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NANYANG TECHNOLOGICAL UNIVERSITY

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A thesis submitted to the Nanyang Technological University
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ABSTRACT

The Chlorella provides possible source of high levels of carbohydrates. Two methods (conventional solvent extraction and macroporous resin based extraction) of extracting carbohydrates from Chlorella were studied. The effects of three factors each were investigated. The results showed that conventional solvent extraction is likely to extract more non-soluble carbohydrates, while macroporous resin based extraction is more efficient in extracting soluble carbohydrates. The carbohydrates extracted were measured by anthrone-sulfuric method.

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1 INTRODUCTION

More than 70% of the earth is covered by oceans, the marine organisms which have a great diversity, have long attracted human beings. Belonging to one of the oldest classes of organisms on earth, marine algae are found to contain abundant amount of biological active compounds of different uses. The remarkable development in the field of application of algae, which is also referred to as seaweeds, in the past decade has raised great public attention. Algae, which can perform photosynthesis just like common plants on the basis of their own chloroplast, are usually divided into two categories: the prokaryotic and the eukaryotic. Up till now, over 3,000 species have been found, among which 70% belong to microalgae. Microalgae, also known as microphytes, are usually referred to, as is shown in the word's spelling, microscopic algae, usually found in marine systems, as well as in freshwater. Microalgae have tiny cell with diverse appearances and great resilience. The first use of microalgae can be dated back to more than 2,000 years ago, by the Chinese people. It is said that Su Wu lived on hair-like seaweed, a kind of Nostoc, when shepherding for the Huns. But the artificial cultivation has only a history of a few decades. All the microalgae that can be put into the application of industrial production belong to the four following phyla: Cyanophyta, Chlorophyta, Chrysophyta, and Rhodophyta. [1]

The manufacture of biological products from microalgae has several advantages. It utilizes solar power, which is highly recommended as a new clean promising energy in the future, as well as CO2, which is commonly believed to be responsible for global warming. Thus, this method can be sustainable as well as environmentally friendly. What

is more, the production is of high efficiency, independent from the productivity of the soil—even the most infertile soil or saline-alkali soil is suitable—easy to be brought into the “assembly line”, needless of complicated pre-treating, and result in various kinds of useful bioactive products.

1.1 Three economical microalgae

Microalgae can be applied to industries of energy, medicine, food, aquaculture, chemistry, environmental protection, agriculture, and aerospace, and so on. They have the ability to be used to produce biofuel to remit energy shortage, bait to be applied in aquaculture, active or supplementary constituent of usual and functional food or even medicines, for instance.There are three species of microalgae that can be recognized as economical: Spirulina, Chlorella, and Dunaliella.

Spirulina [Fig.1A], one of the species with longest history existing in current earth, is the most outstanding resource of natural protein to this day. [2] “There is a need for both national governments and inter-governmental organizations to re-evaluate the potential of Spirulina to fulfill both their own food security needs as well as a tool for their overseas development emergency response efforts”- The UN-Food and Agriculture Organization (FAO) Report on Spirulina 2008. There is also a great amount of vitamins, mineral substance and other essential nutrients in Spirulina, including potassium, calcium, and gamma-linoleic acid, all of which is superior to traditional natural sources like carrots, pork liver and fish. To be even better, Spirulina contains only about 5% fats by weight, without cholesterol. [3]

Dunaliella [Fig.1B] is seen to be orange most of the times owing to the plenty of carotene inside its cell. This kind of microalgae is more likely to survive strict conditions. A news species of Dunaliella was even found living in one of the most extreme environment on earth, Atacama Desert in 2010. [4]

Chlorella [Fig.1C] was first discovered by Doctor M. W. Beyernick, who connected the Greek word “chlor” and Latin word “ella” to name this very kind of single-cell green algae, over a hundred years ago. Tiny as it may be, its photosynthetic efficiency is believed to be comparable with other efficient plants (sugar cane, for example), reaching 8% in theory. [5]Since it is the very three general processes--photosynthesis, respiration, and biosynthesis--that determine the organic composition of a plant, Chlorella was once considered to be able to replace regular stable food like wheat and rice. At the same time, it also consists of attracting essential nutrients such as carbohydrate, minerals and vitamins, among which the protein rate is significantly high. [6] Thus, utilizing Chlorella as potential food resource has been studied, following global fear of food and energy shortage due to the predictable boom of population.

Despite of the similar character that both can be regarded as “super food”, Chlorella do share much in common with Spirulina, however, differences between them can be easily told out. What is the most fundamental difference is that, as is mentioned above, Spirulina is eukaryotic while Chlorella is prokaryotic. Spirulina has been given attention since the very beginning for its high potential to act as functional food, on the other hand, Chlorella was hoped to deal with starvation when the world had hardly recovered from the WWⅡ. Chlorella, unlike Spirulina, has not yet been fully developed nowadays, nevertheless, it has been brought back to focus—since the global

food shortage of the sub war world has been solved before economical Chlorella products came out, a lot of scientists have removed their eyes to other areas. One of the new discover of Chlorella's potential value is that it may contain antitumor elements. [7] Another even more impressing study deals with human being directly, not normal ones but the pregnant. 151 healthy pregnant women was involved in that study, and after the data analysis, it is quite clear that the group having taken Chlorella tablets was generally low in the rate of dioxin transferred into fetuses and nursing infants through placenta. [8]

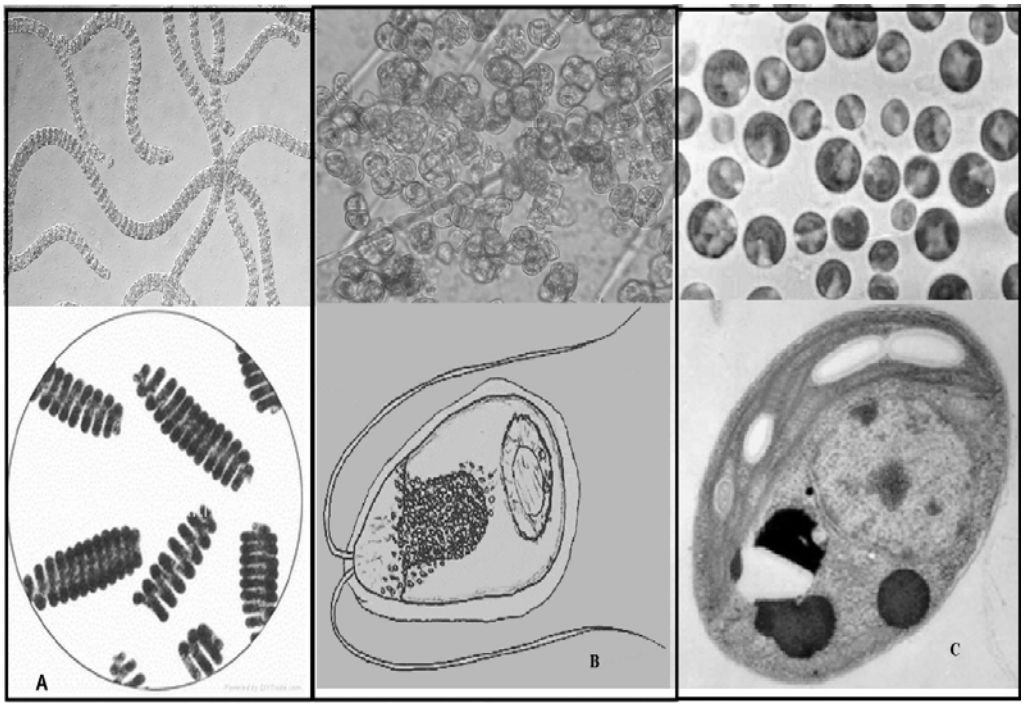

Fig.1 (A) Spirulina under optical microscope (above) and models for their cells (below); (B) Dunaliella under optical microscope (above) and model for its cell (below); (C) Chlorella under optical microscope (above) and model for its cell (below). [9]

In 2003, a comparison between the three nourishing microalgae, which is Spirulina platensis, Chlorella vulgaris, and Isochrisis galbana, was carried out by Ö. Tokusoglu and M.K. Ünal [9]. They used a modified version of the Bligh and Dyer method to determine

the lipids of samples, Kjeldahl protein units to determine crude protein in proximate analysis, all of which was done in triplicate to minimize errors. After the statistical analysis, they suggested that the three kinds of microalgae contains similar rate of stearic acid, while for other nutrients they all have their own advantages. Spirulina leads in the rate of linoleic acid, followed by Chlorella, but for arachidonic acid, Isochrisis is the champion, for example.

1.2 Bioactivity of polysaccharides extracted from microalgae

Without full understanding of all the complicated useful molecules Chlorella may contain, this study is mainly focused on extraction of polysaccharide. Polysaccharide is another bioactive macromolecule that is essential to all cells, apart from protein and nucleic acid. Inside living creatures, polysaccharide is most likely to be found in terms of glycoprotein, glycolipid and proteoglycan. Polysaccharide is highly involved in vital processes. Studies [10-11] have indicated that natural active polysaccharide plays a role of reinforcing agent to the immune system.

Polysaccharide is most capable to carry biological information due to its perfect potential for structural variability, compared with nuclei acids and proteins. That may be the reason that study involving it has been left far behind others. [12] Even worse, most of the traditional studies involving polysaccharide are laid on terrestrial organisms, kelp and red algae, few on microalgae, especially on Chlorella, for it is believed that Chlorella do not contain as much polysaccharide as the species being studied. However, polysaccharide in Chlorella has been demonstrated to have unique benefits inside organic, in acceptable amount. Thus, extraction of polysaccharide from Chlorella is still worth

studying. Chlorella contains various kinds of polysaccharide with different structures which leads to different biological activity. Early in 1972, the very first information about structures of the polysaccharide extracted from Chlorella was reported, though lack of the exact of the structure, and they only isolated some acid after complete as well as partial acid hydrolysis. [13] Just one year later in 1973, one reticuloendothelial system, containing both galactose and arabinose at relatively low degree, was isolated and proven to have the ability to activate polysaccharide. [14] One kind of extracellular polysaccharide, of which arabinose (32.5%) and glucuronic acid (38.3%) are the main components was later isolated from Chlorella. [15] When the isolation of a polysaccharide from Chlorella was reported in 2001 [16], it raised heated discussion since this very polysaccharide was proved to have a potent effect on activating human monocytes/phagocytes. Even in the US patent database, there are also discoveries about extracting polysaccharide from Chlorella. Jaroslav A. holds both the two patents [17-18] of extraction of a kind of hetero-polysaccharide of high viscosity from Chlorella, consisting of uronic acids, rhamnose, mannose and arabinose, and the isolation of extracellular polysaccharide from Chlorella, 80% of which is galactose. As for some new studies, Ogawa et al. [19-20] isolated 2-O-methyl-l-rhamnose, 3-O-methyl-l-rhamnose, 3-O-methyl-d-galactose and mono-O-methylated sugars, yet still low in amounts due to its low content.

1.2.1 Antitumor activity

Because of the increasing global concern about tumor these years, the antitumor activity of the polysaccharide has received most of the attention paid to Chlorella

constituent. Ever since as early as in 1985, Iwao Umezawa, along with Kanki Komiyama, has held a patent [21] claiming that a certain acidic polysaccharide, of which 53% consists of a carbohydrate contain via calculating as glucose to have antitumor activity and even antiviral activity. Although it is still not completely understood how normal cells turned to form tumor, a series of hypothesis has received global proof that free radicals can induce the formation directly. Ever since sulfated polysaccharides from Chlorella have been demonstrated to have the ability to scavenge free agents and prevent oxidative damage, they have gained expectation in developing drugs that can be applied in the treatment of cancer, though no one hold the least hope for them to directly reduce the amount of cancer cells. Studies [22-23] have suggested this expectation to be of considerate possibility, for when tested in vitro, they have significant effect of antiproliferative activity on cancer cell lines and inhibiting the growth of tumor in mice. What is even more impressing, the polysaccharides are likely to isolate cancer cells from their basement membrane, thus able to inhibit the metastatic movement of the cancer cells, though the exact mechanisms of their inhibition has not yet been completely understood. [24] But are the polysaccharides only effective when injected? Luckily, that is not exactly the case. In 1986, Yamamoto et al [25] gave mice oral polysaccharides extracted from various kinds of marine microalgae, and concluded that the decreases in the rate of carcinogenesis have statistical significance.

1.2.2 Immunomodulating activity

Polysaccharides also have immunomodulating activity, mainly on the basis of macrophages modulation. In 2006, Fangmei Y. et al [26] used mice to perform an in vitro

experiment to discover the immunomodulatory activity of polysaccharides from Chloride pyrenoidosa, when partially purified. They concluded that significant rate of B cell was activated by polysaccharides from Chlorella to produce antibody, meanwhile the T-dependent antibody response has an increased sensitivity, which was assumed to be achieved also by the polysaccharides from Chlorella through their additive action with macrophages. Because of the fact that it is well proved that all of the B cells, T cells and macrophages are essential and active elements in the immune system, that study may suggest that polysaccharides from Chlorella have active effect on the system. And this regulation of immune system has been thought to be the main focus for Chlorella when considering about making functional food out of it.

1.2.3 Antiviral activity

Polysaccharides are even thought to have antiviral activity. Huheihel et al [27] found that when highly sulfated, the polysaccharides will show antiviral activity. Several kinds of polysaccharides with different structures have been found to be able to inhibit various kinds of virus, which belong to herpesvirus, togavirus, favivirus, rhabdovirus, arenavirus and orthopoxvirus families [28], though from many species of marine microalgae, not only Chlorella. There have been experiments [29-31] indicating that many elements of the chemical structures may affect the activity, molecular weight and the degree of sulfating, for example. The degree of sulfating especially, is the most crucial active parts of the inhibition for virus, thus, when the degree is not high enough, the activity is likely not so efficient to be applied to real life. [30] Of the entire pathogenic virus group, the Human immunodeficiency virus type-1 (more popular known

as JIV-1) is the most notorious, for it may lead to the serious disease Acquired immunodeficiency syndrome (AIDS). AIDS is such a frightening that it infected over 33million worldwide, calculated till the year of 2008. [32] Not long after the first anti-HIV drugs came out in early, have more than 50% infected people suffer from the drug-resistant strains of virus. [33] This results in increasingly interest in developing a new type of antivirus drugs which can inhibit a wide variety of virus, not only in the pharmaceutical industry but also in the biology and medical field. It is because of the belief that drugs produced from natural compounds is likely to provide more efficiency with less side effects that more and more scientists lay their hope of finding a new generation of anti-HIV drugs on natural compounds and their derivatives. [34-36] Thus polysaccharides extracted from Chlorella may be an alternative in the future. Human beings are the only host for herpes simplex virus, which would lead to herpes simplex, a kind of disease not very serious but hard to treat. Luckily, polysaccharides extracted from seaweeds have been proved to have antiviral activity against HSV-1 and HSV-2 (the virus that are thought to cause herpes simplex), of which the details will be provided below with references. Polysaccharides mainly formed by galactose extracted from Chlorophyceae (Codium fragile [37] and Caulerpa racemosa [38]) have significant EC50 (4.7 and 3.0) on inhibiting HSV-2. Whereas the ones from Phaeophyceae (Sargassum horneri [39], Sargassum patens [40]) have effect on HSV-1, with EC50 at 1.0 and 1.5, but those from Undaria pinnatifida [43] inhibit HSV-2. Polysaccharides from Rhodophyceae have quite different main compounds, galactose for Cryptonemia [41] and mannose for Nemalion helminthoide [42], which may be the cause their different performance on inhibiting HSV-1, yet both can be considered to be effective. For brown seaweeds,

Witvrow et al [28] reported their polysaccharides, mainly consist of fucoidans, have antiviral activity against all of HIV, HSV-1, HSV-2, and even cytomegalovirus, which might lead to cytomegalovirus pneumonia when infected. What is more, the enveloped virus, such as HIV and herpes, will be inhibited by polysaccharides derived from seaweeds when they are trying to break into the cells of the host. Other algal fractions derived from seaweeds are not useless. Reports [43-46] have suggested that they have the ability to inhibit the formation of syncytium or enzyme or virus directly. There is a hypothesis that the sulfate groups are responsible for the activity of the inhibition against virus, for Witvrouw et al [28] reported a serious of figures indicating that when the degree of sulfation increase, the activity will also increase and it is widely agreed that anionic charges are likely to inhibit reverse transcriptase enzyme. Most of the studies are made in vitro. Hence the time when the polysaccharides are added may also affect the efficiency, and this has been proved by experiments [47] dealing with extraction from Splachnidium rugosum, Gigartina atropurpurea, Plocamium cartilagineum and Undaria pinnatifida, against HSV-1 and HSV-2. They suggested that the best time to add is during the first hour after infection, and the efficiency may fall to insignificant when added later. Flavivirus is a great family of virus, also known as arbovirus, containing plus-strand RNA and membrane. The family has many kinds of dangerous virus such as epidemi cecephalitis B, Japanese encephalitis and tick-borneencephalitisvirus (also known as Russian spring-summerencephalitis virus). Among them, dengue and yellow fever viruses have been inhibited both in vitro and in vivo by polysaccharides extracted from red seaweeds. [41, 48] But, until nowadays, there have been no licensed vaccination or anti active agents against dengue virus can be applied in clinic. Fucoidan, a kind of

polysaccharides extracted from brown algae, may have the potential to inhibit the dengue virus inside the patient having got infected via type 2 methods. [49] And it also contribute to the potential for this kind of polysaccharides to be developed as a new type of drugs against dengue virus that then bond between the virus envelope glycoprotein and the polysaccharides are exclusive. What is more, studies [50] also suggest that polysaccharides also provide a perfect potential to be applied in vaginal antiviral treatment without the least possibility to harm the normal bacterial flora and the vaginal epithelial cells.

1.2.4 Anticoagulant activity

Despite the discovery and wide application of heparin, investments on finding new type of anticoagulant drugs are still one of the pharmaceutical focuses, owing to the fact that though has been used in clinical for over fifty years, heparin has several no negligible side effects, including hemorrhagic effect, certain ineffectiveness and incapacity. [51] Even worse, it requires great experience for the doctors to carefully control the dosage. As early as in the year of 1913, Killing et al. [52] reported their discovery of natural anticoagulant properties from the marine organisms. Reports [53-55] about the possibility of marine algae to be used as sources for novel anticoagulant drugs followed. Up till now, anticoagulant has been one of the most popular properties of polysaccharides extracted from the marine algae. [56,57] And polysaccharides extracted from Chlorophyceae, Phaeophyceae, Rhodophyceae, have been proved to have anticoagulant activity, [58-74] and the polysaccharides have different main compounds, including rhamnose, arabinose, glucose, gualactose and fucose, among which two types of polysaccharides, galactans [69,

70,72,74] and fucoidans [75-77], have significant high anticoagulant activity. However, the polysaccharides derived from Chlorella, or even from the entire green algae, are relatively fewer. It was found in 1995 that one subspecies of Chlorophyceae contain anticoagulant-active polysaccharides. [78] Later, Matsubara et al. [63] reported the discovery of another kind of polysaccharides from Codium cylindricum. Moreover, Monostroma nitidum is also found to be able to produce anticoagulant polysaccharides, which is even more active than heparin. [56] But when calculated in general, the polysaccharides derived from red and green algae are lower in the anticoagulant activity than the ones from brown algae. [79] The activity of polysaccharides discussed above has been measured by the following methods. APTT stands for activated partial thromboplastin time, its prolongation is said to be a criterion of the clotting-time dependent on intrinsic pathway. Whereas PT stands for prothrombin time reveals the clotting-time dependent on extrinsic pathway. And TT is short for thrombin time and is considered to be relevant to the inhibition of fibrin polymerization. It has been reported [80] that the function of presence of sulfate groups in polysaccharides is even related to the activity of anticoagulation due to their increase in both non-specific and specific binding to certain proteins with considerable biological activity. Silva et al. [81] studied the critical structures determining the anticoagulant activity in polysaccharides, and suggested that they are the sulfation position and the sugar residue. This completed the study of Pereira et al. [82], which indicated that the O-sulfated 3-linked α-galactans have antithrombin activity. And the molecule weight and sulfate content can also determine the anticoagulant activity. [83]

1.2.5 Other activity

Polysaccharides from marine algae have other biological activities. Fucoidan is the studied most deeply. There has been proved that fucoidan from brown alga is able to increase the differentiation of osteoblastic cell, thus fucoidan can be used as a potential supplements for bone health. [84] Furthermore, it is also able to protect gastric mucousa in acid environment. [85] The matrix metalloproteinase (MMP) is thought to be responsible for protective system in human skin. Therefore, when fucoidan provides the ability to inhibit ultraviolet B (UV B), it is considered to be potential functional compounds in skin cosmetics as sun blockers. Other kinds of polysaccharides also demonstrate impressing ability to be applied in the food, pharmaceutical and cosmetics industry. Reports [86] have shown that one of the efficient compounds that can reduce cholesterol levels is dietary fibers, which can be extracted from ulvan, another kind of polysaccharides derived from marine algae [87,88]. This very kind of polysaccharides also provides potential ability to treat bile-related disorders. [89] Porphyran, another kind of polysaccharides derived from red alga is also very useful. It is proved to have capability of being used as efficient anti-hyperlipidemic agent [90] and cardiovascular treatment, due to its ability of reducing apolipoprotein B100 [91-93]. This apolipoprotein B100 is not only correlated to in vivo lipid synthesis but also cardiovascular diseases. [94]

The application of polysaccharides also relies on the efficiency of extraction as well as a great variety of other factors, and is still not available for manufacturing up till now. This study is aimed to discover a more propitiate method for extraction of polysaccharides from a certain species of Chlorella, or carbohydrates, and the conditions in which the method will provide the highest efficiency. Conventional solvent extraction

is a kind of method which is easy and of low cost, thus widely applied in manufacturing industry in other extractions. In this study a new designed method based on a kind of macroporous resin, XAD-4 is compared with conventional solvent extraction. And each of the method is tested in different conditions in order to avoid the misunderstanding that the low efficiency of certain method is caused by poor matched conditions and decide the better conditions for each method in addition. In this study a productive species of Chlorella was chosen as the raw material and anthranone-sulfuric acid method was chosen to determine the carbohydrates having been extracted through certain method due to its high reliability which has been proved by its long history. Therefore, this study may possibly give some advice on manufacturing of polysaccharides derived from marine algae, especially Chlorella.

2 EXPERIMENTAL PROCEDURES

2.1 Material and equipment

Chlorella used was the second generation of the origin, which is American Type Culture Collection (ATCC) 14854, cultured in another laboratory of the university, originally supplied from American Type Culture (ATCC^{TM}).

All the chemicals involved are D-(+) Glucose, anthrone, sulfuric acid, perchloric acid, ethanol (95% by volume), Amberlite XAD-4, all of which were of analytical reagent, and supplied by Sigma–Aldrich (St. Louis, MO, USA), expect distilled water provided by the laboratory, and corn oil supplied by Fair Price, Singapore.

Three types of centrifuges were used: large bench centrifuge, Thermo Sorvall Legend XTR; middle bench centrifuge, Sigma 3-16; microcentrifuge, Sigma 1-14. The spectrophotometer used was BioSpec-mini provided by Shimadzu, Japan. The three kinds of heaters used are MH-1, belonging to HS/GHS Series, from MRC laboratory equipment, Israel; dry bath incubator from MRC laboratory equipment, Israel; BH-100 from MRC laboratory equipment. The balance involved was AM320 from Shimadzu Cooperation, Japan. The freeze-dry system benchtop supplied by Labconco Corporation with the model of 2.5L. The system used to control the gas speed was supplied by Cole Parme. The required low temperature was achieved with both normal medical and extra low freezer supplied by Sanyo, Japan.

The 50ml, 15ml, 2ml centrifuge tubes were supplied by Greiner Bio-one. And so were the 5000ml, 1000ml, 200ml, 100ml pipettes along with their tips. The test tubes were supplied by Sigma.

2.2 Characterization and preparation of raw materials

2.2.1 Preparation for Chlorella

The Chlorella used in conventional solvent extraction is in the form of dried powder. The culture solution was first centrifuge at 4,000 rpm for 15 minutes. The sediment was washed with distilled water twice, each time isolated in the centrifuge at 4,000 rpm for 10 minutes. Then the sediment was lyophilized to get the dried powder.

The Chlorella used in macroporous resin based extraction is in the condition of suspension. 900ml culture solution, the same as the solution mentioned above, was also centrifuge at 4,000 rpm for 15 minutes. The sediment was collected and added distilled water to 1000ml to form the suspension needed.

2.2.2 Preparation for related chemical solution

The ethyl alcohol used was in the standard form of 80% by volume. The perchloric acid used was 30% by mass. The anthrone-sulfuric solution was 2g/L, which is prepared by adding 2g of anthrone (dry powder) into 1L 98% sulfuric acid, and this very solution requires being prepared the day it is intended to use. The concentration of Chlorella was measured by first heating 2ml of the original liquid to dry and weighed accurately.

2.3 Experimental Procedures

2.3.1 Standard Curve

Weigh accurately, according to Tab.1, standard glucose in 8 test tubes, dissolve with 0.3ml distilled water. Add 10ml anthrone-sulfuric solution into the tubes, quickly put them into the boiling water bath, and keep for 10 minutes. Get the tubes out and let it cool down in cold water for about 10 minutes. Test the OD at 620nm. Use the figures gained to create a graph and regression equation (Fig. 2).

Tab. 1 Figures for Standard Curve with standard glucose

Run	1	2	3	4	5	6	7	8
COG*(μg/ml)	0	10	20	30	40	60	80	100
OD**(620nm)	0	0.046	0.111	0.142	0.195	0.309	0.388	0.473

*Concentration of glucose

**Optical density, also referred to as absorbance

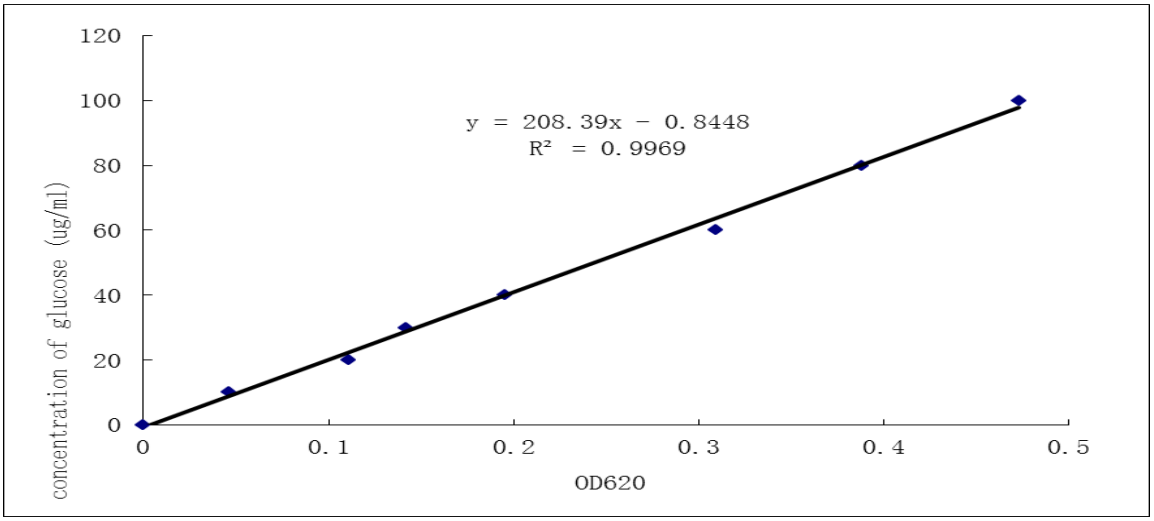

Fig.2 Standard Curve of the OD at 620nm for glucose with regression equation

2.3.2 Extraction Procedures

2.3.2.1 Conventional Solvent Extraction

Conventional solvent extraction was carried out in water bath. About 0.2g each of the powder of Chlorella was weighted accurately and recorded in Tab.2, put into 7 labeled centrifuge tubes and mixed with distilled water. The extraction was carried out referring to conditions in Tab.2. The sediment and supernatant after extraction were separated by centrifugation (8,000rpm, 15 min), and move up to the procedures of analysis of carbohydrate.

Tab. 2 Design for Conventional Solvent Extraction

Run	Mass of Chlorella powder(g)	Extraction time (min)	Extraction temperature (°C)	Ratio of water to raw material (ml/g)
1	0.2046	30	100	30:1
2	0.19075	60	100	30:1
3	0.19725	90	60	30:1
4	0.20145	90	80	30:1
5	0.1947	90	100	30:1
6	0.2045	90	100	20:1
7	0.20155	90	100	40:1

2.3.2.2 Macroporous Resin Based Extraction

Macroporous resin based extraction uses 2g Amberlite XAD-4 in each sample as the main tool to break the cell, and is carried out in a system showed in Fig. 3. The

extraction was carried out according to conditions in Table 3, each sample add a drop of corn oil to reduce bubbles. The speed of gas was measured by calculating the volume of gas in certain period of time using method of draining water. The sediment and supernatant after extraction were also separated by centrifugation (8,000rpm, 15 min), and move up to the procedures of analysis of carbohydrate.

2.3.3 Analysis of Carbohydrate

2.3.3.1 Sediment

Weigh accurately three parallels of about 0.006g from each portion of the freeze-dried sediment, mixed each one with 1.5ml 80% ethanol solution, then keep in water bath at 70° C for 15 minutes. After cooling, centrifuge at 8,000 rpm for 5 minutes. Collect the supernatant separately in labeled tubes and make the sediment repeat the procedure as above with ethanol for twice. Add 1.5ml 30% perchloric acid in each portion and keep them under the condition of continuous vibration for 20 minutes, centrifuge at 10,000rpm for 5 minutes to collect the supernatant in labeled tubes and repeat for twice. After thoroughly incorporated the three batches of supernatant, take 0.3ml out of the supernatant in labeled tubes and mix it with 1.5ml anthrone-sulfuric solution, quickly put into boiling water bath and keep for exactly 10 minutes. After cooling, determine absorbency at 620nm, compare with blank series, which was made before with 0.3ml 30% perchloric acid and 1.5ml anthrone-sulfuric solution and kept in the boiling water bath together with the samples to test.

2.3.3.2 Supernatant

Take 1ml out of each portion of supernatant, dry them up in water bath. Add 1.5ml 30% perchloric acid and keep in the condition of continuous vibration for about 60 minutes, centrifuge at 10,000rpm for 5 minutes to collect the supernatant in labeled tubes. Take three parallels of 0.3ml out of each tube, and mix them with 1.5ml anthrone-sulfuric solution each, quickly put into boiling water bath and keep for exactly 10 minutes. After cooling, determine absorbency at 620nm, compare with blank series, which was made before with 0.3ml 30% perchloric acid and 1.5ml anthrone-sulfuric solution and kept in the boiling water bath together with the samples to test.

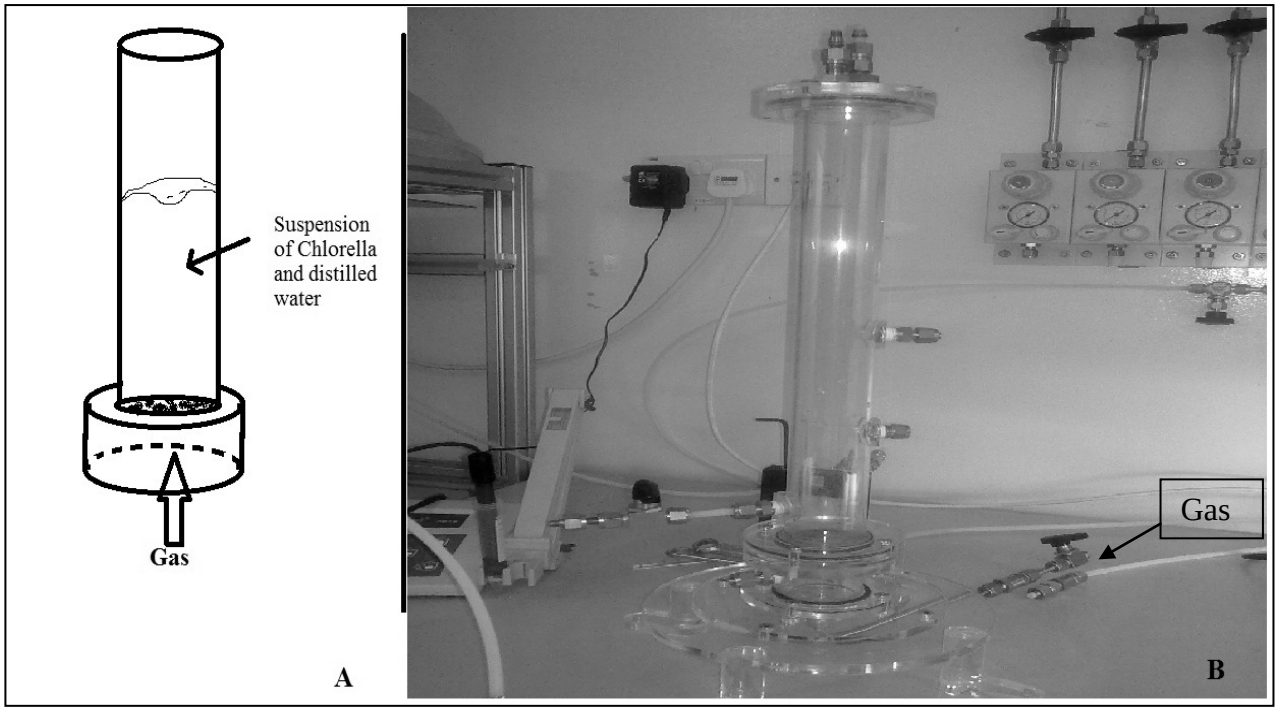

Fig. 3 (A) Sketch map; (B) Object picture of the system for macroporous resin based extraction.

Tab. 3 Design for macroporous resin based extraction

Run	Speed of gas (L/min)	Extraction time (min)	Concentration of sample (g/L)
1	4.37	120	1.0
2	6.12	120	1.0
3	7.65	120	1.0
4	6.12	90	1.0
5	6.12	150	1.0
6	6.12	120	0.5
7	6.12	120	1.5

3 RESULTS AND DISCUSSIONS

3.1 Results

The results were measured by glucose. The figures were recorded and calculated in MSc Excel 2010, and provided in Table 4 for conventional solvent extraction and Table 5 for macroporous resin based extraction.

Tab. 4 Results and standard deviation of conventional solvent extraction

Run	Soluble carbohydrate concentration (g/100g)	Non-soluble carbohydrate concentration (g/100g)	Total carbohydrate concentration (g/100g)	SD-1* (%)	SD-2** (%)	SD-total*** (%)
1	0.20439687	6.46343149	6.66782836	0.00153135	0.10655013	0.10808148
2	0.17063769	6.80395147	6.97458916	0.00375102	0.1675129	0.17126392
3	0.1782752	6.26032843	6.43860363	0.01168244	0.04337338	0.05505582
4	0.18897601	7.30715111	7.49612712	0.00767939	0.15337925	0.16105864
5	0.17590968	8.16730845	8.34321813	0.00234278	0.17757093	0.17991371
6	0.18411131	8.10307975	8.28719105	0.00492567	0.14293503	0.1478607
7	0.21223499	8.84340371	9.0556387	0.01102346	0.04011078	0.05113424

* Standard deviation of soluble carbohydrate

** Standard deviation of non-soluble carbohydrate

*** Standard deviation of total carbohydrate

Tab. 5 Results and standard deviation of macroporous resin based extraction

Run	Soluble carbohydrate concentration (g/100g)	Non-soluble carbohydrate concentration (g/100g)	Total carbohydrate concentration (g/100g)	SD-1* (%)	SD-2** (%)	SD-total*** (%)
1	1.539838	1.37195585	2.91179385	0.09003477	0.1066651	0.19669987
2	2.165008	1.60735685	3.77236485	0.18947071	0.08787921	0.27734992
3	2.831856	1.73265744	4.56451344	0.09003477	0.03843737	0.12847214
4	2.30393467	1.22388755	3.52782222	0.15344952	0.15610431	0.30955383
5	2.65125133	1.27999474	3.93124607	0.13753038	0.05305641	0.19058679
6	1.60930133	0.55248718	2.16178851	0.11950924	0.09301445	0.21252368
7	3.09581667	1.71097214	4.80678881	0.19939728	0.02977	0.22916728

* Standard deviation of soluble carbohydrate

** Standard deviation of non-soluble carbohydrate

*** Standard deviation of total carbohydrate

The graphs were created by Original 8.0 and shown in Fig. 4 for conventional solvent extraction and Fig. 5 for macroporous resin based extraction, each variable separately.

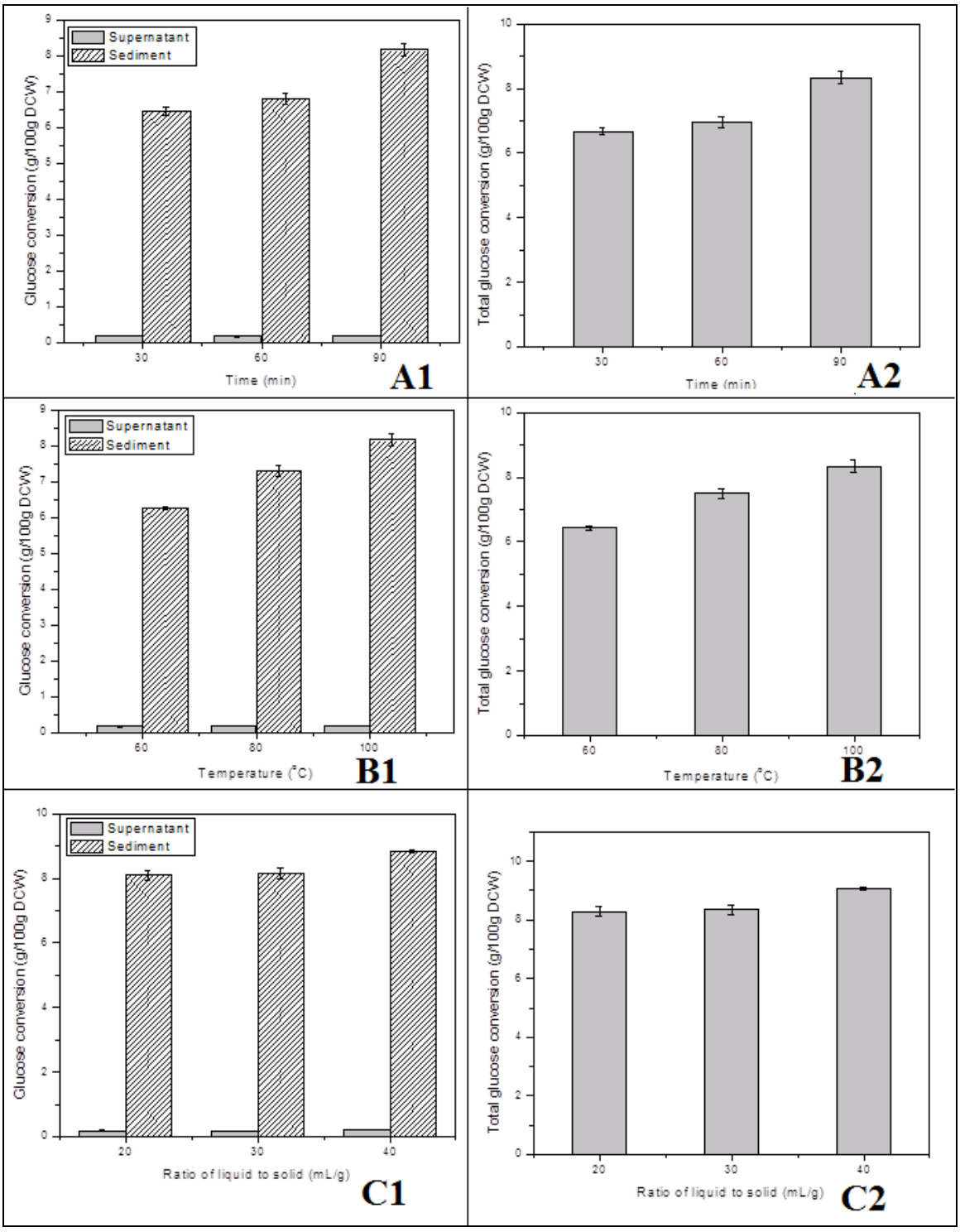

Fig. 4 Graphs for conventional solvent extraction with single variable as (A) time (min), (B) temperature (°C), (C) ratio of liquid to solid (ml/g). The series ended with 1

shows soluble and non-soluble carbohydrate separately and the series ended with 2 is showing total carbohydrate.

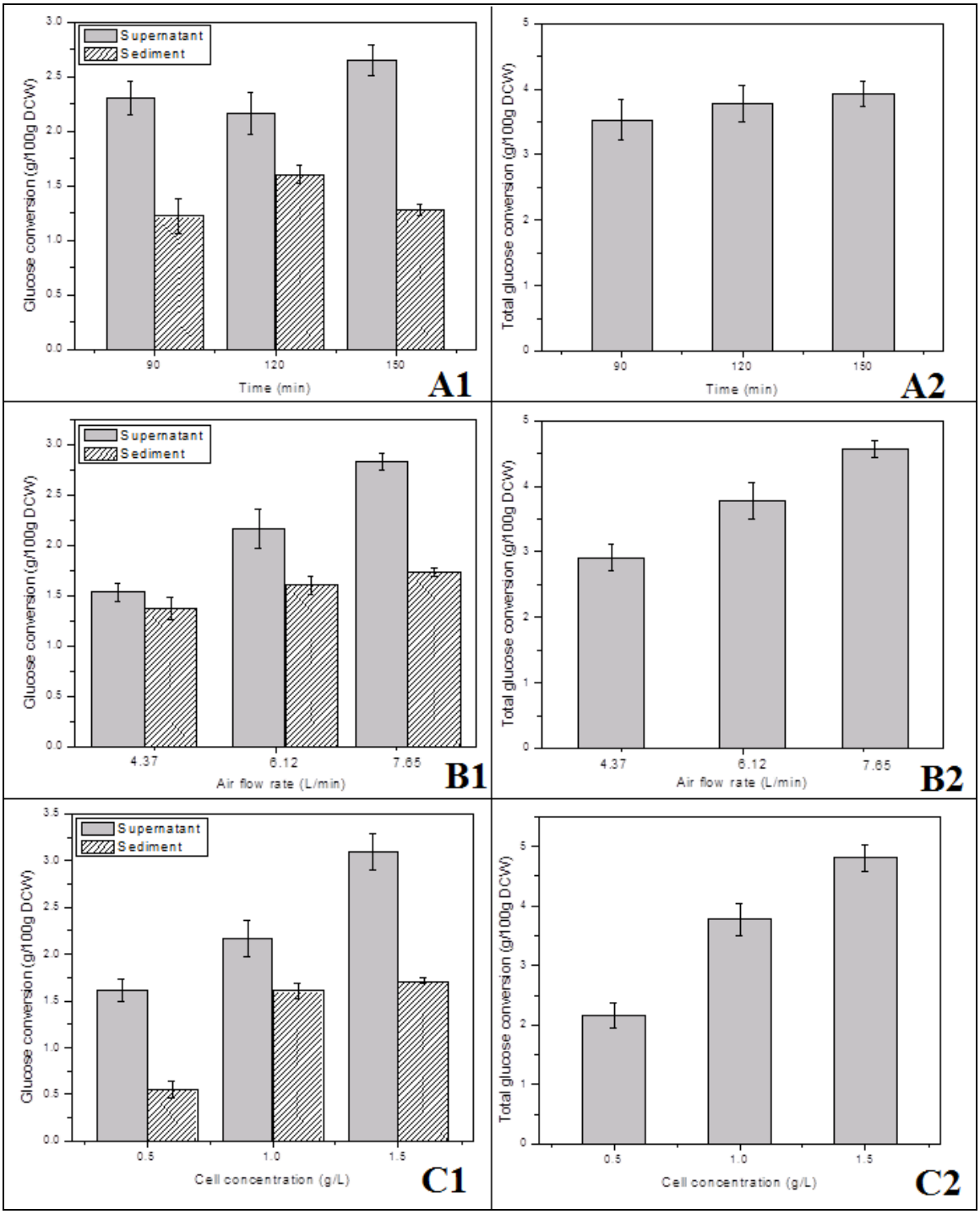

Fig. 5 Graphs for macroporous resin based extraction with variable as (A) time (min), (B) air flow rate (L/min), (C) cell concentration (g/L). The series ended with 1 is showing soluble and non-soluble carbohydrate separately and the series ended with 2 is showing total carbohydrate.

3.2 Discussions

3.2.1 Conventional Solvent Extraction

The results indicate that the efficiency of conventional solvent extraction is in proportion to time, temperature of extraction as well as ratio of water to raw material.

3.2.2 Macroporous Resin Based Extraction

The results indicate that the efficiency of macroporous resin Based Extraction is in proportion to speed of gas, time of extraction, and the concentration of sample.

3.2.3 Discussions

The Table 6, which has been taken from the report [96], shows the estimation of nutrients contained in algae generally. Taken into account that the concentration of every single kind of nutrients contained in individual cell greatly depends on the specie and the environment conditions, figures presented in Table 6 are according to estimates.

Belonging to plants, Chlorella has cell wall to protect each of its cells, which is the main obstacle for process molecules, no matter the nutrients or other biological active molecules, out of the cells, since the cells tend to keep these molecules inside instead of excreting them out. In order to extract the valuable nutrients out of Chlorella, appropriate methods for cell rupture are required, not only in laboratories but also in commercial run.

To select the appropriate method, several main factors need to be taken into consideration; how big is the sample size, how stable is the molecules needed, what are the action needed to be taken after the extraction, how efficient is the method of extraction required.

Tab. 6 General composition of different human food sources and algae (% of dry matter) by estimation [96]

Commodity	Protein	Carbohydrate	Lipid
Bakers' yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Anabaena cylindrica	43-56	25-30	41006
Chlamydomonas rheinhardili	48	17	21
Chlorella vulgaris	51-58	41260	14-22
Dunaliella salina	57	32	6
Porphyridium cruentum	28-39	40-57	41166
Scenedesmus obliquus	50-56	41199	41257
Spirulina maxima	60-71	13-16	41067
Synechococcus sp.	63	15	11

Enzymatic method is a kind of method currently only suitable in laboratory, since the enzyme usually requires special environmental conditions and lacks enough stability to be applied in commercial run. The enzymes that are currently widely used in cell disruptions are lysostaphin, lysozyme and special enzymes.

Another method for cell disruption uses bead, either made of glass or ceram. This is usually pure physical method. To perform such extraction, a system which is able to provide agitation or shake at a level high enough is required. This method is suitable to a wide variety of samples due to its non-chemical mechanism, and when the system has enough energy, this method is able to disrupt cells into very small pieces, thus the efficiency is definitely high.

Recently, though not so new, the method based on sound, especially ultrasound has become more and more popular. This method can also be referred to as “sonication” in general. This method applies sound, usually ultrasound energy to break cell walls and thus extract certain chemicals out of cells. Clean and of high efficiency as this method may be, the disadvantages are not ignorable. The noise caused in this method is so loud that most of the systems require hearing protection to be taken when the process is going on. And because of the high efficiency, the method may extract some unwanted free radicals, which will take reaction with the wanted chemicals and reduce the rate of product.

Cell disruption based on the application of detergent is another popular method in laboratories. This method greatly relies on the cell types, since it uses detergents to break the lipid barrier. The detergents applied in this method are usually not the ionic detergents; since this kind of detergents is likely to denature proteins thus destroy the required proteins. However, even the lowest-destroy detergent has its disadvantages. The cost is relatively high and the choice of detergent requires strong accuracy which can only be achieved through large amount of experiments. Thus this method is usually combined with other methods like mechanical grinding.

Rapid decompression can also bring the goal of cell disruption come true in laboratories. This method is usually described iconically as “cell bomb”. Amazing as it may sound, this method is not widely used due to its significant disadvantages: it can only disrupt those “weak” cells, which are cells without or with tender cell walls; the machines involved in this method have limited capacity and not capable for large scale of samples; the experimenters have higher risk of getting injured than other methods.

Many of the nutrients having been found are likely to become inactive during serious conditions. That is why to extract bioactive nutrients out of cells requires non “harmful” methods.

XAD-4 is a kind of famous macroporous resin, which has been used for more than ten years, especially to isolate lower-molecular-weight hydrophilic acids, and has achieved high reputation for its stable quantity as well as good efficiency. Owing to its great reputation in the field of nutrient extraction, this study aimed to test its capability of applying in carbohydrate extraction. The results indicate that though not as efficient as the traditional solvent extraction in general, the XAD-4 has its advantage in extracting soluble carbohydrates. Taking into account the fact that the non-soluble carbohydrates tested in this study may partly is the product of hydrolyzing cellulose, as well as the fact that XAD- 4 does not break cells in general; the efficiency of traditional solvent extraction may not be as high as it showed in this study.

4 CONCLUSIONS

There are two main goals of this study when this study was first designed. The main goal is to find a new method of extraction involving no or little harmful chemicals. A widely-applied kind of macroporous resin was used to test if it is capable to found the new method. XAD-4 was usually used to extract lipid from microalgae. This study was intended to see if this macroporous resin is able to extract carbohydrates as well as lipids. The second goal is to find appropriate conditions for both the new method and the conventional solvent extraction.

Conventional solvent extraction is an easy and harmless method. It has been widely applied in manufacturing field for quite a long time. In this study, it is able to see that the conditions with highest efficiency is equal to expectation, which is high temperature, long extraction time, large rate of liquid to sediments. This can be an additional piece of evidence to prove that the test method in this study is reliable. In this study, the results indicate that the conventional solvent extraction has higher efficiency in extracting non-soluble carbohydrates, which is involved with a kind of distraction that the "carbohydrates" calculated may partially be the hydrolyzate of cellulose. Moreover, outside the laboratory, more elements require carefully consideration, the attrition of the machines and the cost, for example. In conclusion, conventional solvent extraction does its best job in the conditions of highest temperature, longest extraction time and lowest concentration when possible.

Macroporous resin based method, in general, shows poorer efficiency, but significant higher in extracting soluble carbohydrates. Unlike conventional solvent

extraction, macroporous resin based method is more efficient when the concentration is higher. This may due to the fact that this method relies mainly on the friction among the Chlorella molecules. The XAD-4 is one of the most commonly used macroporous resin and is more often used to extract lipids in the extraction from Chlorella. This study was first conducted to see its potential in applying in carbohydrates extraction, however, despite its good performance in extracting soluble carbohydrates, the rate of carbohydrates extracted in general through this method is not very satisfying. Further study needs to be conducted to find a more effective method in extracting both soluble and non-soluble carbohydrates. Thus the best conditions for macroporous resin based method, when XAD-4 is used, are as long extraction time, as high speed the gas is, as high concentration of raw material as possible.

In conclusion, the conventional solvent extraction does not show significant disadvantages compared to the macroporous resin based method in general. But the latter is more suitable for extracting soluble carbohydrates which is possibly more useful in following application due to its complete bioactive structures and substructures.

NOTATIONS

α	Alpha
μ	Miu

REFERENCES

[1] Pauline Spolaorea, Claire Joannis-Cassana, Elie Duranb, Arsène Isamberta, 'Commercial applications of microalgae', Journal of Bioscience and Bioengineering 2006; 2:87-96.

[2] O Ciferri, 'Spirulina, the edible microorganism', Microbiol. Rev. 1983; 47(4):551-551.

[3] A. S. Babadzhanov, N. Abdusamatova, F. M. Yusupova, N. Faizullaeva, L. G. Mezhlumyan and M. Kh. Malikova, 'Chemical Composition of Spirulina platensis Cultivated in Uzbekistan', Chemistry of Natural Compounds 2004;3:276-279.

[4] Clara Moskowitz, "Extreme Microbe Drinks Dew on Spiderwebs to Live" our Amazing planet Sep 20, 2010 < http://www.ouramazingplanet.com/365-extreme-microbe-drinks-dew-on-spiderwebs-to-live.html>

[5] Israel Zelitch, 'Photosynthesis, Photorespiration and Plant Productivity', New York, Academic Press, 1971; 275-275.

[6] Belasco, W., 'Algae Burgers for a Hungry World?', The Rise and Fall of Chlorella Cuisine 1997; 38:608–634.

[7] Miyazawa Y, Murayama T, Ooya N, Wang LF, Tung YC, Yamaguchi N.,'Immunomodulation by a unicellular green algae (Chlorella pyrenoidosa) in tumor-bearing mice.', J Ethnopharmacol. 1988 Dec; 24(2-3):135-46.

[8] Shiro Nakano, Taketoshi Noguchi, Hideo Takekoshi, Go Suzuki, Masuo Nakano,' Maternal-fetal distribution and transfer of dioxins in pregnant women in Japan, and attempts to reduce maternal transfer with Chlorella (Chlorella pyrenoidosa) supplements', Chemosphere (2005); 61: 1244–1255.

[9] Ö. Tokusoglu and M.K. Ünal,' Biomass Nutrient Profiles of Three Microalgae: Spirulina platensis, Chlorella vulgaris, and Isochrisis galbana', Journal of Food Science (2003); 68(4): 1144-1148.

[10] Aurora Galván, Emilio Fernández, 'Transgenic Microalgae as Green Cell Factories', Rosa León (Ph. D.), Aurora Galván, Emilio Fernández, editors. Springer USA, 2007: 102-105.

[11] Yim Joung Han, Son Eunwha, Pyo Suhkneung, et al. 'Novel sulfated polysaccharide derived from red-tide micrualga Gyrodinium impudicum strain KG03 with immunostimulating activity in vivo', Marine Biotechnology (2005); 7: 331-338.

[12] Erick Reyes Suáreza, Jaroslav A. Kralovec, Miguel D. Noseda, H. Stephen Ewart, Colin J. Barrow, Michael D. Lumsden, T. Bruce Grindley, 'Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of Chlorella pyrenoidosa', Carbohydrate Research (2005); 340(8): 1489-1498.

[13] R.C. White, G.A. Barber,' An acidic polysaccharide from the cell wall of Chlorella pyrenoidosa', Biochimica et Biophysica Acta (BBA) (1972); 264(1): 117-128.

[14] Kojima, M.,Shishido, K.,Kobayashi, S.,' A Chlorella polysaccharide as a factor stimulating RES activity' , RES Journal of the Reticuloendothelial Society (1973); 14(2) :192–208.

[15] I. Yalcin, Z. Hicsasmaz, B. Boz, F. Bozoglu,' Characterization of the Extracellular Polysaccharide from Freshwater Microalgae Chlorella sp.', Food Science and Technology (1994); 27(2): 158–165.

[16] Pugh, N., Ross, S.A., Eisohly, H.N.,Eisohly, M.A., Pasco, D.S.,' Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity from Spirulina platensis, Aphanizomenon flos-aquae and Chlorella pyrenoidosa', Planta Medica (2001); 67(8): 737-742.

[17] Jaroslav A. Kralovec, Halifax (CA). Chlorella Composition Having High Molecular Weight Polysaccharides andPolysaccharide Complexes. US Patent No. 6974576 B2, 2005.

[18] Jaroslav A. Kralovec, Halifax (CA). Fractions of Chlorella Extract Containing Polysaccharide Having Immunomodulating Properties. US Patent No. 6551596 B2, 2003.

[19] K. Ogawa, M. Yamaura, I. Maruyama,' Isolation and Identification of 3-O-Methyl-D-galactose as a Constituent of Neutral Polysaccharide of Chlorella vulgaris', Bioscience, Biotechnology, and Biochemistry (1994); 58(5): 942-944.

[20] K. Ogawa, M. Yamaura, I. Maruyama, 'Isolation and identification of 2-O-methyl-L-rhamnose and 3-O-methyl-L-rhamnose as constituents of an acidic polysaccharide of Chlorella vulgaris', Bioscience, Biotechnology and Biochemistry (1997); 61(3): 539-540.

[21] Iwao Umezawa, Kanki Komiyama. Acidic polysaccharide CH-1 isolated from Chlorella pyrenoidosa and the use thereof. US patent No. 4,533,548, 1985.

[22] Rocha de Souza, M. C., Marques, C. T., Dore, C. M. G., Ferreira da Silva, F. R., Rocha, H. A. O., & Leite, E. L.,' Antioxidant activities of sulphated polysaccharides from brown and red seaweeds', Journal of Applied Phycology (2007); 19:153–160.

[23] Ye, H., Wang, K., Zhou, C., Liu, J., Zeng, X.,' Purification, antitumor and antioxidant activities in vitro of polysaccharides from the brown seaweed Sargassum pallidum', Food Chemistry (2008); 111: 428–432.

[24] Rocha, H. A., Franco, C. R., Trindade, E. S., Veiga, S. S., Leite, E. L., Nader, H. B., et al, 'Fucan inhibits Chinese hamster ovary cell (CHO) adhesion to fibronectin by binding to the extracellular matrix', Planta Medica (2005); 71: 628–633.

[25] Yamamoto, I., Maruyama, H., Takahashi, M., & Komiyama, K.,' The effect of dietary or intraperitoneally injected seaweed preparations on the growth of sarcoma-180 cells subcutaneously implanted into mice', Cancer Letters (1986); 30(2): 125–131.

[26] Fangmei Yang, Ying Shi, Jianchun Sheng, Qiuhui Hu,' In vivo immunomodulatory activity of polysaccharides derived from Chlorella pyrenoidosa', Eur Food Res Technol (2006); 224: 225–228.

[27] Huheihel, M., Ishanu, V., Tal, J., & Arad, S. M., Activity of Porphyridium sp.'polysaccharide against herpes simplex viruses in vitro and in vivo', Journal of Biochemical and Biophysical Methods (2002); 50: 189–200.

[28] Witvrouw, M., & De Clercq, E., Sulfated polysaccharides extracted from sea 'algae as potential antiviral drugs', General Pharmacology (1997); 29: 497–511.

[29] Adhikari, U., Mateu, C. G., Chattopadhyay, K., Pujol, C. A., Damonte, E. B., & Ray, B,' Structure and antiviral activity of sulfated fucans from Stoechospermum marginatum', Phytochemistry (2006); 67: 2474–2482.

[30] Damonte, E. B., Matulewicz, M. C., & Cerezo, A. S.,' Sulfated seaweed polysaccharides as antiviral agents', Current Medicinal Chemistry (2004); 11: 2399–2419.

[31] Luscher-Mattil, M.,' Polyanions—a lost chance in the fight against HIV and other virus diseases?' Antiviral Chemistry and Chemotherapy (2000); 11: 249–259.

[32] Artan, M., Li, Y., Karadeniz, F., Lee, S. H., Kim, M. M., & Kim, S. K.,' Anti-HIV-1 activity of phloroglucinol derivative, 6,6'-bieckol, from Ecklonia cava', Bioorganic and Medicinal Chemistry (2008); 16: 7921–7926.

[33] Stoddart, C. A., & Reyes, R. A.,' Models of HIV-1 disease: A review of current status', Drug Discovery Today: Disease Models (2006); 3: 113–119.

[34] Schaeffer, D. J., & Krylov, V. S.,' Anti-HIV activity of extracts and compounds from algae and cyanobacteria', Ecotoxicology and Environmental Safety (2000); 45: 208–227.

[35] Singh, I. P., Bharate, S. B., & Bhutani, K. K.,' Anti-HIV natural products.' Current Science (2005); 89: 269–290.

[36] Tziveleka, L. A., Vagias, C., & Roussis, V.,' Natural products with anti-HIV activity from marine organisms', Current Topics in Medicinal Chemistry (2003); 3: 1512–1535.

[37] Ohta, Y., Lee, J. B., Hayashi, K., & Hayashi, T., 'Isolation of sulfated galactan from Codium fragile and its antiviral effect', Biological Pharmaceutical Bulletine (2009); 32: 892–898.

[38] Ghosh, P., Adhikari, U., Ghosal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B., et al, 'In vitro anti-herpetic activity of sulfated polysaccharide fractions from Caulerpa racemosa', Phytochemistry (2004); 65: 3151–3157.

[39] Hoshino, T., Hayashi, T., Hayashi, K., Hamada, J., Lee, J. B., & Sankawa, U., 'An antivirally active sulfated polysaccharide from Sargassum horneri (TURNER) CAGARDH', Biological Pharmaceutical Bulletine (1998); 21: 730–734.

[40] Zhu, W., Chiu, L. C. M., Ooi, V. E. C., Chan, P. K. S., & Ang, P. O., Jr., 'Antiviral property and mechanisms of a sulphated polysaccharide from the brown alga Sargassum patens against Herpes simplex virus type 1', Phytomedicine (2006); 13: 695–701.

[41] Talarico, L. B., Zibetti, R. G. M., Faria, P. C. S., Scolaro, L. A., Duarte, M. E. R., Noseda, M.D., et al., 'Anti-herpes simplex virus activity of sulfated galactans from the red seaweed Gymnogongrus griffithsiae and Cryptonemia crenulata', International Journal of Biological Macromolecules (2004); 34: 63–71.

[42] Recalde, M. P., Noseda, M. D., Pujol, C. A., Carlucci, M. J., & Matulewicz, M. C., 'Sulfated mannans from the red seaweed Nemalion helminthoides of the South Atlantic', Phytochemistry (2009); 70: 1062–1068.

[43] Thompson, K. D., & Dragar, C.,' Antiviral activity of Undaria pinnatifida against herpes simplex virus', Phytotherapy Research (2004); 18: 551–555.

[44] Ponce, N. M., Pujol, C. A., Damonte, E. B., Flores, M. L., & Stortz, C. A., 'Fucoidans from the brown seaweed Adenocystis utricularis: extraction methods, antiviral activity and structural studies', Carbohydrate Research (2003); 338: 153–165.

[45] Pujol, C. A., Estevez, J. M., Carlucci, M. J., Ciancia, M., Cerezo, A. S., & Damonte, E. B., 'Novel DL-galactan hybrids from the red seaweed Gymnogongrus torulosus are potent inhibitors of herpes simplex virus and dengue virus', Antiviral Chemistry and Chemotherapy (2002); 13: 83–89.

[46] Schaeffer, D. J., & Krylov, V. S., 'Anti-HIV activity of extracts and compounds from algae and cyanobacteria', Ecotoxicology and Environmental Safety (2000); 45: 208–227.

[47] Harden, E. A., Falshaw, R., Carnachan, S. M., Kern, E. R., & Prichard, M. N., 'Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus', Antiviral Research (2009); 83: 282–289.

[48] Ono, L., Wollinger, W., Rocco, I. M., Coimbra, T. L. M., Gorin, P. A. J., & Sierakowski, M.R., 'In vitro and in vivo antiviral properties of sulphated galactomannans against yellow fever virus (BeH111 strain) and dengue 1 virus (Hawaii strain)', Antiviral Research (2003); 60: 201–208.

[49] Hidari, K. I. P. J., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K., & Suzuki, T., 'Structure and anti-dengue virus activity of sulfated polysaccharide from amarine alga, Biochemical and Biophysical Research Communications (2008); 376: 91–95.

[50] Beress, A., Wassermannv, O., Bruhn, T., & Beress, L., 'A new procedure for the isolation of anti-HIV compounds (polysaccharides and polyphenols) from the marine alga Fucus vesiculosus, Journal of Natural Products (1993); 56: 478–488.

[51] Pereira, M. S., Melo, F. R., & Mourao, P. A. S., 'Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans?', Glycobiology (2002); 12: 573–580.

[52] Killing, H., 'Zur biochemie der meersalgen. Zeitschrift fur Physiologische', Chemie (1913); 83: 171–197.

[53] Church, F. C., Meade, J. B., 'Treanor, E. R.,&Whinna, H. C.. Antithrombin activity of fucoidan. The interaction of fucoidan with heparin cofactor II, antithrombin III, and thrombin', Journal of Biological Chemistry (1989); 264: 3618–3623.

[54] Matsubara, K., 'Recent advances in marine algal anticoagulants', Current Medicinal Chemistry (2004); 2: 13–19.

[55] Nishino, T., Yamauchi, T., Horie, M., Nagumo, T., & Suzuki, H., 'Effects of fucoidan on the activation of plasminogen by u-PA and t-PA', Thrombosis Research (2000); 99: 623–634.

[56] Costa, L. S., Fidelis, G. P., Cordeiro, S. L., Oliveira, R. M., Sabry, D. A., Camara, R. B. G., et al, 'Biological activities of sulfated polysaccharides from tropical seaweeds', Biomedicine and Pharmacotherapy (2010); 64: 21–28.

[57] McLellan, D. S., & Jurd, K. M., 'Anticoagulants from marine algae', Blood Coagulation and Fibrinolysis (1992); 3: 69–80.

[58] Mao, W., Li, H., Li, Y., Zhang, H., Qi, X., Sun, H., et al., 'Chemical characteristics and anticoagulant activity of the sulfated polysaccharide isolated from Monostroma latissimum (Chlorophyta)', International Journal of Biological Macromolecules (2009); 44: 70–74.

[59] Maeda, M., Uehara, T., Harada, N., Sekiguchi, M., & Hiraoka, A., 'Heparinoidactive sulfated polysaccharides from Monostroma nitidum and their distribution in the Cholorophyta', Phytochemistry (1991); 30: 3611–3614.

[60] Mao, W., Zhang, X., Li, Y., & Zhang, H., 'Sulfated polysaccharides from marine green algae Ulva conglobata and their anticoagulant activity', Journal of Applied Phycology (2006); 18: 9–14.

[61] Hayakawa, Y., Hayashi, T., Lee, J. B., Srisomporn, P., Maeda, M., Ozawa, T., et al., 'Inhibition of thrombin by sulfated polysaccharides isolated from green algae', Biochimica Biophysica Acta (2000); 1543: 86–94.

[62] Matsubara, K., Matsuura, Y., Hori, K., & Miyazawa, K., 'An anticoagulant proteoglycan from the marine green alga, Codium pugniformis', Journal of Applied Phycology. (2000); 12: 9–14.

[63] Matsubara, K., Matsuura, Y., Bacic, A., Liao, M. L., Hori, K., & Miyazawa, K., 'Anticoagulant properties of a sulfated galactan preparation from a marine green alga Codium cylindricum', International Journal of Biological Macromolecules (2001); 28: 395–399.

[64] Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J., 'An anticoagulative polysaccharide from an enzymatic hydrolysate of Ecklonia cava', Carbohydrate Polymers (2006); 66: 184–191.

[65] Nishino, T., Aizu, Y.,&Nagumo, T., 'The influence of sulfate content and molecular weight of a fucan sulfate from the brown seaweed Ecklonia kurome on its antithrombin activity', Thrombosis Research (1991); 64: 723–731.

[66] Wang, J., Zhang, Q., Zhang, Z., Song, H., & Li, P., 'Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from Laminaria japonica', International Journal of Biological Macromolecules (2010); 46: 6–12.

[67] Nardella, A., Chaubet, F., Boisson-Vidal, C., Blondin, C., Durand, P., & Jozefonvicz, J., 'Anticoagulant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from the brown seaweed Acosphyllum nodosum', Carbohydrate Research (1996); 289: 201–208.

[68] Chandia, N. P., & Matsuhiro, B., 'Characterization of a fucoidan from Lessonia vadosa (Phaeophyta) and its anticoagulant and elicitor properties', International Journal of Biological Macromolecules (2008); 42: 235–240.

[69] Pushpamali, W. A., Nikapitiya, C., De Zoysa, M.,Whang, I., Kim, S. J., & Lee, J., 'Isolation and purification of an anticoagulant from fermented red seaweed Lomentaria catenata', Carbohydrate Polymers (2008); 73: 274–279.

[70] Carlucci, M. J., Pujol, C. A., Ciancia, M., Noseda, M. D., Matulewicz, M. C., Damonte, E. B., et al., 'Antiherpetic and anticoagulant properties of carrageenans from the red seaweed Gigartina skottsbergii and their cyclized derivatives: Correlation between structure and biological activity', International Journal of Biological Macromolecules (1997); 20: 97–105.

[71] Zuniga, E. A., Matsuhiro, B., & Mejias, E., 'Preparation of a low-molecular weight fraction by free radical depolymerization of the sulfated galactan from Schizymenia binderi (Gigartinales Rhodophyta) and its anticoagulant activity', Carbohydrate Polymers (2006); 66: 208–215.

[72] Sen, A. K., Sr., Das, A. K., Banerji, N., Siddhanta, A. K., Mody, K. H., Ramavat, B. K., et al., 'A new sulfated polysaccharide with potent blood anti-coagulant activity from the red seaweed Grateloupia indica', International Journal of Biological Macromolecules(1994); 16: 279–280.

[73] Zhang, Z., Zhang, Q., Wang, J., Song, H., Zhang, H., & Niu, X., 'Regioselective syntheses of sulfated porpyrans from Porphyra haitanensis and their antioxidant and anticoagulant activities in vitro', Carbohydrate Polymers (2010); 79: 1124–1129.

[74] Kolender, A. A., Pujol, C. A., Damonte, E. B., Matulewicz, M. C., & Cerezo, A. S., 'The system of sulfated □-(1 → 3)-linked D-mannans from the red seaweed Nothogenia fastigiata: Structures, antiherpetic and anticoagulant properties', Carbohydrate Research (1997); 304: 53–60.

[75] Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A. M., et al., 'Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity', Carbohydrate Research (1999); 319: 154–165.

[76] Colliec, S., Fischer, A. M., Tapon-Bretaudiere, J., Boisson, C., Durand, P., & Jozefonvicz, J., 'Anticoagulant properties of a fucoidan fraction', Thrombosis Research (1991); 64(2): 143–154.

[77] Dobashi, K., Nishino, T., Fujihara, M., & Nagumo, T., 'Isolation and preliminary characterization of fucose-containing sulfated polysaccharides with blood-anticoagulant activity from the brown seaweed Hizikia fusiforme', Carbohydrate Research (1989); 194: 315–320.

[78] Jurd, K. M., Rogers, D. J., Blunden, G., & McLellan, D. S., 'Anticoagulant properties of sulphated polysaccharides and a proteoglycan from Codium fragile ssp. Atlanticum', Journal of Applied Phycology (1995); 7: 339–345.

[79] Patankar, M. S., Oehninger, S., Barnett, T., Williams, R. L., & Clerk, G. F., 'A revised structure for fucoidan may explain some of its biological activities', Journal of Biological Chemistry (1993); 268: 21770–21776.

[80] Hayakawa, Y., Hayashi, T., Lee, J. B., Srisomporn, P., Maeda, M., Ozawa, T., et al., 'Inhibition of thrombin by sulfated polysaccharides isolated from green algae', Biochimica Biophysica Acta (2000); 1543: 86–94.

[81] Melo, F. R., Pereira, M. S., Foguel, D., & Mourao, P. A. S., 'Antithrombin-mediated anticoagulant activity of sulfated polysaccharides', Journal of Biological Chemistry (2004); 279: 20824–20835.

[82] Silva, F. R. F., Dore, C. M. P. G., Marques, C. T., Nascimento, M. S., Benevides, N. M. B., Rocha, H. A. O., et al., Anticoagulant activity, paw edema and pleurisy induced carrageenan: Action of major types of commercial carrageenans', Carbohydrate Polymers (2010); 79: 26–33.

[83] Shanmugam, M., & Mody, K. H., 'Heparinoid-active sulfated polysaccharides from marine algae as potential blood anticoagulant agents', Current Science (2000); 79: 1672–1683.

[84] Cho, Y. S., Jung, W. K., Kim, J. A., Choi, I. W., & Kim, S. K., 'Beneficial effects of fucoidan on osteoblastic MG-63 cell differentiation', Food Chemistry (2009); 116: 990–994.

[85] Nagaoka, M., Shibata, H., Kimura-Takagi, I., Hashimoto, S., Aiyama, R., Ueyama, S., et al., 'Anti-ulcer effects and biological activities of polysaccharides from marine algae', Biofactors (2000); 12: 267–274.

[86] Moon, H. J., Lee, S. R., Shim, S. N., Jeong, S. H., Stonik, V. A., Rasskazov, V. A., et al., 'Fucoidan inhibits UVB-induced MMP-1 expression in human skin fibroblasts', Biological and Pharmaceutical Bulletin (2008); 31(2): 284–289.

[87] Guillon, F., & Champ, M.. Structural and physical properties of dietary fibres, and consequences of processing on human physiology. Food Research International (2000); 33: 233–245.

[88] Yu, P., Ning, L., Xiguang, L., Gefei, Z., Quanbin, Z., & Pengchen, L., 'Antihyperlipidemic effects of different molecular weight sulfated polysaccharides from Ulva pertusa (Chlorophyta)', Pharmacological Research (2003); 48: 543–549.

[89] Yu, P., Quanbin, Z., Ning, L., Zuhong, X., Yanmei, W., & Zhi'en, L., 'Polysaccharides from Ulva pertusa (Chlorophyta) and preliminary studies on their antihyperlipidemia activity', Journal of Applied Phycology (2003); 15: 21–27.

[90] Lahaye, M., 'Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some "sea vegetables"', Journal of the Science of Food and Agriculture (1991); 54: 587–594.

[91] Ren, D., Noda, H., Amano, H., Nishino, T., & Nishizawa, K.,' Study on antihypertensive and antihyperlipidemic effects of marine algae', Fisheries Science (1994); 60: 33–40.

[92] Tsuge, K., Okabe, M., Yoshimura, T., Sumi, T., Tachibana, H., & Yamada, K., 'Dietary effect of porphyran from Porphyra yezoensis on growth and lipid metabolism of Sprague-Dawley rats', Food Science and Technology Research (2004); 10: 147–151.

[93] Inoue, N., Yamano, N., Sakata, K., Nagao, K., Hama, Y., & Yanagita, T., 'The sulfated polysaccharide porphyran reduces apolipoprotein B100 secretion and lipid synthesis in HepG2 cells', Bioscience, Biotechnology and Biochemistry (2009); 73(2): 447–449.

[94] Huff, M. W., & Burnett, J. R., '3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and hepatic apolipoprotein B secretion', Current Opinion in Lipidology (1997); 8: 138–145.

[95] Becker, W., 'Microalgae in human and animal nutrition', In Richmond, A. (ed.), Handbook of microalgal culture, Blackwell, Oxford (2004) :312-351.