1), 113-120.

Tømmeraas, K., and Melander, C. (2008). kinetics of hyaluronan hydrolysis in acidic solution at various pH values. *Biomacromol, 9*, 1535–1540.

DOI: http://dx.doi.org/10.17509/xxxx.vxix
p- ISSN 2775-8400 e- ISSN 2775-9857

Ünlüer, Ö. B., Ersöz, A., Denizli, A., Demirel, R., and Say, R. (2013). Separation and purification of hyaluronic acid by embedded glucuronic acid imprinted polymers into cryogel. *Journal of Chromatography B, 934*, 46-52.

Vázquez, J. A., Rodríguez-Amado, I., Montemayor, M. I., Fraguas, J., González, M. D. P., and Murado, M. A. (2013). Chondroitin sulfate, hyaluronic acid and chitin/chitosan production using marine waste sources: Characteristics, applications and eco-friendly rrocesses: A review. *Marine Drugs, 11*(3), 747-774.

Volpi, N., and Maccari, F. (2003). Purification and characterization of hyaluronic acid from the mollusc bivalve mytilus galloprovincialis. *Biochimie, 85*(6), 619–625.

Weissmann, B., and Meyer, K. (1954). The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord1, 2. *Journal of the American Chemical Society, 76*(7), 1753-1757.

Zainudin, N. H., Sirajudeen, K. N. S., and Ghazali, F. C. (2014). Marine sourced glycosaminoglycans'gags'. *Journal of Advanced Laboratory Research in Biology, 5*(3), 46–53.

Zakeri, A., Rasaee, M. J., and Pourzardosht, N. (2017). Enhanced hyluronic acid production in streptococcus zooepidemicus by over expressing hasa and molecular weight control with niscin and glucose. *Biotechnology Reports, 16*, 65-70.

Zhou, H., Ni, J., Huang, W., and Zhang, J. (2006). Separation of hyaluronic acid from fermentation broth by tangential flow microfiltration and ultrafiltration. *Separation and Purification Technology*, *52*(1), 29-38.

DOI: http://dx.doi.org/10.17509/xxxxt.vxix
p- ISSN 2085-9783 e- ISSN 2549-6123