CHEMICAL ENGINEERING TRANSACTIONS
VOL. 37, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Eliseo Ranzi, Katharina Kohse- Höinghaus

ISBN 978-88-95608-28-0; **ISSN** 2283-9216
DOI: 10.3303/CET1437065

Unique Marine Organism: Identification of some Methods for Biomaterial Production

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In this work, one dominant species of tunicates in Scandinavian Ocean called *Ciona intestinalis* has been investigated by extraction and characterization of its cellulose, oil, and amino acids. Potential applications have been evaluated for production of pure crystalline cellulose, biodiesel, and by analysis of amino acid composition of the samples. Pure tunicate cellulose (TC) has been prepared via chemical treatment, using acid, alkali, and the subsequent bleaching. The cellulose percentage, and yield for the chemically pure cellulose obtained were around 96% and 54% respectively. Oil extraction has been done using petroleum ether as the extraction solvent, and then chemical structure and fatty acid composition of the obtained oil have been characterized by NMR (Nuclear Magnetic Resonance Spectroscopy), FTIR (Fourier Transform Infrared Spectroscopy), and GC-MS (Gas Chromatography-Mass Spectrometry). Tunicate's oil has shown high content of free fatty acids together with very low content of glycerol, the latter being the common oil from vegetable origins. Moreover, the composition profile of Tunicate fatty acids seems to be similar to fish oil. However, amino acid composition has shown similarity to egg albumin, implying tunicate to have the capability to be considered as other marine organisms' feed in the near future.

1. Introduction

The name of "Tunicates" comes from "Tunic" which mostly seems to be the extracellular layer covering the epidermal surface of tunicates (Okamoto et al., 1996). Tunicates are the only animals that perform cellulose biosynthesis (Sasakura et al., 2007).Chemically aspects, inside the tough outer tunic, there would be some components such as water, protein, and carbohydrates in various proportions, connective tissue, muscles and blood vessels (Van Daele et al.,1992).One interesting characteristic of Tunic is having "Tunicin", a composite structure of cellulose fibrils connected to protein matrix, to some extent attached together through serine, threonine and the other hydroxylated amino acids (Smith and Dehne, 1971).Large width and highly crystalline structure are two outstanding characteristics of tunicate's cellulose, which consist of almost pure monoclinic cellulose I (the Iß allomorph). (Zhang et al., 2013)
Rånby (1952) showed that cellulose in the tunic is aggregated in the form of microfibrils similar to those of plant cellulose, while, the aggregation and dimension of cellulose fibrils are extremely different among the species of tunicates (Kimura and Itoh, 2007). Tunicates have been attracted a lot of attention in medical application, nanomaterial production, food market, and water pollution issues due to their consisting of chemical compounds such as cellulose, amino-sugars, and proteins or protein-polysaccharide complexes e.g. collagen, glycosaminoglycan, chitin, scleroprotein, iodine-binding proteins, and elastin (Jackson et al., 1993).Nowadays, novel pharmacological or commercially useful products extracted from tunicates have been provided. Anti-cancer, and anti-viral therapeutics using some chemicals like Didemnins, Aplidine, Trabectedin, Palmerolide, and others, have been frequently proved (Carver et al., 2006).Moreover, some tunicates such as *Halocynthia roretzi*, *Halocynthia aurantium* from Japan and *Styela clava* from Korea shown to be used in nutritional point of view. Additionally, cellulose whiskers produced by tunicates is one of the newest exciting applications of tunicate's cellulose (Van den Berg et al., 2007). Cellulose whiskers with the width of around 15 nm, and lengths between 1160 and 2000 nm has been provided recently. High specific surface area, high crystallinity of 95%, and high aspect ratio of cellulose whiskers due to hydroxyl

Please cite this article as: Hassanzadeh M., 2014, Unique marine organism: identification of some methods for biomaterial production, Chemical Engineering Transactions, 37, 385-390 DOI: 10.3303/CET1437065

groups, can lead them to have the high amount of stiffness, mechanical properties, and composite reinforcement (Siqueira et al., 2010). More recently, potential biofuel production from marine organisms has been also investigated. Bioethanol (derived from cellulosic or lignocellulose) and Biodiesel (a mixture of alkyl esters of long chain fatty acids derived from vegetable oils or animal fats), were the first biofuels and have certainly been valuable in developing the biofuel market. However, their production from starch, sugars, and vegetable oils induces competition with food production and can thus hardly deliver the large volume required for worldwide transportation (Palkovits, 2010). To avoid this problem, it is the main interest to produce biofuel from marine creatures.

In this work, the principle objection was evaluation of one dominant species in Norwegian coast called *Ciona intestinalis* which belongs to the family of Ascidiacea (ascidians), to see its capability for conversion to different bio-products. For that purpose, some processing treatments have been conducted with the aims to fractionate tunicate biomass components or enhance the cellulose accessibility and reactivity for its further possible application. Moreover, analysis of amino acid composition and oil structure of the samples has been performed for nutritional purpose and biodiesel production.

2. Materials and methods

2.1 Sample preparation

The tunicate sample subjected to this study was *Ciona intestinalis*, which has been collected from Norwegian coast as the dominant ascidian sample, and acclimatized to the lab situation. The outer layer of tunicate sample (Tunic) was separated manually from the internal organs followed by rinsing with fresh water. The samples were put inside freeze dryer (Scanvac Cool Safe freeze dryer model CC300-8V) for two days to let them completely dried. The dried ascidian tunicate was reduced to powder by using a knife mill. The size of ascidian sample powder used was less than 40 meshes, and the whole organ (Tunicate), internal layer, and external layer (Tunic) have been used in different experiments.

2.2 Cellulose preparation

Cellulose preparation has been done via three-step sequence, meaning acid, alkali, and NaOCl application respectively.5 g ascidian tunicate dried sample was dipped in the acidic aqueous solution of H_2SO_4 (0.9 wt %) in a stainless steel vessel, following by stirring, and heating to 180°C, and then treating for 2 h under gentle stirring. The ascidian tunic was then filtered by using a glass filter, washed with acetone/water, and dried under vacuum at 75°C. For the next step, the acid treated sample was treated with alkali aqueous solution of NaOH/Na_2S (9/3 wt %) for 2 h at 180°C, and then also filtered, washed, and dried in the same process. The alkali-treated sample was treated with aqueous NaOCl solution (2.9 wt %) as a bleaching agent for 1 h at 75°C, followed by filtering, washing, and drying. The final yield has been measured, and carbohydrate analysis has been done for measuring cellulose content according to tappi standard TAPPI T 249 cm-09.

2.3 Nitrogen content analysis

The protein content of the samples treated by three-step sequence was deducted from the nitrogen concentration obtained by pyro-chemiluminiscent analysis on an ANTEK 7000NS analyzer (Antek Instrument, USA). The instrument was calibrated with urea and the nitrogen concentration was multiplied by the common conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) used for estimation of the protein content in samples (Gunnarsson and Tunlid, 1986).

2.4 Amino acid content analysis

Chinese national standard GB/T 5009.124-2003 was followed.10-20mg of the smashed dried tunicate samples from the whole body, internal, and external part (Tunic) of *C. intestinalis* were separately weighed and added 10mL 6 mol/L HCl and 3 drops of phenol. After freezing the resulting suspension for 3-5 minutes and replacing all of the air with nitrogen, the suspension was heated at 110 degree for 22 hours, followed by filtration and analyzed by amino acid analyzer (Hitachi L-8800 high speed amino acid analyzer) using external amino acid standards.

2.5 Oil Extraction

The samples were put inside freeze dryer for two days to let them completely dried. After that, it has been crushed into smaller pieces, transferred into the extractor chamber. Then, the extraction solvent (petroleum ether: p.e) has been added (6 hours; 30-60 °C) into the flask which was fitted into an electric heater. Then, filtration for separation of solution from solid tunicate materials was required. Once it has been done, the remaining p.e solvent was evaporated under vacuum using rotary evaporator. Furthermore, chemical structures and fatty acid compositions of the obtained oil have been characterized by NMR (recorded on a Bruker Avance 400 MHz instrument using the ^{1}H-NMR standard Bruker pulse

programs), FTIR (recorded on a Perkin-Elmer 2000 spectrometer), and GC-MS (Thermoquest CE instrument Trace GC 2000 series equipped with Finnigan Trace MS recording at 70 eV while using DB-5MS column).

3. Results and discussion

Cellulose yield and purity of the sample after three step treatment were shown in Table 1. Pure cellulose was obtained (Figure1) at a yield of 3.6 wt % based on weight of the starting material, while no ash has been seen in the produced pure cellulose. The yield from each step based on weight of dried starting material after each treatment was 21, 33(data hasn't been shown), and 54 wt % after first (H2So4), second (NaOH) and last treatment (oxidation/bleaching: NaClO) respectively, and cellulose content via carbohydrate analyses showed cellulose in an amount of greater than 95 wt %.

Table 1: Yield (based on weight of dried sample after alkali treatment), ash content, cellulose percent, other C5, C6 percent, and cellulose yield after three step (Acid- alkali- NaClO) treatment.

Pretreatment	Ash content (%)	Cellulose (%)	Other C5, C6 Sugars (%)	Yield (%)	Cellulose yield (%)
H_2SO_4-NaOH -NaClO	~0	96	0	54	3.6

Protein content of treated sample was analyzed by ANTEK-analyzer and the result was shown in Table 2.According to that, after Acid-, Alkali-, and Hypochlorite treatments, there has been a step by step decreasing to reach near zero percent of protein by NaClO. The alkali treatment (NaOH) here seems to be the most effective for protein removal.

Table 2: Changes of protein content during three step treatment (H_2SO_4-NaOH-NaClO) measured by ANTEK nitrogen analyzer

Treatments	**Three-step sequence**		
	H_2SO_4	NaOH	NaClO
Protein (%)	23.90	2.70	0.60

Figure 1(a) shows a macroscopic image of pure crystalline cellulose, while Figure 1(b) illustrated a SEM image of the pure cellulose prepared from the three step treatment conditions. The 96% pure cellulose obtained has a high capacity to be served as a potential source of pure cellulose whiskers for bio/nano composite materials, and for producing ethanol (Pirani and Hashaikeh, 2013).

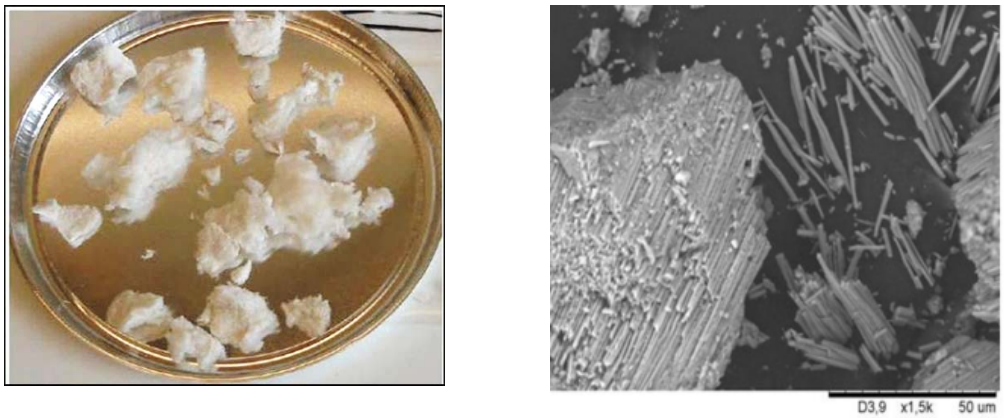

Figure 1(a) (Left): Macroscopic image of the pure cellulose after three step treatment. 1(b) (Right): SEM image of the pure cellulose after treatment.

The protein content and amino acids in the tunicate sample was determined and compared with egg albumin (Table 3). There were 17 amino acids, 9 essential amino acids (EAA) and 8 non-essential amino

acids (NEAA). The highest and lowest amount of total amino acids was related to inner organ, and outer layer (tunic) with 51.09%, and 25.49% respectively. The results showed that tunicate samples contain all types of amino acids found in egg albumin (Lewis et al., 1950). Most of the ratio of tunicate amino acids to egg albumin was around 1 or higher. The high quality of protein sample especially in inner part of the body can be used as an animal (like fish) feed.

Table 3: Amino acid composition of inner organ, tunic (external layer of tunicate), and tunicate (Whole body) sample, in g/100g dry weight has been measured. Amino acids specified with * are EAA, and the rest are NEAA. *Tunicate# is based on g/1oog protein calculated from Tunicate (whole body) amino acids.*

Amino Acid	*Inner Organ*	*Tunic*	*Tunicate (g/100g dry weight)*	*Tunicate# (g/100g protein)*	*Egg albumin (g/100g protein)*	*Ratio (Tunicate#/Egg)*
Thr*	2.66	1.83	2.17	5.13	4.00	1.30
Val*	2.08	1.35	1.70	4.01	8.80	0.50
Met*	1.09	0.38	0.54	2.10	5.40	0.40
Ile*	1.72	0.95	1.28	3.32	7.10	0.50
Leu*	3.79	1.75	2.54	7.31	9.90	0.70
Phe*	1.73	0.81	1.13	3.34	7.50	0.40
Lys*	3.41	0.90	1.84	6.58	6.40	1.00
His*	1.17	0.67	0.90	2.26	2.41	0.90
Arg*	3.63	1.24	2.13	7.00	5.90	1.20
Asp	5.76	3.22	4.18	11.11	9.20	1.20
Ser	3.00	1.69	2.18	5.79	8.50	0.70
Glu	8.52	3.20	5.27	16.43	15.70	1.00
Gly	3.81	1.52	2.31	7.35	3.20	2.30
Ala	2.57	1.33	1.81	4.96	5.70	0.90
Cys	1.70	1.93	1.93	3.28	0.00	0.00
Tyr	1.98	1.36	1.56	3.82	3.75	1.00
Pro	2.47	1.36	1.77	4.76	3.80	1.30
Total(%)	51.09	25.49	35.24	98.53	107	

FTIR spectra of tunicate oil fraction were presented in Figure 2. It can be seen that unlike other oils which contain mainly glycerol ester structures (FTIR peak at 1745 cm^{-1}), the tunicate lipids contain mainly free fatty acids, close to phospholipid (1709cm^{-1}).This is supported by ^{1}H-NMR (Figure3)where the glycerol content is less than 10% of the total fatty acid contents.

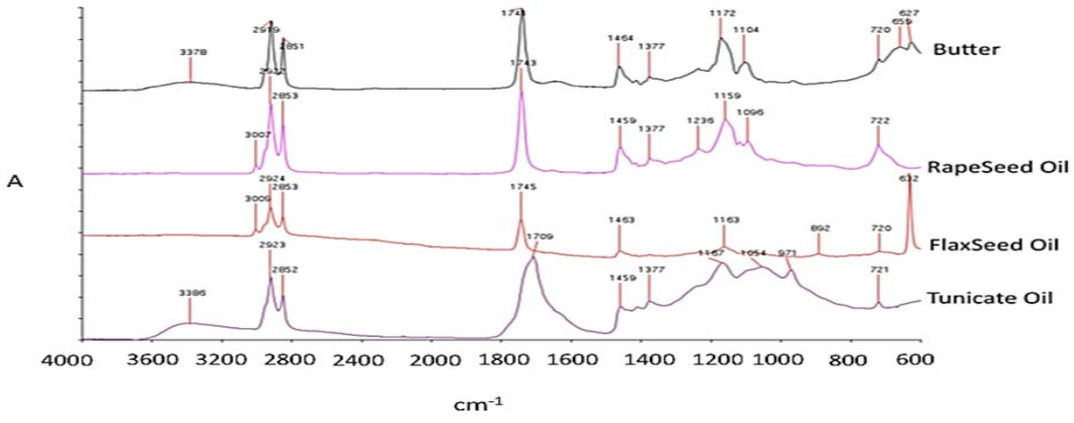

Figure2. The FTIR spectrum of tunicate oil and other feedstocs

^{1}H-NMR spectroscopy gives information about classes of unsaturated and saturated fatty acids. Based on the integral values from the ^{1}H-NMR spectra(Figure 3) the composition of tunicate oils was estimated on two classes: unsaturated and saturated. There were a lot of free fatty acids floating around inside tunicate oil. Glycerol part of the oil shows in around 4.2 ppm which is very low as it has been proved by FTIR. The

hydrogen adjacent to multiple double bonds shows the signal at 2.8 ppm indicating the presence of highly unsaturated lipids (Bratu et al., 2013). High content of free fatty acids together with very low content of glycerol (which is a byproduct of biodiesel) can be considered as advantage for biodiesel production (Yang et al., 2012).

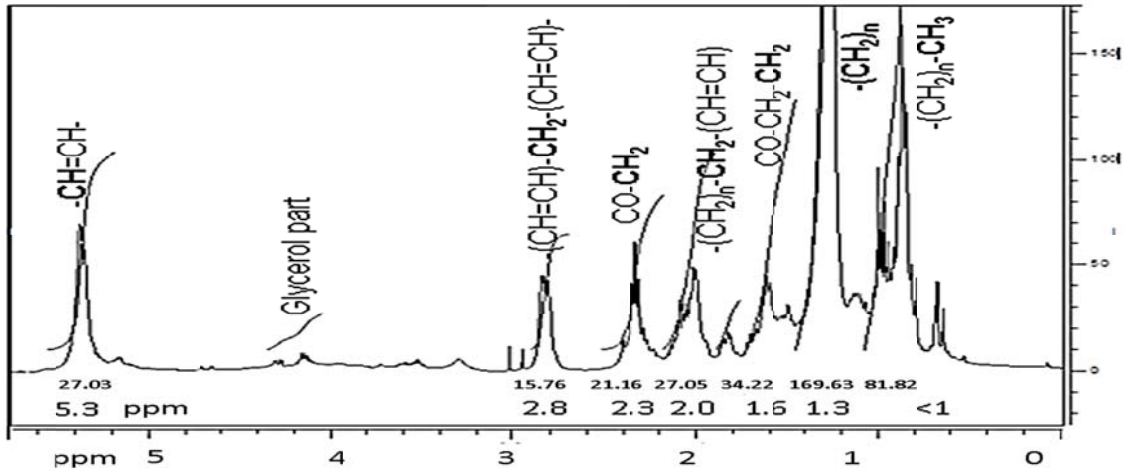

Figure 3: ^{1}H-NMR of tunicate oil

Gas-chromatography coupled with mass spectrometry was used to determine the composition of fatty acids present in tunicate in a comparison with fish oil (Figure 4). According to the figure, it can be seen that tunicate lipids have a fatty acid profile very similar to fish oil. Around fourteen individual fatty acids were identified using GC-MS technique. The n3 symbol indicates that the fatty acid is an omega-3 fatty acid which plays important roles in the human body, such as in the synthesis of specific active compounds, in the brain and eye development of infants or in reducing cholesterol and thus in the prevention of the coronary heart disease (Caponio et al., 2011). As it can be remarked from Figure 4, tunicate oils have a very high amount of ω-3 polyunsaturated fatty acids especially EPA (20:5 ω3; eicosapentaenoic acid) and DHA (22:6 ω3; docosahexaenoic acid), which are the two principal ω3 fatty acids in marine oils. As we expected, small amounts of ω-6 fatty acids have also been determined. (C22:5)

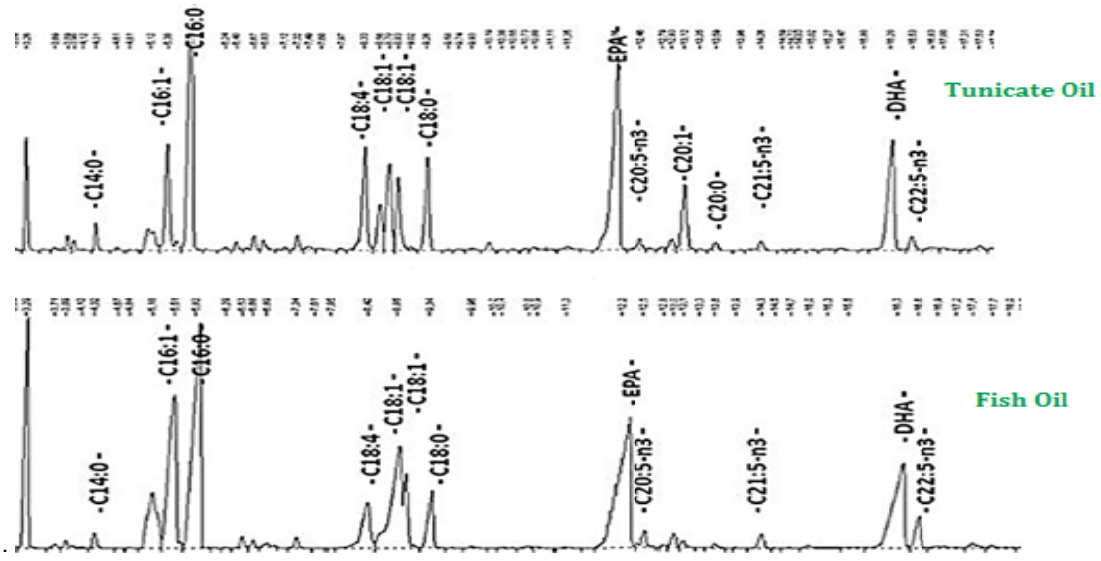

Figure 4: GC chromatography of Tunicate lipids in comparison with fish oil.

4. Conclusions

From tunicate sample harvested from Norwegian coast, the capability for preparation of crystalline pure cellulose which is capable for productions of cellulose whiskers has been shown. Three step treatment (H_2SO_4-NaOH-NaOCL) has proved to have a good combination of a significantly high cellulose percentage (96%) and a high protein removal percentage when aiming at cellulose extraction. Additionally, more concerns can be paid on protein and polysaccharide degradation during the biofuel production by a better possible route of combinations of chemical pretreatments. The oil components inside tunicate showed to be different from plant oils. The fatty acid composition looks more similar to fish oil and even with similar abundance of various types of fatty acids. Using GC-MS technique fourteen individual fatty acids present in tunicate oil samples were identified , while based on ^{1}H-NMR spectroscopy the composition of fish oils was determined on two classes of fatty acids (unsaturated as total-of them ω-3 and DHA individually- and saturated). The ratio of most tunicate amino acids over the egg albumin is all around 1 or more, suggests a good potential to be exploited as feed for animals.

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