CHEMICAL STUDIES ON THE POLYSACCHARIDES OF INDIAN SEAWEEDS WITH SPECIAL REFERENCE TO AGAR

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By

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To

My Beloved Parents

केन्द्रीय नमक व समुद्री रसायन अनुसंधान संस्थान

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CERTIFICATE

This is to certify that the contents of this thesis entitled "CHEMICAL STUDIES ON THE POLYSACCHARIDES OF INDIAN SEAWEEDS WITH SPECIAL REFERENCE TO AGAR" is the original research work of ***MR RAMAVATAR MEENA***, carried out under my supervision.

I further certify that the work has not been submitted either partly or fully to any other University or Institution for the award of any degree.

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DECLARATION

I hereby declare that the work incorporated in the present thesis is original and has not been submitted to any University or Institution for the award of diploma or degree. I further declare that the results presented in this thesis and consideration made herein, contribute in general to the advancement of knowledge in chemistry and in particular to the "*Chemical Studies on the Polysaccharides of Indian Seaweeds with Special Reference to Agar*".

Ramavatar Meena

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ABREVIATIONS

3,6-AG	3,6-Anhydro galactose
D_2O	Deuterated water (Deuterium oxide)
TMS	Tetramethyl silane
cP	Centipoise
DMSO	Dimethyl sulphoxide
KCl	Potassium chloride
$CaCl_2$	Calsium chloride
AAm	Acrylamide
kC	Kappa-carrageenan
IPA	Isoporpanol
s	Second
κ-	Kappa-
NaCl	Sodium chloride
ES	Equilibrium swelling
FT-IR	Fourier Transform Infra Red
ADP	Adenosin diphosphate
ATP	Adenosin triphosphate
NaSCN	Sodium thiocyanate
A	1, 4- α-L-3, 6-anhydrogalactose
G	1, 3-β-D-galactose
NR	Not reported
ND	Not detected
h	Hours
AR	Analytical reagent
G′	Storage modulus
D	Dalton
G″	Loss modulus
ICP	Inductively coupled plasma spectrometer
KPS	Potassium persulphate
LC-MS	Liquid chromatography-mass selection.

NMR	Nuclear Magnetic Resonance
CP-MAS	Solid state NMR spectra
μs	Micro second
[η]	Dynamic viscosity
Pa	Pascal
PAAm	Polyacrylamide
p	Probability
rpm	Rotation per minute.
Spp.	Species
SEM	Scanning electron microscope
TGA	Thermogravimetric analysis
M_w	Weight average molecular weight
T_{gel}	Gelling temperature
T_m	Melting temperature
UV-Vis	Ultra violet visible
XRD	X-Ray differaction
Ø	Porosity
G	Gelation degree
WSP	Water soluble polymers

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PART-I

REVIEW ON AGAR AND VALUE ADDITION OF INDIAN AGAROPHYTES

I.1 INTRODUCTION

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I.1 INTRODUCTION

A Japanese innkeeper discovered agar serendipitously in the mid seventeenth century. He threw away the surplus seaweed soup, which transformed into a gel by the night's freezing cold. In 1882, Koch was the first to use agar in microbiology. Agar was traditionally used in Europe for preparing jams and jellies. The term 'agar-agar' is of Malayasian origin and used to be referred to extracts from *Eucheuma*, which is a source of carrageenan, not agar. By the early 1900s, agar became the gelling agent of choice instead of gelatin. Agar was found more suitable because it remained solid at the temperatures required for growth of human pathogens and was resistant to breakdown by bacterial enzymes.

Agar is a phycocolloid, a water soluble polysaccharide, extracted from a group of red marine algae (Class Rhodophyceae) including *Gelidium*, *Pterocladia*, *Gracilaria and Gelidiella* spp.. These marine algae are widely distributed throughout the world in temperate and tropical regions. Agar is a polymer of galactose, having molecular weight in the order of 10^5 daltons, with repeating units of 1,3-linked β-D-galactose and 1,4-linked α-L-3,6-anhydrogalactose (Figure I.1).

Agar production by modern industrial freezing techniques was initiated in 1921 in California, U. S. A. by a Japanese person named Matsuoka. Now the biggest agar factory in the U. S. A. is the American Agar Company in San Diego, California. In Japan, some two-thirds of the agar makers still rely on the natural winter weather to produce strip agar and square agar. The rest have modern equipped factories using the mechanical freeze-thaw process. In China, the agar factories in the North make agar in winter relying on the natural freezing conditions. In other seasons they use diffusion and press techniques to produce agar powder.

In India, the agar factories are situated mainly in the southern region and they use the mechanical freezing process in all seasons because the natural winter freezing process is not possible in India.

I.2 SOURCE

Agar is obtained from various genera and species of the red-purple seaweeds, belonging to the class Rhodophyceae, where it occurs as a structural polysaccharide in the cell walls

and probably also performs a function in ion exchange and dialysis processes. Distribution of important agarophytes is shown in Table I.1 [1].

TABLE I.1 World distribution of agarophytes

Species	Location
Gelidiella acerosa	Japan, India, China
Gelidium amansii	Japan, China
Gelidium cartilagineum	U. S. A., Mexico, South Africa
Gelidium corneum	South Africa, Portugal, Spain, Morocco
Gilidium litulum	Japan
Gelidium lingulatum	Chile
Gelidium pacificum	Japan
Gelidium pristoides	South Africa
Gelidium sesquipedale	Portugal, Morocco
Gracilaria spp.	South Africa, Philippines, Chile, China, Taiwan, India, U. S. A.
Pterocladia capilacea	Egypt, Japan, New Zealand
Pterocladia lucida	New Zealand
Ahnfeltia plicata	U. S. S. R.

I.3 SEAWEED RESOURCES OF INDIA

India has a long coastline extending to 5700 km and the seaweeds are confined mostly to narrow littoral and sublittoral belts of the marine environment. On the coastline we have about 8.5 million hectares of coastal expanse in the form of sheltered bays and lagoons, which are ideal for mariculture activities. Seaweeds are one of the commercially important living marine resources. These grow abundantly along the Tamil Nadu and Gujarat coasts, and in Lakshadeep, Andaman and Nicobar Island. There are also rich seaweed beds around Mumbai, Ratnagiri, Goa, Karwar, Varkala, Vizhinjam and Pulicat in Tamil Nadu and Chilka in Orissa. Out of the ca. 700 species of marine algae in different parts of the Indian coast, nearly 7 agarophytes are commercially important and

can be utilized as raw material for production of agar for food, manure and pharmaceuticals. Estimated quantities of agarophytes that are occurring naturally in India have been reported: 29 tonnes from Gujarat coast, 1.048 from Tamil Nadu coast and 961-2074 tonnes from Laksadweep Islands [2].

Reports are available in the literature on the distribution, resource assessment utilization and cultivation of seaweeds of the Indian coast [3-9]. The commonly available agarophytes in India are *Gelidiella acerosa*, *Gracilaria dura*, *Gracilaria crassa* and *Gracilaria edulis*.

Standing crop of seaweeds in different maritime states [4] of Indian region is as follows: in Gujarat, Maharastra, Goa, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa and Lakshadweep state is 20509, 20000, 2000, Negligible, 1000, 22044, 7500, 5 and 19345 tones wet wt. of seaweeds respectively. The natural biomasses of *Gelidiella acerosa* during 1979-1983 (100-550 tones dry wt.) and *Gracilaria edulis* during 1981-1991 (117-982 tonnes dry wt.) from Tamil Nadu coast have been reported [10].

I.4 SEAWEED FARMING IN INDIA

In India seaweed farming is in a very formative and experimental stage [10]. The overexploitation of certain natural seaweed resources from spcific localities, especially *Gelidiella acerosa* from the Tamil Nadu coast, has resulted in depletion and shortage of raw material. This situation has encouraged development of seaweed resources through cultivation. Additionally, conservation and judicious harvest strategies for sustainable production and utilization of marine algae are now advocated. As a consequence, The CSMCRI initiated programmes on seaweed cultivation have developed technical expertise for the large-scale cultivation of edible, pharmaceutically important green seaweed and economically important brown and red seaweeds [10]. *Gelidiella acerosa* and *Gracilaria dura* have been cultivated in large scale in the sea using artificial substrata as well as other techniques [11]. It has been observed that agars extracted from the cultivated lot have qualities comparable to those obtained from the natural habitat and Sigma (A0576) agarose [12]. Farmed seaweeds are expected to ensure quality and quantity of biomass as well as production of agars having reproducible quality, contrary to the uncertainties associated with naturally occurring seaweeds.

I.5 HARVESTING SEAWEEDS AND ITS EFFECT ON THE STANDING CROP

In India, the collection of seaweeds or agarophytes was made by hand picking during the low water, sometimes necessitating diving in shallow water (one to two meter). The plants were pulled out from their attachments, some coming completely. Therefore, some guide lines for harvesting of seaweeds should be adopted:

(1) Harvesting should be restricted to seasons of maximum vegetative growth of the seaweed concerned,

(2) The alga should be cut at the base to leave fragments for the regeneration of the plants,

(3) Pulling out of the plants should be avoided, and

(4) Batch harvesting should be adopted.

I.6 VARIABILITY

The agar content of seaweeds varies greatly. The agar content mostly depends on specific seaweed and environmental/seasonal changes (i.e. concentration of carbon dioxide, oxygen tension, temperature of water and intensity of solar radiation). Gelation (i.e. gelling and melting) temperatures and gel strength of the agars by different extraction methods from different seaweeds show variation and some parameters are species specific. Moreover, we have found that variation of process parameters appears to be one of the most important factors in the variability of the products. For example, *Gracilaria dura*, in India and elsewhere in the world, has not been known as a source of good quality agar. We have prepared agarose directly from *Gracilaria dura* and *Gelidiella acerosa* of Indian waters using an improved extraction process [11, 12].

I.7 WORLD MARKET OF AGAR

Major agar producing countries are Japan, Spain, Chile, Mexico, China, and Korea. World production per annum: 110,000 dry tons, a total of USD100-200 million. Agar producing seaweeds are *Ahnfeltia, Gelidium, Gelidiella, Gracilaria, Pterocladia* species. Agar is produced mainly from *Gelidium* and *Pterocladia*. *Gelidium* occurs in Indian waters sparsely, with no report of *Pterocladia* occurring. The major applications bacteriological and microbiological (ca.5% of the total sale) and the remaining find use in food industry as standard thickener. The cheapest food grade agar is ca.USD50.00 per kg.

The bacteriological variety is the most expensive costing up to USD25, 000 per kg. The market of agar has an estimated growth of 5-10% per annum [13].

I.8 SEAWEED INDUSTRY IN INDIA

The seaweed industry in India is chiefly a cottage industry and is based mainly on the natural stock of agar yielding red seaweeds, such as *Gelidiella acerosa* and *Gracilaria edulis*. Production of total dry agarophytes in 2000 was approximately 880-1100 tons and India produces 110-132 tons of dry agars annually. Furthermore, many agar and algin extracting industries have been established in different places in maritime states of Tamil Nadu, Andhra Pradesh, Kerala, Karnataka and Gujarat [14]. Production of 100-160 tonnes/year agar in India from 800-1300 tonnes of seaweeds *Gelidiella acerosa* and *Gracilaria* species has been reported in an FAO document [1].

I.9 AGAR EXTRACTION

Extraction of agar from seaweeds involves the following steps:

(i) Washing and cleaning of seaweed with water;
(ii) pretreatment of seaweeds with acid or alkali depending on the nature of the agarophyte followed by removal of acid or alkali;
(iii) pressure extraction of the seaweed;
(iv) filtration of the hot extractive;
(v) clarification of the hot extractive;
(vi) gelling of the agar extractive at ambient temperature;
(vii) freeze-thawing of the agar gel for purification;
(viii) isolation of the agar after thawing the frozen gel;
(ix) drying and pulverizing the agar by conventional methods;
(x) optionally spray drying the agar.

There exist many reports on the various extraction conditions of agar from the seaweed. During our work, it was recognized that whichever process is followed the

process water requirement in the extraction of agar is usually very high since agar is a gelling material in fairly low concentrations, forming viscous matter during the filtration process thereby substantially slowing down the process causing great difficulties. During the course of the work it was found that extraction of agar requires large volume of process water. Therefore, it is necessary to keep the concentration of agar at an optimized low to ensure easy and quick filtration of the hot extractive. This problem was circumvented using a non-ionic surfactant (Brij 35) along with the agar extractive, which lowers the viscosity of agar sol. The surfactant additive was easily removed during the subsequent freeze-thaw cycles. It has been demonstrated that using non-ionic surfactant during agar extraction the quantity of process water can be curtailed by about 50% [15].

Another crucial step in the agar extraction process is pretreatment of seaweed which involves choice of acid, alkali as well as temperature and duration of treatment in the pretreatment steps. It has been realized that an optimized condition keeping all the factors in view can produce superior quality agarose even from seaweed which has not been reported to produce good agar [11, 12]. An eco-friendly method [11, 12], was developed during present dissertation work for producing agarose in 20-22% yield with gel strength 2200 g cm^{-2} in 1.0% gel at 20°C; gelling point 35±1°C; melting point 98±1°C from *Gracilaria dura* occurring in the west and south east coast of India (Tables I.2 & I.3). This process was also useful for other agarophyte species, because superior quality agarose has also been prepared during this study from the Indian *Gelidiella acerosa* occurring from the natural and artificial sources of west and south east coast of India in 14-16% yield having gel strength 2400 g cm^{-2} in1.5% gel at 20°C; gelling point 41±1°C; melting point 85±1°C (Tables I.3).

To our knowledge, this is the first example of producing agarose having such high gel strength and low gelling point from Indian *Gracilaria* species [11]. The highest known gel strength of agar (ca. 1600 g cm^{-2} in 1.5% gel) that has been reported from *Gracilaria* spp. in the public domain is from *Gracilaria cornea* occurring in Mexican waters [16].

I.10 VALUE ADDITION OF INDIAN AGAROPHYTES

There are many literature reports on the extraction of agar from Indian agarophytes. These reports describe extraction of agar from *Gelidiella acerosa* and *Gracilaria* spp. collected from the natural habitat on the Indian coasts. Review of literature reveals that the quality of agar that was extracted was not very good as these agars have gel strength

in the range <100 g/cm^2 to 300 g/cm^2 [2, 17-30], along with a lone report of agar from cultured *Gelidiella acerosa* giving agar with gel strength 790 g/cm^2, which does not describe the process of extraction [31]. Mairh et al. [32] reported culture studies on *Gelidium pusillum* from which agar having yield 22% and gel strength 210 g/cm^2 was obtained. It has also been widely reported that alkali pretreatment of the seaweed improves upon the gel strength of agar through desulphation reaction thereby improving the overall quality of agar. However, even with alkali pretreatment the maximum gel strength of agar that has been reported from Indian sources is 300 g/cm^2 [cf. 25, 29].

Therefore, the existing information tells us that Indian agarophytes are not the source of good quality agar. In CSMCRI work have been under progress to develop process for extraction of agar from agarophytes growing in Indian waters with a focus on *Gelidiella acerosa* and *Gracilaria* spp. Using an improved procedure involving alkali modification, that has been developed in our laboratory during my dissertation work, superior quality agar and agarose from *Gelidiella* and *Gracialria* spp. were prepared (Tables I.2 & I.3) [11, 12, 33]. The agarose of *Gracilaria dura* prepared in our lab was found to have specifications comparable to several grades of agarose mentioned in the Sigma-Aldrich and Fluka catalog (Table I.3). The agarose that has been prepared from *Gelidiella acerosa* and *Gracilaria dura* has been found to be suitable for DNA electrophoresis (unpublished results).

TABLE I.2 Agar and agarose from Indian agarophytes

Seaweed	Yields[a] (%)	Products	Gel strength (g/cm^2) [b]	T_{gel} (^oC) [c]
Gelidiella acerosa	25-30	Native agar	800±50	41±1
Gelidiella acerosa	16-19	Agarose	2400±50	41±1
Gracilaria edulis	25	Native agar	≤100	37±1
Gracilaria edulis	16	Alkali treated agar	450±50	37±1
Gracilaria crassa	25	Native agar	250±50	36±1
Gracilaria crassa	15	Alkali treated agar	800±50	36±1
Gracilaria corticata	20	Native agar	<100	37±1

Gracilaria corticata	10	Alkali treated agar	<100	37±1
Gracilaria fergusonii	22	Native agar	<100	38±1
Gracilaria fergusonii	12	Alkali treated agar	135±50	38±1
Gracilaria dura	27	Native agar	250±50	34±1
Gracilaria dura	23	Agarose	2200 [d]	35±1

[a]Yield (%) with respect to as received seaweed containing ca. 10% moisture; [b]in 1.5% gel at 20°C, unless otherwise stated; [c]in 1.5% gel; [d]in 1% gel at 20°C.

TABLE I.3 Comparison of CSMCRI Agarose with commercially available agarose

Source	Agarose	Gel strength (g/cm^2) [a]	T_{gel} (°C) [b]	Sulphate (%)
Gracilaria dura	CSMCRI Agarose	2200 [c]	35±1	≤0.25
Gelidiella acerosa	CSMCRI Agarose	2400 [b]	41±1	≤0.28
Sigma-Aldrich	Product No. A0576	≥1800 [c]	36±1.5	≤0.12
Sigma-Aldrich	Product No. A9918	>1000 [c]	36±1.5	<0.25
Sigma-Aldrich	Product No. A9668	>1100	36±1.5	<0.30
Fluka	Product No. 05068	≥1500	34-37	≤0.60
Fluka	Product No. 05070	≥1400	40-43	≤0.50

[a] In 1.5% gel at 20°C, unless otherwise stated; [b] in 1.5% gel at 20°C; [c] in 1% gel at 20°C.

I.11 BACTERIOLOGICAL AGAR

Bacteriological grade agar is used in clinical applications, auxotrophic studies, bacterial and yeast formation studies, bacterial molecular genetics applications as well as in mammalian and plant tissue cultures. Agars are used in final concentrations of 1-2% for solidifying culture media. Smaller quantities of agar (0.05-0.5%) are used in culture media for motility studies (0.5% w/v) and growth of anaerobes (0.1%) and microaerophiles.

We have prepared superior quality agar, named S*A*gar, from *Gelidiella acerosa* of Indian waters which were found to be suitable for bacteriological and molecular biology works [15] (Table I.2).

I.12 AGAROSE

Agar is composed of two principal components e.g. agarose and agaropectin. Agarose is the gelling component; agaropectin has only a low gelling ability. There are several methods of producing agarose; mainly by removing the agaropectin from the agar. There are only a small number of processors who produce purified high quality agarose for a small but growing market, mainly in biotechnological applications. These processors use good quality agar as starting material rather than seaweeds, are often not in the seaweed processing business [1].

During this dessertatrion work superior quality agaroses have been prepared directly from *Gelidiella acerosa* and *Gracilaria dura* of Indian waters employing an improved method of extraction (Table I.3). This method is eco-friendly and cost-effective [11, 12]. Agarose has high end applications such as in molecular biology, protein electrophoresis, cell culture works in the R&D labs, pharma and biotech industries world over.

I.13 APPLICATIONS

Agar is used predominantly for its stabilizing and gelling characteristics. It has the unique ability of holding large amounts of moisture. It is mainly employed as a stabilizer in pie fillings, piping gels, icings, cookies, cream shells etc. Agar is useful in low-calorie breads or biscuits since they are nonnutritive, because it acts as a bulking agent.

Agar, which is extracted from different seaweeds by simple and unmodified methods, could be used for food applications because they have low gel strength, high gelling temperature, high metal ions concentration and high sulphate contents. Moreover, some *Gracilaria* species of Indian waters produce good quality food grade agar [33].

Microbiological and bacteriological agars are the most valuable in microbiology and the ideal agar is low in metabolizable or inhibitory substances, debris, and thermoduric spores; has a gelation temperature of 35-40°C, and melting temperature of 75-100°C. Agar is also used in prosthetic dentistry, forensic medicine, pharmaceutical, electrophoresis, photographic stripping films, cosmetics, lotions, papers, as well as biodegradable thin films for wide variety.

I.14 AGAR COMPOSITES

Hydrogels from gelatin (protein), agar and κ-carrageenan (polysaccharides) have good properties, due to their natural origin, low cost, good biocompatibility [34-37] Composites of agarose-maize starch [38], agar-gelatin and agar-κ-carrageenan have been studied [37]. Hydrogels of these blends will have applications in drug delivery systems.

I.15 NEW APPLICATIONS

Recent new applications of agar harness its viscosity enhancing property and hydrophilicity in microfluidic and visible image receiving devices [39, 40].

1.16 BIOSYNTHESIS OF POLYSACCHARIDES AND AGAR

In brief the biosynthesis of polysaccharides involves first the formation of the appropriate precursors, secondly, the polymerization process and, finally in some cases, modifications of the polysaccharide molecule by substitution or other reactions [41].

Enzyme preparations isolated from *Porphyra umbilicalis* [42], and from *Gigartina stellata* [43], converted L-galactose-6-sulphate and D-galactose-6-sulphate respectively into the corresponding 3, 6-anhydrogalactose. These results have been ascribed to the removal of a "kink" in the polysaccharide chain and permitting thereby more extensive

double helix formation to give a more compact and rigid gel framework. This adaptation allows the plant to produce stiffer gels when, for example, it is exposed to more severe wave action. Little is known of the biosynthesis of the macromolecule of these galactans. Radioactive studies with ^{14}C indicated that uridine diphosphate glucose is converted into UDP-galactose and this is utilized in the synthesis of these galactans [44]. Production of ADP-D-glucose from ATP and α-D-glucose-1-phosphate using a *Chlorella* ADP-glucose pyrophosphorylase was also reported earlier [45].

Agar is a galactan polymer consisting of 1,3-β-D-galactopyranosyl 1,4-3,6-anydro-α-L-galactopyranosyl repeating units containing substantial amounts of methylated and sulphated units (Figure I.1).

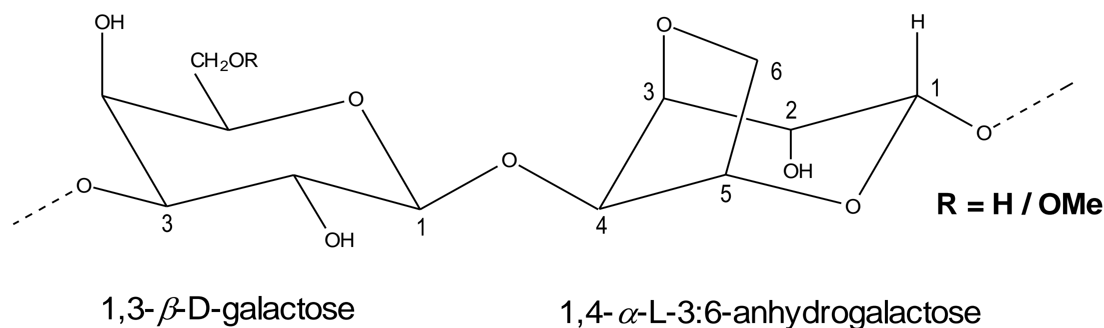

Figure I.1 Agar (Repeating units)

The substitutions are primarily at O-6 and/or methylation giving 3-linked 6-O-methyl-β-D-galactopyranosyl residues and sulphation giving 4-linked 6-O-sulpho-α-L-galactopyranosyl residues.

It is generally believed that chains of alternating D- and L-galactopyranosyl residues are assembled on primer molecules in the Golgi apparatus [46]. Sulphation of L-galactopyranosyl residues is believed to occur in the Golgi at an early stage in the biosynthesis, while ring closure and methylation may occur somewhat later. At some stage in the biosynthesis, migration out of the Golgi into the cell wall matrix takes place and further modification of the agar polysaccharides can occur as the new tissue ages. Floridean starch, a branched glucan similar to amylopectin having some α- 1→3 branchings, and floridoside [α-D-galactopyranosyl-(1→2)-glycerol] act as dynamic

carbon pool for glucose and galactose, which can be used in the dark for cell processes, one of which may be agar biosynthesis. A tentative pathway for this biosynthesis has been proposed. This involves degradation of floridean starch to its precursor glucose 1-phosphate, via a *phosphorylase*, with subsequent formation of UDP-D-galactose and GDP-L-galactose, the precursors of the agarobiose repeating unit. Glucose 1-phosphate is also a precursor of floridoside, which is formed via UDP-D-glucose and UDP-D-galactose, while floridean starch is formed via ADP-D-glucose [47, 48]. These processes have been validated by Hemmingson et al. [46], by introducing ^{13}C-enriched $NaH^{13}CO_3$ into samples of the red seaweed *Gracilaria chilensis* Bird, McLachlan et Oliveira, and subsequently identifying the metabolites using GC/MS techniques.

I.17 FUTURE PROSPECTS

It is possible to prepare superior quality agar and agarose from agarophytes other than only *Gelidium* spp., a source of superior quality agars, as opposed the prevailing perception. It is also possible to prepare superior quality agarose from the Indian agarophytes e.g. *Gelidiella acerosa*, *Gracilaria* spp. and *Gelidium* spp. It is extremely necessary to have a composite strategy which involves bioprospecting for new agarophyte species, value addition of seaweed sources through extraction process improvements as well as large scale cultivation of seaweeds.

The work that has been done in CSMCRI is expected to bring about a change in the scenario of seaweed based industries in India creating renewed investment and employment opportunities in the country’s coastal districts for the fisher folk who would participate in the large scale cultivation activities of value added seaweeds.

I.18 CONCLUSIONS

Agar is a phycocolloid isolated from several red marine algae. It is a very useful gelling biopolymer of plant origin, which is obtained only from certain red seaweeds, called agarophytes. Agar is a galactan polymer with average molecular weight ca. 10^5 D. Agar’s application started as a gelling agent in foods. Later on it was extensively used in microbiological applications. Recent new applications harness its gelling property and hydrophilicity in microfluidic devices besides other applications involving agar and agarose composites.

Indian agarophytes have not been known to be sources of very good quality agar. In this account we have endeavoured to capture the developments that have taken place in our laboratory during this dissertation work, in an ongoing programme of value addition of seaweeds, highlighting the fact that Indian agarophytes can be used for preparation of superior quality agar and agarose. This review is due to be published in a monograph published by CSMCRI [49].

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PART II

CHAPTER II.1

PREPARATION OF AGAROSE FROM *GRACILARIA DURA*

II.1.1 INTRODUCTION

II.1.2 EXPERIMENTAL

II.1.2.1 Materials
II.1.2.2 Native agar preparation
II.1.2.3 Agarose preparation
II.1.2.4 Physical properties
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II.1.2.8 Weight average molecular weight (M_w)
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II.1.3 RESULTS

II.1.3.1 Yield (%)
II.1.3.2 Physical properties
II.1.3.3 Weight average molecular weight
II.1.3.4 Chemical properties
II.1.3.5 FTIR Spectra
II.1.3.6 ^{13}C-NMR spectroscopy
II.1.3.7 Dynamic viscosity measurement
II.1.3.8 Oscillatory Measurements

II.1.4 DISCUSSION

II.1.5 REFERENCES

II.1.1 INTRODUCTION

Natural occurrence of the red seaweed *Gracilaria dura* (C. Agardh) J. Agardh has been reported from the west coast of India [1]. The low gel strength agars from *Gracilaria dura* of Indian waters [2] and from other regions of the world were reported in the literature [3-6].

Agar and agarose (Figure II.1.1) are one of the most used polysaccharides in biotechnological applications [7-8]. Fluka Catalog (2003-2004) mentions the greatest gel strength agarose (Product No. 05071) with gel strength ≥1800 g cm^{-2} (in 1.5% gel), gelling temperature in the range of 40-43°C and sulphate content ≤0.30%. Sigma Catalog of 2004-2005 mentions agarose having gel strength in the range of 100-1800 g cm^{-2} (1.0% gels), gelling temperatures 36-42°C and sulphate contents 0.10-0.30%. Numerous processes and studies have been done on the agarose preparation from the high quality agars and from the low-grade agarose using complex or multi-step purification processes. They used high quality agar or low quality agarose for the preparation of high quality agarose by further purification e.g. either by chromatographic procedure or by fractionation using organic solvents [9-12]. Partially purified agarose was prepared by precipitating of the charged impurities using quaternary base [13]. Fractionation of galactans isolated from *Gracilaria dura* collected in the Black Sea and the analytical results, which are slightly different from those reported by the present authors, were published earlier [14].

Agarose is an industrially important high value material and is extensively used in biotechnology and molecular biology applications. In a continuing program of value addition of Indian seaweeds in our laboratory, the present study demonstrates that Indian *Gracilaria dura*, an agarophyte which has not been reported as a source of good quality agar, can be used for producing agarose in a cost-effective and environment friendly method.

We report herein, for the first time, the preparation of agarose from the agarophyte *Gracilaria dura*, using an improved and cost-effective method [15, 16], and characterization of the agarose. Comparison of the physicochemical properties of this

agarose was done with those of the commercially available products of Sigma and Fluka for benchmarking, which were found to be comparable.

II.1.2 EXPERIMENTAL

II.1.2.1 Materials

The agarose polymer investigated was obtained from specimens of *Gracilaria dura* (C. Agardh) J. Agardh (Gracilariaceae, Rhodophyta), growing in Indian waters. The agarose preparation process corresponded to a patent specification [15, 16]. Thalli of this species were collected from its natural habitat (during November to July) at the west coast of India (20·54° N 70·22° E). Harvested plants were brought to the laboratory, air dried and stored in plastic bags. Sample specimen of the seaweed after identification was submitted to the CSMCRI Herbarium. For comparative study, agarose was purchased from Sigma-Aldrich, USA (Cat. No. A0576), because of its low sulphate content and gelling point as well as high gel strength.

II.1.2.2 Native agar preparation

Native agar (without alkali pre-treatment), was prepared from *Gracilaria dura* (100 g dry) by soaking the seaweed in tap water for 1 h at room temperature and then heated in tap water at 80°-90°C on a water-bath for 1.5 h. The soaked seaweed was autoclaved with demineralised water (1:35 w/v or 350 ml DM water for 10 g of seaweed) for 1.5 h at 120°C. The extractive was homogenized; the homogenate was boiled and filtered hot through Celite bed under reduced pressure. The filtrate was frozen (at -20°C for 15 h) and thawed; after removing the thawed liquid the agar was dried in the air followed by drying in the oven at 50°C for 4 h to get the native agar.

II.1.2.3 Agarose preparation

The agarose polymer was prepared in the laboratory and pilot plant scale, using a cost effective, direct, solvent free and simple, an improved method [15, 16]. Dry *Gracilaria dura* samples (100g-1.5 kg dry each) were soaked in tap water for 1h at room temperature and alkali treated with 10% aqueous NaOH at 85°C for 2 h. The excess alkali was removed from the pretreated seaweed which was then autoclaved in water to obtain extractive, treating the extractive with charcoal and Celite, vacuum filtering the hot extractive over a Celite bed, freezing the filtrate and thawed the mass, straining the product to remove thawed liquid and thereafter squeezed to obtain agarose, which was dried and ground.

II.1.2.4 Physical properties

Gel strength measurement: A 1.0 % solution (50 ml) of agar and agarose was prepared in an autoclave at 120°C. After the formation of gel at room temperature the gel was kept at 10oC overnight in a refrigerator. Gel strength was measurement at 20oC on a Gel Tester (Kiya Seisakusho, Ltd., Tokyo, Japan). Gelling and melting temperatures of gel samples were measured following the method described by Craigie et al. [13]. For measurement of gelling temperature, 10 ml sol of agar or agarose was allowed to cool gradually and a thermometer was emerged in the sol. Temperature at which thew thermometer was fixed to the gel was noted. For melting temperature the gel was heated on a water bath and one iron ball (ca 1 g of weight) was placed on the surface of gel. The temeparature at which the ball touched the bottom of the tube was noted. The gelling temperature of agarose gel samples was also confirmed on the basis of rheological signatures as described by Prasad et al. [17].
Apparent viscosity measurement: Apparent viscosity (in 1.0% agar or agarose samples at 80°C) was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072), using Spindle No.1 at a speed of 60 rpm.

Optical rotation measurement: Optical rotation was measured in 0.25% agarose sol at 45°C, on a Rudolph Digi pol -781 Polarimeter (Rudolph Instruments Inc, NJ, USA).

II.1.2.5 Chemical properties

Estimation of 3, 6-anhydrogalactose (3, 6-AG): The 3, 6-anhydrogalactose (3, 6-AG) was estimated by improved phenol-resorcinol method using fructose as standard [18]. Range of the method 8 µg-32 µg. Accurately weighed agar samples were solubilised in distilled water (5 mg/5 ml).

Reagents:

1. Standard fructose solution: Stock solution: 27 mg of AR grade fructose in 50 ml of benzoic acid saturated distilled water (Prior to this water was warmed to dissolve benzoic acid).

   Working solution: 3.0 ml of solution to 100 ml with distilled water.

2. Acetal solution: Stock solution: 82 mg acetal (100 µl) in 10 ml distilled water. Working solution: 1.0 ml of stock solution to 25 ml with distilled water.

3. Resorcinol solution: Stock solution: 150 mg AR grade resorcinol in 100 ml distilled water.

4. Resorcinol-acetal reagent: 100 ml concentrated HCl was added to 9.0 ml of resorcinol solution.1.0 ml working acetal solution was added.

Method: For standard chart aliquots from 0.5 to 2.0 ml were taken and volume was made up to 2.0 ml with distilled water. Tubes were cooled in an ice bath. 10 ml of resorcinol-acetal reagent was added to each of the test tubes. Kept at 20°C for 4 min. These tubes were transferred to water bath at 80°C for 10 min. Further the test tubes were cooled in an ice bath. Redish colour developed. UV absorbance was measured at 555 nm taking a blank containing water of same volume and other reagents. This gave a standard chart. For analyses of the samples same procedures were applied, this give concentration of fructose, to convert fructose to 3, 6-anhydrogalactose, the values were multiplied by a factor 1.087.

Ash content measurement: The ash content of the native agar and agarose samples was estimated in the residue that was obtained after igniting the agar or agarose at 550°C for 4 h.

Determination sulphate and metal ions: Metal ions analyses were carried out after ignition of known weight of agarose and resultant ash were digested using acid solution.

After this make the volume of digested samples up to 100 ml with triple distilled water. Metal ion and sulphate contents analyses (ICP) were carried out on a Perkin-Elmer ICP-OES Optima 2000DV machine following the method described by Wolnik [19].

II.1.2.6 FTIR spectra

Infrared spectrum was recorded on a Perkin-Elmer Spectrum GX, FT-IR System, USA in KBr (by taking 2.0 mg of agarose in 600 mg of KBr to prepare the pellet) and compared with the IR spectrum of Sigma agarose (A0576) [20].

II.1.2.7 ^{13}C NMR

Noise-decoupled ^{13}C NMR spectra were recorded on a Bruker Advance DPX 200 Spectrometer, Switzerland, at 50 MHz. *Gracilaria dura* and Sigma agarose samples were dissolved in D_2O (50 mg/ml) and the spectra were recorded at 70°C with 5400-5500 accumulations, pulse duration 5.9 μs, acquisition time 1.2059 s and relaxation delay 6 μs using DMSO as internal standard (ca. δ 39.5). The solid state spectroscopy (CP-MAS ^{13}C NMR) used magic angle spinning of 4 KHz and cross-polarization techniques employing contact and repetition times of 16 ms and 5 s respectively, and 450-550 scans were collected. Samples were used directly and spectra were recorded at ambient temperature. Chemical shifts were referenced to adamantane run as an initial sample and are quoted relative to tetramethylsilane (TMS).

II.1.2.8 Weight average molecular weight (M_w)

Intrinsic viscosities [η] were determined at 32°C using an Ostwald viscometer. Sols of agarose samples were prepared in 1.0 M NaCl at a concentration 0.02% to 0.12%. Weight average molecular weight was calculated from the intrinsic viscosity using the Mark–Houwink equation for agarose as described by Rochas & Lahaye [21].

$$[\eta] = 0.07\ M^{0.72}$$

where [η] is intrinsic viscosity in ml/g and,
M is the average molecular weight

II.1.2.9 Rheological measurements

Dynamic rheological measurements of sol and gel samples of both agarose samples were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany). The cone/plate (60 mm diameter, 1° rad angle) geometry was selected for dynamic viscosity measurement at 45°C. The plate/plate (35 mm diameter) geometry was selected for oscillation measurements of agarose gel samples in the controlled deformation mode with a strain value 0.05%, the temperature of gel being maintained at 25°C using the DC50 water circulator. Measurements of G' and G" were performed over 60 minutes. Subsequent measurements were carried out immediately after placing gel sample on the plate. For measurements at all temperatures the exposed part of the samples were covered with silicone oil to minimize losses due to evaporation. All rheological data present were means of three replicate measurements.

II.1.2.10 Statistical analyses

Data were analyzed using one way analysis of variance (ANOVA). Results were considered statistically significant when $p<0.05$. Calculations were performed using Origin Software, Version 6 (Microcal Software Inc. MA, USA). To carry out the analysis of the variance (ANOVA) four replications (n = 4) of each parameter in three groups were made.

II.1.3 RESULTS

II.1.3.1 Yield (%)

Yields were calculated on the basis of as received dry seaweed containing nil moisture (Table II.1.1). The yield of native agar was 27±0.81% for different naturally occurring *Gracilaria dura* samples collected from the west coast. The yields of agarose samples, which were obtained with the 10% NaOH alkali pre-treatment, were 23±0.45% for all the seaweed samples investigated in this study (Table II.1.1). The yields obtained with alkali

pre-treatment using various NaOH concentrations ranged from 22-25% (Table II.1.1). The ANOVA test showed that there was no significant variation in the yield of native agar and agarose samples with the temporal collection of the seaweed samples ($p>0.05$).

II.1.3.2 Physical properties

The optical rotation of *Gracilaria dura* agarose was $[\alpha]_{589}^{45}$ -22° (c 0.25, H_2O), and that of Sigma agarose (A0576) was -21° (c0.25, H_2O). Apparent viscosities of the native agar and agarose were 32 ± 0.5 cP and 44 ± 0.81 cP in 1.0% sol at 80°C. The gel strength of native agar was 250±8.16 g cm^{-2} and those of agarose samples of *Gracilaria dura* and of Sigma (A0576), were 2200±25 g cm^{-2} and >1800 g cm^{-2}, respectively (Table II.1.2). The analysis of variance revealed that the gel strength of *Gracilaria dura* agarose was significantly greater than those of Sigma and Fluka agarose gel samples ($p<0.05$). The variations in the gel strengths from 500 to 2200 g cm^{-2} with alkali concentrations are shown in Table II.1.1. The gelling and melting temperatures of native agar gel are 34 ± 0.57^{o}C and 88 ± 0.52^{o}C, and those of agarose gel were 35 ± 0.55^{o}C and 98± 0.76^{o}C, respectively (Table II.1.1 and II.1.2).

II.1.3.3 Weight average molecular weight

The weight average molecular weights of native agar and agaroses were determined and detailed given in Table II.1.1. Molecular weights of agarose polymers decreased with increase in the concentration of alkali used in the alkali pretreatment step (Table II.1.1). The greatest weight average molecular weight, (3.15 ± 0.07) x10^{5} g . mol^{-1}, was observed for native agar sample, and the lowest was (1.02 ±0.01) x10^{5} g . mol^{-1} , for the agarose polymer obtained by 15% NaOH pretreatment, while agarose prepared with 10% NaOH pretreatment, had M_w (1.23 ±0.079) x 10^{5} g . mol^{-1}.

II.1.3.4 Chemical properties

The 3,6-anhydrogalactose content increased from 15 ± 0.95% for native agar (i.e. with no alkali pre-treatment) to 42±0.84% which was associated with decrease in the sulphate contents (from 3.32% to 0.25 %) for agarose obtained with 10% NaOH pre-treatment (Table II.1.1).

Table II.1.1 Properties of agarose extracted from *Gracilaria dura*[a] under different alkali pretreatment conditions

NaOH (%)	Yield[b] (%); ±SD	Gel strength (g cm^{-2}); ±SD	M_w (g . mol^{-1}); ±SD	3,6-AG (%); ±SD	Ash (%); ±SD	Sulphate (%); ±SD
0	27± 0.81	250±8.1	(3.15 ± 0.07) x 10^5	15 ± 0.95	8.16± 0.12	3.32± 0.057
1.5	25± 0.57	280±9.5	(3.00± 0.06) x 10^5	24± 0.57	5.28± 0.12	2.12± 0.057
3	25± 0.50	700±15.0	(2.98 ± 0.04) x 10^5	32± 0.57	3.43± 0.076	1.84± 0.11
5	24± 0.57	1600±19	(1.5 ± 0.05) x 10^5	37± 0.5	2.02± 0.019	0.50± 0.028
7	23± 0.57	1875±11	(1.25 ± 0.04) x 10^5	39± 0.57	1.58± 0.04	0.30± 0.024
10	23± 0.45	2200±25	(1.23 ±0.079) x 10^5	42±0.84	0.90± 0.033	0.25±0.006
15	22± 0.95	2200±25	(1.02± 0.09) x 10^5	42± 0.5	0.88± 0.024	0.25± 0.006

[a] All samples of *Gracilaria dura* were collected during November to July from the natural stock,
[b] Yields were calculated on the basis of as received dry seaweed containing nil moisture,
[c] Gel strength of all samples were measured in 1.0% gel at 20°C

The metal ion analyses using inductively coupled plasma spectrophotometry (ICP) of the native agar and agarose samples were carried out and compared with those of Fluka agar and agarose (Table II.1.3). The metal ion contents of *Gracilaria dura* agarose were identical with those of Fluka agarose (Table II.1.3). The native agar of *Gracilaria dura* showed higher calcium and magnesium ion contents than that of the Fluka agar. In case of sodium ion, Fluka products showed higher value than those of the native agar and agarose of *Gracilaria dura* studied herein (Table II.1.3). The analysis of

variance revealed that the sulphate and metal ion contents of *Gracilaria dura* native agar was significantly greater than that of agarose sample ($p<0.05$).

Table II.1.2 Comparison of native agar and agarose of *Gracilaria dura* with Sigma and Fluka agaroses

Agar/Agarose source	Gel strength[a] (in g cm^{-2} at 20 ^{o}C); ±SD	Gelling temperature (^{o}C); ±SD	Sulphate content (%); ±SD	Ash content (%); ±SD
Gracilaria dura (Native agar)	270 ±10.84	34 ± 0.57	3.32 ± 0.057	8.5 ± 0.054
Gracilaria dura (Agarose)	2200[b] ±25	35 ± 0.5	0.25 ± 0.006	0.9 ± 0.08
Sigma[c] (A 0576)	>1800[b]	36 ± 1.5	≤0.12	≤0.25
Sigma[c] (A 9918)	>1000[b] >2000 (1.5%)	36 ± 1.5	<0.25	≤0.5
Sigma[c] (A 9793)	>750[b] >1000 (1.5%)	36±1.5	<0.25	≤1.1
Sigma[c] (A 9668)	>700[b] >1100 (1.5%)	36±1.5	<0.30	≤1.5
Sigma[c] (A 3643)	≥650[b]	36±1.5	≤0.25	NR[d]
Sigma[c] (A 3768)	≥800[b]	42±1.5	≤0.30	NR[d]
Fluka[c] (05068)	≥1500(1.5%)	34-37	≤0.60	≤1.0
Fluka[c] (05070)	1400 (1.5%)	40-43	≤0.50	≤1.0
Fluka[c] (05071)	≥1800(1.5%)	40-43	≤0.30	≤1.0
Fluka[c] (05077)	≥2000 (1.5%)	40-43	≤0.30	≤1.0

[a] Gel strength was measured in 1.5% gel, unless otherwise stated; [b]In 1% gel; [c]As mentioned in the Sigma and Fluka catalogue 2004-2005; [d] NR=Not reported.

Table II.1.3 Comparison of metal ion contents in native agar and agarose of *Gracilaria dura* with those of Fluka products[a]

Metal ions	Native Agar of *Gracilaria dura*	Agarose of *Gracilaria dura* (USP: 2005/0267296 A1)	Agar (Fluka Cat. No. 05038)	Agarose (Fluka Cat. No. 05068)
Ca	≤3933	≤680	≤1000	≤500
Cd	≤0.45	ND	≤ 5	≤10
Co	≤0.45	ND	≤ 5	≤10
Cr	≤0.89	ND	≤ 5	≤10
Cu	≤ 8.48	≤0.078	≤ 5	≤10
Fe	≤104	ND	≤50	≤10
K	≤13,495	≤22.3	≤1000	≤500
Mg	≤3463	≤200	≤200	≤10
Mn	≤9.8	ND	≤5	≤10
Na	≤7058	≤233	≤5000	≤5000
Ni	≤5.35	≤0.15	≤5	≤10
Pb	≤1.34	ND	≤5	≤10
B	≤58.9	≤1.30	NR	NR
As	Nil	ND	NR	NR
Al	≤141.1	≤0.76	NR	NR
Zn	≤266	≤3.77	≤10	≤10

[a] All values are in ppm; ND=Not detected; NR=Not reported

II.1.3.5 FTIR Spectra

The FTIR spectra of the *Gracilaria dura* and Sigma agaroses were carried out and depicted in Figure II.1.2. The principal IR bands for *Gracilaria dura* and Sigma agaroses were identical and good agreement with previous report [22].

II.1.3.6 ^{13}C-NMR spectroscopy

^{13}C-NMR spectra of the agarose samples as well as their solid state spectra (CP-MAS) are presented in Figures II.1.3 & II.1.4, respectively. The chemical shifts of the 12 carbon atoms (Figure II.1.3) of the disaccharide repeating units of agarose (Figure II.1.1) were comparable with those reported in the literature [23-25] (Table II.1.4). The solid state spectra (CP-MAS) exhibited five peaks at 62.59, 69.90, 75.67, 79.83 and 99.55 ppm for *G. dura* agarose while the Sigma agarose showed peaks at 62.45, 69.79, 75.38, 79.35 and 100.08 ppm (Figure II.1.4), which was similar to those reported by Rochas & Lahaye [21].

Table II.1.4 Chemical shift assignments for ^{13}C NMR spectra of *Gracilaria dura* agarose[a]

Unit	^{13}C chemical shifts (ppm)						
	C1	C2	C3	C4	C5	C6	References
G	102.4	70.2	82.2	68.8	75.3	61.4	Lahaye, Yaphe, Viet, & Rochas, 1989
A	98.3	69.9	80.1	77.4	75.7	69.4	
G	102.3	70.1	82.2	68.6	75.2	61.3	Usov, Yarotsky, & Shaskov, 1980
A	98.2	69.7	80.0	77.2	75.5	69.7	
G	102.07	69.99	81.93	68.53	75.10	61.20	Truus *et al.*, 2006 (Sigma agarose)
A	98.05	69.56	79.77	77.04	75.25	69.07	
G	102.08	69.98	81.93	68.52	75.10	61.20	Truus *et al.*, 2006 (LKB agarose)
A	98.06	69.55	79.77	77.04	75.25	69.06	
G	102.23	70.00	81.96	68.49	75.10	61.16	Sigma Agarose (A0576) of present study
A	97.92	69.75	79.91	77.03	75.40	69.14	
G	102.05	69.76	81.70	68.23	74.94	60.92	Agarose from *G. dura* of present study
A	97.66	69.59	79.72	76.75	75.13	68.90	

[a]G: 1, 3-β-D-galactose and A: 1, 4- α-L-3, 6-anhydrogalactose

II.1.3.7 Dynamic viscosity measurement

The variations in dynamic viscosity of gels of *Gracilaria dura* and Sigma (A0576) agaroses are shown in Figure II.1.5. The dynamic viscosity of the both gels was decreased when increased the shear rate. Non-Newtonian or shear thinning behavior was observed in both the agarose gels (Figure II.1.5).

II.1.3.8 Oscillatory Measurements

The temperature dependence of storage (G') and loss (G") moduli of *Gracilaria dura* and Sigma (A0576) agarose gels were studied (Figure II.1.6). The storage modulus increased with decreasing temperature for both agarose gel samples. Slightly higher values of the G' for *Gracilaria dura* agarose indicated more rigidity than that of the Sigma agarose (A0576) gel. The sudden increase in G' value and cross the G" near the gelling point also confirmed the low gelling point of the gel samples, which was measured by manual method described by Craigie & Leigh [13] (Figure II.1.6). The time dependence of storage and loss moduli were also studied at constant temperature 25°C (Figure II.1.7).

II.1.4 DISCUSSION

Superior quality agarose polymer was prepared from *Gracilaria dura*, an agarophyte of Indian waters, using an improved method, developed during this dissertation work [15, 16]. To our knowledge, this is the first report of direct, cost-effective and solvent-free process for preparation of agarose from an agarophyte. More particularly, this constitutes the first report of a low-gelling agarose having the greatest gel strength among those reported till date in the public domain. This agarose has the specifications comparable to some commercially available superior quality agaroses (Tables II.1.1 and II.1.2). In this study, the quality of native agar has been significantly improved by the present method. The pre-treatment conditions and concentration of NaOH were optimized and it was found that the agarose obtained with 10% NaOH pre-treatment was superior in yield and quality. The yield of native agar of *Gracilaria dura* was greater than that of the agarose obtained from *Gracilaria dura* samples with 10% NaOH pretreatment (Table II.1.1). These results are in agreement with the fact that the yield loss was due to polymer degradation caused by alkaline hydrolysis [2, 26, 27]. The alkali mediated desulphation of the native agar has led to a superior quality agarose with the careful control of pH (>7) in the post alkali treatment step ensuring minimum degradation of the acid sensitive galactan polymer.

There was no significant temporal difference in the quality of agarose polymers which were prepared from the different natural samples of *Gracilaria dura* as well as from the samples that were cultivated in the sea both in the southeast and west coasts of India. This observation has unfolded the ruggedness of this particular renewable seaweed resource of Indian waters. Our observation is particularly significant *vis a vis* the literature reports on *Gracilaria* [28, 29] describing that the species dependence is not the only factor of variations of the yield and quality of agars [30], but the environmental factors, such as seasonal variations [31] and extraction methods [13, 27, 32] also influence the properties of agar as well.

The gel strength of native agar increased and the molecular weight decreased with increasing concentration of alkali in the pretreatment stage (Table II.1.1). In other words, the weight average molecular weight of native agar was ca. 3-fold greater than that of the agarose polymer obtained with 10% alkali pre-treatment ($p<0.05$). Similar trend was

reported by Murano et al. [4]. The agarose used in the present investigation had the greatest gel strength (2200 g cm^{-1}, in 1.0% gel) amongst those reported from the same seaweed as well as from other *Gracilaria* species. Marinho-Soriano & Bourret [6] reported 600 g cm^{-1}as the gel strength for *Gracilaria dura* in 1.5% agar gel. Rochas & Lahaye [21] used 0.75 M NaSCN for the measurement of [η]. In this investigation 1.0 M NaCl was used for the measurement for preventing gelation at the measuring temperature 32°C, and it was found that there was no difference in the mobility of the sols when measured in 0.75 M NaSCN, which was actually used by Rochas & Lahaye [21]. They reported that "same molecular weight was obtained when different solvents were used e.g. 0.1 M $NaNO_3$; 0.1, 0.5, or 0.75 M NaSCN, for which the conformational ordering and consequently the aggregation are completely different at room temperature". The weight average molecular weight of *Gracilaria dura* agarose prepared in this investigation was in good agreement with those of commercial agaroses [FMC, USA (Reference No. 291402 & 92364); Colab Laboratories, USA (A 37); IBF, France (FF 2743); Sigma, USA (VI) and Oxoid, England (LII)], reported by the Rochas & Lahaye [21].

This agarose was characterized by measuring the gel strength, viscosity, gelling temperature, metal ion contents, optical rotation, rheological properties, IR and ^{13}C NMR spectra. This was found to be of similar specifications when compared with Sigma (A0576) and Fluka agaroses. In the FTIR spectra of both the agarose polymers of this investigation and Sigma (A0576) no band in the region 845-850 cm^{-1} corresponding to C-O-S stretching was detected, indicating the absence of C_4-sulphate in the galactopyranose moiety [33]. The carbon resonances in the ^{13}C NMR and the CP-MAS spectra of both these agarose polymers differed marginally and showed no peak at ca. 59.0 ppm indicating absence of $-OCH_3$ group. It should be mentioned here that the source of Sigma agarose (A0576) is not known. In the CP-MAS spectra of these agaroses, 5 distinct peaks appeared, with a single peak appearing at ca. 100 ppm corresponding to the anomeric carbons of G and A moieties of the agarose repeating units (Figure II.1.1). In agar, these two carbon atoms appear as two distinct peaks ca. 100 ppm [24], possibly because of the presence of sulphate groups in agar resulting in anisotropy around these two carbons. The general agreement of the carbon resonances of the agarose of present investigation with those reported in the literature is presented in Table II.1.4 (cf. Figure II.1.3).

Low sulphate and metal contents are desirable attributes of a superior quality agarose. The sulphate and metal contents of the agarose in the present investigation were measured and compared with those reported (Tables II.1.1 and II.1.3 respectively), showing excellent compatibility of this agarose with those available commercially.

In a continuing program of value addition of Indian seaweeds in our laboratory, the present study demonstrates that Indian *Gracilaria dura*, an agarophyte which has not been reported as a source of good quality agar, can be used for producing agarose in a cost-effective and environment friendly method.

The novelty of the improved method [15, 16], described herein lies in elimination of acids, organic solvents and chromatographic techniques from the entire process of preparing agarose, which were widely reported in the prior art.

II.1.5 CONCLUSIONS

Agarose was prepared from a red alga *Gracilaria dura* occurring in the Arabian Sea at the west coast of India. The agarose has been characterized by studying its physicochemical properties as well as by FTIR, ^{13}C NMR and CP-MAS spectra, inductively coupled plasma (ICP) spectrophotometric and rheological measurements. This agarose had gel strength 2200 g cm^{-2} (in 1% gel), gelling temperature $\leq 35^{o}C$, sulphate content $\leq$0.25%, $[\alpha]_{589}^{45}$ -22° and M_w 1.25 x10^5 g.mol^{-1}. These properties were benchmarked against those of the commercially available agarose products of Sigma (A0576) and Fluka (05068), and were found to be comparable.

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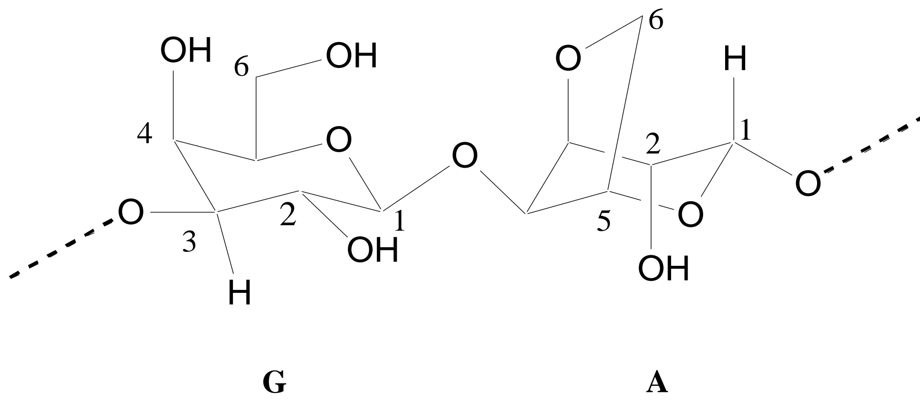


Figure II.1.1 Basic disaccharide repeating units of agarose, G: 1, 3-β-D-galactose and A: 1, 4- α-L-3, 6-anhydrogalactose.

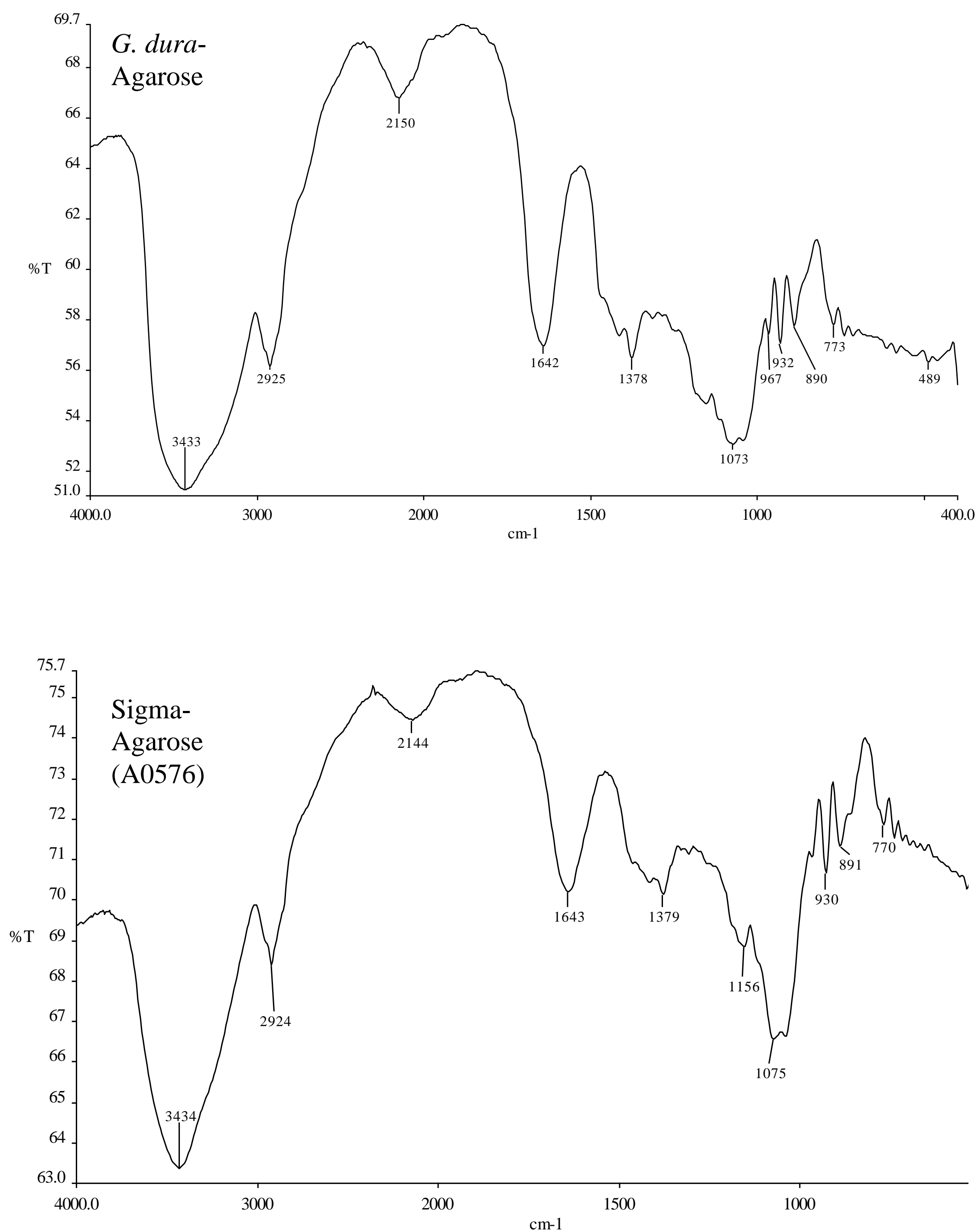


Figure II.1.2 FT-IR spectra of the *Gracilaria dura* and Sigma (A0576) agaroses.

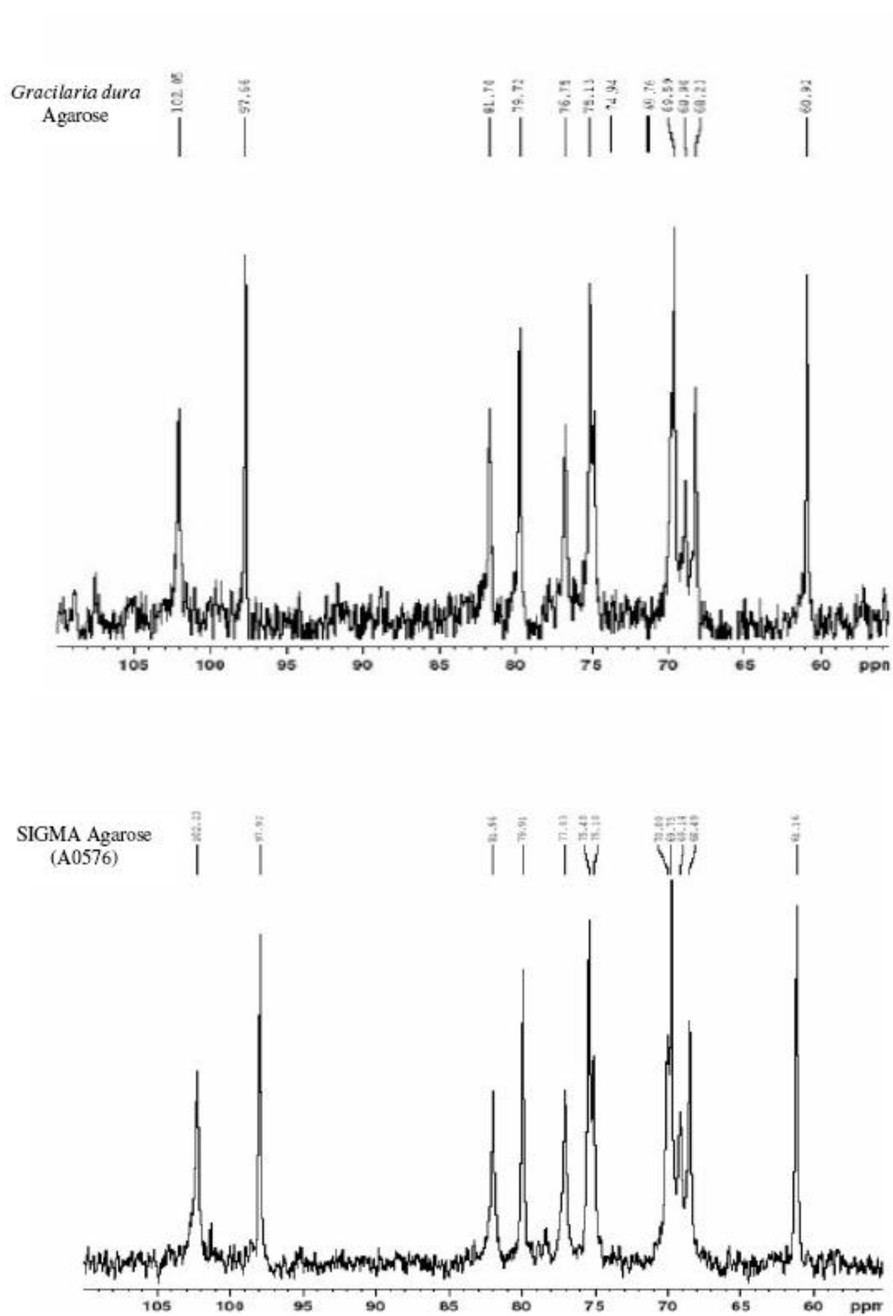
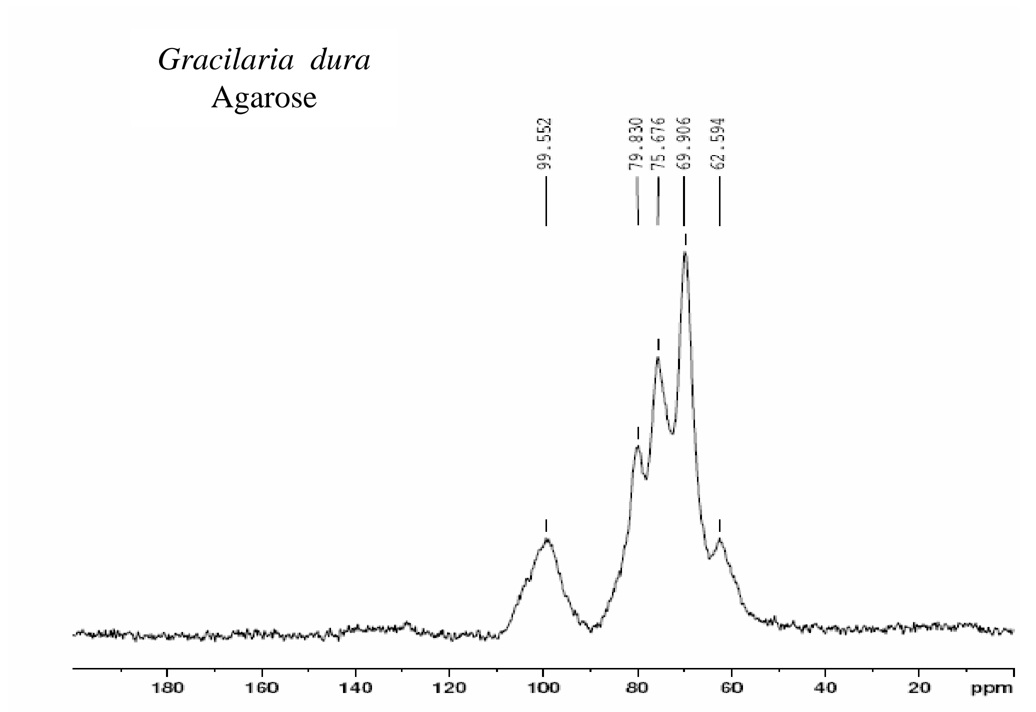


Figure II.1.3 ^{13}C NMR spectra of the *Gracilaria dura* and Sigma (A0576) agaroses.



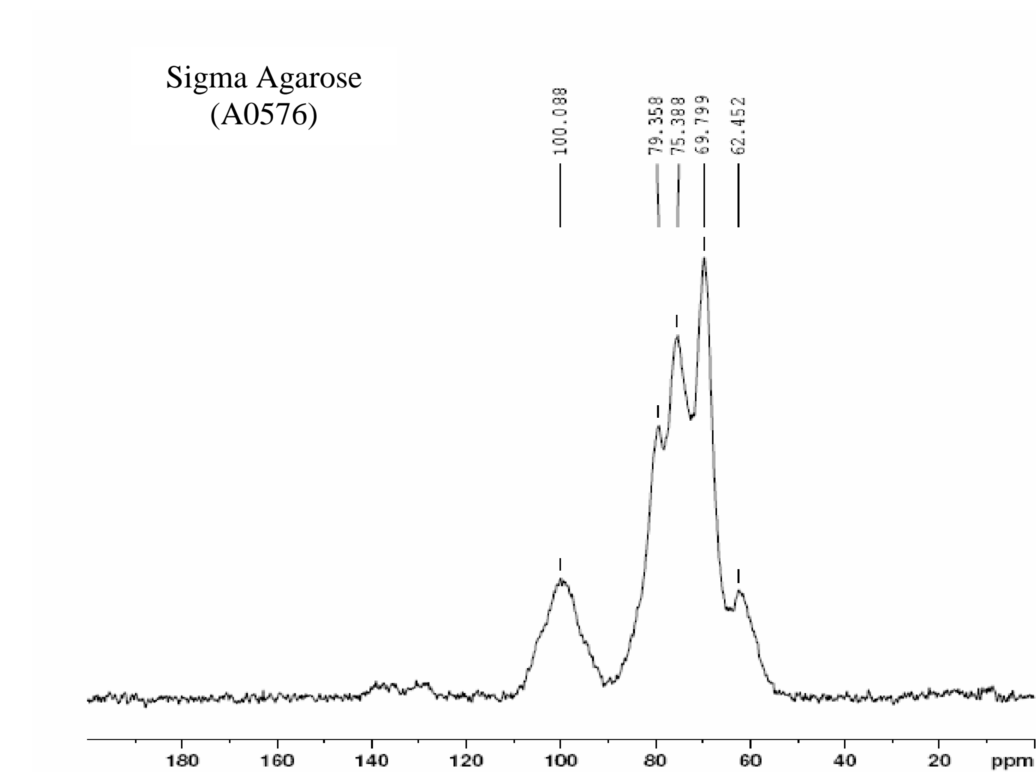


Figure II.1.4 Solid state ^{13}C NMR (CP-MAS) spectra of the *Gracilaria dura* and Sigma (A0576) agaroses.

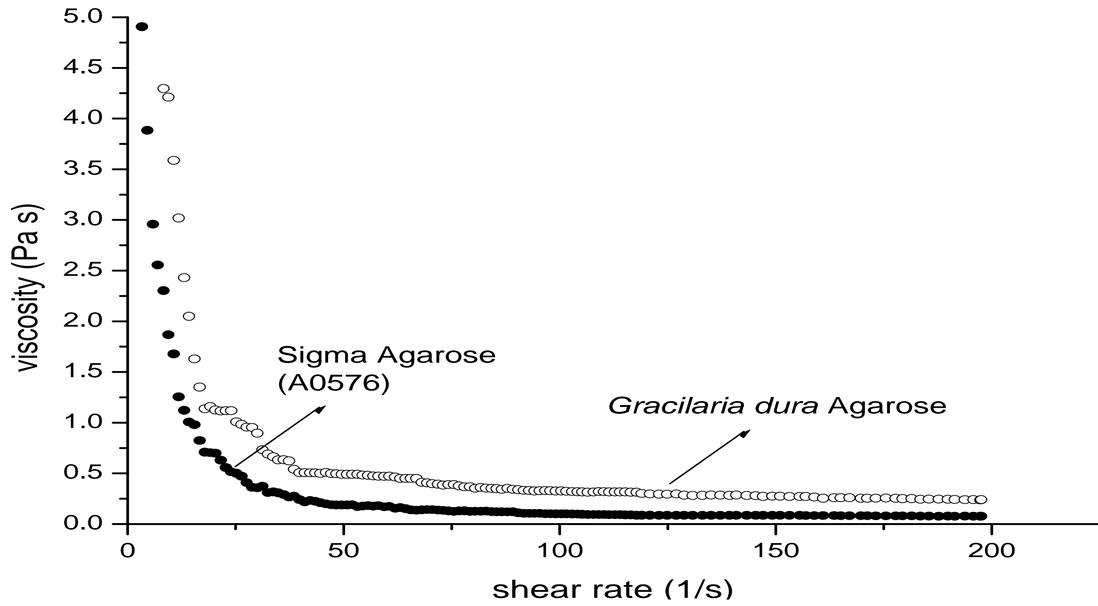


Figure II.1.5 Shear rate vs. dynamic viscosity of *Gracilaria dura* and Sigma (A0576) agarose gel samples.

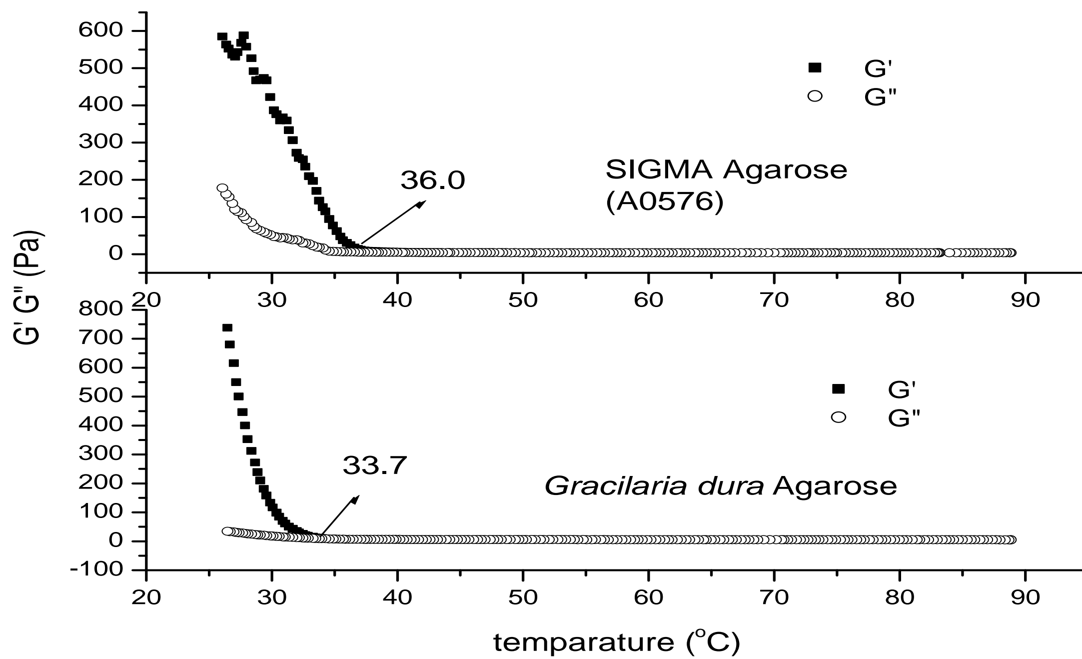


Figure II.1.6 Temperature dependence of G' and G" of *Gracilaria dura* and Sigma (A0576) agarose gel samples at a strain value 0.05.

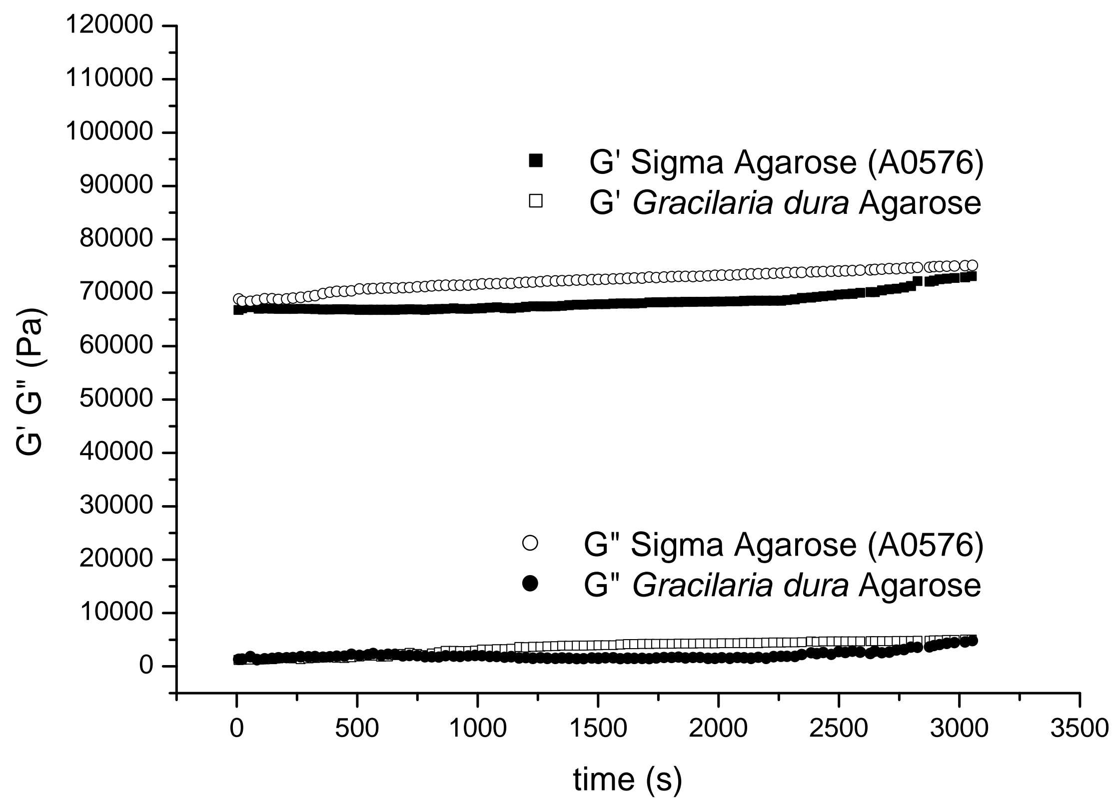


Figure II.1.7 Time dependence of G' and G" of *Gracilaria dura* and Sigma agarose (A0576) gel samples.

CHAPTER II.2

VALUE ADDITION OF INDIAN AGAROPHYTES I: PREPARATION OF SUPERIOR QUALITY AGARS FROM *GRACILARIA* SPP. COLLECTED FROM GULF OF MANNAR, INDIA

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II.2.1 INTRODUCTION

The agarophyte *Gracilaria* has been widely studied and reported in the literature [1]. The species dependence is not the only factor of variations of the yield and quality of agars [2]. Environmental factors, such as seasonal variations [3] and extraction methods [4, 5, 6], have been reported to influence the properties of agar as well.

Six species of *Gracilaria* (e.g. *G.edulis*, *G.crassa*, *G. foliifera*, *G. corticata*, *G. millardetii* and *G. fergusonii*) occurring in Indian waters have been reported to be a potential source of agar [7]. Among these, *Gracilaria corticata, G. crassa* and *G. edulis* are the most common ones. These *Gracilaria* species are available on the southeast coast of India, Gulf of Mannar (Tamil Nadu). Therefore, *G. edulis, G. crassa, G. foliifera* and *G. corticata* were selected for this study. Many authors have reported extraction of agar from different *Gracilaria* spp. e.g. *Gracilaria edulis, G. corticata, G. dura, G. fergusonii, G. foliifera*, *G. confervoides* and *Gelidiella acerosa* of Indian waters in particular [cf. 8-11]. Villanueva & Montano [12], have reported alkali treatment of *G. edulis* at 90°C for 2 hours, followed water and acid washing, and the best quality agar from *G. edulis* was obtained with 10% NaOH treatment at 90°C. Siddhanta et al. [13] have also optimized alkali concentration and treatment temperature for *Gracilaria dura* and were 10% NaOH and 90°C. Praiboon et al. [15] have mentioned that agars extracted from Thai and Japanese *Gracilaria* spp. including *G. edulis* with and without alkali treatments could be used for food applications due to low gel strength. Numerous reports were also present in the literature for alkali treatment up to 10% NaOH at 90°C up to 3 hours [16, 17]. But there were no reports on the optimization of alkali concentrations for *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. corticata*. In this study, we have done systematic studies and optimized the concentration of alkali for obtaining the best quality agars from *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. corticata* studied herein.

Gelidiella acerosa and *Gracilaria edulis* are the two principal agarophytes utilized by the Indian agar industry. Recently, in a continuing program of value addition of Indian agarophytes we have prepared bacteriological agar as well as superior quality agarose with gel strengths of 800 g cm^{-2} (1.5% gel at 20°C) and 2200 g cm^{-2} (1.0% gel at 20°C) from Indian waters *Gelidiella acerosa* and *Gracilaria dura* respectively [13, 14, 18, 19].

Meena et al. [20], has been reported that agars extracted from Indian agarophytes showed significant sugar reactivity with sucrose and glucose. There has been no report of

native and alkali treated agar from *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. corticata,* having gel strength more than 250 g cm^{-2}. In this communication, we report the preparation of superior quality agars having high gel strengths after alkali modification from *Gracilaria edulis* and *G. crassa* collected from the Gulf of Mannar. The analysis results of agars extracted from *G. edulis* and *G. crassa* after alkali modification indicates that these agars can be used for industrial use.

II.2.2 MATERIALS AND METHODS

II.2.2.1 Materials

Four *Gracilaria* spp. e.g. *Gracilaria edulis* (S. Gmelin) P. Silva, *G. crassa* Harvey ex J. Agardh, *G. foliifera* (Forsskal) Børgesen and *G. corticata* (J. Agardh) J. Agardh, samples were studied and they were collected from the natural stocks of the Gulf of Mannar Tamil Nadu, India (8. 46 to 9.14 N and 78.90 to 79.14 E), during September 2001 to 2004. Harvested plants were shade dried and packed in the gunny bags, transported to our laboratory by road transport. The dry seaweed samples were stored as received, separately in plastic bags. The dry seaweed measured 7-10% moisture content. Before extraction of agar, the seaweed was washed thoroughly with tap water to remove the epiphytes and extraneous impurities. All the sample specimens were submitted with the Central Salt and Marine Chemicals Research Institute, Bhavnagar, Herbarium (AS0416905, AL0201908, AL0204301 and AL0204109) after identification.

II.2.2.2 Native agar extraction

Different samples of dry *Gracilaria edulis, G. crassa, G. foliifera* and *G. corticata* (20 g each) were soaked in tap water for 1 h at room temperature followed at 90°C in a water bath for 2 h. The soaked seaweed was cooked in an autoclave with distilled water for 1.5 h at 120°C. The cooked seaweed was then homogenized in grinder mixture, boiled with Celite and charcoal and filtered through Celite bed under vacuum to obtained clear extract. The filtrate kept to room temperature for gel formation and gelled material was then frozen in the freezer at -15°C for 15 h and thawed to obtain the native agar. Finally the thawed agar was air dried for 24 h at ambient conditions and then dried in an oven at 50°C for 2 h.

II.2.2.3 Alkali treated agar extraction

Alkali pre-treatment of the *Gracilaria* species was carried out using 3%, 4%, 6%, 8%, 10% and 15% aqueous NaOH solutions as described in our previous work on *Gracilaria dura* [13, 14]. Different samples of *Gracilaria edulis, G. crassa, G. foliifera* and *G. corticata* (20 g dry each) were soaked in 400 ml tap water for 1 h at room temperature and then treated with 400 ml various concentrations of aqueous NaOH solutions at 90°C in a water-bath for 2 h. After the alkali treatment, excess was removed by water washing until the washing showed pH in the range of 7-8. The seaweed was then autoclaved with distilled water (1:30 w/v) at 120°C for 1.5 h. Afterwards the alkali-treated agar was obtained by using similar process as mentioned for native agar extraction.

II.2.2.4 Physical analyses

Ash content estimation: The ash content of the native and alkali treated agar samples was estimated in the residue that was obtained after igniting the agar at 550°C for 4 h.

Gel strength measurement: A 1.5% (w/v) solution of agar was prepared in an autoclave at 120°C to minimize the water evaporation. After the formation of gel at room temperature, it was kept at 10°C overnight in a refrigerator. Gel strength, gelling and melting temperatures [4], and apparent viscosity were measured. The detailed methods for gel strength, gelling and melting temperatures and apparent viscosity measurements were given in Chapter II.1, Section: **II.1.2.4 Physical properties.** Loss on drying was calculated in the solid powdered agar (5-7 g) samples on a moisture analyzer (Sartorius AG, Germany), at 100°C. The pH of the agar solution (1.5%, w/w) samples was measured by Digital pH Meter 335 Systronics, at 70°C.

II.2.2.5 Chemical analyses

Estimation of 3, 6-AG: The 3,6-anhydrogalactose content was estimated using a phenol-resorcinol reagent employing the improved method using fructose as a standard [21]. Detail method has been mentioned in Chapter II.1, Section: **II.1.2.5 Chemical properties.**

Estimation of sulphate: Sulphate content of the agar samples was estimated by turbidimetric method using potassium sulphate as standard described by Dodgson et al. [22].

Reagents

1. Stock solution: 360 mg potassium sulphate in 100 ml distilled water (working solution: 1:2 dilution of stock solution gives approximately 99 μg of sulphate per 0.1 ml).

2. Barium chloride- Gelatin solution : 100 ml of 0.5% gelatin solution was prepared and kept at 4oC for overnight and 0.5 g of Barium chloride was added to that and allowed to stand for 3—4 h before use.

3. 4% Trichloroacetic acid (TCA) in distilled water.

Method: Aliquots of potassium sulphate working solution were taken up to 0.2 ml and volume of each aliquot was adjusted with distilled water to 0.2 ml 3.8 ml of 4% TCA was added to each aliquot followed by addition of 1 ml barium chloride-gelatin solution. Blank was prepared in the same way replacing potassium sulphate solution by 0.2 ml distilled water. After incubation for 15-20 min at r.t., UV absorbance was measured at 360 nm. In the first set of experiment (A), each experimental solutionwas prepared with a particular aliquot of agar sample and volume was made upto 0.2 ml with 1 N HCl and TCA (3.8 ml) was added followed by gelatin-barium chloride solution (1 ml). The UV absorbance was measured (E) at 360 nm against a blank solution containing 1 N HCl (0.2 ml), TCA (3.8 ml) and gelatin-barium chloride solution (1 ml).

In the second set of experiment, each solution was prepared with same sample aliquots volume which was used for set "A" experiment and volume was made upto 0.2 ml with 1 N HCl and addition of 3.8 ml TCA followed by 1 ml of gelatin solution. The UV absorbance were measured (B) against a blank solution containing 1 N HCL (0.2 ml), TCA (3.8 ml) and gelatin solution (1 ml). Absorption due to sulphate is equal to E-B, B denotes the absorption quantum arising out if the UV- active components in the hydrolyzed agar sample is other than sulphate.

Metal ion determination: Metal ion and sulphate contents analyses (ICP) were carried out on a Perkin-Elmer ICP-OES Optima 2000DV machine following the method described by Wolnik [23]. Detail method has been mentioned in Chapter II.1, Section: **II.1.2.5 Chemical properties.**

II.2.2.6 Rheological measurements

Dynamic rheological measurements of these samples were carried out on a rheometer (RS1, HAAKE Instru- ments, Karlsruhe, Germany). The cone/plate (60 mm diameter, 1 rad. angle) geometry was selected for dynamic viscosity measurements of sol at 45°C, with 3-5 ml sample volume. The plate/plate (35 mm diameter) geometry was selected for oscillation measurements of agar gel in the controlled deformation mode with 0.05% strain, the temperature of gel being maintained at 25°C using the DC50 water circulator. The subsequent measurements were carried out immediately after placing gel sample on the plate/plate. For measurements at all temperatures the exposed part of the samples were covered with silicone oil to minimize losses due to evaporation. All rheological data present were means of three replicate measurements. Under the experimental conditions selected, no notable syneresis or gel slippage that can be judged by abrupt decrease in G' was observed for the systems measured.

II.2.2.7 Statistical analyses

Analysis of variance (one way ANOVA test) was carried out by using Microcal Origin, Version 6 software (Microcal Software Inc. MA, USA). To carry out the analysis the variance four replications ($n = 4$) of each parameter in three groups were made. Mean and standard deviation were calculated using the Microsoft Excel 2000, software.

One-way ANOVA test was conducted for significant differences (when $p < 0.01$) between native agar (without NaOH pre-treatment) and best quality alkali treated agars (with optimum NaOH pre-treatments) in agar yield, gel strength, 3,6-anhydrogalactose content, sulphate content and ash content from the four *Gracilaria* species.

One-way ANOVA test was also conducted for no significant differences (when $p > 0.01$) in the properties (e.g. agar yield, gel strength, 3,6-anhydrogalactose, sulphate, ash etc.) in the same treatment group.

II.2.3 RESULTS

II.2.3.1 Yields

The effect of NaOH concentration on the yield of agars is presented in Table II.2.1-4, for *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. corticata*, respectively. While, one way ANOVA test for significant differences between native agar (without NaOH pre-treatment) and best quality alkali treated agars (with optimum NaOH pre-treatment) in agar yield from four *Gracilaria* species was shown in Table II.2.5. The mean values of the agar yield for native agar (without NaOH treatment) ranged 16±0.77 to 25±0.76 %, with the greatest value (25%) obtained for *G. edulis* (Table II.2.1) and the lowest (16%) was obtained for *G. corticata* (Table II.2.4). The yield of agar decreased with the increase in alkali concentration for all the *Gracilaria* species studied herein. The mean values of the agar yield for alkali treated agar ranged between 9.5±0.80 to 23±0.89% (Table II.2.1-4). The greatest (16%) yield for the best quality alkali-treated agar was obtained for *Gracilaria edulis* with 8% NaOH pre-treatment and the lowest (9.5%) was obtained for *G. corticata* Table II.2.1 & 4, respectively. No significant differences in the yield ($p >$ 0.01) of agars was observed in the same treatment group (Table II.2.1-4), while significant differences was noted (Table II.2.5) between native agar (without NaOH pre-treatment) and best quality alkali treated agar (with optimum NaOH concentrations) yield for the four *Gracilaria* species ($p < 0.01$).

II.2.3.2 Gel strength

The gel strengths of native agars varied significantly among the seaweed species, which ranged 100±6.19 g cm^{-2} to 250±15.2 g cm^{-2} (Table II.2.1-4), with the greatest value of the gel strength 250±15.2 g cm^{-2} for *G. crassa* and the lowest 100±6.19 g cm^{-2} for *G. corticata* (Table II.2.2 & 4). Generally, the gel strength of the native agars (without NaOH pre-treatment) was increased when increased the concentration of NaOH (Table II.2.1-4). The significant increase in the gel strengths of the native agars with alkali pre-treatments was observed for the *G. edulis* and *G. crassa* (Table II.2.1 & 2). The significant increase (p<0.01) in the gel strength (490±8.16 g cm^{-2} and 800±15.4 g cm^{-2}), with alkali pre-treatment was observed for *G. edulis* and *G. crassa* with 8% and 10% NaOH, respectively (Table II.2.1& 2). The lowest increase in the gel strength (from 100±6.19 g cm^{-2} to 110±7.93 g cm^{-2}) with alkali pre-treatment was observed for *G.*

corticata agar samples (Table II.2.4). The gel strengths of the best quality alkali treated agars were significantly higher ($p<0.01$) than those of the native agars obtained for *G. edulis* and *G. crassa* Table II.2.5. The native and best quality alkali treated agars of *Gracilaria crassa* exhibited greatest gel strengths 250±15.2 g cm^{-2} and 800±15.4 g cm^{-2}, among the four *Gracilaria* species studied herein (Table II.2.2 & 5).

II.2.3.3 Gelling Properties

The gelling and melting temperatures of the native and alkali treated agars are shown in Table II.2.1-4, for *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. corticata* respectively. The mean values of the gelling temperature for native agars ranged 34±0.73^{o}C to 39±0.81^{o}C, with greatest value 39±0.81^{o}C obtained for *Gracilaria edulis* and the lowest value 34±0.73^{o}C obtained for *G. crassa*. The slight increase in the gelling temperature was observed for alkali treated agars obtained for *G. edulis*, *G. crassa* and *G. foliifera* (Table II.2.1-3), while no change in gelling temperature was observed for *G. corticata* (Table II.2.4). The best quality alkali treated agar of *G. crassa* had the lowest value 35±0.76^{o}C of gelling temperature (Table II.2.2) and low gelling temperature is useful for biological works. While, highest value 41±0.80^{o}C of gelling temperature was observed for the best quality alkali treated agar of *G. edulis* (Table II.2.1). The highest value 87 ±0.82^{o}C of melting temperature was obtained for *G. crassa* best quality alkali treated agar and the lowest value 75±0.77^{o}C for *Gracilaria corticata* native agar (Table II.2.5).

II.2.3.4 Ash content

Significant variation was detected between the ash contents of native agar (without alkali) and best quality alkali treated agars (with optimum NaOH concentration) for four *Gracilaria* species, investigated herein ($p < 0.01$), Table II.2.5. In general, ash content was decreased when increase the concentration of NaOH for four *Gracilaria* species (Table II.2.1-4). The ash contents for native agars e.g. 6.7±0.23 %, 6.0±0.41 %, 7.5±0.08 % and 8.0±0.22 %, were twice or more than twice of those found for alkali-treated agars e.g. 3.0±0.17%, 2.5 ±0.08%, 3.6±0.08 % and 4.0±0.09 %, of *Gracilaria edulis, G. crassa, G. foliifera* and *G. corticata* respectively (Table II.2.5).

II.2.3.5 Chemical properties

Generally, increase in 3,6-anhydrogalactose content was observed when increased the concentration of NaOH for four *Gracilaria* species. The 3,6-anhydrogalactose contents for the alkali-treated agars obtained from the four *Gracilaria* spp. e.g. *G. edulis*, *G. crassa*, *G. foliifera* and *G. corticata*, ranged 25±0.83% to 40±0.77% (Table II.2.1-4). The maximum increase, 22% to 40% in the 3,6-anhydrogalactose content was observed for the best quality alkali treated agar of *Gracilaria crassa* (Table II.2.2 & 5). In general, the sulphate content of the native agar was decreased when increase the concentration of NaOH (Table II.2.1-4). The sulphate contents for the alkali-treated agars obtained from the four *Gracilaria* spp. e.g. *G. edulis*, *G. crassa*, *G. foliifera* and *G. corticata*, ranged 1.4±0.07% to 6±0.15% (Table II.2.1-4). The lowest sulphate content 1.4±0.07 % was observed in best quality alkali treated agar of *Gracilaria edulis* (Table II.2.5). ANOVA test showed significant differences between native agars (without alkali) and best quality alkali treated agars (with optimum alkali) in the 3, 6-anhydrogalactose contents and sulphate contents from the four *Gracilaria* species, $p < 0.01$ (Table II.2.5).

II.2.3.6 Apparent viscosity

The apparent viscosity of native agars increased when increase the concentration of alkali for four *Gracilaria* species (Table II.2.1-4). The greatest apparent viscosity (45±0.52 cP s) was observed in the best quality alkali treated agar of *G. crassa* (Table II.2.2) and lowest (15±0.74 cP s) was obtained in native agar of *Gracilaria corticata* (Table II.2. 4). Loss on the drying was slightly different in native and alkali treated agar samples and was ranged between 8.2-8.9% for all the agar samples (Table II.2.6). Solution pH was greater for alkali treated agar samples than those of the native agar samples (Table II.2.6).

II.2.3.7 Metal ion analyses

The metal ion analyses using inductively coupled plasma spectrophotometry (ICP) of the native agar and alkali treated agar samples were carried out and compared with those of Fluka agar (Table II.2.6). The metal ion contents of *G. edulis and G. crassa* agars were identical with those of Fluka agar (Table II.2.6). The native agars of *G. edulis and G. crassa* showed higher metal ion contents than that of the alkali treated agars and Fluka agar. In case of sodium ion, Fluka products showed higher value than those of the agars

of *G. edulis and G. crassa* studied herein (Table II.2.6). The analysis of variance revealed that the sulphate and metal ion contents of *G. edulis and G. crassa* native agar samples were significantly greater than that of alkali treated agars samples (p<0.05).

II.2.3.8 Dynamic rheological measurement

The comparison of the steady rheological characterization of the four best quality alkali treated agar (with optimum alkali) gel samples is shown in Figure II.2.1. Dynamic viscosity was measured under applied shear rate from 40-130 1/s (Figure II.2.1). The dynamic viscosity (η) decreased with increasing shear rate (γ), corresponding to a shear-thinning behavior and all the gel samples showed non-Newtonian or pseudoplastic behaviour. The effect of shear was greater on the agar gel sample of the *G. corticata* in comparasion to those of the agar gel samples of *Gracilaria edulis*, *G. crassa* and *G. foliifera* (Figure II.2.1). This indicates that gel sample prepared from the best quality alkali treated agar (with 10% NaOH pre-treatment) from *Gracilaria crassa* having the lowest gel thinning property among the all *Gracilaria* species agar gel samples studied herein.

II.2.3.9 Visco-elasticity measurement

Change in dynamic moduli (G' and G'') of the best quality alkali treated agar gels of four *Gracilaria* species, during cooling are shown in Figures II.2.2a & b. On cooling, both G' (storage modulus, Figure II.2.2a) and G'' (loss modulus, Figure II.2.2b) increased rapidly near the gelling temperatures of the agar gels. The storage modulus increased with decreasing temperature with all agar gel samples. The greatest values of the G' was observed for the best quality alkali treated agar gel of *G. crassa*, indicating more rigidity and stable gel network than the other agar gels (Figure II.2.2a). The G' values of agar gels followed the increasing order: *G. crassa* > *G. edulis* > *G. foliifera* > *G. corticata* as illustrated in Figure II.2.2a.

II.2.4 DISCUSSION

There is a strong dependence of agar quality with the alkali treatment and temperature of treatment [10, 13, 14, 24-26]. Pre-treatment with alkali causes elimination of the 6-sulphate

group leading to the formation of a 3,6-anhydro bridge [4, 27], presumably via an SN^{i}-type mechanism through the participation of the 3-hydroxyl group of the L-galactopyranosyl moiety resulting in the improvement of the gelling properties [10, 28]. The greatest 3, 6-anhydrogalactose content (Table II.2.2 & 5) in *Gracilaria crassa* was observed in the agar that was extracted with 10% NaOH pretreatment. We reported earlier [13] that the gel strength of agar depends on the post alkali treatment step, wherein the best result was obtained, if the pH of the seaweed was maintained in the range 7-8. This prevents degradation of acid sensitive galactan polymer backbone present in the cell wall containing the 3,6-anhydrogalactose moieties that is responsible for the high gel strength, presumably due to the build-up of local concentration of acid during neutralization.

The agar yield varied among the species studied, with a minimum obtained with *G. corticata*. The agar content obtained from *G. crassa* was the highest at all alkali concentrations than the *Gracilaria edulis*, *G. foliifera* and *G. corticata*. The agar yield decreased when increasing the concentration of NaOH in all the seaweed species studied. ANOVA test for significant differences between native agar (without alkali) and best quality alkali treated agar (with optimum alkali concentration) in agar yield, gel strength, 3,6-anhydrogalactose, ash and sulphate contents from the four *Gracilaria* species, shown in Table II.2.5. This ANOVA results indicates significant variation in the properties of native agar and best quality alkali treated agars from the *Gracilaria* species, $p < 0.01$ (Table II.2.5). ANOVA test also carried out in the same treatment group, and analysis of variance in the same treatment group for same seaweed indicates that the yield, gel strength, 3, 6-anhydro-galactose, ash and sulphate contents etc. of the agar was not significantly different in the same treatment group for any particular seaweed species, $p > 0.01$ (Tables II.2.1-4). The decrease in yield of alkali-treated agar compared to that of native agar might be attributed to the possible degradation and losses of the polysaccharide in the alkaline liquor [13, 16]. Comparative study of these agars with commercially available Fluka (05038) agars showed similar characteristics was shown in Table II.2.6. The ash contents, sulphate contents, gel strengths and metal ions in *G. edulis* and *G. crassa* agar samples were identical with Fluka (05038) agar. The native agars of *G. edulis* and *G. crassa* showed significantly higher concentrations of calcium and magnesium ions than the agar sample of Fluka (Table II.2.6). In case of sodium ion, Fluka products showed significantly higher value than the native and alkali treated agars of Indian agarophytes studied herein. Therefore, the agars derived from these Indian agarophytes were comparable with commercially available Fluka agars. It may be mentioned here that the sources and method of the commercially available agar that have

been mentioned here are not known. Thus, the agars that are reported herein will be suitable for high end uses.

In view of the improved characteristics of alkali-treated agars from *Gracilaria edulis* and *G. crassa* as compared to those of *G. corticata* and *G. foliifera*, it would be reasonable to conclude that the former two seaweeds contain alkali labile sulphate group leading to formation of the 3,6-anhydrogalactose moiety thereby enhancing the gel strength. The two-seaweed species *Gracilaria foliifera* and *G. corticata* contained a lower degree of alkali labile sulphate as evidenced by the marginal lowering of sulphate contents with no significant increase in gel strength. Further, the optimum concentration of alkali that was used for alkali pre-treatment of different *Gracilaria* spp., that has been studied herein, is species specific (Table II.2.1-4). Similar observations were reported by Freile-Pelegrin and Robledo [26]. The dynamic viscosity decreased with increasing shear rate, corresponding to a shear-thinning behavior in agar samples as expected. The effect of shear was greater on the agar sample from *G. edulis*, *G. corticata* and *G. foliifera* in comparison to the agar sample from *G. crassa* (Figure II.2.1). Agar extracted from *G. crassa* showing low gel thinning property than those of *G. edulis, G. foliifera* and *G. corticata* obtained from the Gulf of Mannar. It indicates more firmness of *G. crassa* agar under applied shear rate. Viscoelasticity measurement in1.5% agar gels during cooling are shown in Figures II.2.2a & b. On cooling, both G′ (storage modulus) and G″ (loss modulus) increased rapidly. It has been observed that the increment in G′ was higher for the agar gel sample from *G. crassa*, followed by *G. edulis, G. foliifera* and *G. corticata*, indicating thereby a greater elasticity of the former agar gels in comparison to those of *G. edulis*, *G. foliifera* and *G. corticata* (Figure II.2.2a). The increments are almost similar and least for *G. foliifera* and *G. corticata* indicating weaker gel structure for these agars [19]. It may be due to the low sulphate content in agar of *G. crassa* as well as presence of more junction zones in the gel sample of *G. crassa* [29]. It can also explain on the basis of 3, 6-anhydrogalactose amount which was greatest in *G. crassa* agar and it was well known that gel strength was increased when increased the 3, 6-anhydrogalactose.

This work dispelled the myth of prevalent perception of poor quality status of a certain agarophyte sourced from the Indian waters. Thus the agarophyte species namely, *Gracilaria edulis* and *G. crassa* occurring in Indian waters could be used for producing superior quality agars. This is the first report of such superior quality agars that have been prepared from these two *Gracilaria* species. In this study, all species showed high

melting temperatures (84-86°C) and low gelling temperatures (35-40°C) which fall in the range of United States Pharmacopoeia (USP) standards, indicates its commercial importance [17]. The gelling temperature of alkali treated agar of *G. crassa* was ca. 35±0.76^{o}C, the lowest of the lot, would be useful for bacteriological and biotechnological applications. A new grade of agars is "sugar reactive" has been reported by Freile-Pelegrin and Robledo [26], in which the gel strength increases as sugar is added to the agar solution. This effect can be produced by high molecular weight agar of low sulphate content. Commercially, agars possessing this characteristic are derived from Indian *Gracilaria edulis* and *G. crassa* [20]. On this regard, *G. edulis* and *G. crassa* agars those were extracted by the alkali pre-treatments with 5% to 8% NaOH having good gel strength, low metal contents and low sulphate content could be used in food and biological applications, as well as in the area where are sugar reactive agar were used.

The result of the present investigation indicates that these algal species were being used as commercial stocks and used in the Indian agar industry. Results also suggested that industry could be used single or blended seaweeds to produce the desired final agar product. The results reported here would be beneficial for bio-prospecting of Indian agarophytes.

II.2.5 CONCLUSIONS

Gracilaria edulis, *G. crassa*, *G. foliifera* and *G. corticata* are naturally occurring agarophytes of Indian waters. These agarophytes were evaluated for their agar contents. The effect of different concentrations of NaOH in the alkali treatment was studied. These four *Gracilaria* species of Indian waters produced agars, both native and alkali treated, with different properties confirming the heterogeneity of the agar polymers in this genera, as one would expect. *G. edulis* and *G. crassa* produces agar polymers with high gel strengths 490±8.16 g cm^{-2} and 800±15.4 g cm^{-2} with 8% and 10% NaOH treatment, respectively. The poor gelling ability of agar polymers from *G. foliifera* and *G. corticata*, indicated low immediate commercial importance of these species. *G. edulis* and *G. crassa* produced superior quality agars by alkali treatment, and may be considered for commercial exploitation from the Indian resources [30].

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Table II.2.1 Effect of alkali (NaOH) concentration on the physicochemical properties of agar extracted from *Gracilaria edulis*, and one way ANOVA test for variation in the same treatment group.[a]

Physicochemical properties of *Gracilaria edulis* agar samples								
NaOH (%)	Yield [b] (%;±SD)	Range	*F*-ratio	Prob. (*p*)	Gel strength [c] (g cm^{-2}; ±SD)	Range	*F*-ratio	Prob. (*p*) [e]
Native agar	25±0.76	24 to 26	0.0869	0.9658	100±7.30*	90 to 110	0.2682	0.8470
3	23±0.89	22 to 24	0.1052	0.9554	135±7.71	125 to 145	0.0547	0.9823
4	20±0.86	19 to 21	0.2682	0.8470	220±14.60	200 to 240	0.2105	0.8871
6	18±0.83	17 to19	0.0869	0.9658	340±8.56	330 to 350	0.1052	0.9554
8 [d]	16±0.87	15 to 17	0.0731	0.9732	490±8.16*	480 to 500	0.0731	0.9732
10	13±0.73	12 to 14	0.2682	0.8470	490±7.72	480 to 500	0.3636	0.7804
15	11±0.77	10 to 12	0.2105	0.8871	490±8.34	480 to 500	0.0612	0.9792
NaOH (%)	3,6-AG (%;±SD)	Range	*F*-ratio	Prob. (*p*)	Sulphate (%; ±SD)	Range	*F*-ratio	Prob. (*p*)
Native agar	19±0.80	18 to 20	0.0731	0.9732	5.4±0.23	5.1 to 5.7	0.2677	0.8474
3	22±0.85	21 to 23	0.1333	0.9383	3.6±0.15	3.4 to 3.8	0.0802	0.9694
4	25±0.73	24 to 26	0.0857	0.9665	2.7±0.16	2.5 to 2.9	0.1449	0.9309
6	28±0.77	27 to 29	0.1052	0.9554	2.1±0.077	2.0 to 2.2	0.2105	0.8871
8 [d]	29±0.83	28 to 30	0.3584	0.7865	1.4±0.073	1.3 to 1.5	0.2682	0.8470
10	29±0.85	28 to 30	0.2666	0.8481	1.3±0.085	1.2 to 1.4	0.1600	0.9211
15	30±0.80	29 to 31	0.2682	0.8470	1.3±0.082	1.2 to 1.4	0.0638	0.9779
NaOH (%)	Ash content (%; ±SD)	Range	*F*-ratio	Prob. (*p*)	Gelling T. (°C; ±SD)	Range	*F*-ratio	Prob. (*p*)
Native agar	6.7±0.23	6.4 to 7.0	0.0085	0.9988	39±0.81	38 to 40	0.0857	0.9665
3	5.6±0.15	5.4 to 5.8	0.0519	0.9836	39±0.79	38 to 40	0.0731	0.9731
4	4.9±0.15	4.7 to 5.1	0.0678	0.9759	40±0.73	39 to 41	0.1052	0.9554
6	3.7±0.14	3.5 to 3.9	0.0253	0.99402	40±0.70	39 to 41	0.0666	0.9765
8 [d]	3.0±0.17	2.8 to 3.2	0.0201	0.9959	41±0.80	40 to 42	0.1111	0.9519
10	2.9±0.07	2.8 to 3.0	0.0666	0.9765	41±0.82	40 to 42	0.4285	0.7362
15	2.9±0.08	2.8 to 3.0	0.2727	0.8439	41±0.83	40 to 42	0.3548	0.7865

[a] All value are calculated as means of four replicates in three groups
[b] The yield was calculated on the basis of bone dry seaweeds.
[c] Gel strength was measured in 1.5% gel at 20°C.
[d] Optimized NaOH concentration for *Gracilaria edulis*.
[e] All value are greater than >0.01, indicates no significant differences in the same group of treatment.
*Indicates significant increase in the gel strength with alkali.

Table II.2.2 Effect of alkali (NaOH) concentration on the physicochemical properties of agar extracted from *Gracilaria crassa*, and one way ANOVA test for variation in the same treatment group.[a]

NaOH (%)	Yield [b] (%;±SD)	Range	F-ratio	Prob. (p)	Gel strength [c] (g cm^{-2}; ±SD)	Range	*F*-ratio	Prob. (*p*) [e]
Native agar	23±0.86	22 to 24	0.0857	0.9665	250±15*	230 to 270	0.0909	0.9636
3	22±0.73	21 to 23	0.0731	0.9732	330±12.65	315 to 345	0.0281	0.9932
4	21±0.93	20 to 22	0.0857	0.9665	420±17.12	400 to 440	0.0802	0.9694
6	18±0.75	17 to19	0.2105	0.8871	640±16.68	620 to 660	0.4222	0.7405
8 [d]	16±0.86	15 to 17	0.1052	0.9554	780±14.60	760 to 800	0.1578	0.9225
10 [d]	15±0.80	14 to 16	0.0909	0.9636	800±15*	780 to 820	0.1743	0.9117
15	12±0.84	11 to 13	0.0731	0.9732	800±16.12	780 to 820	0.2181	0.8819
NaOH (%)	**3,6-AG (%;±SD)**	**Range**	**F-ratio**	**Prob. (p)**	**Sulphate (%; ±SD)**	**Range**	***F*-ratio**	**Prob. (*p*)**
Native agar	22±0.73	21 to 23	0.1333	0.9383	3.2±0.15	3.0 to 3.4	0.0240	0.9946
3	26±0.86	25 to 27	0.0869	0.9658	2.9±0.23	2.6 to 3.2	0.0097	0.9986
4	32±0.83	31 to 33	0.4074	0.7505	2.4±0.15	2.2 to 2.6	0.1519	0.9264
6	38±0.77	37 to 39	0.0857	0.9665	2.0±0.080	1.9 to 2.1	0.1052	0.9554
8	40±0.80	39 to 41	0.2682	0.8470	1.8±0.086	1.7 to 1.9	0.2682	0.8470
10 [d]	40±0.77	39 to 41	0.1739	0.9119	1.6±0.077	1.5 to 1.7	0.2105	0.8871
15	40±0.83	39 to 41	0.4222	0.7405	1.5±0.040	1.45 to 1.55	0.4285	0.7362
NaOH (%)	**Ash content (%; ±SD)**	**Range**	***F*-ratio**	**Prob. (*p*)**	**Gelling T. (°C; ±SD)**	**Range**	***F*-ratio**	**Prob. (*p*)**
Native agar	6.0±0.41	5.5 to 6.5	0.0528	0.9832	34±0.73	33 to 35	0.1052	0.9554
3	5.6±0.31	5.2 to 6.0	0.2382	0.8680	34±0.36	33.5 to 34.5	0.2727	0.8439
4	4.6±0.15	4.4 to 4.8	0.0193	0.9961	34±0.38	33.5 to 34.5	0.3333	0.8015
6	3.1±0.04	3.0 to 3.2	0.2682	0.8470	35±0.76	34 to 36	0.2105	0.8871
8	2.5±0.07	2.4 to 2.6	0.6129	0.6195	35±0.73	34 to 36	0.2682	0.8470
10 [d]	2.5±0.07	2.4 to 2.6	0.1052	0.9554	35±0.76	34 to 36	0.3333	0.8015
15	2.5±0.08	2.4 to 2.6	0.3548	0.7865	35±0.78	34 to 36	0.4074	0.7505

[a] All value are calculated as means of four replicates in three groups; [b] The yield was calculated on the basis of bone dry seaweeds; [c] Gel strength was measured in 1.5% gel at 20°C; [d] Optimized NaOH concentration for *Gracilaria crassa*; [e] All value are greater than >0.01, indicates no significant differences in the same group of treatment.
*Indicates significant increase in the gel strength with alkali.

Table II.2.3 Effect of alkali (NaOH) concentration on the physicochemical properties of agar extracted from *Gracilaria foliifera,* and one way ANOVA test for variation in the same treatment group.[a]

NaOH (%)	Yield [b] (%;±SD)	Range	*F*-ratios	Prob. (*p*)	Gel strength [c] (g cm^{-2}; ±SD)	Range	*F*-ratios	Prob. (*p*) [e]
Native agar	22±0.80	21 to 23	0.0857	0.9665	100±8.56*	90 to 110	0.1052	0.9554
3	20±0.85	19 to 21	0.3548	0.7865	100±8.06	90 to 110	0.0731	0.9732
4	18±0.83	17 to 19	0.2727	0.8439	100±8.34	90 to 110	0.3142	0.8148
6	16±0.77	15 to 17	0.1052	0.9554	120±8.56	110 to 130	0.0731	0.9732
8 [d]	15±0.73	14 to 16	0.0909	0.9636	135±7.63	125 to 145	0.4444	0.7256
10	14±0.79	13 to 15	0.0731	0.9732	135±7.74	125 to 145	0.2985	0.8258
15	12±0.76	11 to 13	0.2105	0.8871	135±8.00*	125 to 145	0.1791	0.9085
NaOH (%)	3,6-AG (%;±SD)	Range	*F*-ratios	Prob. (*p*)	Sulphate (%; ±SD)	Range	*F*-ratios	Prob. (*p*)
Native agar	15±0.73	14 to 16	0.0909	0.9636	5.7±0.154	5.5 to 5.9	0.1142	0.9500
3	19±0.85	18 to 20	0.0731	0.9732	5.2±0.085	5.0 to 5.2	0.1052	0.9554
4	24±0.85	23 to 25	0.0869	0.9658	4.6±0.152	4.4 to 4.8	0.0193	0.9961
6	29±0.83	28 to 30	0.2682	0.8470	4.1±0.085	4.0 to 4.2	2105	0.8871
8 [d]	31±0.79	30 to 32	0.1052	0.9554	3.8±0.085	3.7 to 3.9	0.2682	0.8470
10	31±0.74	30 to 32	0.2105	0.8871	3.7±0.073	3.6 to 3.8	0.2105	0.8871
15	31±0.77	30 to 32	0.2682	0.8470	3.7±0.068	3.6 to 3.8	0.2352	0.8700
NaOH (%)	Ash content (%; ±SD)	Range	*F*-ratios	Prob. (*p*)	Gelling T. (°C; ±SD)	Range	*F*-ratios	Prob. (*p*)
Native agar	7.5±0.08	7.4 to 7.6	0.1052	0.9554	37±0.73	36 to 38	0.2682	0.8470
3	6.6±0.07	6.5 to 6.7	0.2352	0.8700	37±0.77	36 to 38	0.3636	0.7804
4	5.3±0.08	5.2 to 5.4	0.1600	0.9211	38±0.83	37 to 39	0.0731	0.9732
6	4.1±0.07	4.0 to 4.2	0.2682	0.8470	39±0.89	38 to 40	0.3793	0.7696
8 [d]	3.6±0.06	3.5 to 3.7	0.2105	0.8871	39±0.75	38 to 40	0.3333	0.8015
10	3.6±0.08	3.5 to 3.7	0.2727	0.8439	39±0.68	38 to 40	0.5135	0.6806
15	3.6±0.08	3.5 to 3.7	0.2352	0.8700	39±0.62	38 to 40	0.4444	0.7256

[a] All value are calculated as means of four replicates in three groups
[b] The yield was calculated on the basis of bone dry seaweeds.
[c] Gel strength was measured in 1.5% gel at 20°C.
[d] Optimized NaOH concentration for *Gracilaria foliifera*.
[e] All value are greater than >0.01, indicates no significant differences in the same group of treatment.
* Indicate slight increase in the gel strength with alkali.

Table II.2.4 Effect of alkali (NaOH) concentration on the physicochemical properties of agar extracted from *Gracilaria corticata*, and one way ANOVA test for variation in the same treatment group.[a]

NaOH (%)	Yield [b] (%;±SD)	Range	*F*-ratios	Prob. (*p*)	Gel strength [c] (g cm^{-2}; ±SD)	Range	*F*-ratios	Prob. (*p*) [d]
Native agar	16±0.77	15 to 17	0.5238	0.6440	100±6.19*	90 to 110	0.3793	0.7696
3	14±0.81	13 to 15	0.2727	0.8439	100±8.06	90 to 110	0.2352	0.8700
4	13±0.77	12 to 14	0.2105	0.8871	100±8.85	90 to 110	0.1052	0.9554
6	12±0.85	11 to 13	0.2727	0.8439	100±7.72	90 to 110	0.0731	0.9732
8	11±0.72	10 to 12	0.0666	0.9765	110±6.29	100 to 120	0.0666	0.9765
10	10±0.85	9 to 11	0.0731	0.9732	110±7.93	100 to 120	0.2820	0.8373
15	9.5 ±0.77	8.5 to 10.5	0.0204	0.9958	110±8.94*	100 to 120	0.2682	0.8470
NaOH (%)	**3,6-AG (%;±SD)**	**Range**	***F*-ratios**	**Prob. (*p*)**	**Sulphate (%; ±SD)**	**Range**	***F*-ratios**	**Prob. (*p*)**
Native agar	16±0.73	15 to 17	0.1818	0.9066	6.8±0.23	6.5 to 7.1	0.4390	0.7292
3	19±0.83	18 to 20	0.5238	0.6740	6.0±0.15	5.8 to 6.2	0.0810	0.9690
4	22±0.73	21 to 23	0.4074	0.7505	5.6±0.17	5.4 to 5.8	0.0857	0.9665
6	25±0.77	24 to 26	0.5714	0.6444	4.7±0.08	4.6 to 4.8	0.3548	0.7865
8	28±0.80	27 to 29	0.4222	0.7405	4.2±0.08	4.1 to 4.3	0.1052	0.9554
10	28±0.73	27 to 29	0.2727	0.8439	4.2±0.08	4.1 to 4.3	0.2352	0.8700
15	28±0.80	27 to 29	0.2682	0.8470	4.2±0.07	4.1 to 4.3	0.0810	0.9690
NaOH (%)	**Ash content (%; ±SD)**	**Range**	***F*-ratios**	**Prob. (*p*)**	**Gelling T. (^{o}C; ±SD)**	**Range**	***F*-ratios**	**Prob. (*p*)**
Native agar	8.0±0.22	7.7 to 8.3	0.1463	0.9300	38±0.62	37 to 39	0.4074	0.7505
3	6.8±0.15	6.6 to 7.0	0.0857	0.9665	38±0.70	37 to 39	0.5135	0.6806
4	6.3±0.15	6.1 to 6.5	0.1142	0.9500	38±0.77	37 to 39	0.2727	0.8439
6	4.9±0.08	4.8 to 5.0	0.1052	0.9554	38±0.75	37 to 39	0.6000	0.6272
8	4.2±0.08	4.1 to 4.3	0.3548	0.7865	38±0.77	37 to 39	0.4222	0.7405
10	4.0±0.08	3.9 to 4.1	0.0731	0.9732	38±0.71	37 to 39	0.2973	0.8267
15	4.0±0.09	3.9 to 4.1	0.2224	0.8775	38±0.65	37 to 39	0.0769	0.9712

[a] All value are calculated as means of four replicates in three groups
[b] The yield was calculated on the basis of bone dry seaweeds.
[c] Gel strength was measured in 1.5% gel at 20°C.
[d] All value are greater than >0.01, indicates no significant differences in the same group of treatment.
* Indicate negligible increase in gel strength with alkali.

Table II.2.5 Analysis of variance (one way ANOVA test) for significant differences between native agar (without NaOH) and best quality agar (obtained with optimum NaOH pre-treatment) in agar yield, gel strength, 3, 6-anhydrogalactose, sulphate and ash contents from the *Gracilaria* species.

Properties	Agar	Value (±SD)	Range	*F*-ratios	Prob. (*p*)
Gracilaria edulis					
Yield %	Native agar	25 (±0.76)	24-26	204.05	<0.01
	Alkali treated agar	16 (±0.87)	15-17		
Gel strength ($g\ cm^{-2}$)	Native agar	100 (±7.30)	90-110	4563.00	<0.01
	Alkali treated agar	490 (±8.16)	480-500		
3,6-AG %	Native agar	19 (±0.80)	18-20	265.42	<0.01
	Alkali treated agar	29 (±0.83)	28-30		
Sulphate %	Native agar	5.4 (±0.23)	5.1-5.7	960.00	<0.01
	Alkali treated agar	1.4 (±0.07)	1.3-1.5		
Ash %	Native agar	6.7 (±0.23)	6.4-7.0	1050.00	<0.01
	Alkali treated agar	3.0 (±0.17)	2.8-3.2		
Gracilaria crassa					
Yield %	Native agar	23 (±0.86)	22-24	192	<0.01
	Alkali treated agar	15 (±0.80)	14-16		
Gel strength ($g\ cm^{-2}$)	Native agar	250 (±15.2)	230-270	2186.91	<0.01
	Alkali treated agar	800 (±15.4)	780-820		
3,6-AG %	Native agar	22 (±0.73)	21-23	972	<0.01
	Alkali treated agar	40 (±0.77)	39-41		
Sulphate %	Native agar	3.2 (±0.15)	3-3.4	768	<0.01
	Alkali treated agar	1.6 (±0.08)	1.5-1.7		
Ash %	Native agar	6.0 (±0.41)	5.5-6.5	282.69	<0.01
	Alkali treated agar	2.5 (±0.27)	2.4-2.6		
Gracilaria foliifera					
Yield %	Native agar	22 (±0.80)	21-23	192	<0.01
	Alkali treated agar	14 (±0.79)	13-15		
Gel strength ($g\ cm^{-2}$)	Native agar	100 (±8.56)	90-110	36.75	<0.01
	Alkali treated agar	135 (±7.74)	125-145		
3,6-AG %	Native agar	15 (±0.73)	14-16	768	<0.01
	Alkali treated agar	31 (±0.74)	30-32		
Sulphate %	Native agar	5.7 (±0.15)	5.5-5.9	1200	<0.01
	Alkali treated agar	3.7 (±0.07)	3.6-3.8		
Ash %	Native agar	7.5 (±0.08)	7.4-7.6	4563	<0.01
	Alkali treated agar	3.6 (±0.08)	3.5-3.7		
Gracilaria corticata					
Yield %	Native agar	16 (±0.77)	15-17	121.78	<0.01
	Alkali treated agar	9.5 (±0.8)	8.5-10.5		
Gel strength ($g\ cm^{-2}$)	Native agar	100 (±6.19)	90-110	1.421	>0.01 (0.2782)
	Alkali treated agar	110 (±7.93)	100-120		
3,6-AG %	Native agar	16 (±0.73)	15-17	432	<0.01
	Alkali treated agar	28 (±0.73)	27-29		
Sulphate %	Native agar	6.8 (±0.23)	6.5-7.1	1610.68	<0.01
	Alkali treated agar	4.2 (±0.08)	4.1-4.3		
Ash %	Native agar	8.0 (±0.22)	7.7-8.3	960	<0.01
	Alkali treated agar	4.0 (±0.09)	3.9-4.1		

Table II.2.6 Comparison of gel strength, ash content, sulphate content, pH values and metal ion contents in native agars and alkali treated agars of *G. edulis and G. crassa* with that of Fluka (05038, *BioChemika* for microbiology) agar [a]

Properties	*G. crassa*		*G. edulis*		Fluka
	Native agar	Alkali treated agar	Native agar	Alkali treated agar	Agar (Product No. 5038)
Gel strength (g cm^{-2})	250 (±15.2)	800 (±15.4)	100 (±7.30)	490 (±8.16)	>300
Ash (%)	6.0 (±0.41)	2.5 (±0.27)	6.7 (±0.23)	3.0 (±0.17)	≤5
Sulphate (%)	3.2 (±0.15)	1.6 (±0.08)	5.4 (±0.23)	1.4 (±0.07)	≤0.9
Loss on drying (%)	≤8.5	≤8.9	≤8.2	≤8.6	≤10
pH	7.1	7.8	7.2	7.6	5-8
Ca	≤3433	≤1280	≤4350	≤1440	≤1000
Cd	≤0.45	Nil	≤0.62	≤0.22	≤ 5
Co	≤0.45	Nil	≤0.84	Nil	≤ 5
Cr	≤0.89	Nil	≤0.87	Nil	≤ 5
Cu	≤ 8.48	≤0.078	≤11.0	≤1.66	≤ 5
Fe	≤104	≤0.32	≤123.2	≤0.53	≤50
K	≤1895	≤420.3	≤2140	≤558.9	≤1000
Mg	≤3463	≤190	≤3703	≤243	≤200
Mn	≤9.8	≤0.92	≤32	≤1.5	≤5
Na	≤4058	≤1233	≤3742	≤1652	≤5000
Ni	≤5.35	≤0.15	≤3.5	≤1.2	≤5
Pb	≤1.34	Nil	Nil	Nil	≤5
B	≤58.9	≤1.30	≤19.6	≤2.2	NR [b]
As	Nil	Nil	Nil	Nil	NR [b]
Zn	≤266	≤3.77	≤145.6	≤3.9	≤10

[a] All values of metal ion contents are in ppm; [b] NR=Not reported

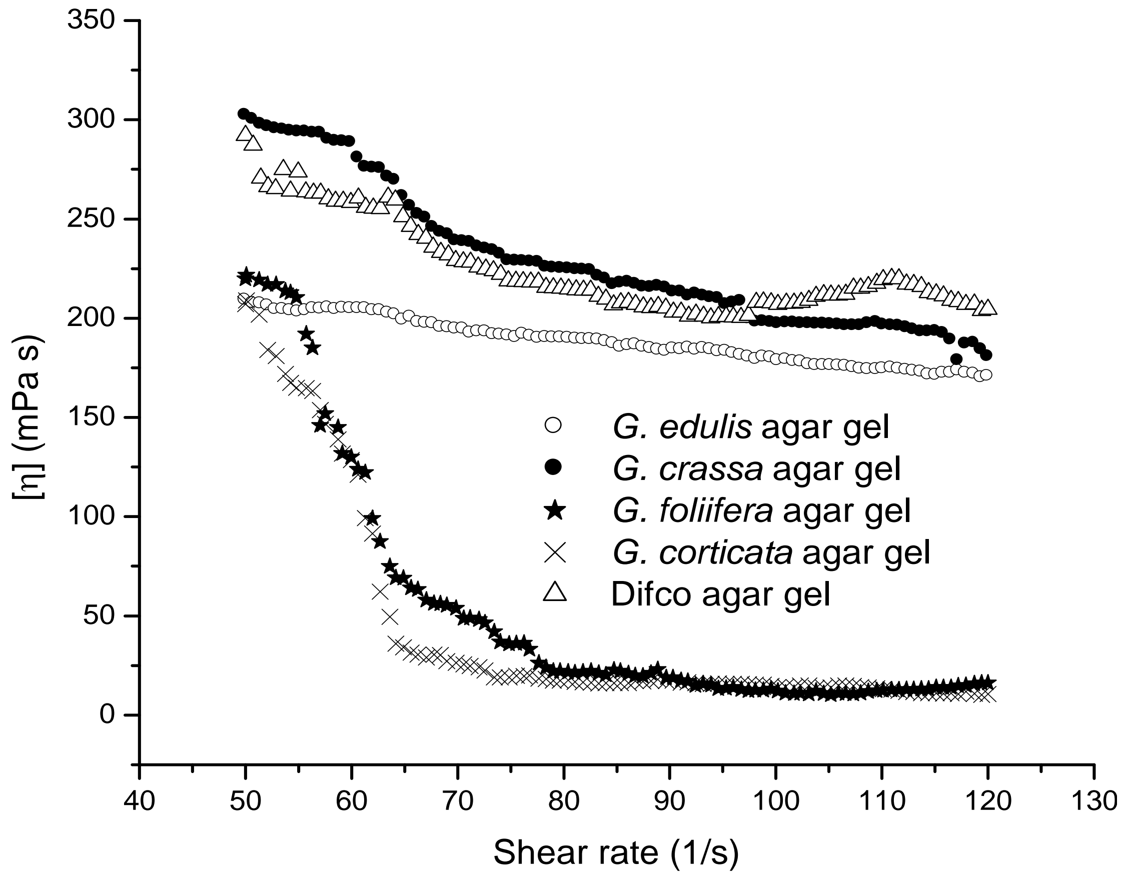

Figure II.2.1 Dynamic viscosity profile of alkali treated agar gel samples extracted from four *Gracilaria* species and Difco agar used as reference agar gel sample.

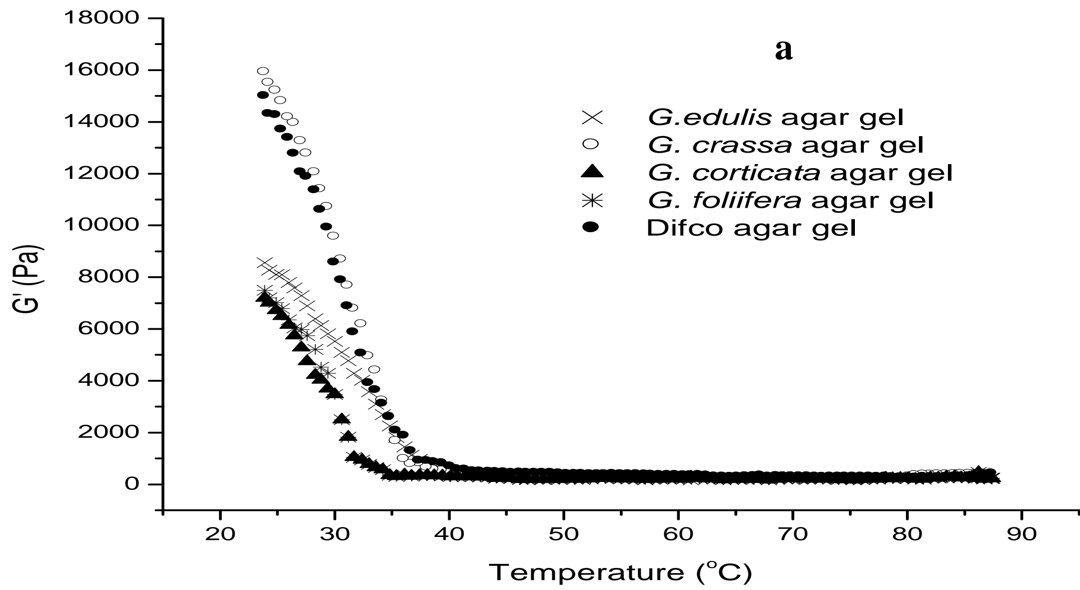

Figure II.2.2a Temperature dependence of storage modulus (G') of alkali treated agar gel samples extracted from four *Gracilaria* species and Difco agar used as reference agar gel sample.

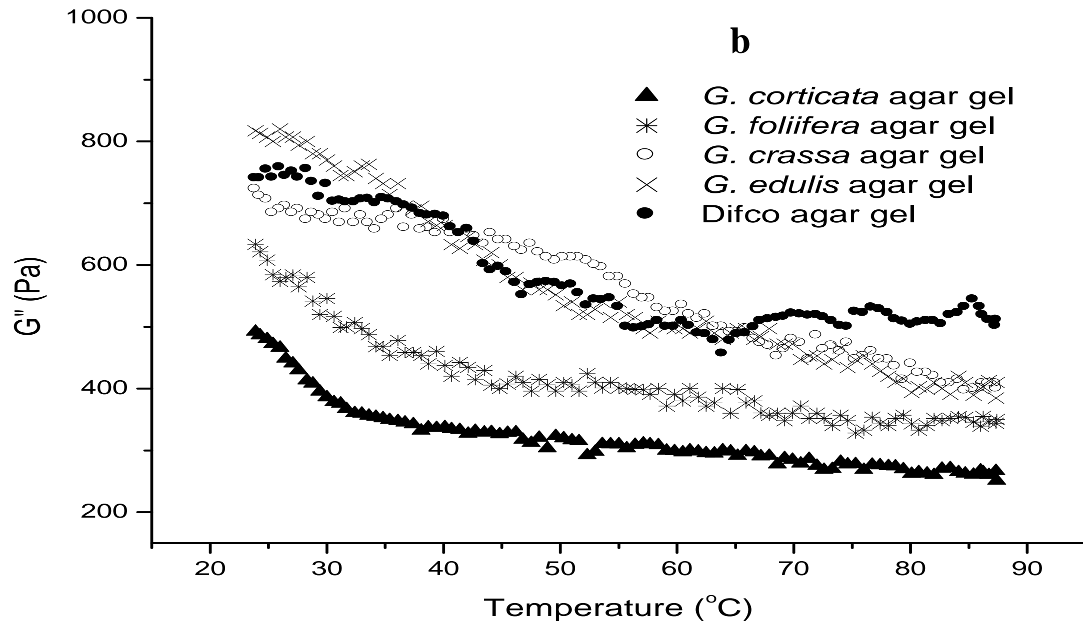

Figure II.2.2b Temperature dependence of loss modulus (G") of alkali treated agar gel samples extracted from four *Gracilaria* species and Difco agar used as reference agar gel sample.

CHAPTER II.3

AGAROSE FROM *GELIDIELLA ACEROSA* OF INDIAN WATERS

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II.3.1 INTRODUCTION

Value addition of Indian *Gracilaria* spp. have been reported earlier in the Chapters 1 & 2 of Part II this dissertation, wherein it has been reported that Indian *Gracilaria dura* could be used as a source of superior quality agarose, and *G. edulis* and *G. crassa* be used for extracting superior quality agars just by using improved methods of extraction (cf. Siddhanta et al. Meena et al [1, 2]. Recently, preparation of superior quality agar from *Gelidiella acerosa* has been reported in the literature [3, 4]. Various factors such as environmental factors and the extraction process parameters have been reported to influence the quality of agar from the species of *Gelidiales*, *Pterocladia* and *Gelidium* [5-8].

In this Chapter, superior quality agarose polymer was prepared from *Gelidiella acerosa* collected from the southeast coast of India. Agarose was produced in 12-15% yields, having gel strength between 2000-2100 g cm^{-2} (in 1.5% gel). The result of the present study demonstrated that Indian *Gelidiella acerosa*, an agarophyte which has been reported so far as a source of agar can be used for producing agarose in a cost-effective and environment friendly method.

II.3.2 EXPERIMENTAL

II.3.2.1 Materials

The agarose polymer investigated in this chapter was obtained from specimens of *Gelidiella acerosa*, collected from southeast coast of India (Mandapam). The agarose preparation process corresponded to a patent specification [1, 2]. Harvested plants were brought to the laboratory, air dried and stored in plastic bags. Sample specimen of the seaweed after identification was submitted to the CSMCRI Herbarium. For comparative study, agarose was purchased from Sigma-Aldrich, USA (Cat. No. A0576), because of its low sulphate content and gelling point as well as high gel strength.

II.3.2.2 Extraction of native agar

Native agar was extracted from *Gelidiella acerosa* in the lab as well as in the pilot plant taking 20 g to 1.5 kg dry seaweed respectively per batch. Dry seaweed was soaked in tap water for 1 h, the wet seaweed was soaked in 0.5% acetic acid solution for 2 h. The excess acid from soaked seaweed was removed by water washing [cf. 7]. This washed seaweed was then autoclaved at 120°C for 1.5 h with distilled water (seaweed-water, 1:30 w/w). The extract was processed using the method as described in Chapter II.1, *Section*: II.1.2.2. The percentage yield of dried agar was calculated on the basis of bone dry seaweed.

II.3.2.3 Preparation of agarose

Alkali pre-treatment of *Gelidiella acerosa* was carried out using 3%, 4%, 6%, 8%, 10% and 15% aqueous NaOH solutions as described in our previous patented work on *Gracilaria dura* (Siddhanta et al.; Meena et al. [1,2]. Agarose samples were prepared in the lab as well as in the pilot plant taking 20 g to 1.5 kg dry seaweed per batch, respectively. For agarose preparation 20 g to 1.5 kg dry *Gelidiella acerosa* were soaked in 200 ml to 20 liters tap water for 1 h at room temperature and then treated with 200 ml to 20 liters various concentrations of aqueous NaOH solutions at 80°C in a water-bath for 2 h. After the alkali treatment, excess was removed by water washing until the washing showed pH in the range of 7-8. The seaweed was then autoclaved with distilled water (1:20 w/v) at 120°C for 1.5 h. Afterwards the extract was processed using the method as described in Chapter II.1.

II.3.2.4 Physical analyses

The ash content of the native agar and agarose samples was estimated in the residue that was obtained after igniting the agar at 550°C for 4 h. For measuring the physical properties, 1.5% (w/w) solutions of agar and agarose were prepared in an autoclave at 120°C. After the formation of gel at room temperature, it was kept at 10°C overnight in a refrigerator. Gel strength was measured at 20°C using a Nikkansui type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). Gelling and melting temperatures were measured

according to the method described by Craigie & Leigh [9]. Apparent viscosity in the1.5% agar sol was measured at 80°C on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072); using spindle No.1 at 60 rpm. The pH of the agar solution (1.5%, w/w) samples was measured by Digital pH Meter 335 Systronics, at 70°C. Detailed for all measurements have been described in the Chapter II.1.

II.3.2.5 Chemical analyses

The 3, 6-anhydrogalactose content was estimated using a phenol-resorcinol reagent employing the improved method using fructose as a standard [10]. Metal ion and sulphate contents analyses (ICP) were carried out on a Perkin-Elmer ICP-OES Optima 2000DV. Detailed methods for measurements of 3, 6-AG and metal ions have been described in Chapter II.1.

II.3.2.6 Rheological measurements

Dynamic rheological measurements of *Gelidiella acerosa* and Sigma (A0576) agarose gel samples were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany), as described in the Chapter II.1.

II.3.2.7 FTIR Spectra

The FTIR spectra of *Gelidiella acerosa* and Sigma (A0576) agarose were recorded, as described in Chapter II.1.

II.3.2.8 Weight average molecular weight (M_w)

Weight average molecular weight was calculated from the intrinsic viscosity using the Mark–Houwink equation for agarose as described by Rochas & Lahaye [11]. Detail process has been described in Chapter II.1.

II.3.2.9 Statistical analyses

Analysis of variance (one way ANOVA test) was carried out by using Microcal Origin, Version 6 software (Microcal Software Inc. MA, USA). To carry out the analysis the variance four replications ($n = 4$) of each parameter in three groups were made. Mean and standard deviation were calculated using the Microsoft Excel 2000, software.

One-way ANOVA test was conducted for significant differences (when $p < 0.01$) between native agar (without NaOH pre-treatment) and agarose (with optimum NaOH pre-treatments) in agar yield, gel strength, 3,6-anhydrogalactose content, sulphate content and ash content from the *Gelidiella acerosa*.

II.3.3 RESULTS AND DISCUSSION

II.3.3.1 Physical Properties

Yields were calculated on the basis of as received dry seaweed containing nil moisture (bone dry) (Figure II.3.1b). The yield of native agar was 28.0±0.5% for *Gelidiella acerosa* samples collected from the south east coast of India. The yields obtained with alkali pre-treatment using various NaOH concentrations ranged from 12-24% (Figure II.3.1b). The optimum alkali concentration for the pretreatment was identified on the basis of the yield and quality of the product as discussed below. The variation in agar yield is not only affected by extraction time, temperature but also by the pre-extraction treatments. Alkali treatment was observed to soften the wiry and tough thalli of seaweeds, and acid treatment facilitated extraction of the polysaccharide by disrupting crosslinks occurring in the algal cell wall structure [12].

The optical rotation of *Gelidiella acerosa* agarose was $[\alpha]_{589}^{45}$ -23° (c 0.25, H_2O), and that of Sigma agarose (A0576) was -21° (c 0.25, H_2O). Apparent viscosities of the native agar and agarose were 26 ± 0.5 cP and 31 ± 0.81 cP in 1.5% sol at 70°C, and the gel strengths were 800±15 g cm^{-2} and 2000±25 g cm^{-2}, respectively (Figure II.3.1a). The variations in the gel strengths from 1050 g cm^{-2} to 2100 g cm^{-2} with alkali concentrations are given in Figure II.3.1a. The gelling and melting temperatures of agarose gel were 41± 0.55^{o}C and 85± 0.5^{o}C, respectively.

Among the *Gelidiales*, alkali modified agar from *Gelidium serrulatum* J. Agardh, *G. floridamum* Taylor, and *Pterocladia capilacea* (S.G. Gmelin) Bornet have improved quality in terms of gel strength (relative to the decrease in percent sulphate and increase in 3,6-anhydrogalactose contents) compared to their untreated counterparts [6]. Likewise, alkali modified agar samples of *Gelidiella acerosa* have significantly higher gel strength (189 g cm^{-2}) than the native samples (33 g cm^{-2}) [11]. The characteristics of *Gelidiella acerosa* agarose (alkali modified agarose) in terms sulphate (%) and 3, 6-anhydrogalactose contents are depicted in Figure II.3.1b. The sulphate contents of native agar decreased on pretreatment with alkali (Figure II.3.1b). The sulphate content ranged 0.3-1.04% with alkali concentrations while sulphate content was 1.114% in the native agar samples (Figure II.3.1b). Examination of Fig. 3 revealed that agarose prepared with the 10% alkali pretreatment afforded 15% yield containng 0.36% sulphate, and 42% 3,6-anhydrogalatose, with gel strength 2000 g cm^{-1}, which was by far the best profile amongst the ones of agaroses obtained from different other alkali pretreatment conditions, in view of the sulphate content and the yield. Thus it was decided that 10% alkali pretreatment was the optimized concentration of alkali which would give the best quality agarose from *Gelidiella acerosa*.

The weight average molecular weights of the native agar and agarose were determined and depicted in Figure II.3.1c. Molecular weights of native agar decreased with increased concentration of alkali used in the alkali pretreatment step (Figure II.3.1c). The greatest weight average molecular weight, $(2.94 \pm 0.07) \times 10^5$ Dalton, was observed for native agar sample, and the lowest was $(1.89 \pm 0.01) \times 10^5$ Dalton, for the agarose polymer obtained by 15% NaOH pretreatment, while agarose prepared under optimized alkali concentration (10% NaOH) was M_w $(1.94 \pm 0.079) \times 10^5$ Dalton. This M_w values had a direct consequence on gel strength; it was noted that agarose of greatest gel strength showed the lowest value of M_w – similar observation was reported in the literature by Meena et al. and Murano et al. [2, 13]**.** This observation is in line with those reported by several authors describing that the degree of sulphation is inversely correlated with gel strength [6, 14, 15, 16, 17]. It may be noted that alkali pretreatment gives rise to the formation of 3, 6-AG on the L-galactose-6-sulphate moiety [14], which had direct consequence on enhanced gel strength of agrose obtained under optimized pretreatment conditions (Figure II.3.1a & b).

The analysis of variance revealed that yield, gel strength, sulphate, 3,6-AG and ash contents of *Gelidiella acerosa* agarose were significantly greater than those of the native agar ($p<0.05$) [cf. Chapter II.2].

II.3.3.2 Metal Ions

Metal ion analyses of the native agar and agarose samples were carried out using inductively coupled plasma spectrometry (ICP) [18] and the values were compared with those of Fluka agar and agarose samples. Metal ion concentrations were significantly different in the native agar and agarose samples, obtained from *Gelidiella acerosa* (Table II.3.1). Native agar and agarose samples from *G. acerosa* showed higher concentrations of calcium and magnesium ions than those of the reference samples of Fluka agar and agarose. In case of sodium ion, Fluka agar and agarose samples showed significantly higher values.

Table II.3.1 Comparison of metal ion contents in native agar and agarose of *Gelidiella acerosa* with those of Fluka products [a]

Metal ions	Native Agar of *Gelidiella acerosa*	Agarose of *Gelidiella acerosa*	Agar (Fluka Cat. No. 05038)	Agarose (Fluka Cat. No. 05068)
Ca	≤6350	≤2900	≤1000	≤500
Cd	≤0.42	≤0.27	≤ 5	≤10
Co	≤0.84	ND	≤ 5	≤10
Cr	ND	ND	≤ 5	≤10
Cu	≤38.0	≤2.16	≤ 5	≤10
Fe	≤4.2	≤2.7	≤50	≤10
K	≤2140	≤90.3	≤1000	≤500
Mg	≤3703	≤687	≤200	≤10
Mn	≤32	ND	≤5	≤10
Na	≤2942	≤683	≤5000	≤5000
Ni	≤1.5	≤0.27	≤5	≤10
Pb	ND	ND	≤5	≤10
B	≤41.1	≤9.2	NR	NR
As	≤5.3	ND	NR	NR
Al	≤7.6	≤3.7	NR	NR
Zn	≤19.6	≤3.78	≤10	≤10

Note: [a] Values in ppm; NR = Not reported; ND=Not Detected

II.3.3.3 Spectral analysis (FTIR)

The spectrum of agarose samples extracted from *Gelidiella acerosa* was very similar to that of the Sigma agarose (A0576) sample, in the region 500-1500 cm^{-1}. Both showed characteristics band at 740, 773 and 930 cm^{-1} due to β-galactose skeletal bending. FT-IR band at 890 cm^{-1}band is specific for agar and can be attributed to anomeric C-H of β-galactose residues. Other characteristic bands observed were at 3434 and at 3415 (s), indicates O-H stretching, 1631 and at 1640 (s, CO-NH-stretching) at 1412 and at 1414 (sh) appears due to H-C_6-O local symmetry, at 1382 and at 1375 (m) as well as at 1153 and at 1154 (sh), attributed due to S=O stretching; at 1068 and at 1073 (s) due to C-O-C stretching, at 933 and at 931 (m) attributed due to 3,6-AG ring vibration, 893 and at 891 (m, β-anomeric-CH- deformation); at 775 and at 771 (w, β-galactose skeletal bending); at 741and at 740 (w, β-galactose skeletal bending); at 714 and at 715 cm^{-1} (w, β-galactose skeletal bending), observed for the agarose samples extracted from *Gelidiella acerosa* (Figure II.3.2).

II.3.3.4 Rheological Measurments

The stability of modulus during storage at 25°C was investigated for Sigma (A0576) and *Gelidiella acerosa* agarose gels (Figure II.3.3a). The G'' values for the both agarose gel samples increased with time indicating absence of syneresis in the agarose gel at 1.5% concentration [19]. The slightly higher values of the storage modulus (G') for the Sigma (A0576) agarose under stress for long time indicates more stability of Sigma (A0576) agarose gel than the *Gelidiella acerosa* agarose gel.

Temperature dependencies of G' and G'' for Sigma (A0576) and *Gelidiella acerosa* agarose gel samples are summarized in Figure II.3.3b. This experiment was conducted by decreasing temperature from 90°C to 20°C. The value of loss modulus (G'') decreased with decreasing temperature and a reverse trend was observed for storage modulus (G') values. At the gelling point i.e. ca. 41°C in *G. acerosa* and ca.36°C in Sigma agarose gel samples, the values of G' increased dramatically and crossed the G'' value at these points. Similar observation was reported in the literature [20].

The effect of frequency (0.1-10 Hz) on storage and loss moduli (G' and G") of agarose for *Gelidiella acerosa* and Sigma (A0576) are shown in Figure II.3.4a. The G' values were significantly greater than the G" values at all the measured frequencies. This indicated gel nature of both the agarose samples. Slightly higher value in this experiment for Sigma agarose also indicated greater rigidity of the Sigma agarose gel.

The variations in dynamic viscosity of gels of *Gelidiella acerosa* and Sigma (A0576) agaroses are shown in Figure II.3.4b. The dynamic viscosity of both gels decreased with increasing shear rate. Non-Newtonian or shear thinning behavior was observed in both the agarose gels [cf. 21].

II.3.4 CONCLUSONS

Superior quality agarose polymer was prepared from *Gelidiella acerosa*, an agarophyte of Indian waters, using an improved method [1, 2]. To our knowledge, this is the first report of direct, cost-effective and solvent-free process for preparation of agarose from *Gelidiella acerosa*. The present study demonstrates that the agarophyte species namely, *Gelidiella acerosa* occurring in Indian waters could be used as a source of agarose polymer. The gelling temperatures and gel strength of agarose samples prepared directly from the *Gelidiella acerosa* were 41°C and 2000 g cm^{-2} respectively. Agarose prepared herein would be useful for bacteriological and biotechnological applications. This work would be beneficial for bio-prospecting of Indian agarophytes.

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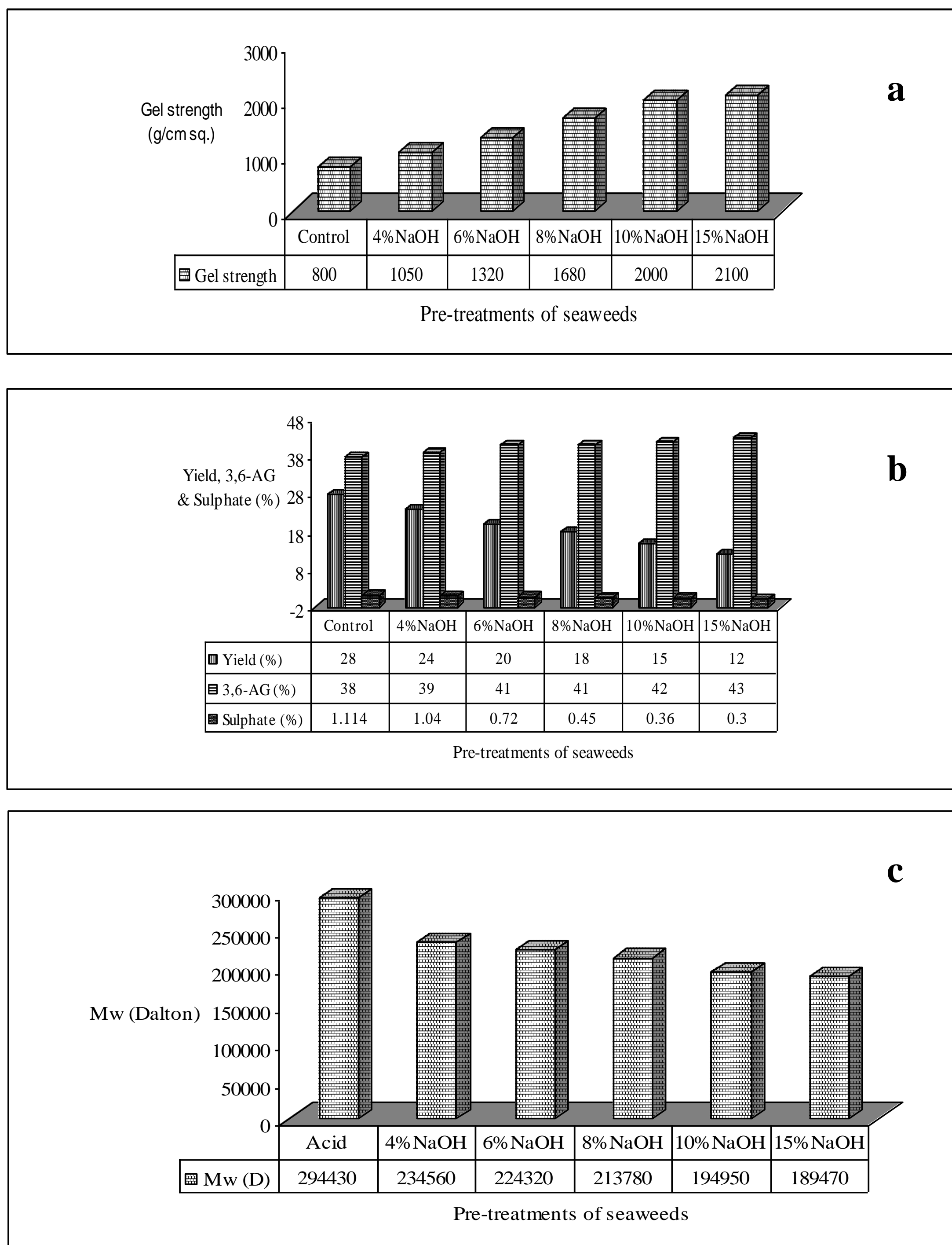

Figure II.3.1a-c Effect of alkali (NaOH) concentration on the physicochemical properties of agar (a) gel strength; (b) yield, 3,6-anhydrogalactose and sulphate contents (%), and (c) weigth average molecular weight (Mw)

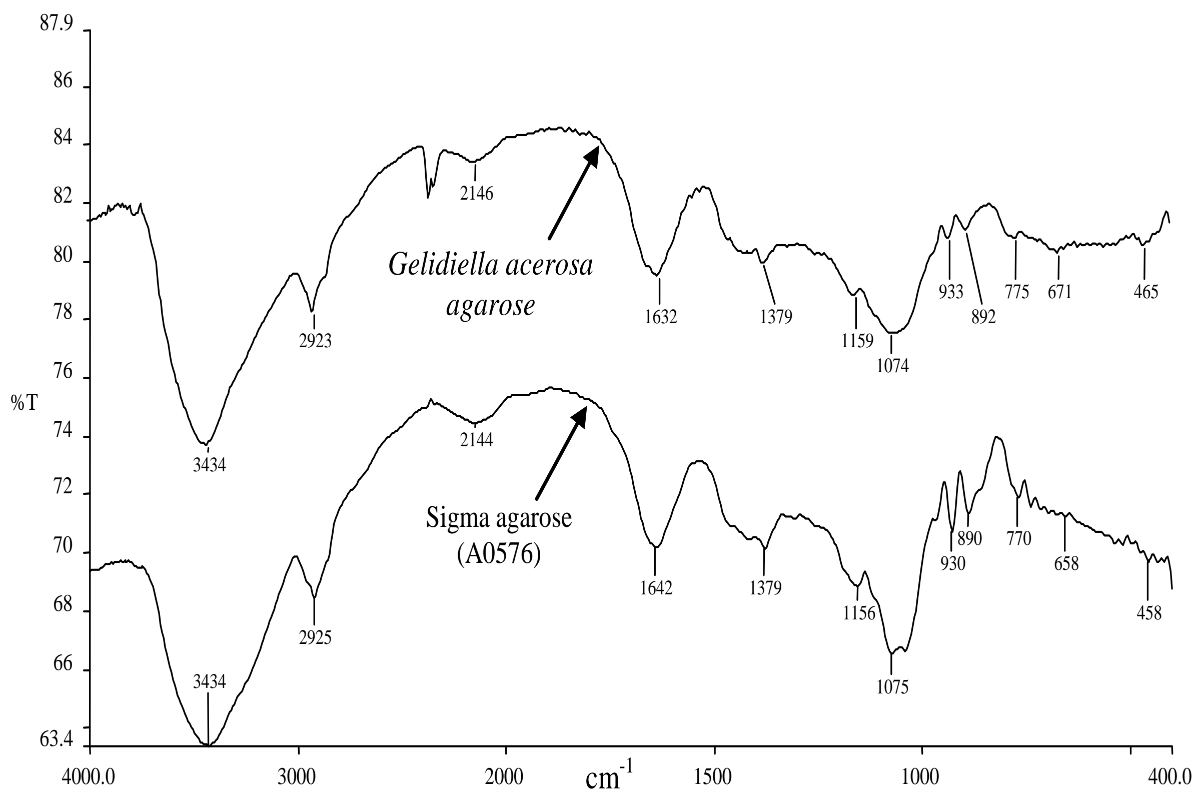

Figure II.3.2 FT-IR spectra of agarose samples of *Gelidiella acerosa* and Sigma Agarose (A0576)

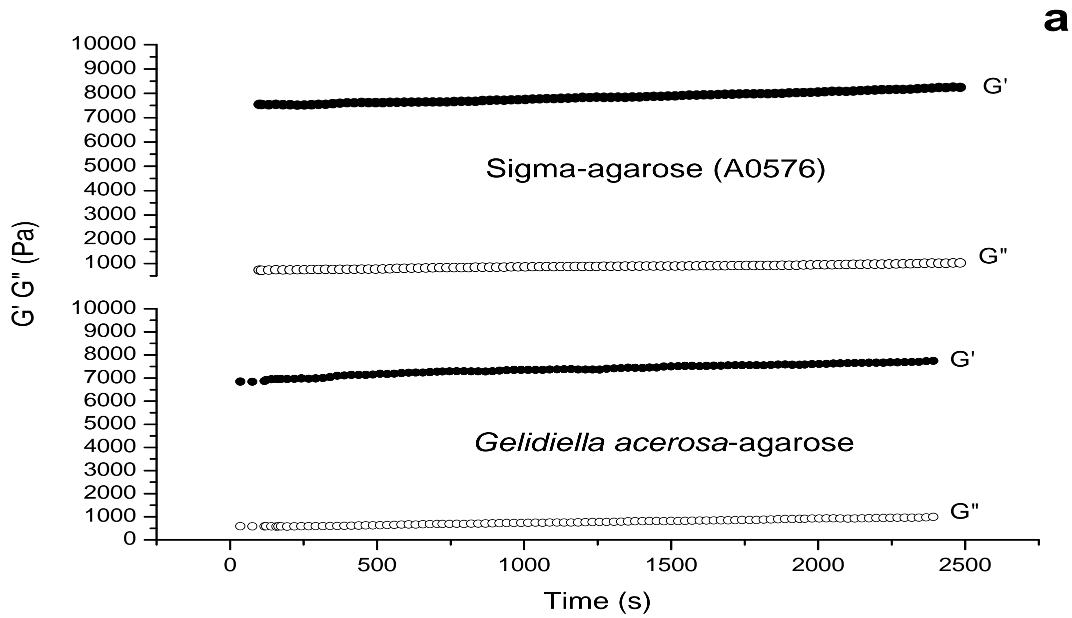

Figure II.3.3a Variation in storage and loss modulus (G′ and G″) of *Gelidiella acerosa* and Sigma agarose gel samples with time

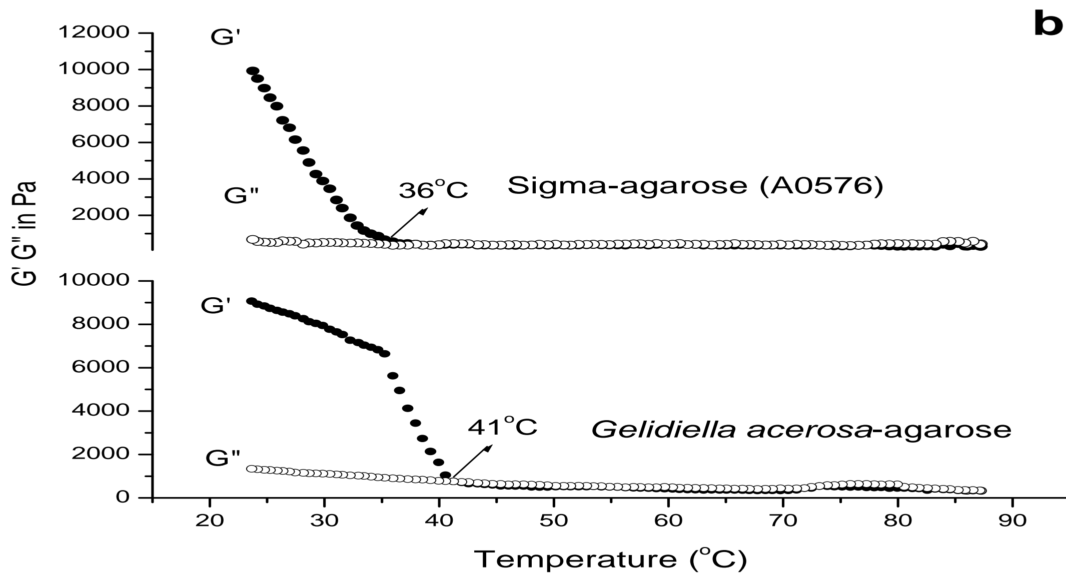

Figure II.3.3b Variation in storage and loss modulus (G′ and G″) of *Gelidiella acerosa* and Sigma agarose gel samples with temperature

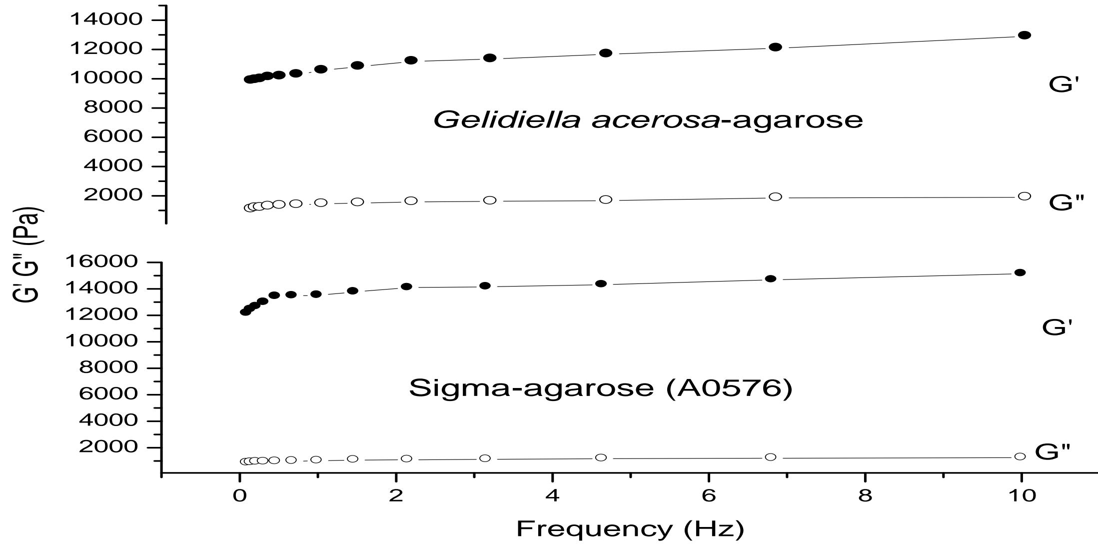

Figure II.3.4a Variation in storage and loss modulus (G′ and G″) of *Gelidiella acerosa* and Sigma agarose gel samples with frequency

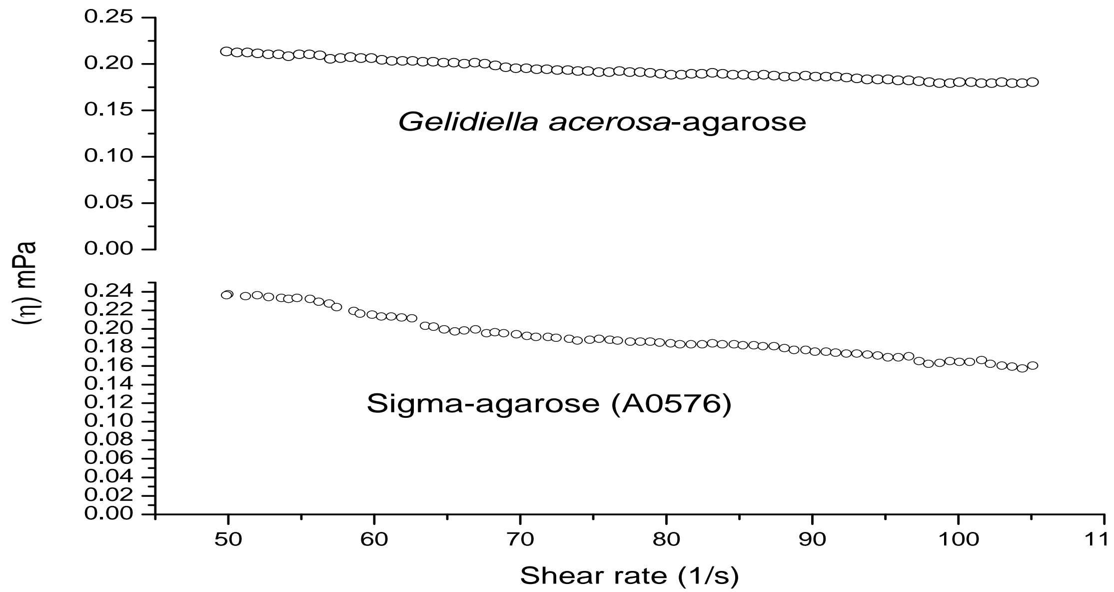

Figure II.3.4b Gel thinning behaviour of agarose gel samples of *Gelidiella acerosa* and Sigma

PART III

CHAPTER III.1

STUDIES ON "SUGAR REACTIVITY" OF AGARS EXTRACTED FROM SOME INDIAN AGAROPHYTES

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III.1.1 INTRODUCTION

The principal sources of commercial agar include the Gelidiales and Gracilariales that are aquatic plants found primarily in Asia [1, 2]. Due to differences in species, habitat, harvesting season, extraction and processing conditions during extraction, various 3,6-anhydro-galactose (3,6-AG) contents and molecular sizes are found for agar extracts, which accordingly possess different gel characteristics [1]. Agar has a wide variety of uses as human and animal foods in addition to having numerous industrial applications. In the food industry, agar is employed predominantly for its stabilizing and gelling characteristics. The thickening effect of agar when dispersed in water medium is the basis of its use as bulking, stabilizing and emulsifying agent in foods. Its gels are used as texture modifier. Furthermore, it has the unique ability of holding large amounts of moisture whereby preventing quick dehydration of confectionary products [3].

Soft and elastic gels used in the food industry are obtained from *Gracilaria* spp. Armisen & Galatas [4] indicated that addition of sugar in high concentration (above 50%) to *Gracilaria* agar increases its gel strength much more than it does to that of *Gelidium* agar. Murano described this type of agar as "sugar-reactive" [5], which was considered the most expensive phycocolloid today [6].

Studies have been done on the effect of sucrose on the gelling properties of phycocolloids. Nishinari et al. reported that addition of increasing amounts of sucrose (up to 60%) increased melting temperature [7]. Matsuhasi has reported doubling of gel strength when 50% sucrose was added [1]. On the other hand, Romero et al. reported decrease of gel strength of *Gelidiella acerosa* agar after adding 50% sucrose and increase of gel strength in case of agar from *Gracilaria* spp. [8]. Herein we report the results of the studies on the sugar reactivity of agar extracted from four agarophytes of Indian waters [9]. To our knowledge, this is the first report on "sugar reactivity" of agars extracted from Indian agarophytes. Further it is the first report describing glucose monosaccharide having caused reactivity of agars.

III.1.2 MATERIALS AND METHODS

III.1.2.1 Materials

Three agarophytes (*Gelidiella acerosa, Gracilaria crassa and Gracilaria edulis*) samples were collected from the south east coast of India (Mandapam), one agarophyte (*Gelidium pusillum*) sample was collected from the west coast of India (Veraval), and using Qualigens Fine Chemicals, powdered Oxoid Agar No.1 (Oxoid, UK; Lot/Ch.-B: 810501-2) as reference material for all the measurements in this study. It may be mentioned here that the source seaweed of Oxoid agar is not known. Sucrose (AR) and glucose (AR) were procured from Ranbaxy Fine Chemicals Ltd., India. Samples of the dried material were washed in tap water to remove epiphytes and sand, cut in to small pieces, air dried and then oven dried at 60°C to constant weight before agar extraction. Specimen of each sample has been deposited with the CSMCRI Herbarium.

III.1.2.2 Agar extraction

Agar was extracted from dried samples of *Gelidiella acerosa* following the method described by Roleda et al. [10]. Alkali modification was done prior to extraction of agar samples from *Gracilaria crassa*, *Gracilaria edulis* and *Gelidium pusillum,* as described by Meena etal. [11]. The hot homogenized extracts were filtered under reduced pressure through Celite bed and filtrate was allowed to gel at room temperature and kept at –20°C for 15 h. The frozen gel was thawed, the thawed liquid was squeezed off, and then the gel was washed with distilled water and air dried for 24 h at room temperature (30°C) followed by drying in the oven at 60°C for 4 h. The yield of agars was calculated on the basis of as received dry seaweed.

III.1.2.3 Gel preparation

Three types of gels were prepared. The control gel was made of 1.12% (w/w) aqueous agar solution. The sucrose-agar and glucose-agar gels were prepared by incorporating sucrose and glucose to constitute 50% sucrose/glucose and 1.12% agar in the gel.

III.1.2.4 Gel strength

The gel strength (in g cm^{-2} at 20^oC) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). The measurements were performed on a 1.12% w/w agar solution (previously cured overnight at 10^oC), Hutardo-Ponce & Umezaki [12], using a solid cylindrical plunger of 1 cm in diameter.

III.1.2.5 Gelling and melting temperature

Gelling and melting temperatures of agar gel was measured as described by Craigie & Leigh [13].

III.1.2.6 Syneresis index

The amount of water exuded from the gel samples after standing for a certain period of time was determined and quantified using a modified method [14]. The syneresis index values of the gel samples were taken as the difference between the initial weight of the gel and its final weight after 2 h. This value indicated the water holding capacity of the gel.

III.1.2.7 Apparent Viscosity

Apparent viscosity was measured using a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No.1 was used for measuring apparent viscosity at speed of 60 rpm.

III.1.2.8 Chemical Analysis

The amount of 3,6-anhydrogalactose present in agar extracts of the different agarophytes were estimated by improved phenol-resorcinol reagent method [15] using fructose as standard, while sulphate content in the agar samples was estimated by turbidimetric method [16].

III.1.2.9 FT-IR Spectra

Infrared spectra were recorded on a Perkin-Elmer Spectrum GX (FT-IR System, USA), by taking 2.0 mg of agar in 600 mg of KBr (quantitative IR). All spectra are average of two counts with 10 scans each and a resolution of 5 cm^{-1}.

III.1.2.10 Weight average molecular weight

Intrinsic viscosities [η] were determined at 32°C using an Ostwald viscometer. Solutions of agar were prepared in 1M NaCl at a concentration range 0.02% to 0.1%. Viscosity average molecular weights were calculated from the intrinsic viscosity using the Mark–Houwink equation for agarose as described by Rochas & Lahaye [17].

III.1.2.11 Thermal Analysis (TGA)

Sample of gels were prepared as mentioned above. Thermal analysis (TGA Toledo Mettler TGA system, Switzerland) was done in (15-20 mg) gel samples, using a temperature programme 30°C to 600°C (@10°C per min).

III.1.2.12 Rheological measurements

Samples of sol and gel were prepared as described above. Dynamic rheological measurements of these samples were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany). The cone/plate (60 mm diameter, 1 rad angle) geometry was selected for dynamic viscosity measurements of sol at 45°C, with 1 ml sample volume. The plate/plate (35 mm diameter) geometry was selected for oscillation measurements of agar gel in the controlled deformation mode with 0.05% strain, the temperature of gel being maintained at 25°C using the DC50 water circulator. Initially, the measurements of G' and G'' were monitored at 5 min intervals over 1 h but no significant variation was observed and subsequent measurements were carried out immediately after placing gel sample on the plate/plate. For measurements at all temperatures the exposed part of the samples were covered with silicone oil to minimize losses due to evaporation. All rheological data present were means of three replicate measurements. Under the experimental conditions selected, no notable syneresis or gel

slippage that can be judged by abrupt decrease in G' was observed for the systems measured [18].

III.1.3 RESULTS AND DISCUSSION

III.1.3.1 Physical Properties

The gel strengths of agar gels from different agarophytes with and without sugars (sucrose and glucose) were measured, and are depicted in Figure III.1.1 and Table III.1.1. The gel strengths of sucrose-agar gels increased (25-45%), which was most pronounced with agar of *Gracilaria crassa* (45%) and the least (25%) was observed with agar of *Gelidium pusillum* Figure III.1.1. The gel strengths of glucose-agar gels also increased (20-34%), wherein the lowest effect (20%) was observed with agar of *Gelidium pusillum*, with agar of *Gracilaria crassa* affording the highest effect with glucose which exhibited 34% increase in gel strength compared to that of control agar gel Figure III.1.1. The gel strength of sugar-Oxoid agar gels increased with sucrose (33%) as well as with glucose (23%) Figure III.1.1. Romero et al. [8], reported "sugar reactivity" of sugar-agar gel in presence of sucrose only, using agars obtained from the seaweeds of the Philippines. Romero et al. also reported that agar of *Gelidiella acerosa* was not sugar reactive. Our studies, however, have shown that agar of *Gelidiella acerosa* of Indian waters are sugar reactive in presence of both sucrose and glucose (Table III.1.1).

The gelling and melting temperatures of the three gel samples e.g. control, sucrose-agar and glucose-agar, are shown in Table III.1.1. The 1.12% control agar solution prepared from *Gelidiella acerosa* and reference (Oxoid) agar had the highest gelling temperatures (41°C), while those of *Gelidium pusillum*, *Gracilaria edulis* and *Gracilaria crassa*, were the lowest (34°-38°C). Sucrose-agar gels of *Gelidiella acerosa*, *Gracilaria edulis* and Oxoid agars had the highest gelling temperature (41°-44°C). However, lowering (2°-3 ^{o}C) in the gelling temperatures was observed with the corresponding glucose-agar gels. Similarly, corresponding melting temperatures increased (2°-4°C) in sucrose-agar gels and decreased (2-3°C) in glucose-agar gels.

The extent of syneresis was determined in case of control; sucrose-agar and glucose-agar gel samples and the values are depicted in Figure III.1.2. The amounts of water exuded from the control gels were significantly higher than those of the sucrose-

agar and glucose-agar gels, whereas the extent of syneresis is higher in glucose-agar gels compared to the sucrose-agar gels. Among the control gels, the *Gelidiella acerosa* agar showed the highest syneresis index while the one from *Gracilaria crassa* was the lowest. The seaweed source of Oxoid agar is unknown; however, it showed similarity with *Gelidium pusillum* and *Gelidiella acerosa* agar gels in terms of the water holding capacity. Syneresis is a spontaneous process by which water is released with the contraction of the gel matrix that may occur upon standing leading to a more stable agar gel network.

The apparent viscosity at 80°C was determined in case of control, sucrose-agar and glucose-agar gel samples and the values are given in Table III.1.1. The viscosities of the control gels were significantly lower than those of the sucrose-agar and glucose-agar gels. Further, viscosities of the glucose-agar gels were lower than those of the sucrose-agar gels. In the control agar gels, the *Gracilaria crassa* agar showed the highest viscosity, *Gelidiella acerosa* agar having the lowest. Similar trend was observed in sucrose-agar and glucose-agar gels.

III.1.3.2 Chemical analysis

Agar analysis by FTIR: The IR spectra of all agar samples extracted from different Indian agarophytes as well as Oxoid agar exhibited similar IR-bands to that reported by Christiaen & Bodard [19] (Figure III.1.3).

The 3, 6-anhydrogalactose contents of the agar of different agarophytes as well as Oxoid agar were estimated and are shown in Table III.1.1. The agar prepared from *Gelidiella acerosa* and *Gelidium pusillum* had the highest 3, 6-anhydrogalactose values (42% & 43%), while that of *Gracilaria edulis* the lowest (29%). The sulphate contents of the agar of the different agarophytes as well as Oxoid agar were estimated and are presented in Table III.1.1. The agar prepared from *Gracilaria edulis* had the highest sulphate content value (2.6%), while *Gelidiella acerosa* and *Gelidium pusillum* that of the lowest (1.114% & 0.96%). It may be mentioned here that the combination of lowest

sulphate and highest 3,6-anhydrogalactose contents gives high gel strength, [5, 20] which is reflected in the high gel strength values of *Gelidiella acerosa* and *Gelidium pusillum* agars.

III.1.3.3 Rheological properties

Dynamic viscosities of control agar, sucrose-agar and glucose-agar gel samples were measured and the values are depicted in Figure III.1.4. The materials showed shear thinning behaviour when a shear rate lower than about 150 s^{-1} was applied. When shear rate exceeded this value, the flow curve gets flat, indicating the Newtonian behavior. But in the same condition sucrose-agar gel samples showed least gel thinning and the control gel samples showed the highest gel thinning behavior in this study. These observations are similar to those reported by Loisel et al. [21]. Therefore, the effect of sugars on the flow properties could be explained by several contributing factors. First, increasing the sucrose content in the binary solvent induces an increase in viscosity. This increase is not linear and a 40-95% enhancement of viscosity was recorded. This effect has been explained by Normand et al. as the reduction in polymer chain mobility, prompting helix nucleation inhibiting thereby the growth process as a result of packing of the helices into thick bundles [22].

The time dependence of G' and G", of agar from different agarophytes with or without sucrose and glucose are depicted in Figure III.1.5. Sucrose-agar gels prepared from *Gracilaria crassa* and *Gelidium pusillum* exhibited the highest G' value followed by *Gelidiella acerosa* and *Gracilaria edulis*. Decrements in the G' value was observed in the similar sequences with the glucose-agar gels. As expected, the G" values followed a reverse pattern. The sucrose-agar gels exhibited the higher value of G' with the time followed by glucose-agar and control agar, and it increases with the time indicating increasing stiffness of the gels. Sucrose being a disaccharide has more flexible molecular geometry and would be favourably accommodated in the agar gel network through hydrogen bonding resulting in stronger gels involving increased number of junction zones as described by Rees [23] in the case of carrageenan and other polysaccharide gels. *Gelidiella acerosa* and *Gracilaria edulis* agar gels are the weaker two compared to those of *Geldium* pusillum and *Gracilaria crassa* agar gels. Similar trend is observed in the gels in presence of sucrose. Of these four the latter two agars are comparable to Oxoid agar in rheological properties with and without sugars.

III.1.3.4 Thermal properties

Thermogravimetric analysis (TGA) of (15-20 mg) control agar gels and the (15-20 mg) sugar-agar gels are shown in Figure III.1.6. The TGA plots of control agar gels show mass loss in one stage. With control agar gel of *Gracilaria crassa* 100% mass loss was observed at 79°C, whereas that of *Gelidiella acerosa* lost 97% mass at 96°C. In sugar-agar gels a two-step mass loss process was observed. In glucose-agar gels, *Gelidium pusillum* agar lost 52% in the first step in the range 130°-196°C where the final step started and finally at 600°C 96% mass loss was observed. In the latter case, there is a brief discontinuity in the final step in the temperature range 398°-420°C involving negligibly small mass loss. In case of glucose-agar gel of *Gelidiella acerosa* 77% mass was lost in the first step in the range 140° -200°C, when the final step of mass loss begins to end up at 99% level at 600°C. The thermograms of the glucose-agar gels of *Gracilaria crassa* and *Gracilaria edulis* followed the similar pattern lying in the ranges mentioned above. In sucrose-agar gels of the four agarophytes no significant differences were observed in the in the two steps mass loss process mentioned above having the following ranges: 80% mass loss at 125°-200 ^{o}C when final step begins to have 100% mass loss at 600°C (Figure III.1.6). In the case of Oxoid agar it was observed that control agar gel exhibited low thermal stability (i.e. it loses 96% mass at 67°C) than the control agar gels of Indian agarophytes. The glucose/sucrose-agar gels of oxoid agar showed higher thermal stability (i.e. it loses 45% and 40% mass in first step at 146°-190°C and 136°-196°C, and finally 89% and 92% mass loses at 600°C, respectively) than the sugar-agar gels of Indian agarophytes studied herein.

Sulphate content has been known to be a function of gel strength of agar, i.e. lower the sulphate, higher is the gel strength and vice versa [5, 20]. It is described in the literature that sulphation at C-6 of the galactose moieties creates kinks, which hinders gelation [23, 24]. Furthermore, the sulphate groups may push agar polymers apart, glucose and sucrose being accommodated in the gel network with varied degree of strengths resulting in higher gel strength and thermal stability with sucrose compared to glucose. Oxoid agar contains ca. 1% suphate whereas the agar of *Gelidium pussilum* and *Gracilaria edulis* agars contain 0.5% and 2.6% sulphate respectively (Table III.1.1). It is interesting to note that in the case of both Oxoid-sugar gels and sugar-agar gels of Indian agarophytes, the thermograms show a reverse stability patterns compared to control agar (Figure III.1.6). However, the agar of *Gelidiella acerosa* also contains ca. 1% sulphate,

and the thermal stability of sugar-agar gels of *Gelidiella acerosa* is less than that of the corresponding sugar-agar gels of Oxoid containing ca. 1% sulphate. This leads one to conclude that there are other contributing factors than sulphate which manifest the observed stability patterns of the gel samples in this investigation. In this study it was further observed that in all other measurements, control agar and sugar-agar gels of Indian agarophytes behaved in a similar fashion to those of Oxoid agar, unlike in TGA measurements. Therefore, it appears that the said dissimilarity in the characteristics of Oxoid agar and agar of Indian agarophytes involves the role of structural geometry of the agar polymers of various origins. Further studies will be needed for addressing this issue.

III.1.4 CONCLUSIONS

Sugar reactivity was observed with in the sugar-agar complexes in presence of sucrose and glucose with agars of Indian agarophytes viz. *Gelidiella acerosa*, *Gracilaria edulis*, *Gracilaria crassa* and *Gelidium pusillum* [9]. The sugar reactivity was more pronounced in presence of sucrose than glucose. Oxoid agar was used as the reference material. Control agar gel contained 1.12% agar (w/w) in water. Sucrose-agar and glucose-agar gels in water consisted of 50% (w/w) sucrose and 50% (w/w) glucose respectively along with 1.12% (w/w) agars of the four seaweeds mentioned above. Addition of sucrose resulted in increase (ca. 25-45%) in gel strength; increase (2-3°C) in gelling and melting temperatures was observed in the gels prepared with agars from all the agarophytes and Oxoid agar. On the other hand, addition of glucose resulted in increase (19-34%) in the gel strength and gelling and melting temperatures of the agar gels of Oxoid as well as of all other agars decrease (2-3°C). Maximum sugar reactivity was observed with the 50% level of sucrose and glucose in agar gels. Rheological and thermogravimetric (TGA) characteristics of these gel samples were studied. The latter showed two patterns e.g. control agar gel of Oxoid agar was thermally less stable than the four control agar samples studied; in sugar-agar gel samples it followed a reverse pattern. To our knowledge, this is the first report of “sugar reactivity” of agar of Indian agarophytes [9]. Sugar reactivity of agar in presence of glucose is also reported herein for the first time. The results of this study will be useful in bioprospecting as well as in exploring new applications [9].

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Table III.1.1 Physicochemical properties of Indian and standard Oxoid agars with and without sugars

Agar	Yield [a] (%; ±SD)	Gel Strength [b] (g cm^{-2}; ±SD)	3,6-AG (%; ±SD)	Sulphate (%; ±SD)	Gelling Temp. (^{0}C; ±SD)	Melting Temp. (^{0}C; ±SD)	Apparent Viscosity [c] (cP; ±SD)	Weight Av. Mol. wt. (±SD)
Oxoid								
Without sugars	NA	680±10	42±0.8	0.92 ±0.03	41±0.44	85 ±0.45	9±0.89	$1.54X10^5$ ±485
With 50% sucrose	NA	920±15	ND	ND	44±0.55	88±0.44	14±0.44	ND
With 50% glucose	NA	850±10	ND	ND	38±0.55	83±0.55	11±0.54	ND
Gelidiella acerosa								
Without sugars	27±0.83	560±25	43±0.4	1.11 ±0.04	42±0.45	84±0.54	22±0.45	$3.24X10^5$ ±534
With 50% sucrose	NA	720±20	ND	ND	44±0.55	87±0.54	43±0.55	ND
With 50% Glucose	NA	665±25	ND	ND	39±0.45	82±0.54	30±0.55	ND
Gracilaria crassa								
Without sugars	18±0.55	500±25	38±0.5	1.3±0.03	35±0.45	82±0.54	42±0.9	$1.84X10^5$ ±604
With 50% sucrose	NA	720±25	ND	ND	38±0.55	85±0.45	62±0.52	ND
With 50% Glucose	NA	670±20	ND	ND	33±0.55	80±0.54	48±0.54	ND
Gracilaria edulis								
Without sugars	16±0.45	50±10	29±0.4	2.6±0.05	39±0.45	76±0.45	29±0.55	$0.64\ 10^5$ ±595
With 50% sucrose	NA	340±15	ND	ND	42±0.55	78±0.5	52±0.54	ND
With 50% Glucose	NA	310±15	ND	ND	37±0.45	72±0.45	33±0.45	ND
Gelidium pusillum								
Without sugars	16±0.55	890±25	43±0.5	0.96 ±0.02	34±0.55	97±0.45	19±0.45	$0.99\ X10^5$ ±374
With 50% sucrose	NA	1120±20	ND	ND	37±0.45	99±0.44	40±0.90	ND
With 50% Glucose	NA	1070±25	ND	ND	31±0.45	94±0.44	26±0.55	ND

[a] Yield was calculated on the basis of as received dry seaweed; [b] Gel strength was measured in 1.12% gel at 20°C; [c]Viscosity was measured at 80^0 C, NA=Not Available; ND=Not Determined

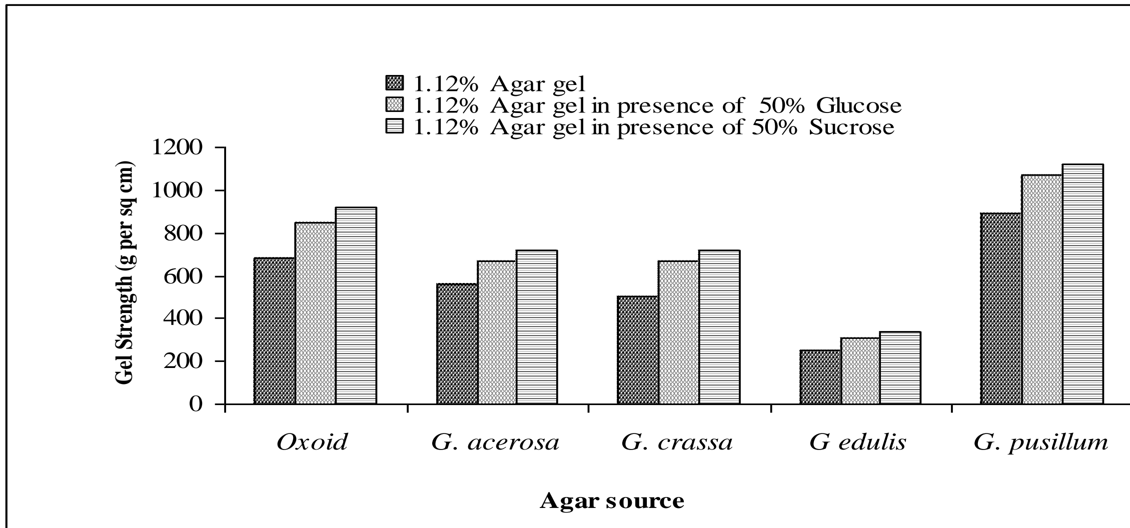

Figure III.1.1 Gel strength of agar gels of different Indian agarophytes and Oxoid agar with optimum amount of sucrose, glucose and without sugars or control agar gels.

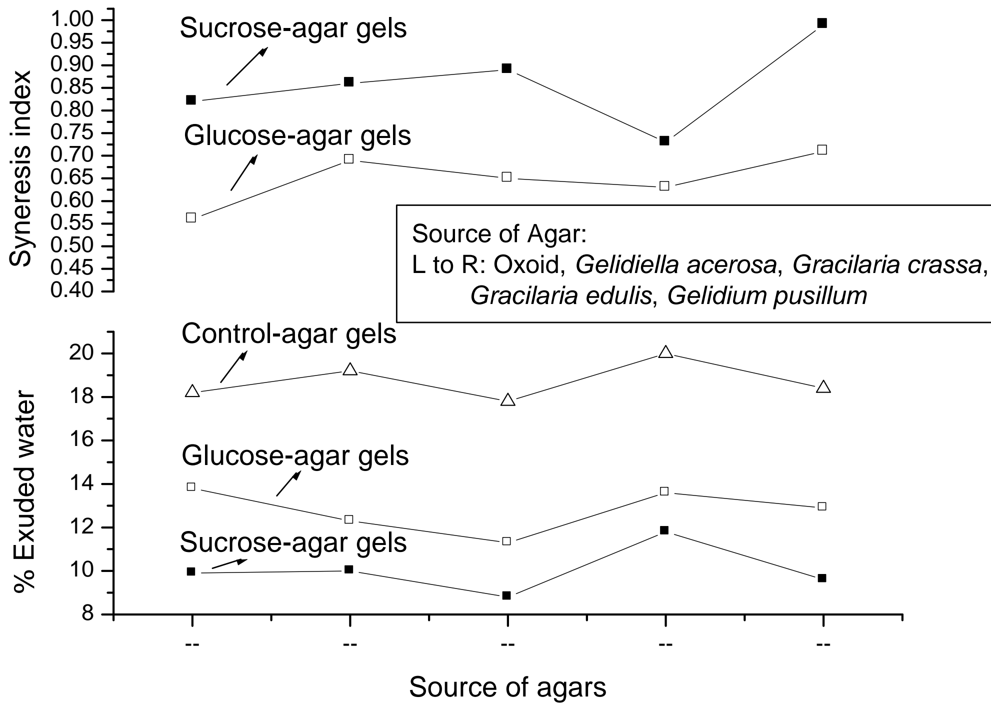

Figure III.1.2 Syneresis index and %exuded water of agar gels of different Indian agarophytes and Oxoid agar with sucrose (o), glucose (□) and control agar gels (■).

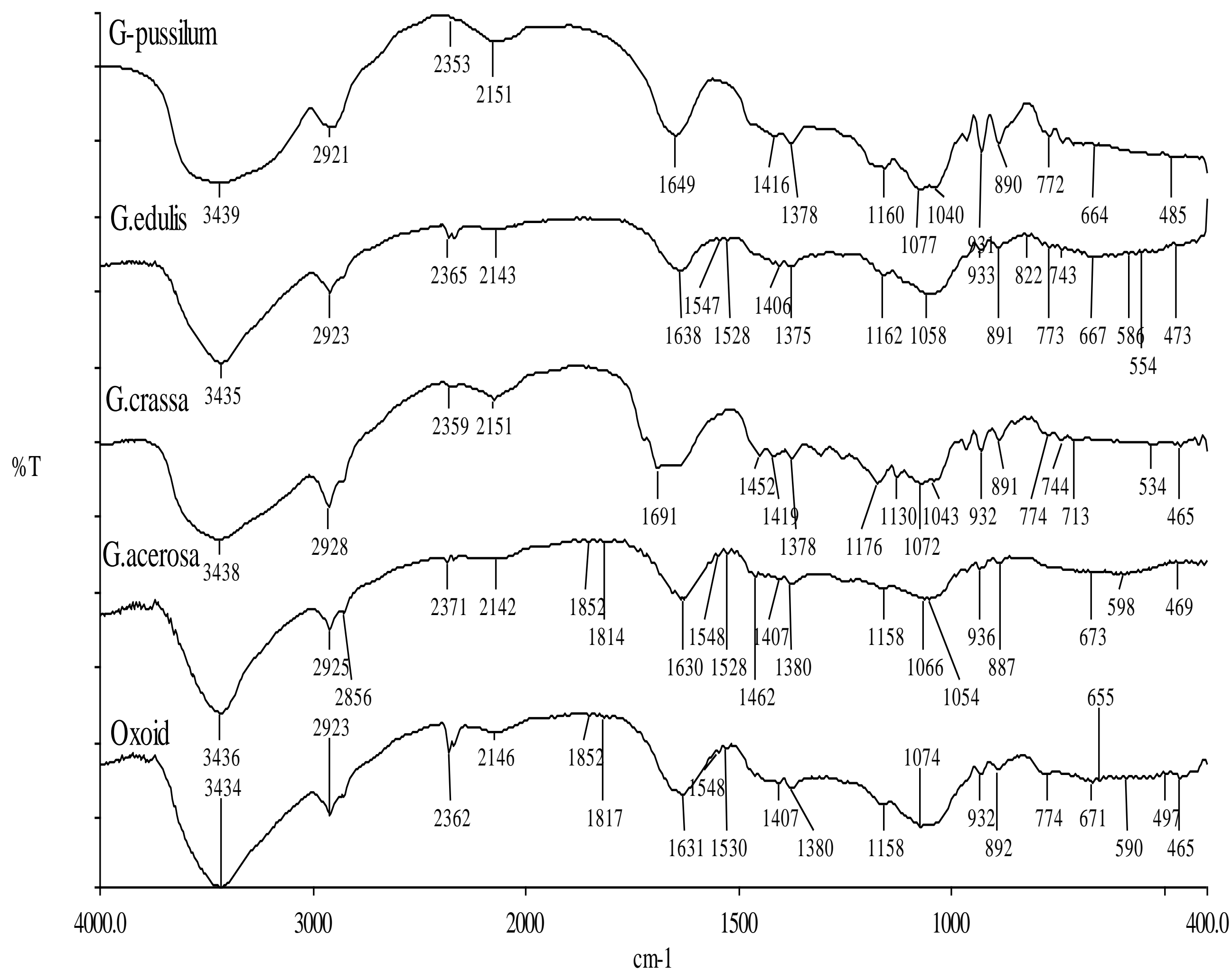

Figure III.1.3 Comparative FT-IR spectra of agar of different Indian agarophytes and Oxoid agar.

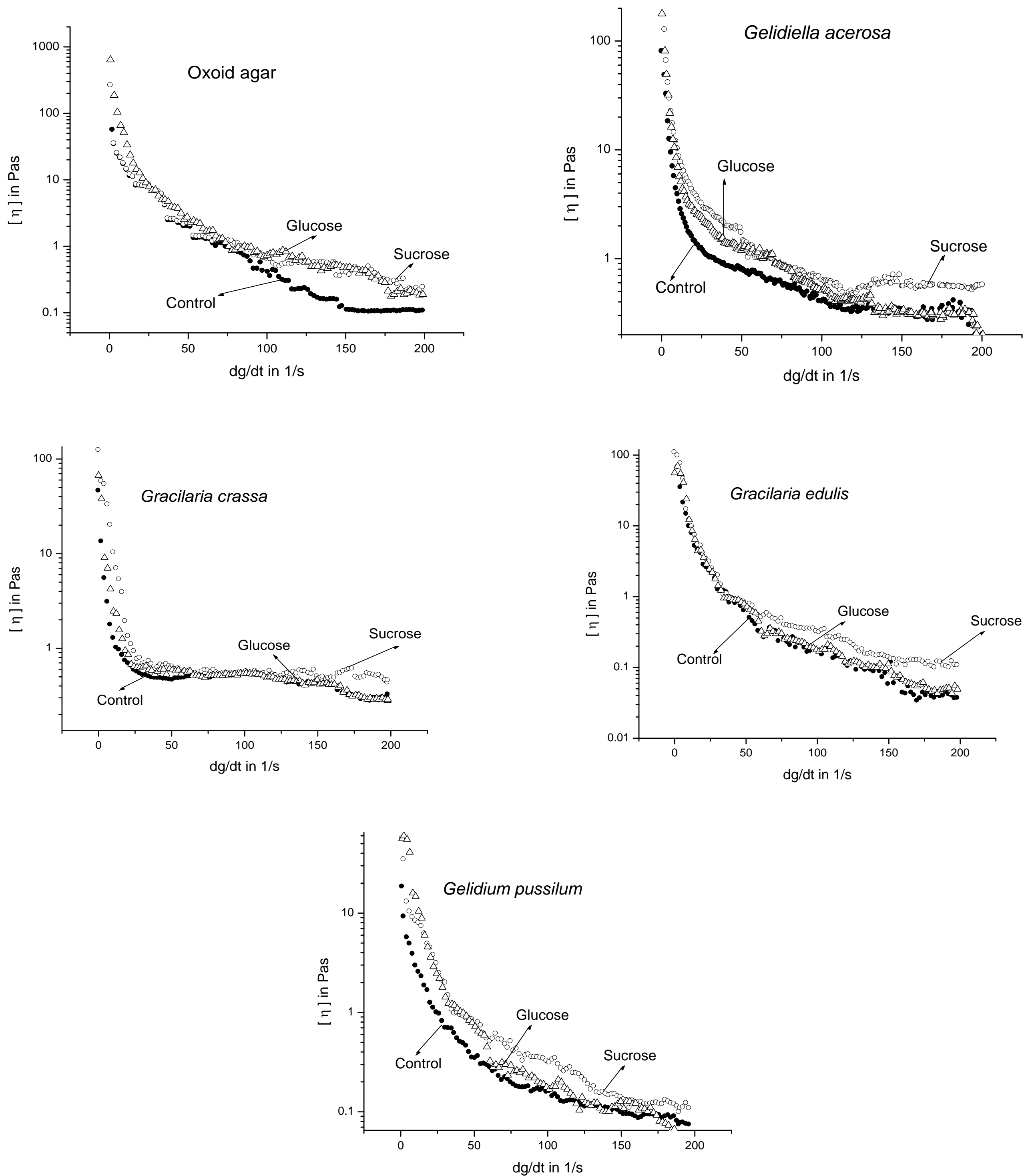

Figure III.1.4 Flow properties of agar gels (at 45°C) of different Indian agarophytes and Oxoid agar with sucrose (O), glucose (Δ) and control agar gels (•).

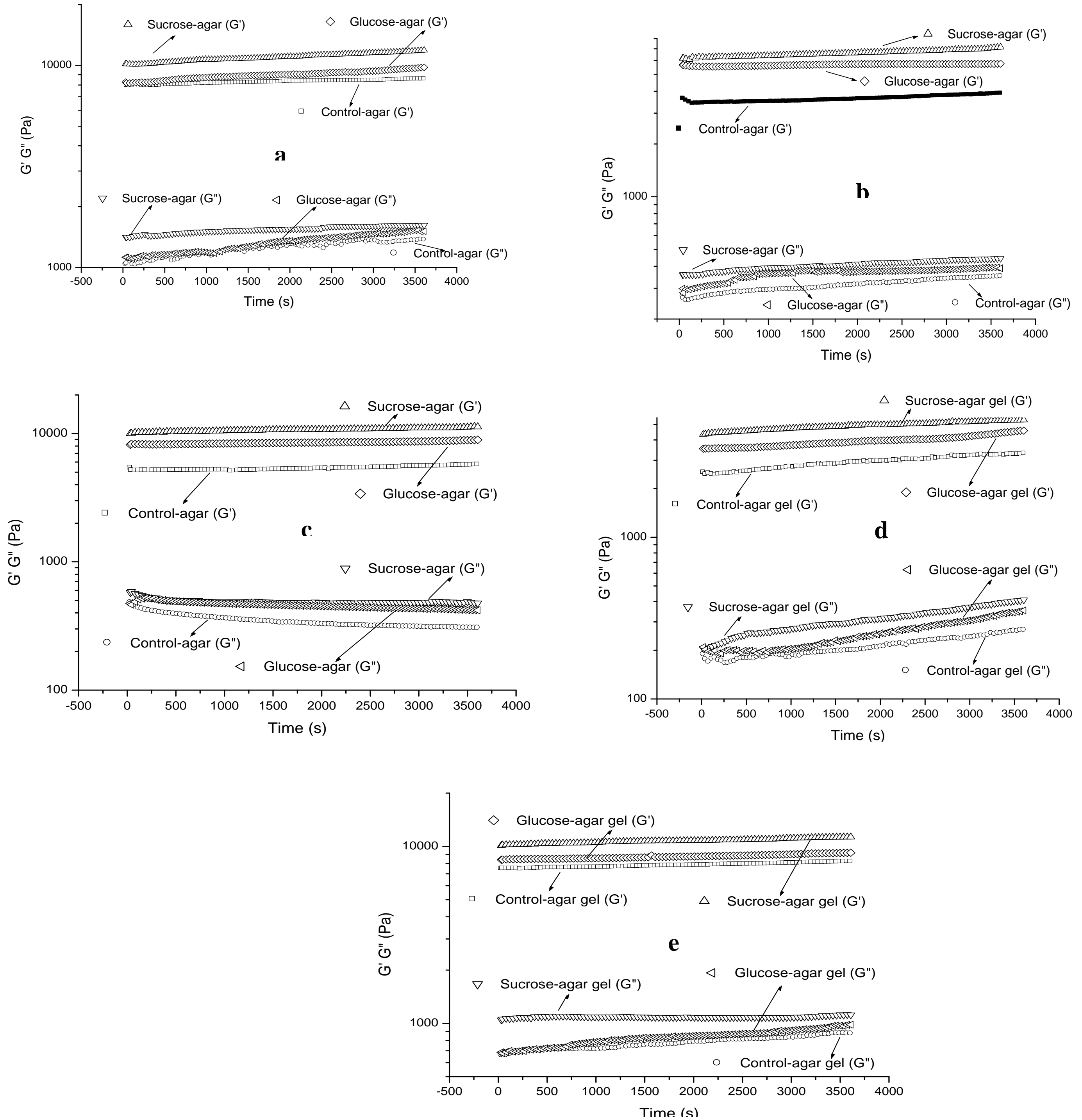

Figure III.1.5a-e Storage (G') and Loss (G") moduli of agar gels (at 25°C) of different Indian agarophytes [*G. acerosa* (**b**); G. *crassa* (**c**); *G. edulis* (**d**); *G. pusillum* (**e**)], and Oxoid agar gels (**a**) with sucrose, glucose and control agar gels.

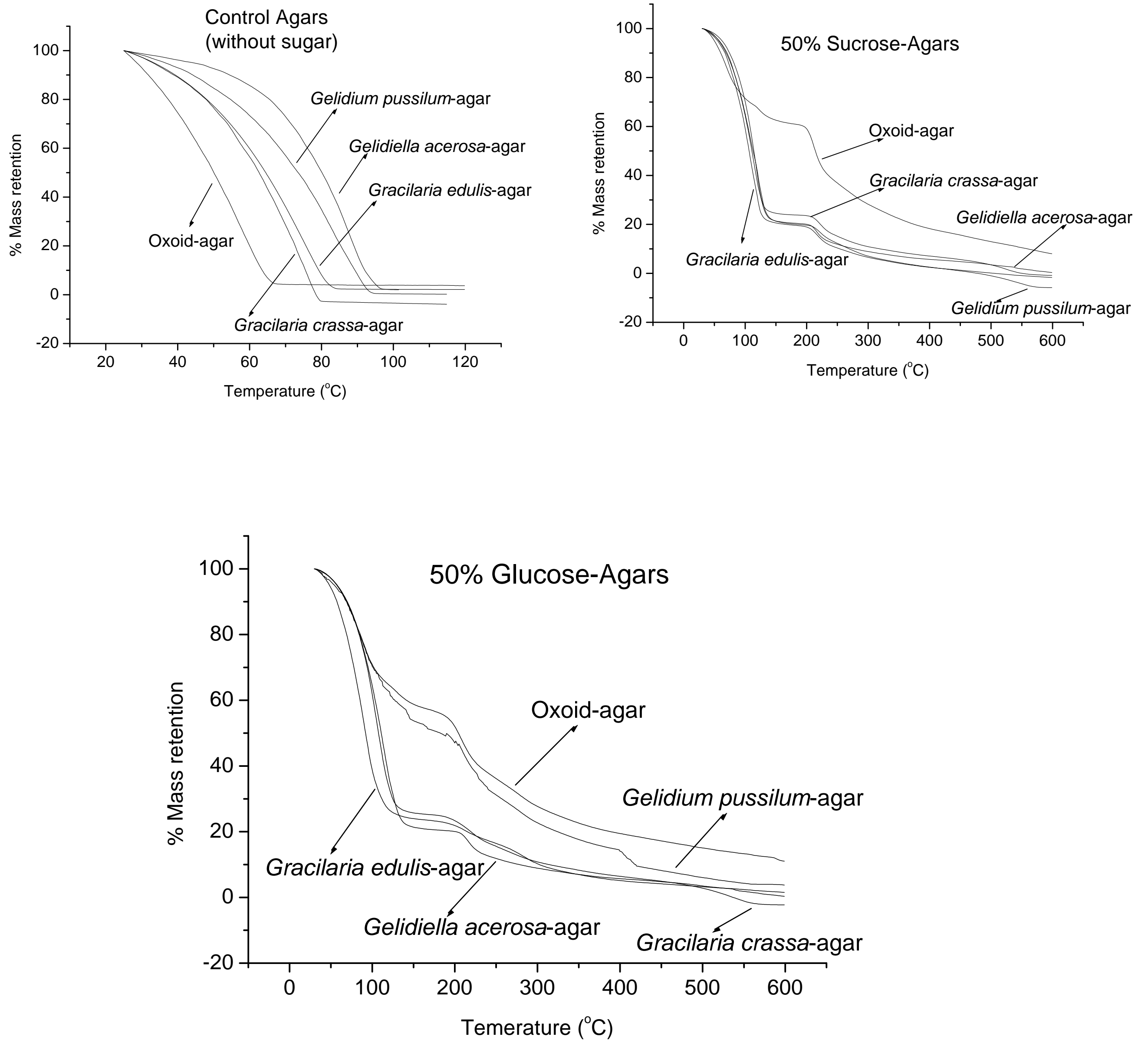

Figure III.1.6 Thermograms of agar gels of different Indian agarophytes and Oxoid agar with sucrose, glucose and control agar gels.

CHAPTER III.2

ONE-POT SYNTHESIS OF A pH RESPONSIVE SUPER ABSORBENT *KAPPA*-CARRAGEENAN AND ACRYLAMIDE BASED COPOLYMER GEL HAVING ADHESIVE PROPERTIES

III.2.1 INTRODUCTION

III.2.2 MATERIALS AND METHODS

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III.2.1 INTRODUCTION

Super absorbent hydrogels are three-dimensional hydrophilic crosslinked networks, which are able to absorb and retain water, saline or biological fluids without dissolution many times over their weight [1, 2]. The first industrial superabsorbent hydrogel was synthesized using this method via ceric-induced graft copolymerization of acrylonitrile onto starch followed by crosslinking alkaline hydrolysis of the nitrile groups of the produced graft copolymer [3]. Physical and chemical modifications of synthetic or natural polymers have been done with a view to value addition of the same targeting new applications [4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. Due to the large application of starch and cellulose in textile, paper and food industry, they are being grafted or crosslinked for producing derivatives having different physicochemical properties [14]. Ushakov first reported cellulose graft copolymers in 1943 [15].

Carrageenan is a seaweed polysaccharide and the backbone of the polymer consists of alternating α-1, 3-linked D-galactopyranose and β-1, 4-linked 3, 6-anhydro-D-galactopyranose (Figure III.2.1) [16]. A biopolymer based super absorbent hydrogel was synthesized through chemical cross linking graft polymerization of acrylic acid on to kappa carrageenan in presence of cross linking agent (N, N'methylene bis acrylamide) and an initiator ammonium persulphate was reported by Pourjavadi et al. [17]. Shchipunov reported a sol-gel biomaterial based on carrageenan and silica [18]. Natural polysaccharides or gums were modified to impart more functional properties [19]. Pourjavadi reported synthesis of carrageenan and sodium alginate based super absorbent hydrogel [20] using vigorous reaction conditions including long duration.

Acrylamide is a chemical intermediate used in the production and synthesis of polyacrylamide [21]. These high molecular weight polymers can be modified to develop nonionic, anionic, or cationic properties for specific uses. The principle end use of acrylamide is in water soluble polymers used as additives for water treatment, enhanced oil recovery, flocculants, papermaking aids, thickeners, soil conditioning agents, sewage and waste treatment, ore processing, and permanent-press fabric. The polyacrylamide mainly used in treatment of municipal drinking water and waste water [21]. It was also used as gelling agents for explosives, binders in addatives, in production of diazo compounds, and for gel chromatography and electrophoresis [21, 22]. To keep these in mind we have chose the acrylamide as monomer and grafted with kappa-carrageenan in the present study. Incedentally, letter was exhibited adasive properties [12]. A material that is capable of sticking to surfaces of other substances and binding them to one another is

called adhesive [23]. Adhesives from vegetable sources are well known; they include natural gums, starch and starch derivatives. They are commonly used for sizing paper and textiles and for labeling, sealing, and manufacturing paper goods [24].

In this Chapter microwave induced one-pot route for graft copolymerisation of acrylamide (AAm) on to kappa-carrageenan (kC) initiated by potassium persulphate, has been described [12]. Resultant copolymer hydrogels exhibited adhesive and super absorbent properties. The aim of the present study was to develop a simple and rapid method for grafting and synthesis of acrylamide copolymers of kappa-carrageenan which would have new applications.

III.2.2 MATERIALS AND METHODS

III.2.2.1 Preparation of κ-carrageenan (kC)

Refined κ-carrageenan that was used in this study was prepared from the dried red seaweed *Kappaphycus alvarezii* (*Euchuema cotonii*) in our lab using the method described by Craigie & Leigh [16]. Potassium persulphate, acrylamide, isopropanol and potassium chloride used were of analytical grade and were purchased from Sigma-Aldrich, Mumbai and Ranbaxy Fine Chemicals Ltd. Mohali, India.

III.2.2.2 Apparatus

LG make domestic microwave oven having temperature range 40-100°C (Magnetrons are set at a frequency of 2450 MHz) [LG Microwave Appliance, Model No. MS-285SD; Made in Korea].

III.2.2.3 Preparation of kC-graft-PAAm

To a dispersion of kC (1-2 g) in 35 ml distilled water was added a solution of acrylamide (AAm) [2-8 g (0.571-1.1428 mol/L)] in 10 ml distilled water. To this mixture a solution of KPS [0.071-0.4 g (0.00518-0.0296 mol/L)] in 5 ml distilled water was added and was stirred. The mixture was then irradiated under microwave (60%) for 90-120 s. The mixture turned viscous and turbid white in colour. This mixture was then added to iso-

propanol (IPA) (2 parts with respect to the reaction mixture, w/w) and was stirred well and allowed to stand for 20 h. The white solid mass that separated out was collected by filtration. The solid samples were washed with 50 ml each of 60% and 80% isopropanol-water mixture to remove the unreacted homopolymer from the products, followed by washing with 100% IPA to remove the remaining water from the final products. The solid product, kC-*graft*-PAAm, was then air dried. The copolymers of kC-*graft*-PAAm, were prepared using in three different ratios of AAm and KPS keeping the amount of kC constant: kC: AAm: KPS (w/w), 1:2.0:0.20; 1:3.0:0.30 and 1:4.0:0.40; the ratio of AAm: KPS being (in mol/L) 0.0148: 0.57; 0.87:0.022 and 0.0296:1.14 respectively].

The kC-*graft*-PAAm based adhesive was prepared by dissolving the copolymer in water (5% w/w) heating at 90-110°C for 10 min. The kC-*graft*-PAAm based gel was prepared by dissolving the copolymers with different nitrogen contents in 1% KCl (5% w/w) by heating at 90-110°C, cooled it at room temperature and kept overnight at 10°C.

III.2.2.4 Swelling Measurement

An accurately weighed dried amount of powdered copolymer with particle size between 5-10 mesh was immersed in the different solutions having pH from 1-13 including distilled water and 1% NaCl, KCl and $CaCl_2$ solutions, in separate experiments. The swollen gel particles were wipe dried with tissue paper to remove the adhering water and weighed at regular intervals until equilibrium was reached. Degree of swelling or equilibrium swelling (ES) capacity was calculated using the following equation:

$ES = W_{(s)} – W_{(d)}/ W_{(d)}$

Where $W_{(s)}$ and $W_{(d)}$ are the weights of the swollen and dry material samples, respectively.

III.2.2.5 Grafting Parameters

The grafting parameters e.g., conversion % (C%), grafting efficiency % (E%), grafting % (G%), were determined according method described by Liu et al. [25], using the following equations:

Grafting percent (G%) = (Wt. of poly AAm grafted/wt. of kC) X 100

Grafting efficiency (E%) = (Wt. of poly AAm grafted/total wt. of poly AAm) X 100

Conversion percent (C%) = (Total wt. of poly AAm/wt. of AAm charged) X 100.

III.2.2.6 Weight of adhesive

Liquid and solid weights of applied adhesive were measured using standard method described in ASTM Handbook [23].

Weight of liquid adhesive

The weight of liquid adhesive was determined using the following equation.

S = (W2 – W1) X 317.5 / N X A (when W1 & W2 are in grams)

Where, S = Wt. of liquid adhesive applied (expressed in pounds per thousand sq. feet of joint or surface area); W2 = Wt. of specimen immediately after application of the adhesive; W1 = Wt of specimen before applying the adhesive; A = Area of test specimen (in sq. inches) and N = number of surfaces spread. 317.5 = Conversion factor.

Weight of Solid adhesive

The weight of solid adhesive was determined using the following equation.

D = (W2 – W1) x 317.5 / N X A (when W1 & W2 are in grams)

Where, D = Wt. of adhesive solids applied (expressed in pounds per thousand sq. feet of joint or surface area); W2 = Wt. of the specimen after application of the adhesive and elimination of solvents; W1 = Wt. of the duplicate uncoated specimen; A = Area of test specimen (in sq. inches) and N = number of surfaces spread; 317.5 = Conversion factor.

In this experiment two weighed pieces of paper, transparency sheet and wood were taken having measured their surface areas under investigation. Then the 5% dispersion of the copolymer adhesive (with %N 11.05) was applied on one of the designated surfaces by uniformly spreading, onto which the other piece without adhesive was placed and stuck. The united pieces were weighed immediately and the weight of the liquid adhesive was calculated as mentioned above. The pieces could not be taken apart intact after 1 hr of application. The united pieces were air dried at $30^{o}C$ for 20h, followed by at $50^{o}C$ for 4 h when the weight of the specimen remained constant. The above experiment was repeated with a standard adhesive available in the market (Fevicol, Pidilite Industries, Mumbai, India), using specimens with identical size and surface areas.

III.2.2.7 Characterization

The analytical characterizations of the copolymer were done as follows: IR spectra in KBr on a Perkin-Elmer Spectrum GX, FT-IR System (USA), X-ray diffractions studies Philips X'pert MPD X-ray powder diffractometer using $2\theta = 5$ to 60°, optical micrographs were recorded on an Optical microscope of Olympus model SZH 10, Japan with 70x magnification, elemental analyses on a Perkin Elmer-2400, CHNS/O Analyser) and thermal analysis on a TGA Toledo Mettler TGA system, Switzerland), rheological measurements were done on a RS1 Rheometer (HAAKE Instruments, Karlsruhe, Germany). Cone/plate geometries (60 mm diameter, 1° rad angle) were selected for rheological measurements in solutions taking 1 ml sol on to the plate of the rheometer, for measurements in the sol and gels, prepared from two hydrogels (i.e. hydrogels with, %N= 6.35 and 11.05) and the results were compared with those of control kC (1% gel in 1% KCl). Viscosities at varying shear rate were studied at 25°C. Oscillation measurements were carried out in controlled deformation mode with 0.05 % strain and plate/plate geometry (35 mm dia) was used. The temperature was maintained using the DC50 water circulator. Rheological data presented are means of three replicate measurements. Under the given experimental conditions no syneresis or slippage of gel was observed, as there was no abrupt decrease in G' values. The apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No.1 and rpm 60 were used for measuring the apparent viscosity. The gel strength (g/cm^2) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). In this study gelling and melting temperatures of copolymer gels were measured as described by Craigie & Leigh [16].

III.2.2.8 ^{13}C NMR

^{13}C NMR spectrum (noise-decoupled) was recorded on a Bruker Advance DPX 200 Spectrometer, Switzerland at 50 MHz. Sample (50 mg/ml) was dissolved in D_2O and spectrum was recorded at 70°C with 9279 accumulations, pulse 5.9 μs, acquisition time 1.015 s and relaxation delay 6 μs using DMSO (ca. δ 39.5) as internal standard.

III.2.3 RESULTS AND DISCUSSION

III.2.3.1 Solubility and swelling

In the solubility measurement we observed that the parent polysaccharide κ-carrageenan (kC) was soluble in cold water and gets degraded in acidic solutions while the samples of the copolymer kC-*graft*-PAAm are not soluble in water, acidic and alkaline solutions, hexane, dichloromethane or CCl_4 at ambient temperature. It was observed that the grafted copolymer swells in water, acid and alkaline solutions, but no swelling was observed in organic solvents. Thus grafting hampers solubility or dispersibility of kC making the backbone less hydrophilic comparison to parent kC. On the other hand grafting of the hydrophilic PAAm moiety on the kC backbone makes the copolymer capable of undergoing extensive inter and intra hydrogen bonding in water resulting in improved networking arrangement manifesting the swelling capacity.

The copolymer samples had increased swelling capacity, the highest being with the copolymer having %N 10.56 at pH 12.5 (Table III.2.1). This may be attributed to an increase in the diffusion of AAm molecules in to the polysaccharide backbones resulting in an increase in swelling capacity and the higher AAm content enhances the hydrophilicity of the hydrogels. However the copolymer with 11.05% N showed a decrease in swelling possibly due to the increase in viscosity and hydrodynamic volume of the polymer chain. The swelling of copolymers decreased in salt solutions compared to that in the alkali solution. This decrease in swelling capacity can be explained on the basis of "charge screening effect" as described by Pourjavadi et al. [20].

Table III.2.1 Swelling properties of copolymer hydrogels at 35°C

Solution media (pH)	Equilibrium swelling[a] (g/g) of			
		kC-*graft*-PAAm copolymers with		
	kC (%N 0.21)	%N 6.35	%N 10.56	%N 11.05
Water (7.0)	N.S.	8.5±0.05	14.5±0.07	12.7±0.06
Alkaline (12.5)	N.S.	12.6±0.06	22.0±0.06	15.4±0.07
Acidic (1.2)	N.S.	8.0±0.05	14.0±0.05	11.0±0.06
1% NaCl (7.0)	N.S.	8.0±0.07	12.6±0.07	10.3±0.05
1% KCl (7.0)	N.S.	9.0±0.06	13.5±0.06	12.8±0.07
1% $CaCl_2$ (7.0)	N.S.	8.5±0.07	12.5±0.06	10.8±0.06

[a]Swelling of control kC and different hydrogels were measured with 10 min intervals and Table 1 shows equilibrium swelling values and equilibrium swellings that were obtained after 190 min in water, 240 min in alkali, 120 min in acid and 220 min for salt solutions respectively; N.S.=Not swelled.

III.2.3.2 Physicochemical Properties

Physical properties of the grafted copolymer kC-*graft*-PAAm having different nitrogen contents and those of control kC are given in Table III.2.2. Apparent viscosity of the copolymer hydrosol increased with increase in %N. The one with %N 11.05 exhibited greatest apparent viscosity (74 cP) at 5% concentration in water at 80°C. The increase in apparent viscosity with the increase in the %N of the products could be explained on the basis of stiffness and the increase in the hydrodynamic volume of the copolymers in the presence of high PAAm or %N [20, 25]. Gel strength of the copolymer hydrogels decreased from 135 g/cm^2 for 5% hydrogel with %N 6.35 to <100 g/cm^2 for 5% hydrogel with %N 11.05. Corresponding decrement in the gelling and melting temperature of the hydrogels were also observed (Table III.2.2). It was also observed that all the copolymer samples did not gel in absence of KCl. The gel formation in the presence of KCl indicated that the parent polysaccharide was present in the copolymer network. The gel strength decreased with increasing %N values in the copolymers, this may be due to the decrease in the quantity of parent polysaccharide which is actually responsible for the gel network formation in presence of KCl. Copolymer samples also showed increase in %N with increasing concentration of AAm in the grafting reaction. The similar observation was reported by Sharma et al. [19].

Table III.2.2 Properties of kC-*graft*-PAAm copolymer hydrogels

Sample[a]	Apparent Viscosity (in cP at 80 ^{o}C)	pH (at 80°C)	Gel Strength (g/cm^2) at 30°C	Gelling T (in ^{o}C)	Melting T (in ^{o}C)
kC (in 5% KCl)	1100±7.4	7.3±0.07	>1400	71±0.54	>100±0.57
kC-*graft*-PAAm[b] gel (in 1% KCl)	65±0.75	6.8±0.05	135±3.3	28±0.45	65±0.67
kC-*graft*-PAAm[c] gel (in 1% KCl)	69±0.75	6.5±0.06	120±5.4	22±0.54	58±0.62
kC-*graft*-PAAm[d] gel (in 1% KCl)	74±0.54	6.2±0.05	<100±5.4	19±0.54	55±0.54

[a] Measured in 5% sol/gel; [b] %N=6.35; [c] %N=10.56; [d] %N=11.05

III.2.3.3 Grafting Parameters and effect of monomer concentration

The weights of unreacted AAm monomer that were recovered from the reaction mixture were 0.27, 0.22 and 0.75 g for kC-*graft*-PAAm having %N= 6.35, 10.56 and 11.05 respectively. The data of G%, E% and C% are given in Table III.2.3. The relative increase in %N and %G of the copolymers was significant when the concentration of AAm increased from 0.57mol/L to 0.87mol/L. These parameters did not change significantly on further increase in the concentration of AAm from 0.87mol/L to 1.14 mol/L. The general increasing pattern was also observed with %E and %C, albeit in smaller extent, except for a decrease in %C when the concentration of AAm was 1.14 mol/L (Table III.2.3). It may be due to the fact that when AAm and KPS are present in higher concentration, there are higher chances of generation of active free radical sites on the kC backbone which would give higher yield of grafted product (higher C%, G% and E% values) as reported in the literature [19,20]. In presence of the initiator in the aqueous solution of kC when the AAm to polysaccharide ratio increased then kC-*graft*-PAAm could play the role of self emulsifier so as to absorb more monomers (AAm) on the polymer (kC) surface, which subsequently enhanced the rate of the grafting reaction, thereby sharply increasing C%, G% and E% values [8]. It was observed that in the absence of the water soluble initiator the reaction did not take place at all.

Table III.2.3 Effect of %N on the grafting parameters

Grafting	%N content		
parameter	%N 6.35	%N 10.56	%N 11.05
G%	170±0.67	272±0.51	320±0.77
E%	87.8±0.60	97.8±0.62	98.5±0.50
C%	76.0±0.53	90±0.67	80.0±0.60

III.2.3.4 Elemental analysis

The average values of C, H, N parentage in the copolymers and those of κ-carrageenan are given in Table III.2.4. It was observed that the %N increased with increase in the ratio (in mol/L) of acrylamide and KPS.

Table III.2.4 Elemental composition of κ-carrageenan and kC-*graft*-PAAm copolymers

Elements (%)	κ-carrageenan	AAm:KPS 0.57:0.0148	AAm:KPS 0.87:0.022	AAm:KPS 1.14:0.0296
C	35.80±0.07	37.76±0.07	38.90±0.05	41.94±0.07
H	6.40±0.074	5.76±0.074	7.10±0.02	7.60±0.023
N	0.21±0.004	6.35±0.02	10.56±0.07	11.05±0.074

III.2.3.5 FT-IR Spectrum of the super water absorbent

The FTIR spectra were recorded with the grafted copolymer with %N 10.56, PAAm, AAm and kC (Figures III.2.2 a, b). The spectrum of the copolymer showed the presence of PAAm, having an absorption band at 1664 cm^{-1} for >C=O stretching and at 1452 cm^{-1} for C-N stretching. Presence of the C-4 sulphate band at 842 cm^{-1} in IR of kC [26], as well as a less intense band at 3416 cm^{-1}, compared to parent kC, appeared for OH group in the copolymer indicating that grafting took place on OH group, rather than on the sulphate moiety.

III.2.3.6 ^{13}C NMR studies

The ^{13}C NMR resonances of κ-carrageenan, PAAm and κ-carrageenan-*graft*-PAAm and their probable assignments are given in Table III.2.5 and Figure III.2.3. The assignments of the parent polysaccharide (kC) that was used in this study, was done by comparing the observed δ-values reported in the literature [27, 28]. The assignments of carbons of the PAAm moiety in the copolymer kC-*graft*-PAAm were done by comparing the δ-values obtained from the PAAm spectrum (Table III.2.5). Presence of the 3,6-anhydrogalactopyranose as well as galactopyranose moieties of the repeating unit of parent kC in the copolymer was indicated by the corresponding ^{13}C NMR resonances. A small downfield shift of all the backbone carbons of the polysaccharide in the copolymer in comparison to those of the parent kC was observed. This may have been due to the introduction of the PAAm moiety onto the polysaccharide, which is expected. The δ-

values of PAAm carbons in the copolymer, however, remained unaffected compared to those of pure PAAm.

Table III.2.5 ^{13}C NMR data (in ppm) observed for parent polysaccharide, PAAm and copolymer with %N 10.56.

Product name	δ (ppm)	Assignment
kappa-carrageenan	61.4 (61.0) [a]	C-6’
	68.7 (69.2) [a]	C-6
	69.4 (69.3) [a]	C-5
	69.8 (69.5) [a]	C-2
	76.1 (76.5) [a]	C-5’
	77.1 (78.0) [a]	C-4
	80.1 (78.9) [a]	C-3
	98.2 (94.9) [a]	C-1
	102.4 (102.2) [a]	C-1’
PAAm	36.54	-*C*H_2
	42.63	-*C*H
	180.18	-*C*ONH_2
kC-*graft*-PAAm (with %N 10.56)	35.69	-*C*H_2 of PAAm
	43.66	-*C*H of PAAm
	62.34	C-6’
	70.76	C-6
	71.11	C-5
	75.11	C-2
	75.85	C-5’
	77.85	C-4
	79.29	C-3
	80.26	C-3’ cf. Ref. [23]
	96.41	C-1
	103.56	C-1’
	180.72	-*C*ONH_2 of PAAm

[a] Values are from Ref. [27]

III.2.3.7 Thermal Analysis

Thermogravimetric analysis (TGA) of κ-carrageenan (kC) and the grafted copolymers (kC-*graft*-PAAm) are shown in Figure III.2.4. The TGA of kC shows mass loss in stages. The first stage ranges between 40°C and 105°C corresponding to 7-9% mass loss. This is presumably due to the loss of adsorbed and bound water molecules. The second stage of the weight loss starts at 190°C and that continued up to 260°C during which there were 20-22% of mass loss followed by up to 455°C showing 100% mass loss. The thermograms of the grafted products indicate that they are more thermally stable than kC, and their thermal stability to be in the order (%N values) 11.05 > 10.56 > 6.35, and are different from that of kC. It was observed that the mass losses in all the copolymers also took place in stages.

III.2.3.8 X-ray diffraction analysis

The X-ray diffraction experiments of kC; AAm and different graft copolymers of kC-*graft*-PAAm were carried out (Figures III.2.5a-d). The diffraction patterns of kC and kC-*graft*-PAAm are not significantly different. The samples of pure kC showed no clear sharp or narrow peaks; the profile appeared to be a signature of amorphous compound showing two less intense and an intense (at $2\theta = 28.53^{o}$) noisy bumps in addition to a feeble bump ca. $2\theta = 8^{o}$. In the copolymers with %N 6.35, 10.56 and 11.05, the intense bump appeared at slightly higher 2θ values e.g. 29.57^{o}, 30.52^{o} and 30.99^{o} respectively, the bump below $2\theta = 10^{o}$, however, did not appear in the copolymers. The copolymer with %N 6.35 four additional peaks appeared at 2θ 9.07^{o}, 22.09^{o}, 35.79 and 43.31^{o}. In the copolymers with %N 10.56 and 11.05 two such additional peaks appeared at ca. 2θ 16.23^{o}, 43.42^{o}. The X-ray diffraction pattern of AAm showed its crystalline nature having sharp peaks at ca. 2θ 11.99^{o}, 24.08^{o} and 28.58^{o}. From this study it can be concluded that considerable modification of the polysaccharide has taken place leading to a change in molecular association in the copolymers when compared with kC and AAm. Xiao et al. reported that the X-ray diffraction pattern of PAAm is amorphous having a very weak broad shaped one [29], which is apparently masked by the strong bumps of that of kC in the copolymers.

III.2.3.9 Morphology

Optical micrographs of acrylamide (AAm), different copolymers were taken with 70 X magnifications and compared with kC (Figures III.2.6a-d). These images revealed that the morphology of kappa-carrageenan (kC) got modified significantly in the copolymers. The optical micrograph of the parent polymer kC appeared fibrous in nature, while those of grafted copolymers appeared to have definite shapes akin to rectangular and/or spheroid geometries. It was also observed that the shapes appeared more spheroidal in the copolymer with higher %N. This reaffirms the enhancement of organized molecular arrangements in the copolymer as indicated by the X-ray diffractogram discussed above.

III.2.3.10 Adhesive property

A comparison of the liquid and solid weights of the copolymer adhesive (kC-*graft*-PAAm with %N 11.05) and Fevicol are shown in Table III.2.6. It appears that the efficacy of the copolymer adhesive is comparable with that of the standard adhesive in about same usage level (cf. solid weight of the adhesives).

Table III.2.6 Liquid and solid weights of copolymer adhesive (5% dispersion of kC-*graft*-PAAm with %N 11.05) and standard adhesive (Fevicol)

Specimens	Liquid and solid wt. of adhesives (in pound per thousand sq. feet of surface area)			
	kC-*graft*-PAAm (%N 11.05)		Fevicol	
	Liquid weight	Solid weight	Liquid weight	Solid weight
Paper sheets	6.55±0.059	1.82±0.059	7.22±0.069	2.56±0.068
Transparency sheets	4.01±0.045	2.1±0.059	4.2±0.059	1.6±0.059
Wood blocks	16.92±0.059	4.7±0.068	18.6±0.055	4.8±0.055

III.2.3.11 Rheological properties

Variations of dynamic viscosity with shear rate (Figure III.2.7) for sol and gel samples prepared from the copolymer with %N 6.35 and 11.05 (5% w/w sols or gels). It was observed that the sol and gel samples showed gel-thinning behavior throughout the shear rate range studied. The result obtained in this study was similar to that reported by Harry-O'Kuru et al. [30]. The stability of modulus during storage at 25°C was investigated and is depicted in Figures III.2.8a and 8b. The storage modulus values of the 5% sols (without KCl) and gels (in 1% KCl) samples were obtained from copolymers having %N 6.35 and 11.05. It was observed that the G'' values for gel samples increased with increment of time indicating absence of syneresis in the gel samples at 5% concentration as described by Ross-Murphy [31]. This observation shows the stability of the gel samples under stress for a long duration. The G' values for 5% gel of the copolymer with %N 6.35 was higher than that of the one with %N 11.05. Moreover, in case of 5% hydrogel sols (without KCl), the modulus exhibited a reverse trend i.e. G'' > G' value and after storage for long duration, values of G' increased and G'' decreased slightly.

III.2.4 CONCLUSIONS

A rapid method of synthesizing copolymers of κ-carrageenan and acrylamide using microwave irradiations has been described [12]. The maximum water absorbency (22 g/g) was achieved in the copolymer with %N 10.56, under the optimized reaction conditions with specific w/w ratios of kC:AAm:KPS, while highest adhesive property was observed with the copolymer having %N11.05. The adhesive property was evaluated with wood blocks, paper and transparency sheets, and found to be comparable with an adhesive (Fevicol) available in the market. That the copolymer was formed with *kappa* carrageenan and PAAm was assessed from the physicochemical, thermal and rheological properties, FTIR, ^{13}C NMR spectra. The changes in the molecular association in the copolymers were observed from the X-ray diffractograms and optical micrographs [10].

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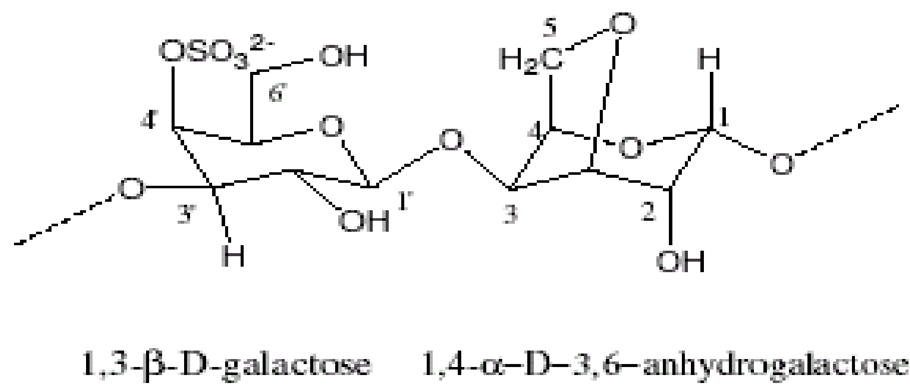

Figure III.2.1 Repeating units of κ-carrageenan

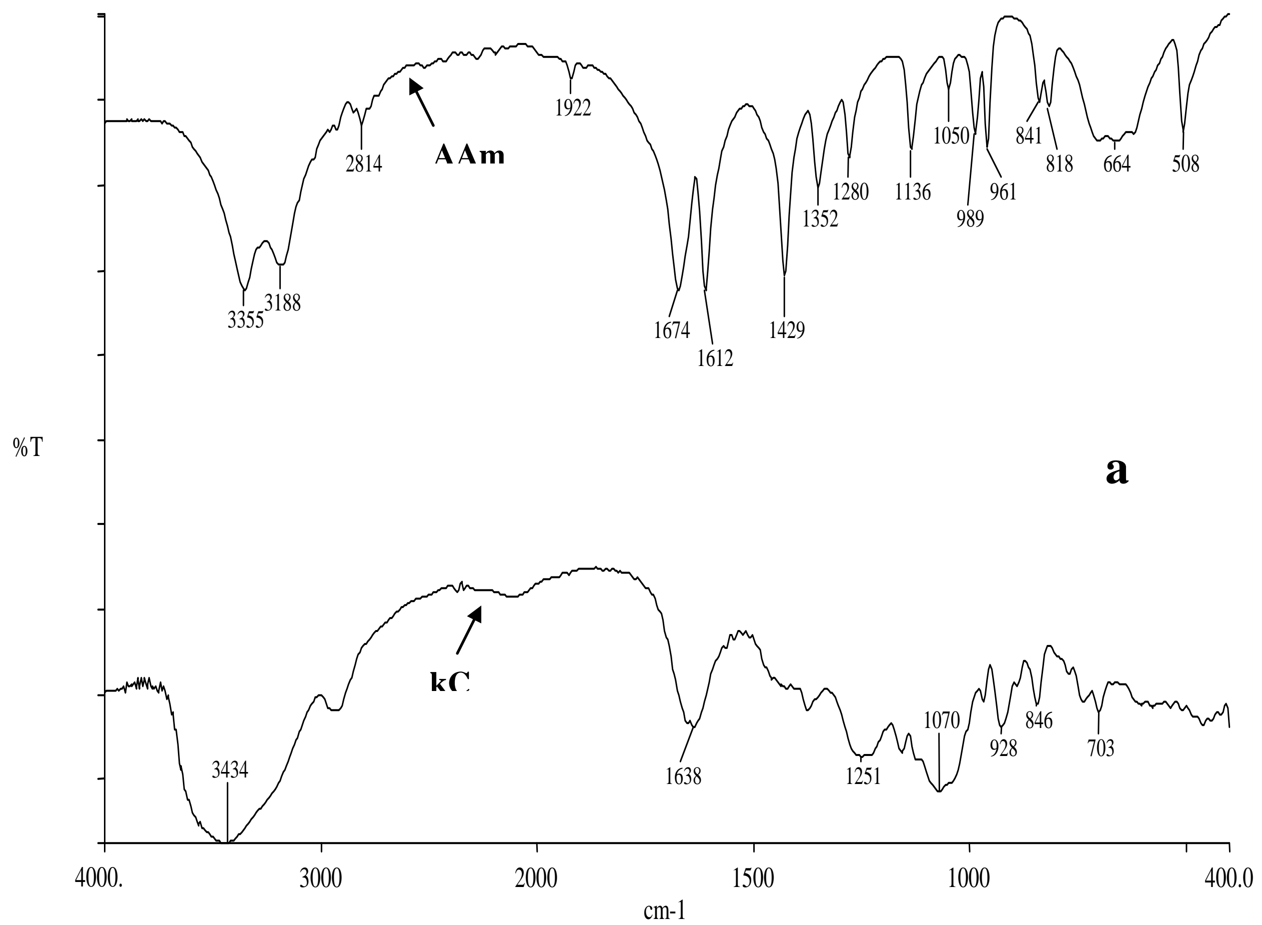

Figure III.2.2a FTIR spectra of acrylamide (AAm) and k-carrageenan (kC)

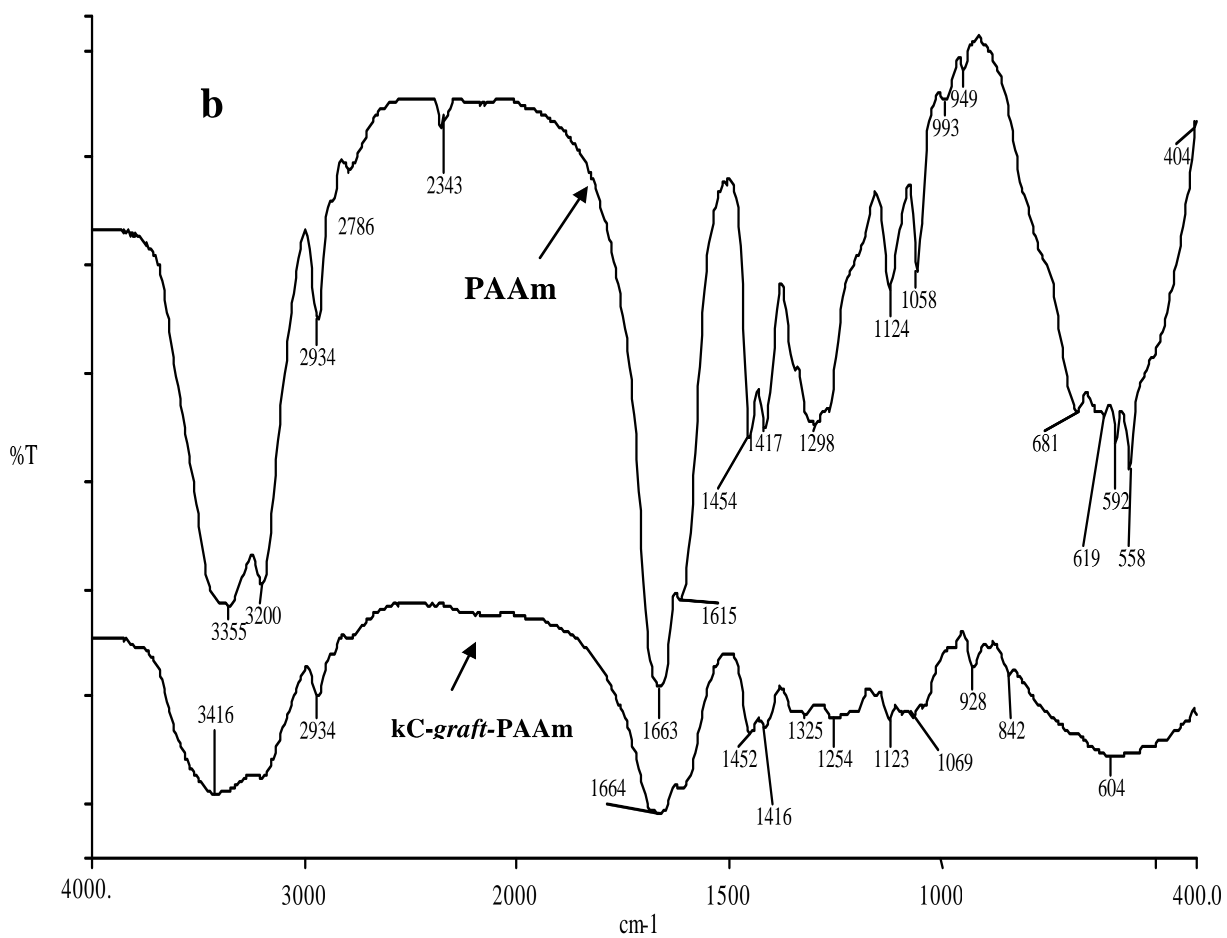

Figure III.2.2b FTIR spectra of (a) AAm and kC, and (b) PAAm and kC-*graft*-PAAm

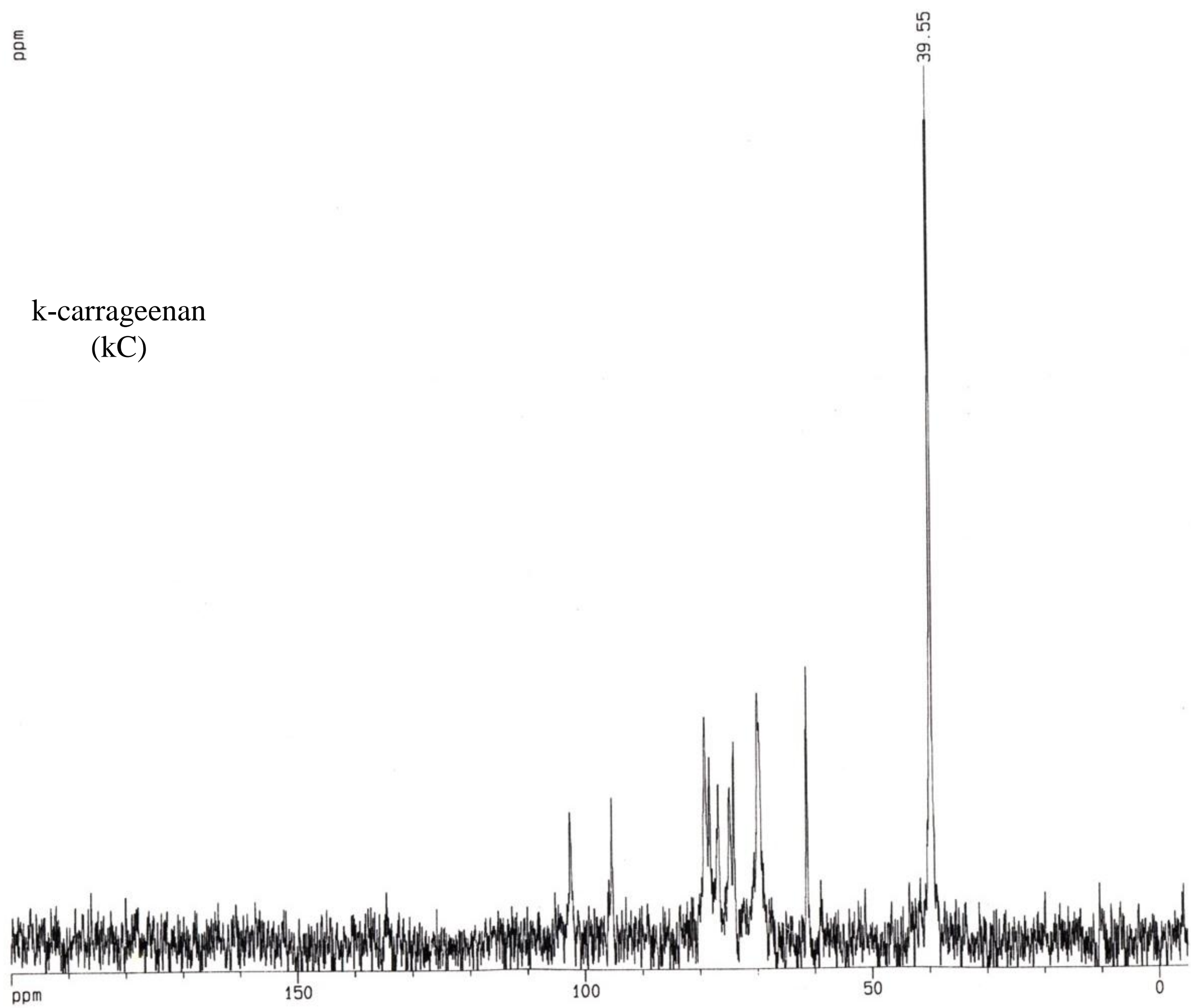

Figure III.2.3a ^{13}C-NMR spectra of k-Carrageenan (kC)

Polyacrylamide
(PAAm)

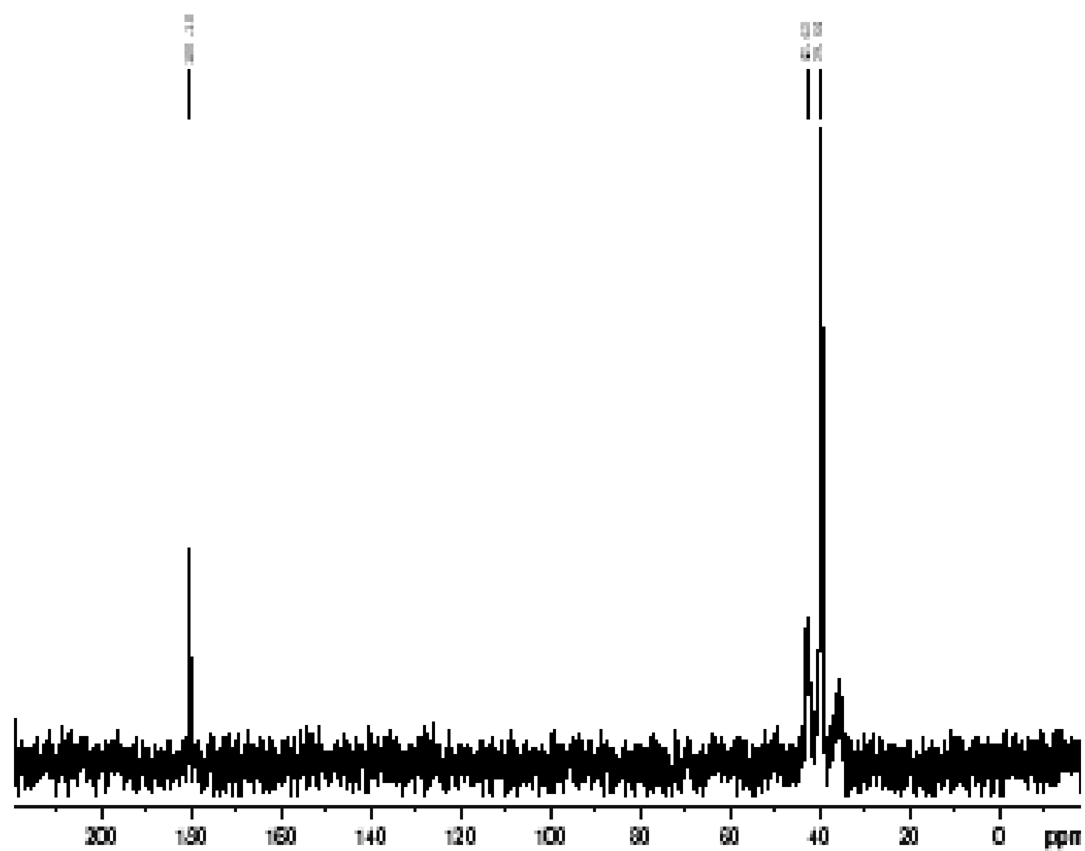

Figure III.2.3b ^{13}C-NMR spectra of Polyacrylamide (PAAm)

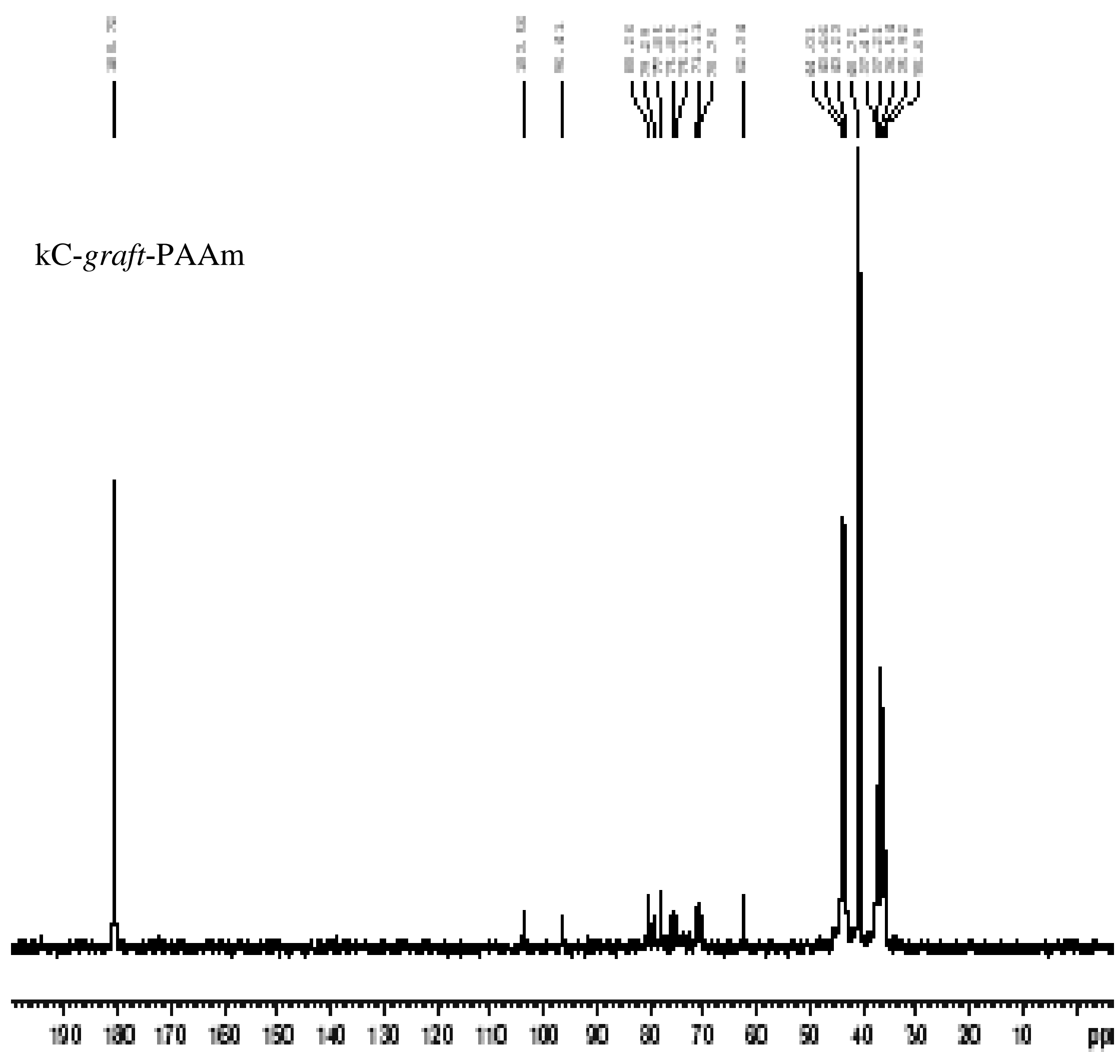

Figure III.2.3c ^{13}C-NMR spectra of kC-*graft*-PAAm

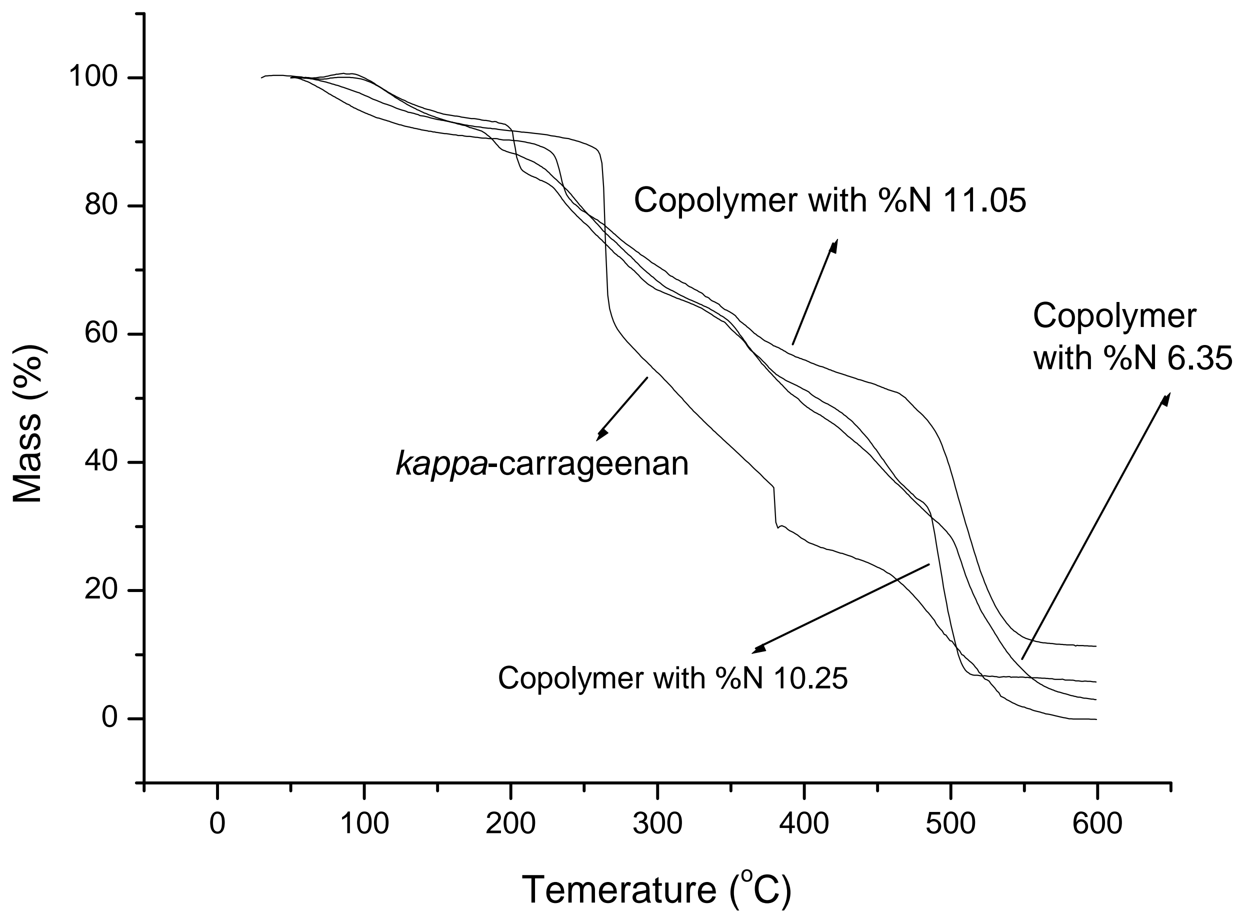

Figure III.2.4 TGA thermograms for kC and different copolymers with %N 6.35, 10.56 and 11.05.

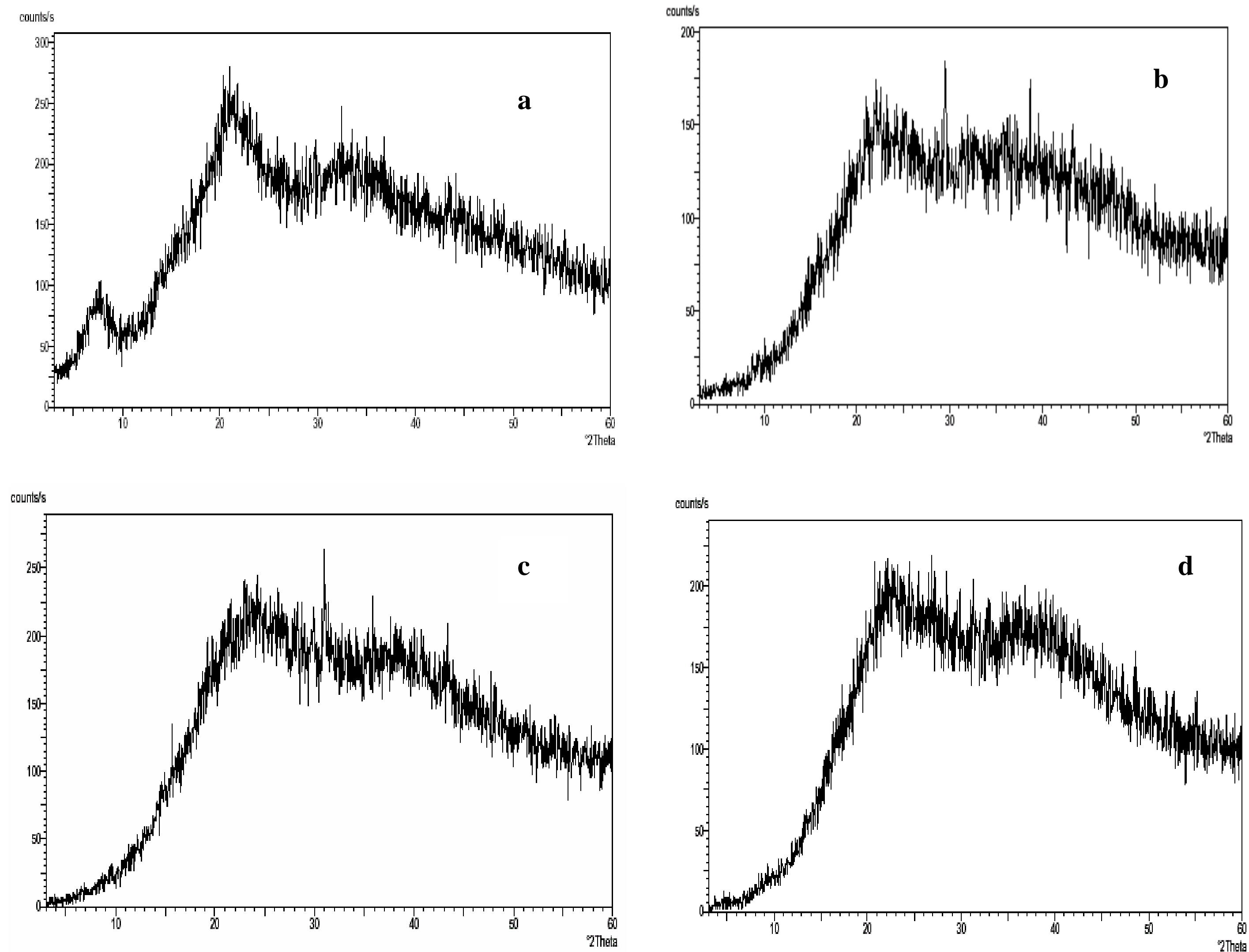

Figure III.2.5a-d X-ray diffractions of the kC (a), and different copolymer hydrogel with %N 6.35 (b), %N 10.25 (c) and %N 11.05 (d)

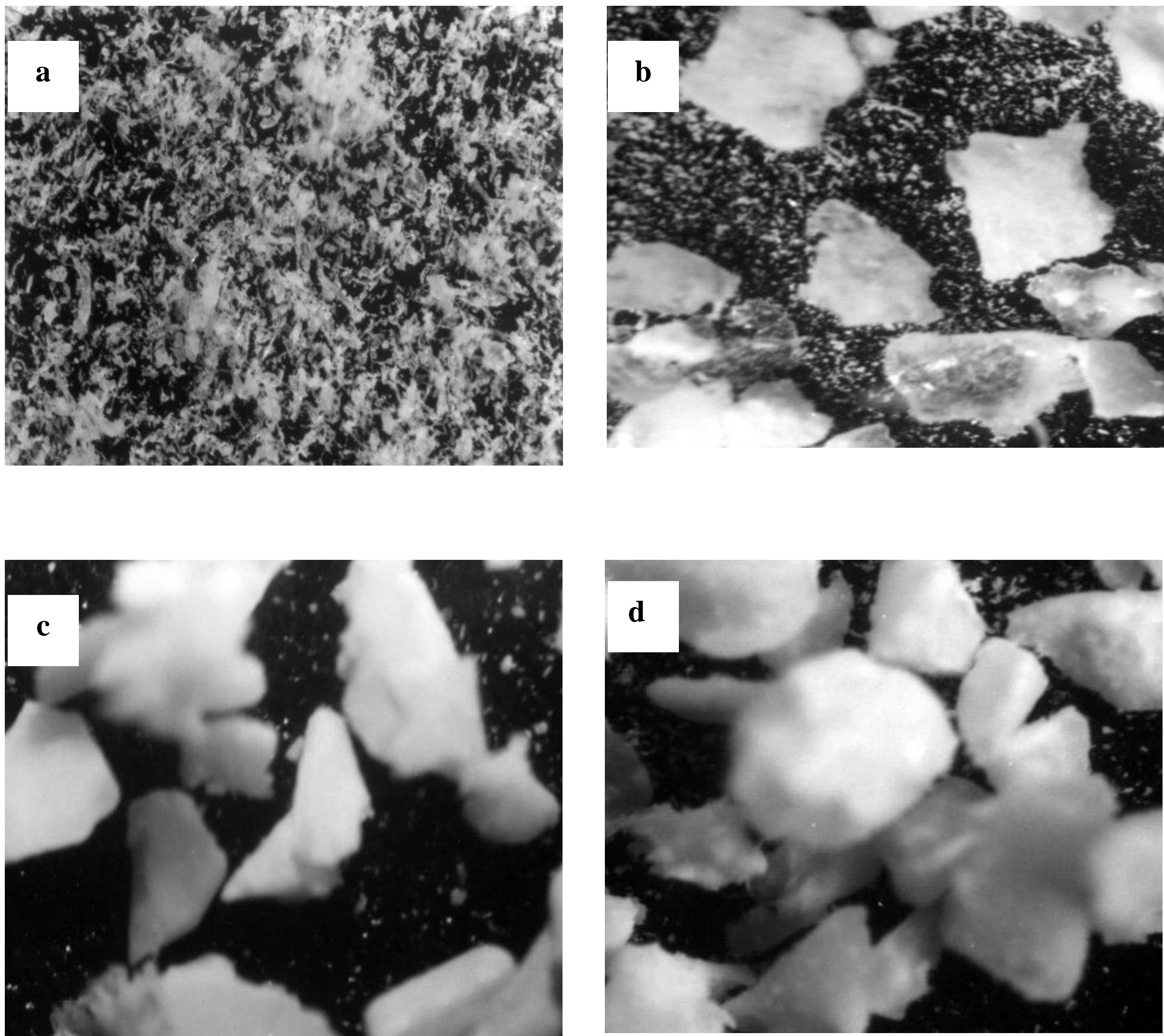

Figure III.2.6a-d Optical micrographs of the kC (a), AAm (b), and different copolymer hydrogels with %N 6.35 (c) and %N 10.56 (d).

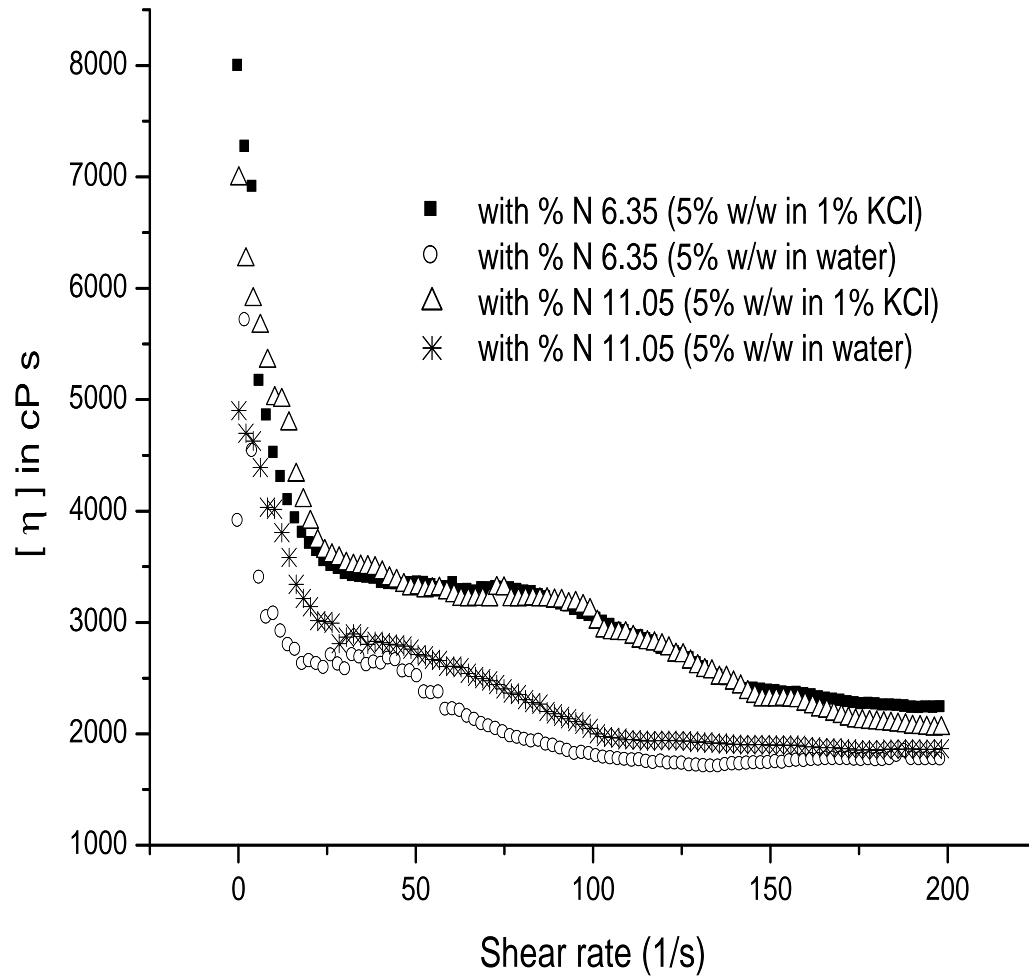

Figure III.2.7 Dynamic viscosity of the copolymers with %N 6.35 and %N11.05 in absence and presence of KCl.

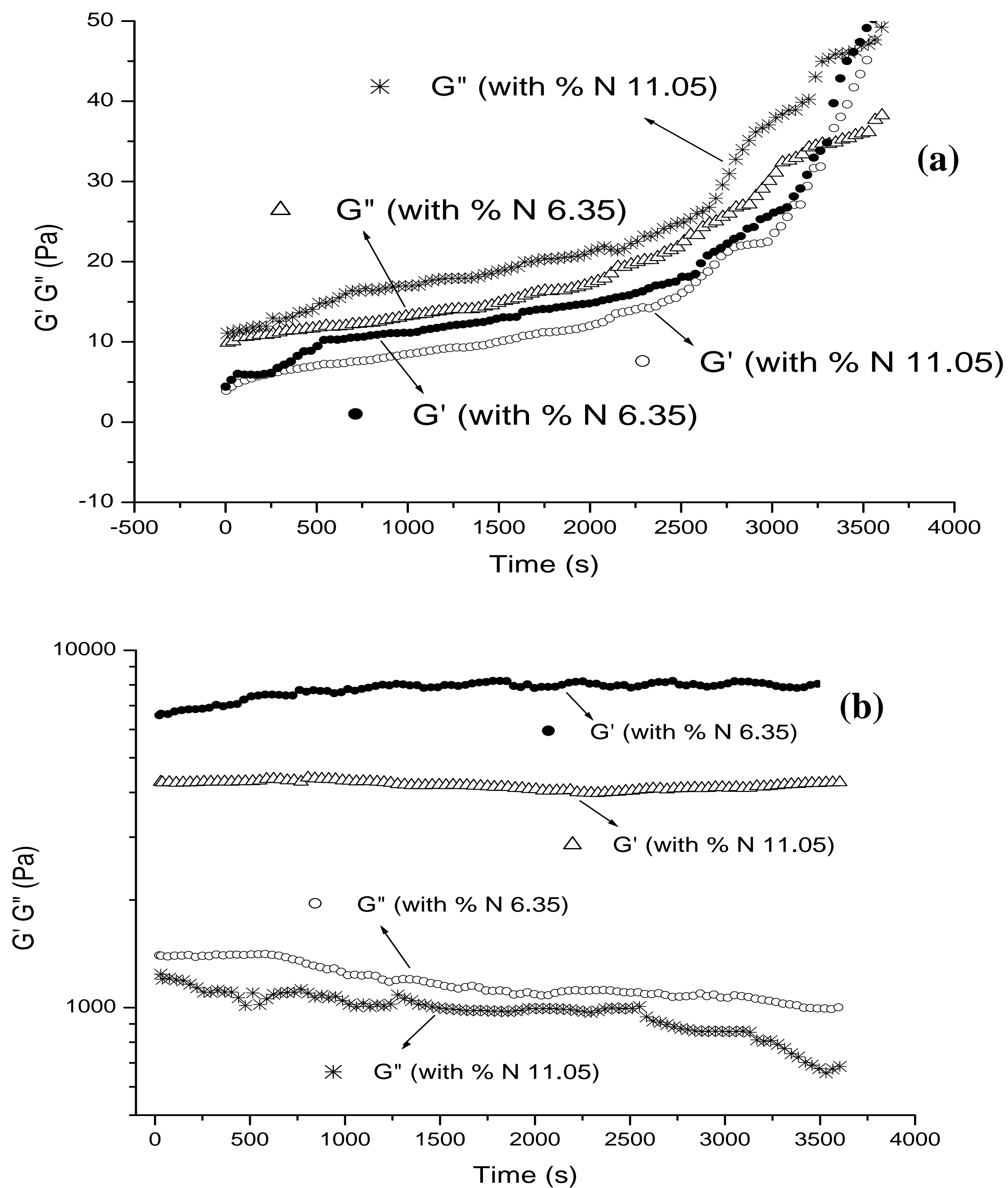

Figure III.2.8a-b Storage and loss modulus with the time for copolymer with %N 6.35 and %N 11.05 in absence (**a**) and presence of KCl (**b**).

CHAPTER III.3

SYNTHESIS OF ROBUST HYDROGELS BASED ON POLYSACCHARIDE BLEND

III.3.1 INTRODUCTION

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III.3.5 CONCLUSION

III.3.6 REFERENCES

III.3.1 INTRODUCTION

In an ongoing program of our laboratory on modification of seaweed polysaccharides for preparing hydrogels with improved properties [1-7], we report herein preparation of a robust hydrogel system based on grafting of Agar/Na-Alg blend with acrylamide (AAm). Pourjavadi et al. [8, 9] reported the synthesis of various polysaccharide based super absorbent hydrogels including graft copolymerization of acrylamide onto κ-carrageenan and sodium alginate backbones [8, 9].

Agar is a seaweed polysaccharide and chemically consists of alternating 3-O-linked D-galactopyranose and 4-O-linked 3, 6-anhydro-L-galactopyranose (Figure III.3.1a). Alginates are linear anionic polysaccharides of (1-4) linked α-guluronate (G units) and β-D-mannuronic acid (M units) residues (Figure III.3.1b). Both the biopolymers are biodegradable and are used in the food industry, cosmetics, pharmaceutical and biological applications. Alginates obtainable from the Indian seaweeds are of low viscosity, which limits the applications of this polysaccharide. To our knowledge, we report herein for the first time the preparation of PAAm copolymer hydrogel based on agar-alginate blend. The grafted product showed considerably enhanced swelling properties, and stability in all pH media, compared to the parent blend polysaccharides. This product may be useful in those applications which demand soft hydrogels with superior water retention ability as well as resilience to acid and alkaline pH media, e.g. in health and personal care, and in agricultural applications.

III.3.2 EXPERIMENTAL

III.3.2.1 Materials and Instruments

Agar and sodium alginate used in this investigation were extracted from the seaweeds namely *Gelidiella acerosa* and *Sargassum wightii* growing in the Indian waters, respectively. The agar and alginate were extracted following the methods as reported in the literature [2, 10]. Potassium persulphate (AR), acrylamide (AR) were purchased from Sigma-Aldrich, Mumbai. LG make domestic microwave oven having temperature range 40-100°C (Magnetrons are set at a frequency of 2450 MHz) [LG Microwave Appliance, Model No. MS-285SD; made in Korea].

III.6.2.2 Synthesis of blend and copolymers

Agar (0.1-1.0 g) and sodium alginate (Na-Alg) (0.2-3.0 g) were dissolved separately in the distilled water, using microwave irradiations. Different blend polysaccharide samples were prepared by mixing agar and Na-Alg in 1:9, 1:3, 1:1 and 3:1 ratios (w/w). The resulting blend samples were allowed to cool forming gels which were dehydrated with isopropanol (IPA) (1:2 w/w) for 24 h. Dewatered hardened gel particles were filtered and dried in air followed by drying in an oven at 50°C. The dried blend samples were kept away from moisture and heat in a closed plastic bottle.

Copolymer hydrogel samples were prepared from Agar/Na-Alg blend (having 25:75 wt% of agar and Na-Alg). This blend was selected on the basis of his greatest swelling ability in all media. 2 g blend was dissolved in 85 ml distilled water by heating under microwave (MW) irradiation. To this was added KPS solution (0.01 to 0.05 g in 5 ml H_2O) with stirring and irradiated (MW) for 30 s, followed by addition of a solution of acrylamide (AAm) (1.2 – 3.6 g in 10 ml H_2O). The reaction mixture was then irradiated for 60 seconds with constant stirring, cooled to room temperature and dehydrated with IPA to isolate the solid graft copolymer (Agar/Na-Alg-*graft*-PAAm). The solid product was filtered off and washed with water to remove the homopolymer. The grafted copolymer was dried at 45°C until a constant weight was obtained.

III.3.3 MEASUREMENTS

III.3.3.1 Grafting Parameters

The grafting parameters e.g., conversion % (C%) and grafting efficiency was determined by the method described by Meena et al. [5], using the following equations:

Grafting percent (G%) = (Wt. of poly AAm grafted/wt. of blend) X 100
Conversion percent (C%) = (Total wt. of poly AAm/wt. of AAm charged) X 100.

III.3.3.2 Equilibrium Swelling (ES)

An accurately weighed sample of dried parent agar polysaccharide, blend polysaccharides, Agar-*graft*-PAAm, Na-Alg-*graft*-PAAm and Agar/Na-Alg-*graft*-PAAm copolymers with particle size between 5-10 mesh was immersed in the different solutions having pH 1.2, 7.0 and 12.5, in separate experiments. The swollen gel particles were wiped with tissue paper to remove the adhering water and weighed at regular intervals

until equilibrium was reached. Equilibrium swelling (ES) capacity was calculated as described by Prasad et al. and Meena et al. using the following equation [3, 5].

$ES = W_s - W_d / W_d$ (1)

Where, W_s and W_d are the weights of the swollen and dry material samples, respectively.

III.3.3.3 Syneresis index

The amount of water exuded from the gel samples after standing for a certain period of time was determined and quantified using a modified method as described by Fiszman and Duran [11].

III.3.3.4 Miscellaneous measurements

Infra red spectra were recorded in KBr on a Perkin-Elmer Spectrum GX, FT-IR System (USA). X-ray diffractions studies were done on a Philips X'pert MPD X-ray powder diffractometer using $2\theta = 5$ to 60^0. Optical micrographs were recorded on an optical microscope of Olympus model SZH 10, Japan with 70X magnification, and thermal analysis was done on a TGA Toledo Mettler TGA system, Switzerland). The apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072) using Spindle No.1 at rpm 60. The gel strength (g/cm^2) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). In this study gelling and melting temperatures of copolymer gels were measured as described by Craigie and Leigh [12].

III.3.3.5 Rheological measurements

Rheological measurements were done on a RS1 Rheometer (HAAKE Instruments, Karlsruhe, Germany), using cone/plate geometries (60 mm diameter, 1° rad angle) in solutions taking 1 ml sample on to the plate of the rheometer. Viscosities at varying shear rate were studied at 25^oC. Oscillation measurements were carried out in controlled deformation mode with 0.05% strain using plate/plate geometry (35 mm dia).

III.3.3.6 Scanning electron microscopy (SEM)

Vacuum oven dried samples were mounted on a sample holder and coated with gold. The samples were examined with a scanning electron microscope (Model Carl-Zeiss Leo VP 1430) at an accelerating voltage of 20 kV and 202 X magnification.

III.3.4 RESULTS AND DISCUSSION

III.3.4.1 Optimization of blend ratios

The ratios of polysaccharides in the blends were optimized by measuring the swelling ability given in the Table III.3.1. The swelling of the blend system increased with increasing amount of sodium alginate up to 75 wt%, and maximum swelling capacity 14 g/g, 12 g/g and 8.5 g/g were obtained in acidic, neutral and alkaline media, respectively (Table III.3.1). Increase in swelling ability with sodium alginate may be due to more hydrophilicity of the sodium alginate. The stability of the blend hydrogels with sodium alginate may be due to the increased entanglements of the macromolecule chains within the blend hydrogel resulting in slower diffusion in the aqueous medium [13].

Table III.3.1 Swelling ability of the blend samples [a]

Products	Swelling at pH 1.2	Swelling at pH 7.0	Swelling at pH 12.5
Agar	6±0.40	5±0.22	5±0.20
Sodium alginate (Na-Alg)	--	--	--
Agar/Na-Alg blend (1:9 w/w or 10%:90% w/w)	9±0.20	8±0.40	6±0.42
Agar/Na-Alg blend (1:3 w/w or 25%:75% w/w)	14±0.65	11±0.40	8.5±0.42
Agar/Na-Alg blend (1:1 w/w or 50%:50% w/w)	12±0.20	10±0.22	8±0.32
Agar/Na-Alg blend (3:1 w/w or 75%:25% w/w)	11±0.22	9±0.40	8±0.32

[a]Data presented are mean of triplicate measurements

III.3.4.2 Physical properties

The physical properties of agar, sodium alginate, Agar/Na-Alg blend and Agar/Na-Alg-*graft*-PAAm copolymer are given in Table III.3.2. The apparent viscosity increased with

the increasing proportion of AAm and KPS with respect to the blend (Table III.3.2). The increase in apparent viscosity when grafted with AAm could be explained on the basis of stiffness and the increase in the hydrodynamic volume of the copolymers in the presence of higher level of PAAm [8, 14]. The gel strength of the blend polysaccharide was decreased after copolymerization with AAm (Table III.3.2). Gel strengths of the copolymer hydrogel samples were decreased when increased the concentrations of AAm and KPS (Table III.3.2). The copolymer hydrogel with maximum swelling ability showed gel strength 120 g/cm^2 (Table III.3.2). After optimum concentrations of AAm and KPS the gelling and melting temperatures, and gel strength of the copolymer hydrogel samples were decreased (Table III.3.2).

Table III.3.2 Physical properties and equilibrium swelling of the parent and blend polysaccharides, as well as Agar/Na-Alg-*graft*-PAAm hydrogel[a,b]

Product	Apparent Viscosity (cP s; at 80°C)	pH (at 60°C)	Gel Strength (g/cm^2)	Gelling Temp. (^{o}C)	Melting Temp. (^{o}C)	Equilibrium Swelling (Es, in g/g)		
						At pH 1.2	At pH 7.0	At pH 12.5
Agar	28±0.55	6.9	800±7.07	41±0.5	84±0.7	7.0	6.0	5.0
Sodium alginate	14±0.5	7.0	N A [c]	N A [c]	N A [c]	NA [c]	NA [c]	NA [c]
Blend [Agar:Na-Alg; 1:3 w/w]	20±0.45	7.0	170±4.5	33±0.5	69±0.5	14.0	12.0	8.5
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:0.6:0.03 w/w)	47±0.54	6.9	140±8.0	30±0.42	63±0.4	20.0	15.0	10.0
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.2:0.03 w/w)	57±0.45	6.7	120±6.5	29±0.4	61±0.5	24.0	18.0	11.0
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.8:0.03 w/w)	62±0.55	6.4	100±8.4	27±0.4	57±0.4	21.0	16.0	10.0
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.2.04 w/w)	63±0.55	6.1	100±6.4	26±0.5	54±0.34	14.6	ND [d]	ND [d]
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.2:05 w/w)	65±0.55	5.7	<100±8	22±0.4	50±0.4	12.5	ND [d]	ND [d]

[a]All measurements were done in 1.5% sol/gel; [b]Data presented are mean of triplicate measurements; [c]NA = Not Applicable; [d]ND = Not determine

The value of syneresis was increased with increased concentration of AAm and KPS (Table III.3.3). The syneresis values for copolymer hydrogel, which was prepared under optimum grafting conditions were 6.2±0.026. Syneresis results indicating lower water

holding capacity of the copolymer hydrogels than that of the blend (Table III.3.3), may be due to modified gel network in presence of PAAm.

Table III.3.3 Degree of Syneresis[a]

Nature of hydrogel	Initial weight (W_o, in g)	Final weight (after 2 h, in g)	Syneresis (ΔW)	Degree of syneresis ($\Delta W/W_o$)
Agar	10±0.059	8.54±0.019	1.46±0.010	0.146±0.0047
Agar/Na-Alg blend (1:3 w/w or 25%:75% w/w)	10±0.059	5.11±0.010	4.89±0.019	0.489±0.0037
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:0.6:0.03 w/w)	10±0.053	4.4±0.022	6.2±0.026	0.620±0.0052
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:1.2:0.03 w/w)	10±0.053	3.8±0.022	6.2±0.026	0.620±0.0052
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.8:0.03 w/w)	10±0.053	3.5±0.022	6.2±0.026	0.620±0.0052
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.2:0.04 w/w)	10±0.054	3.1±0.026	6.9±0.022	0.69±0.0044
Agar/Na-Alg-*graft*-PAAm (Blend:AAm:KPS, 1:1.2:0.05 w/w)	10±0.055	2.7±0.022	7.3±0.032	0.73±0.0045

[a] Data presented herein are mean of triplicate measurements.

These results confirmed that the gelling properties of the parent agar in the blend and grafted copolymers (whereas further more hydrophilic PAAm has been introduced in the gel network) have been modified significantly. It may be explained on the basis of more hydrophilicity of the sodium alginate (which was predominant in the blend) and PAAm, which has been introduced in the copolymer hydrogels after grafting. It could also be explained on the basis of chemical intervention of PAAm in the copolymer hydrogels network. The changes in the gelling properties with increase in the concentration of KPS and AAm may be due to increase in viscosity and hydrodynamic volume of the copolymer induced by PAAm, as well as formation of the weaker gel network caused by acid generated from relatively higher concentration of KPS (Table III.3.2 & 3).

Both the conversion percent (C%) and grafting percent (G%) increased with the increasing concentrations of the monomer (AAm) and the initiator (KPS) (Table III.3.4). The copolymer hydrogel prepared under optimum grafting conditions with C% = 85%

and G% = 104% exhibited greatest swelling abilities 24 g/g, 18 g/g, and 11 g/g in acidic, neutral and alkaline media, respectively (Tables III.3.2 & 4). In the absence of the water soluble initiator the reaction did not take place at all [4, 5]. Grafting percentage (G%) and conversion percentage (C%) increased with increasing the concentration of monomer (AAm). The maximum values of G%=170% and C%=94% were obtained with 1.8 w/w ratio of AAm (Table III.3.2). The decrease in the G% and C% at a higher monomer concentration was apparently due to preferential homopolymer formation [15, 16, 17]. Likewise, G% and C% increased with increasing concentration of KPS up to 0.04 w/w, decreasing thereafter with increasing KPS concentration, as reported in the literature [18]. A plausible reaction mechanism for the formation of the graft copolymer is proposed in Scheme III.3.1 [19].

Table III.3.4 Grafting parameters of the different copolymers [a].

Copolymer products	Conversion percentage (C%)	Grafting percentage (G%)
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:0.6:0.03 w/w)	85±0.42	51±0.80
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:1.2:0.03 w/w)	86.6±0.52	104±0.42
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:1.8:0.03 w/w)	96±0.65	173±0.42
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:2.4:0.03 w/w)	94±0.65	170±0.42
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:1.2.04 w/w)	95±0.81	114±0.62
Agar/Na-Alg-*graft*-PAAm (Agar/Na-Alg:AAm:KPS, 1:1.2:05 w/w)	90.8±0.52	109±0.72

[a] Data presented are mean of triplicate measurements

Greatest swelling ability (24 g/g) of the grafted blend was obtained in acidic solution having pH 1.2 (Table III.3.2). Swelling capacity increased with increasing ratio of KPS up to 0.03 w/w (w.r.to blend polysaccharides), and thereafter it declined (Table III.3.2). Decrease in swelling can be explained on the basis of an increase in terminating step reaction via molecular collision, which was referred to as self-crosslinking by Chen and Zhao [20]. The decrease in swelling ability may be due to the fact that, at higher concentrations, KPS undergoes induced decomposition, leading to a decrease in the radical concentration thereby reducing the initiation processes affecting both the grafting

and swelling ability. The free radical degradation of the blend backbone by sulphate radical-anions is an additional reason for the swelling loss at higher KPS concentration, which has been reported in the literature [21, 22]. Similarly, swelling ability increased with increase in the AAm ratio up to 1.2 w/w (w.r.to blend polysaccharide) (Table III.3.2). The decrease in swelling capacity beyond this may be due to the increased chance of chain transfer to AAm molecules. This led to the enhanced homopolymerization reaction over graft copolymerization [21].

III.6.4.3 Characterization of copolymer hydrogel

The grafting of the PAAm was confirmed by FTIR, TGA, X-ray, and SEM analysis of the samples (Figures III.3.2, 3, 4, & 7, respectively). The IR spectrum of the graft copolymer showed the presence of PAAm, having an absorption band at 1671 cm^{-1} for (C=O stretching) and at 1459 cm^{-1} for C-N stretching (Figure III.3.2).

Thermogravimetric analysis (TGA) also confirmed grafting of blend with PAAm (Figure III.3.3). In agar, the mass loss was in three stages, e.g. up to 105°C, 370°C and 540°C corresponding to 12%, 64% and 97% mass losses, respectively [2]. Likewise, in sodium alginate the stages were 110°C, 295oC and 750oC corresponding to 7%, 50% and 80% mass loss, respectively. In Agar/Na-Alg blend, 14%, 72% and 92% mass losses corresponded to 110°C, 420°C and 750°C, respectively. The mass loss pattern of the blend appears closer to that of agar, while the stages happened at different set of temperatures. Thermogram of the blend copolymer traced its path between those of the blend and agar exhibiting 4%, 65% and 87% mass losses at temperatures 110°C, 430°C and 750°C, respectively (Figure III.3.3). The pattern of the mass losses of the grafted copolymer was different showing lower values than the blend, while the corresponding temperatures were almost identical, indicating the occurrence of grafting. The TGA pattern of the blend copolymer presumably reflects the modified macromolecular entanglements and architecture in the copolymer.

The X-ray diffraction pattern indicated that crystallinity of alginate and acrylamide disappeared in the blend polysaccharide (Figure III.3.4) as well as in the grafted copolymer (Figure III.3.4), indicating grafting resulting in modified macromolecular arrangements.

III.6.4.4 Rheological measurements

The copolymer showed lowest gel thinning behavior and highest viscosity values than agar, Na-Alg and Agar/Na-Alg blend, while parent sodium alginate exhibited greatest gel thinning behavior and lowest viscosity (Figure III.3.5). In the oscillation measurements, the cross-over points of storage and loss moduli confirmed the gelling point [1]. The greatest gelling point (ca. 41°C, Figure III.3.6) was observed for the parent agar gel sample while, the lowest (ca 29°C, Figure III.3.6) was observed for the grafted blend copolymer gel sample. As expected, the G" values for all the gel samples were higher at high temperatures and G' values increased with decrement of temperature, and dramatic increments were noted near the gelling point indicating formation of gel at lower temperature (Figure III.3.6).

III.6.4.5 Morphological analysis

The scanning electron microscopy (SEM) images of the graft copolymer confirmed grafting when compared to those of of parent polysaccharides, PAAm and Agar-Na-Alg blend (Figure III.3.7). The SEM image of the graft copolymer appeared more composed than those of the parent polysaccharides indicating modified molecular associations in the former.

III.6.5 CONCLUSIONS

Non-gelling and readily water soluble seaweed polysaccharide sodium alginate has been stabilized in water as well as wide range of pHs, with agar through blending and grafting of blend with acrylamide, using a simple and water based process under microwave irradiation. The copolymer hydrogel of the Agar/Na-Alg blend having 75% w/w sodium alginate, had superior swelling ability, good gelly strength, relatively lower water holding capacity and enhanced flow or gel thinning properties compared to the blend and parent polysaccharides. Considerable stability, good gelly strength and swelling ability of the copolymer hydrogel in the wide range of pH compared to those of the parent polysaccharides and blend would make it an attractive material for newer applications [23, 24].

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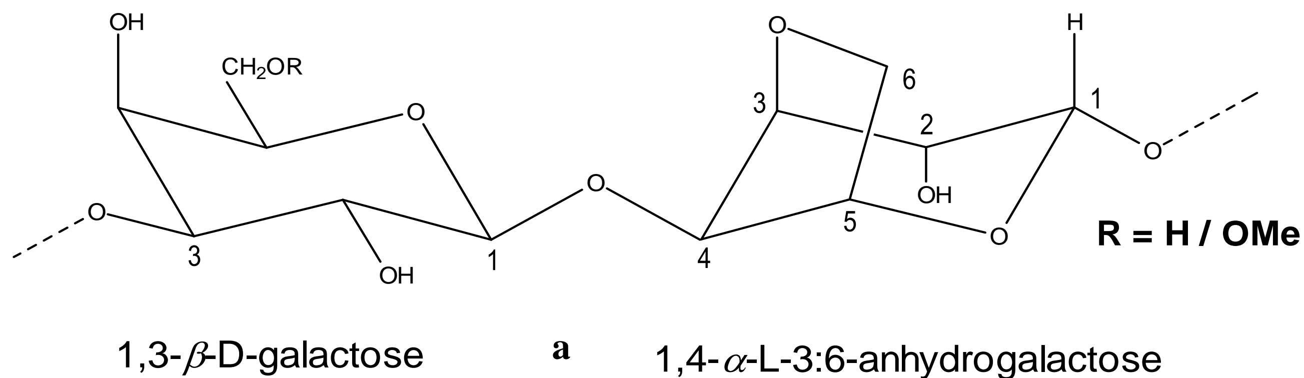


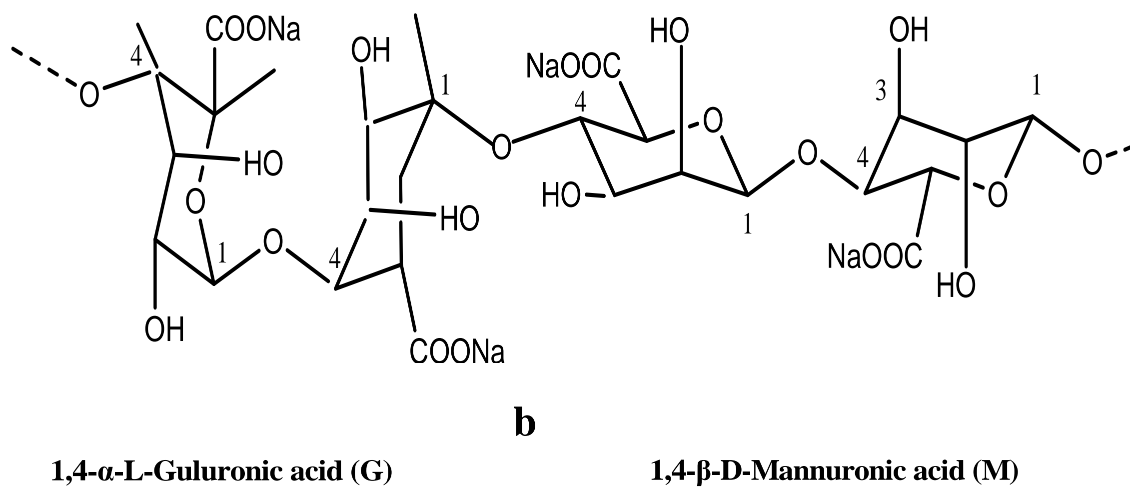


Figure III.3.1a,b Repeating disaccharide units of agar **(a)** and sodium alginate **(b)**

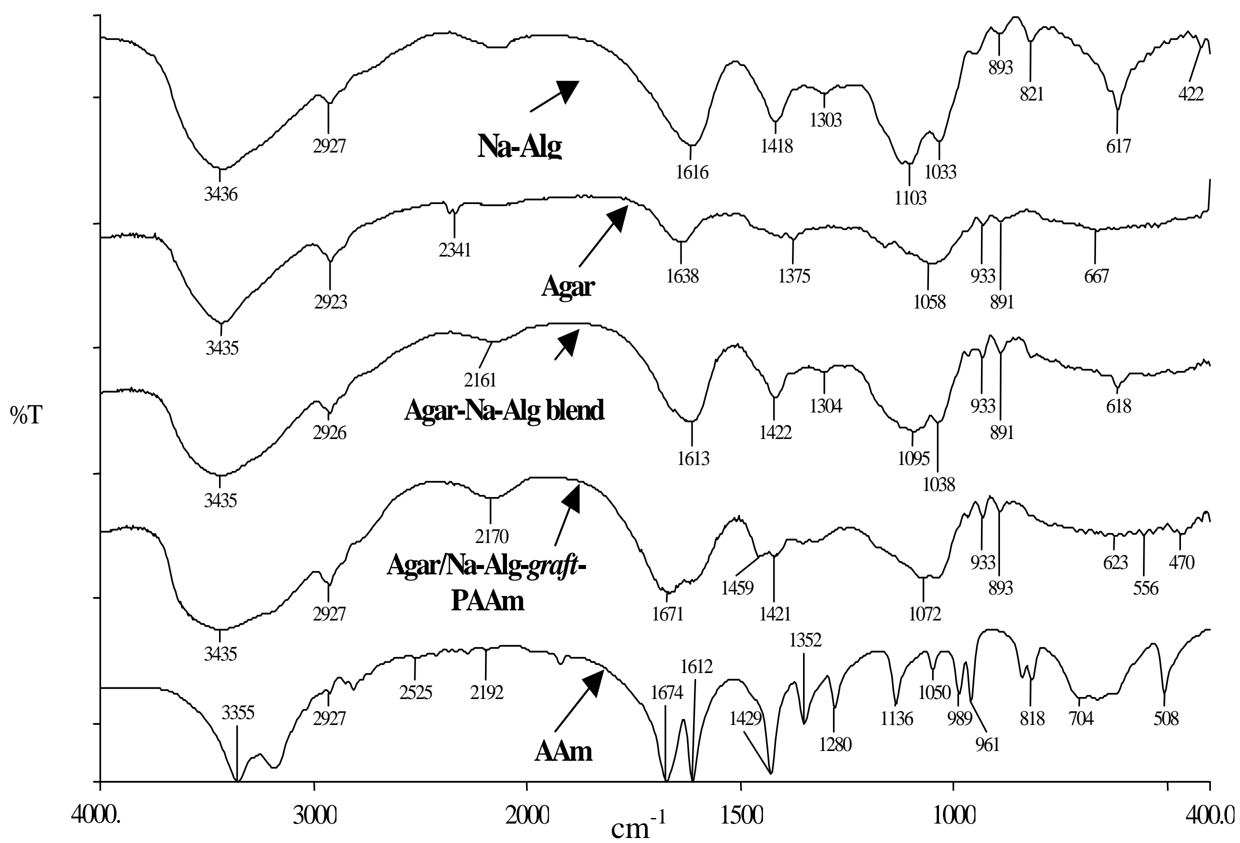


Figure III.3.2 FTIR spectrum of the AAm, agar, Na-Alg, Agar/Na-Alg blend and the graft copolymer.

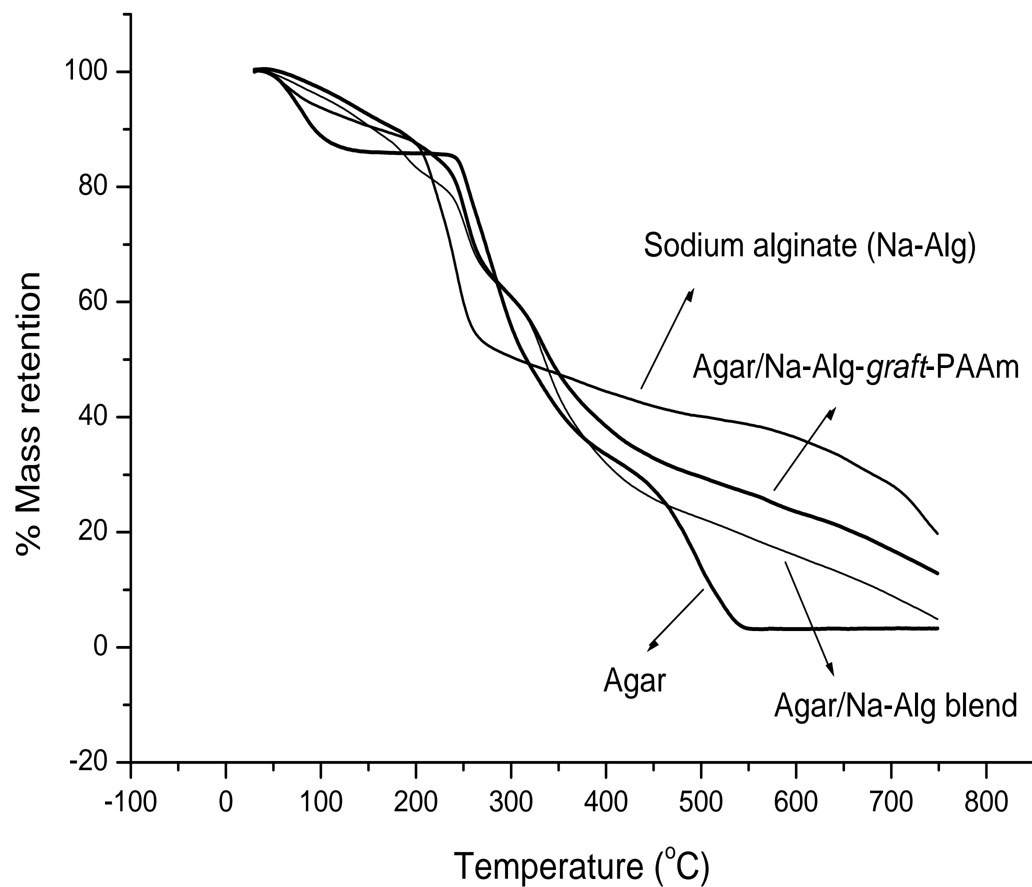


Figure III.3.3 TGA thermograms for agar, Na-Alg, Agar/Na-Alg blend and the graft copolymer.

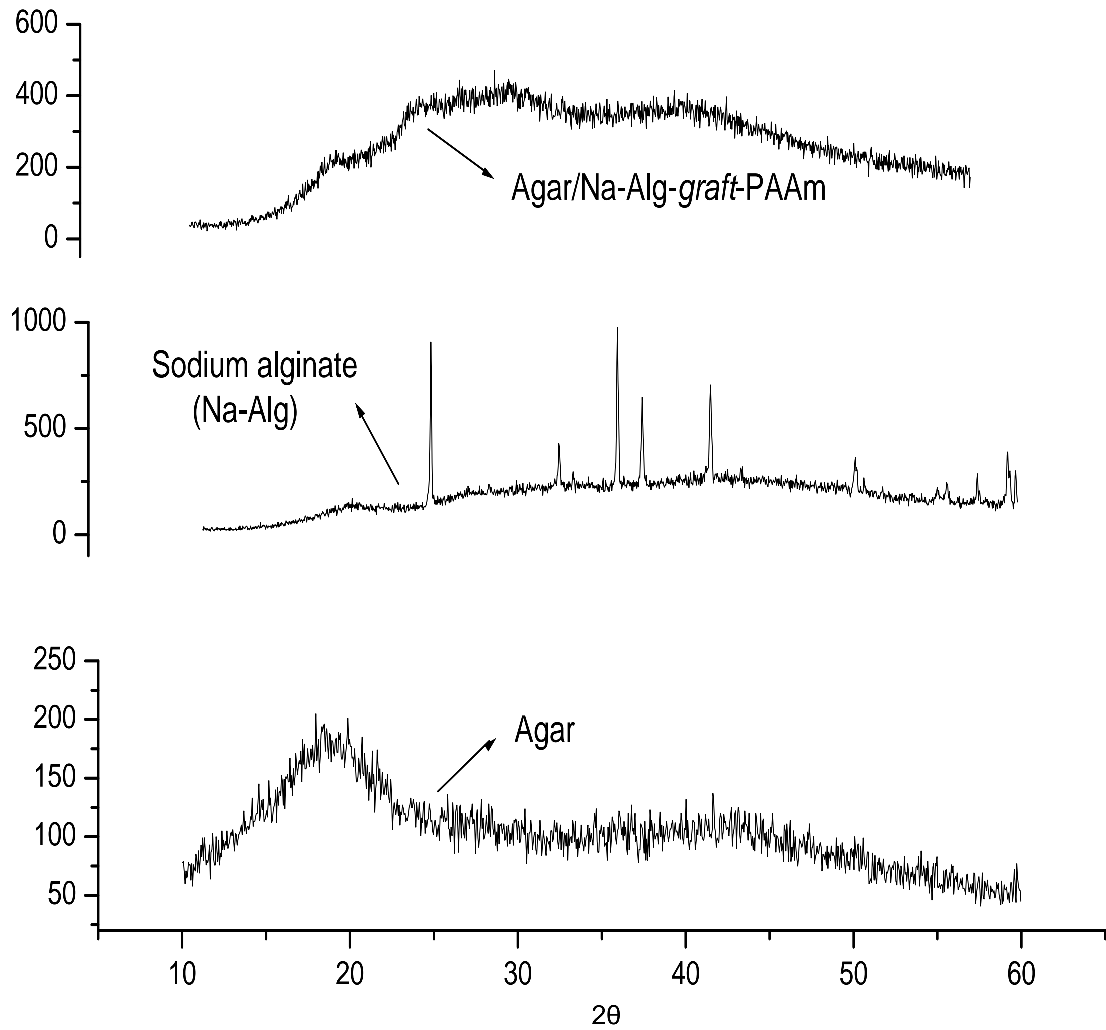


Figure III.3.4 X-ray diffractograms of the AAm, agar, Na-Alg and the graft copolymer

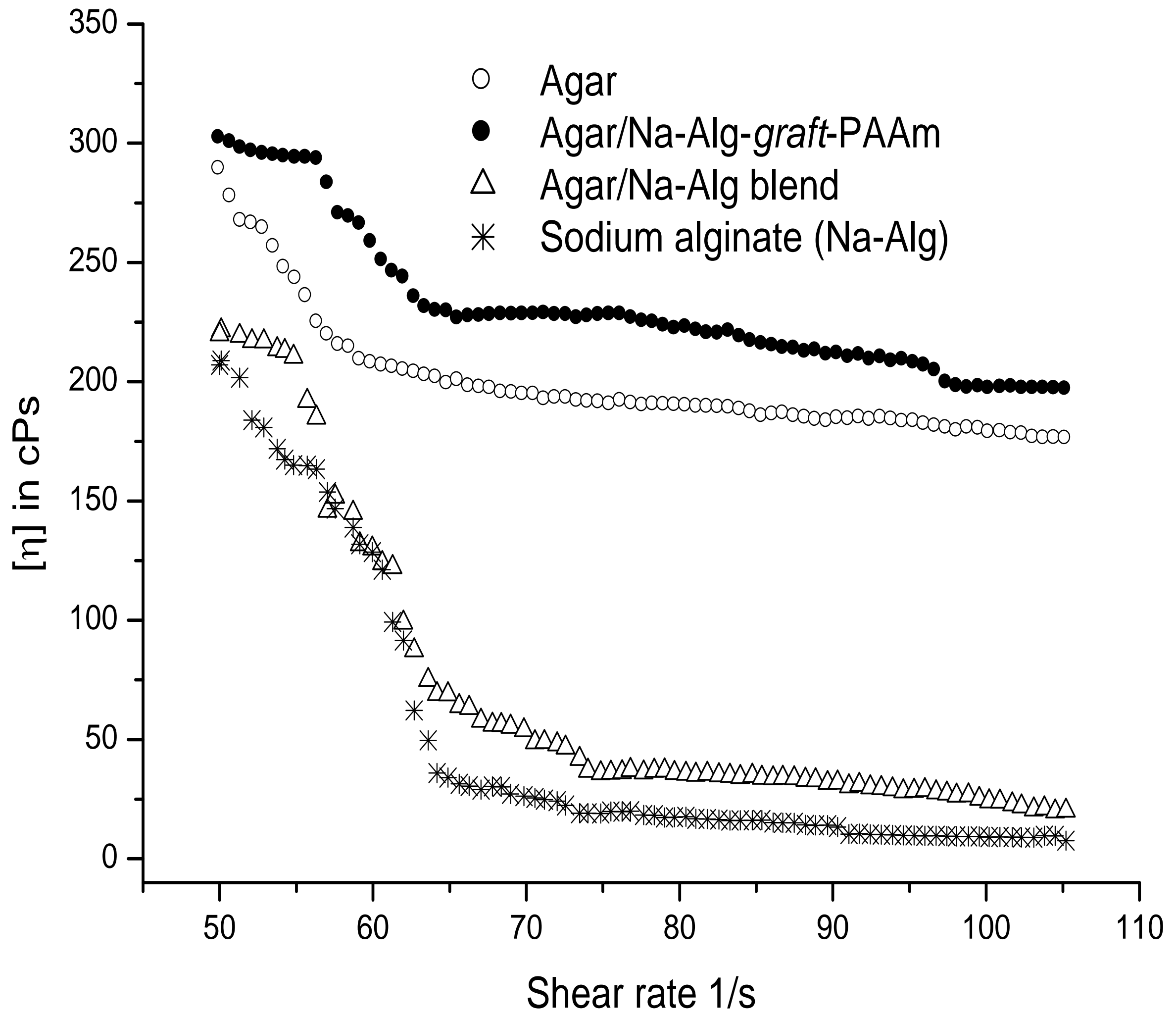


Figure III.3.5 Dynamic viscosity of the agar, Na-Alg, Agar/Na-Alg blend and the graft copolymer gels.

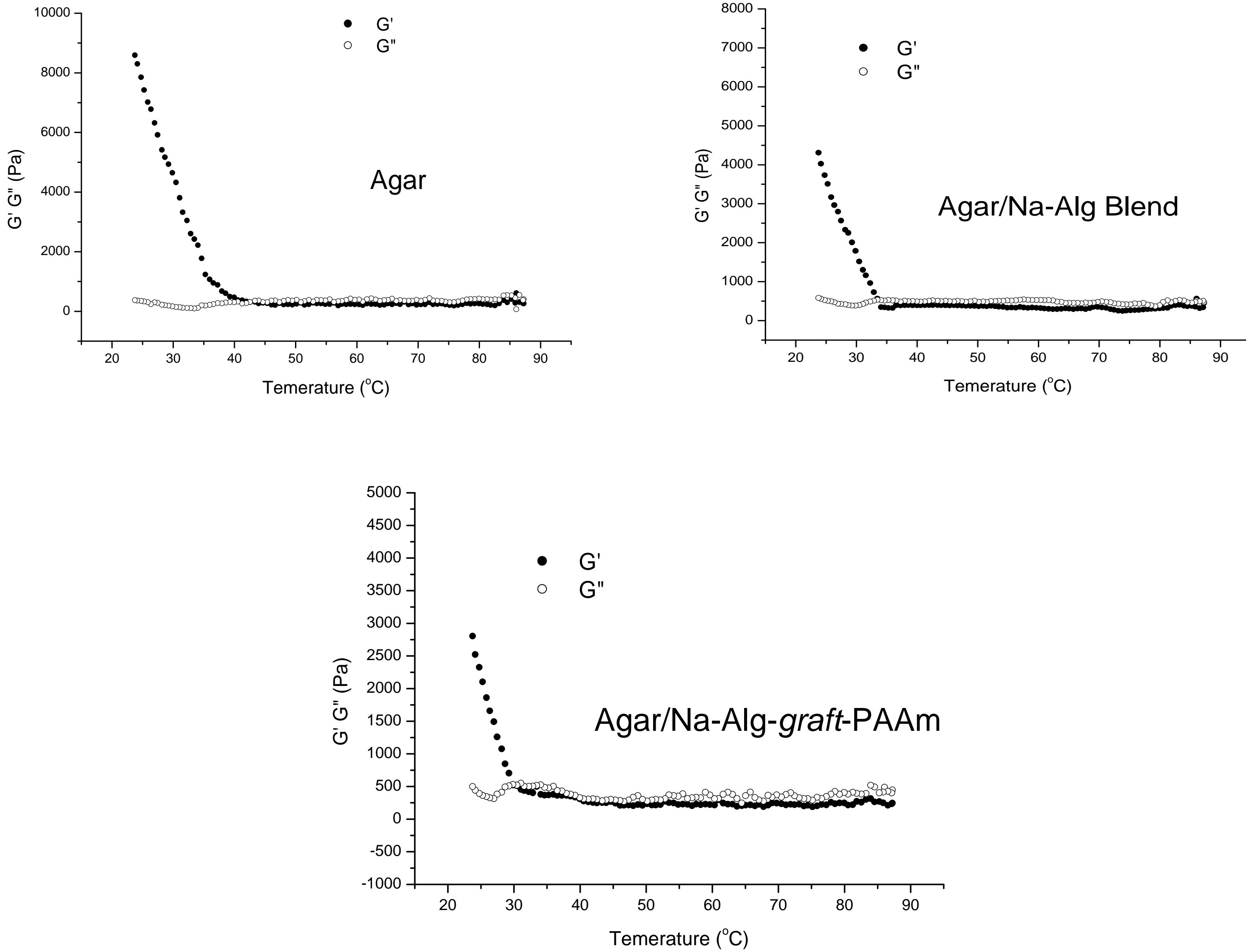
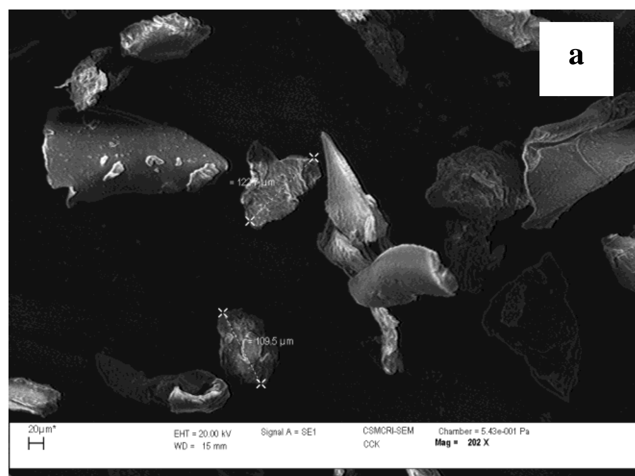
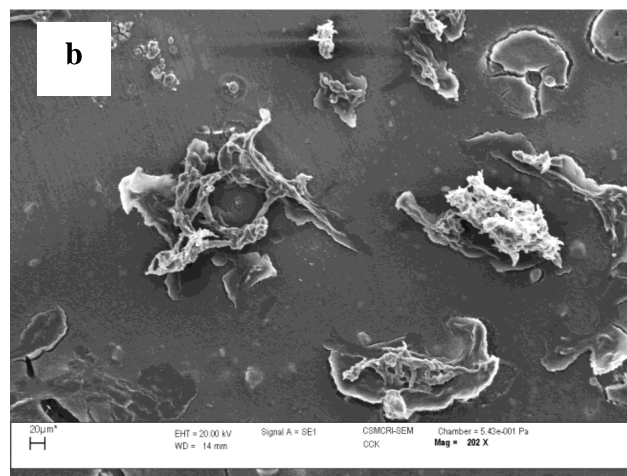
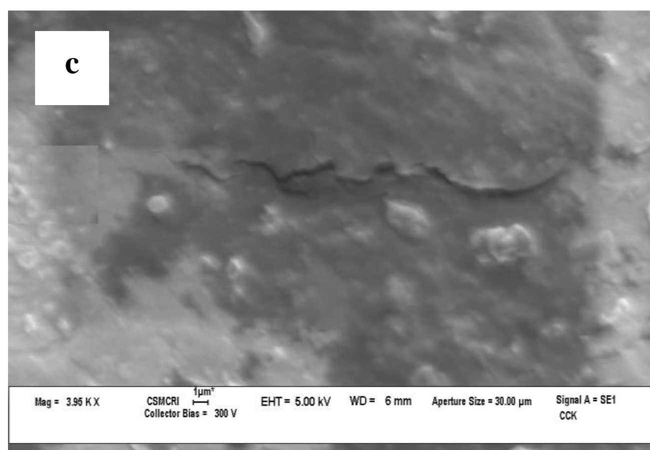
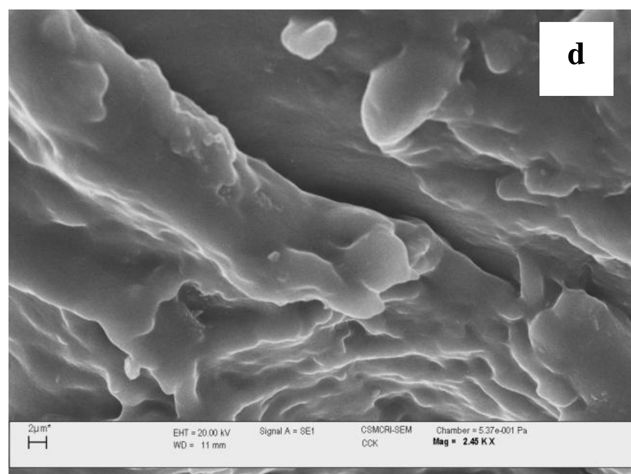


Figure III.3.6 Temperature dependence of the storage and loss modulus for agar, Na-Alg, Agar/Na-Alg blend and the graft copolymer hydrogels.









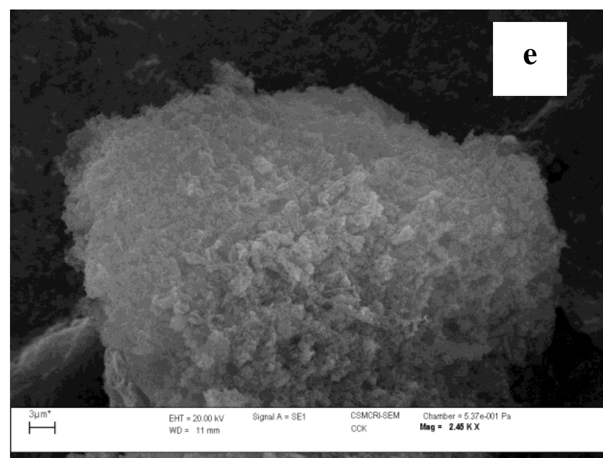
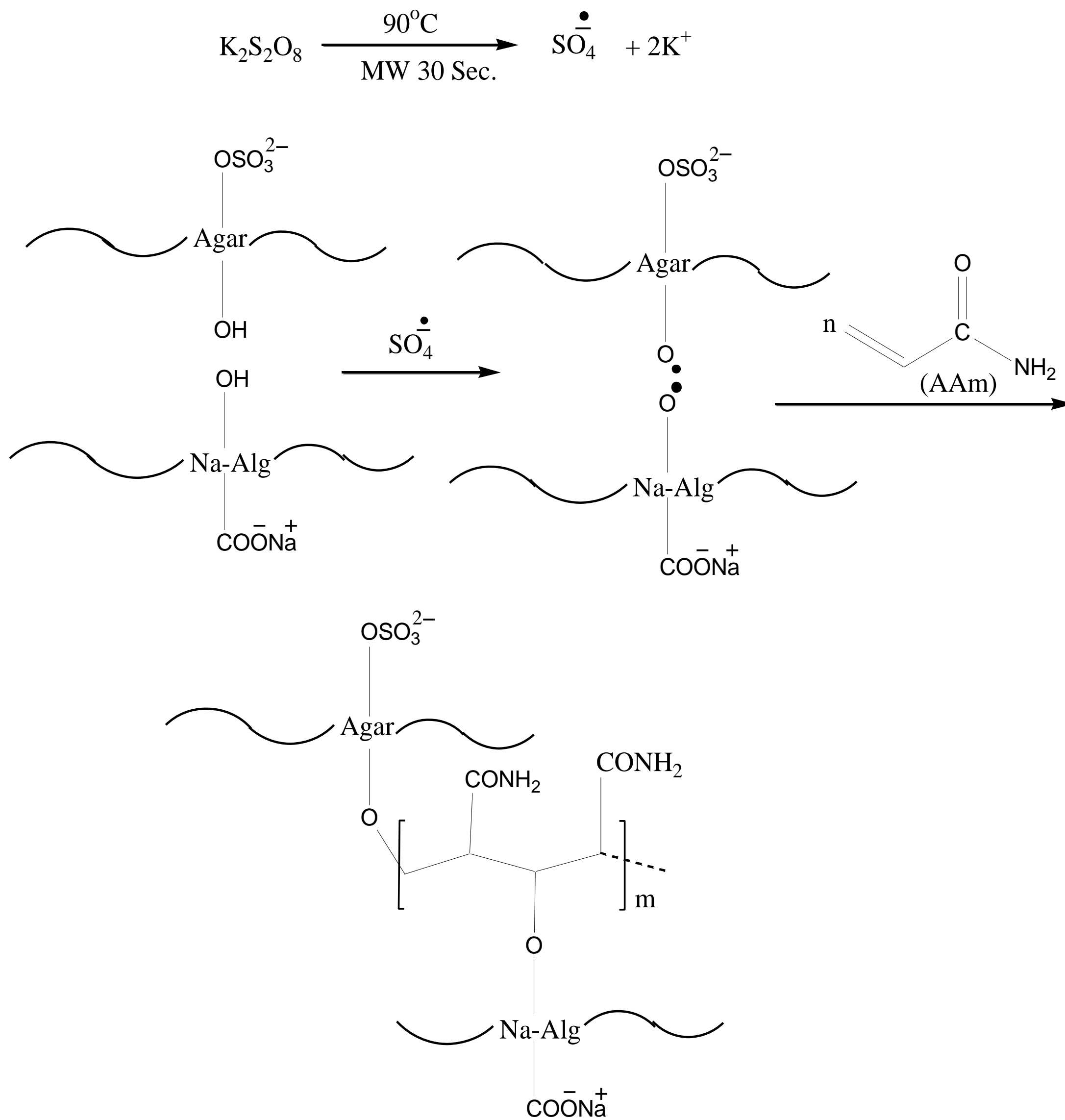


Figure III.3.7a-e Scanning electron microscope images (**a**) agar, (**b**) Na-Alg, (**c**) PAAm, (**d**) Agar/Na-Alg blend and (e) the graft copolymer.



Scheme III.3.1 Outline of the synthesis of the copolymer (Agar/Na-Alg-*graft*-PAAm) hydrogel

CHAPTER III.4

EFFECT OF GENIPIN, A NATURALLY OCCURRING CROSSLINKER, ON THE PROPERTIES OF AGAROSE

III.4.1 INTRODUCTION

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III.4.4 CONCLUSIONS

III.4.5 REFERENCES

III.4.1 INTRODUCTION

Hydrophilic gels are in increasing demand in the biomedical and pharmaceutical applications due to their biocompatibility [1-3]. Most of these applications in the past have made use of synthetic water soluble polymers (WSP) such as polyacrylic acids, polyacrylamides, polyethelene oxide, polyvinyl alcohols and polyvinyl pyrrolidones. Subsequently, these hydrogels were modified by blending some natural polymers with WSP [4]. The resultant hydrogels exhibit different properties than those of the original polymers [5, 6]. The improved properties of the hydrogels were harnessed either as super absorbents or as controlled delivery systems. The natural polymers that have been widely used for this purpose are cellulose, starch, chitin, carrageenan, agar and alginates [7]. Genipin is a naturally occurring cross-linking agent, having much less toxicity and widely used in herbal medicine [8], and the dark blue pigments obtained by its spontaneous reaction with amino acids or proteins have been used in the fabrication of food dyes [9]. It was reported that porcine pericardia crosslinked with genipin led to the formation of stable crosslinked products [10]. It has also been reported that the gelatin-derived bioadhesives display higher biocompatibility and less cytotoxicity when crosslinked with genipin than with other agents, such as formaldehde, glutaraldehyde and epoxy compounds [11, 12]. Gerard et al. [13] has described estimation of amino acid concentrations in various polymeric matrices including agar, agarose and carrageenan. Protein levels in bacteriological agar were estimated on the basis of nitrogen content of the polysaccharide [14]. Agarose is a hydrophilic polymer and widely used in biomedicinal applications and bioengineering. The chemical structure of genipin (Figure III.4.1a) and the basic disaccharide repeating units of agarose are (1→ 3) linked β-D-galactose and (1→ 4) linked α-L-3, 6-anhydrogalactose (Figure III.4.1b), are reported in the literatures [15, 16].

In this paper we report the effect of genipin, a naturally occurring cross linking agent, on agarose and preparation of absorbent genipin-fixed agarose hydrogel, which is thermally more stable and degrades slowly in Ringer's solution compared to agarose. The product was characterized by thermogravimetric analysis (TGA), gelation degree, swelling ability and rate of degradation studies. The reaction mechanism of genipin with amino groups and schematic illustration (Figure III.4.2) of the formation of genipin-fixed agarose network has been proposed [cf. 15].

III.4.2 EXPERIMENTAL

III.4.2.1 Materials

The phycocolloid, agarose was extracted from the red seaweed *Gracilaria dura* occurring in Indian waters. Genipin was purchased from Challenge Bioproducts Co., Ltd. Taiwan. Iso-propanol (Laboratory Reagent grade) was procured from Ranbaxy Chemicals Ltd., Mohali (Punjab), India.

III.4.2.2 Preparation of agarose

Agarose was prepared from the red seaweed *Gracilaria dura* using an improved procedure described by Siddhanta et al. and Meena et al. [17, 18].

III.4.2.3 Preparation of genipin-fixed agarose hydrogels

In a typical batch, the agarose sol was prepared by dissolving 2 g of agarose in 50 ml of distilled water at 120°C for 20 min in an autoclave. A stock solution (10%) of genipin was prepared in 10 ml of 60% aqueous ethanol. The aqueous solution of the agarose was then mixed with different volumes of the genipin stock solution at 40°C to obtain the final polymer agarose-genipin mixture with weight percentages of genipin lying in the range 0.05 to 1.5 wt%. The homogeneous viscous solutions were kept at room temperature (30°C) and allowed to react for different durations e.g. 10, 20, 30, 40, 50, 60, 65, 70, 75, 80, 85, 90, 95 and 100 h, in fourteen different experiments. The reaction mixture started assuming light blue color after 120 min and the color intensified with the passage of time becoming deep blue in color after 60 h. The gelled reaction mixture in each experiment was worked up to obtain the genipin-fixed agarose hydrogel by dehydrating the gel with iso-propanol (1:2.5 w/w) for 24 h. After dehydration, the gel sample was air dried followed by drying at 50°C for 2 h.

III.4.2.4 Gelation degree

The gelation degree G was calculated adopting the method reported by Lendlein, et al. [19], using the following equation 1.

$G = m_d / m_{iso}$ ------------------ (Equation 1)

Where, m_d = dry weight of the gel and m_{iso} = weight of isolated gel.

A known weight of the dry genipin-fixed agarose hydrogel was taken, which was put into the different pH media (e.g. pH 1.2, 7.0 and 12.5) for equal time duration of 10 h, in separate experiments. The weight of the isolated gel samples were determined (m_{iso}). The isolated gel samples were dehydrated with a 100-fold excess of isopropanol overnight, washed with isopropanol carefully drying at room temperature under reduced pressure, the sample was weighed again (m_d).

III.4.2.5 Swelling ratio measurements

The swelling ratio of agarose and genipin-fixed agarose with different weight percentage 0.5, 0.8 and 1.0% of genipin (with respect to the parent polysaccharide) were measured in this study. In the swelling measurements, the dry hydrogel was weighed (W_0) and immersed in aqueous media having different pHs e.g. 1.2, 7.0 and 12.5, separately. After the designated soaking time had elapsed, the wet samples were wiped dry with filter paper to remove excess liquid, and weighed (W_t). The swelling ratio ΔW (%) was calculated by the following Equation 2.

$$\Delta W(\%) = [(W_t - W_o) \div W_o] \times 100 \quad \text{---- (Equation 2)}$$

III.4.2.6 Degradation rate measurement

The degradation rate patterns of genipin-fixed agarose and agarose were measured in Ringer's solution (prepared in our lab: sodium chloride 8.6 mg ml^{-1}; potassium chloride 0.3 mg ml^{-1} and calcium chloride dihydrate 0.33 mg ml^{-1} under aseptic conditions), a reconstituted physiological medium [20]. The agarose and genipin-fixed agarose hydrogels (having 0.5, 0.8 and 1.0 wt% of genipin) were weighed (Wo) and placed in a capped plastic aseptic tubes with 25 ml Ringer's solution. The ion concentrations are similar to those in human physiological environments. The test samples were immersed in the Ringer's solution, as simulated body fluid, to measure the rate of degradation *in vitro*. Samples were incubated at 37°C in an incubator, after soaking for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 days, all the mixtures was drawn from the Ringer's solution, dried to remove water and weighed (Wt). The weight loss ratio ΔW *(%)* was then calculated using the Equation 3 [20].

$$\Delta W(\%) = [(Wo - Wt) \div Wo] \times 100 \quad \text{................ (Equation 3)}$$

III.4.2.7 Optimization of the crosslinking reaction time

Optimization of the reaction time was done on the basis of swelling capacity of the genipin-fixed agarose samples in the different pHs solutions.

III.4.2.8 Optimization of genipin quantity

Optimization of the genipin concentration was done on the basis of swelling capacity of the genipin-fixed agarose samples in the different pHs solutions.

III.4.2.9 Characteristics of genipin-fixed agarose hydrogel

The agarose and genipin-fixed agarose hydrogel were characterized using different techniques. Optical microscopy was recorded on an Olympus model SZH 10, Japan with 70X magnification and thermal analysis (TGA were done on a TGA Toledo Mettler TGA System Switzerland. Apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072) using Spindle No.1 at rpm 60. UV-Vis spectra were recorded on a Varian CARY 500 Scan UV-Vis-NIR spectrophotometer. Optical rotation was measured on a Rudolph Digi pol-781 Polarimeter (Rudolph Instruments Inc, NJ, USA) in 0.025% aqueous solution at 45°C.

Scanning electron microscopy (SEM) of the vacuum oven dried samples of the powder of the non-modified and modified agarose were mounted on a sample holder and coated with gold. The samples were examined with a scanning electron microscope (Model Carl-Zeiss Leo VP 1430) at an accelerating voltage of 20 kV and 202 X magnification.

III.4.2.10 Elemental analysis and protein estimation

The elemental analyses were done on a Perkin Elmer-2400, CHNS/O Analyser and total nitrogen was estimated by Kjeldahl method [21] on a KEL PLUS-KES 20L Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN Equipments, Chennai, India). Crude protein content was calculated multiplying the nitrogen content by the approximate factor 6.25 [21].

III.4.2.11 Rheological measurements

Dynamic rheological measurements were done on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany). The measuring geometries selected were a cone/plate (60 mm diameter, 1° rad angle) for measurements in 1% sols taking 1 ml sol on to plate of the rheometer, for measurements in the sol, prepared from genipin-crosslinked agarose hydrogel with 0.8 wt% genipin and compared with agarose. Viscosities at varying shear rate were studied at $45^{o}C$. Oscillation measurements were carried out in controlled deformation mode with 0.05 % strain and plate/plate geometry (35 mm dia) was used. The temperature was maintained using the DC50 water circulator. Rheological data presented are means of three replicate measurements.

III.4.3 RESULTS AND DISCUSSION

III.4.3.1 Optical microscopy

The genipin-crosslinked agarose hydrogels was blue in appearance caused by the cross linking reaction between genipin and agarose. The agarose and genipin were colorless before mixing together, but light blue color appeared after 120 min of mixing and the mixture became dark blue after 60 h. Optical micrograph of the powdered product, blue in colour, is given in Figure III.4.3 [22]. The protein content in parent agarose was estimated to be 0.25%. Gerard et al. estimated the amino acid concentration of glycine and amino acids (excluding glycine) to be in the range of 290 ng/mg (0.029%) and 376 ng/mg (0.037%) respectively [13]. It is, therefore, apparent that genipin reacted with the amino acids present in the polysaccharide affording the crosslinked product. It was further observed that genipin reacted with gelatin producing dark blue color within minutes of mixing while in the present investigation it took about 60 h to produce dark blue color indicating that the amino acids present in agarose in trace amounts.

III.4.3.2 Gelation degree

The gelation degree (G) of the non-modified agarose decreased after crosslinking with genipin. The G values for agarose 0.l62, 0.169 and 0.2 were observed at 1.2, 7.0 and 12.5 pH media respectively. The gelation degree of genipin-fixed agarose deacreased significantly with optimum 0.8 wt% genipin to 0.023, 0.024 and 0.027 at 1.2, 7.0 and 12.5 pH media, respectively (Figure III.4.4).

III.4.3.3 Swelling behavior

The genipin-fixed agarose, obtained with 0.8 wt% genipin after 85 h crosslinking reaction time, showed the swelling ability in the following order in pH media 1.2 >7.0 >12.5, having maximum swelling ratios (%) 4800, 4300 and 3600 in the solutions of pHs 1.2, 7.0 and 12.5, respectively (Table III.4.1 and Figures III.4.5a-c, 6 & 7). On the other hand, the parent agarose exhibited swelling ratios (%) ca. 600 in all the pH media, clearly indicating the improved network system in the genipin-fixed agarose hydrogel (Table III.4.1, Figure III.4.5a-c).

Table III.4.1 Equilibrium swelling ratios of the genipin-fixed agarose and parent agarose in the different pHs media.

Equilibrium swelling ratios (%), of the genipin-fixed agarose (with 0.0, 0.5, 0.8 and 1.0 wt% genipin), after ca 1100 min at											
pH 1.2 (Figure 5a)				pH 7.0 (Figure 5b)				pH 12.5 (Figure 5c)			
0.0 wt%	0.5 wt%	0.8 wt%	1.0 wt%	0.0 wt%	0.5 wt%	0.8 wt%	1.0 wt%	0.0 wt%	0.5 wt%	0.8 wt%	1.0 wt%
600	3000	4300	4000	600	2900	4200	4000	500	2800	3700	3600

One important observation is the remarkable stability of the genipin-fixed agarose hydrogel in acidic pH, while in such acidity agarose gets readily depolymerised and dispersed (Figure III.4.5a). Agarose swells up to ca. 60 min and then achieves a steady value, whereas the product swells gradually up to ca. 1100 min and then achieves an equilibrium value, which was followed by very slow degradation only in pH 1.2 (Figure III.4.5a). The swelling ratio of genipin-fixed agarose with crosslinking reaction time (Figure III.4.6) and with concentration of genipin (Figure III.4.7) indicates that the minimum reaction time may be 85 h for agarose and genipin is 0.8 wt%. Thus the swelling ratio of the crosslinked agarose had an inverse relationship with the gelation degree.

III.4.3.4 Degradation rate measurement

The mass loss ratio of non-modified agarose and genipin-fixed agarose (with 1.0, 0.8 and 0.5% wt% genipin) was measured in Ringer's solution, wherein 50%, 32%, 33% and 38% mass loss was observed for agarose, genipin-fixed agarose (with 1.0 wt% genipin),

genipin-fixed agarose (with 0.8 wt% genipin) and genipin-fixed agarose (with 0.5 wt% genipin), respectively (Figure III.4.8). The mass loss ratio indicated that there are no significant variations in the mass loss between genipin-fixed agarose prepared with 1.0 wt% and 0.8 wt% genipin.

III.4.3.5 Thermogravimetric analysis

The TGA curve for agarose, genipin and genipin-fixed agarose polymer are shown in Figure III.4.9. The TGA curve of agarose shows three stages of weight loss. The first weight loss (12%) stage between 30-110°C was due to the loss of water, the second weight loss (62%) stage between at 240-350°C and complete weight loss (100%) was observed between 350-520°C. The weight loss in genipin-fixed agarose polymer was also obtained in three stages. In the first stage weight loss 10% between 30-121°C, in second stage weight loss was 30% between 180-250°C and in third stage ca. 90% weight loss was observed up to 750°C. The latter shows enhance thermal stability of the product.

III.4.3.6 UV-Spectroscopy and Optical Rotation

Specific rotation values of agarose and genipin were - 21.6^{o} and +111.1^{o} respectively, while that of genipin-fixed agarose was - 12.2^{o}. This considerable change in the optical rotation also suggests substantial modification in the molecular geometry of the parent agarose that came about in the genipin-fixed polysaccharide. The UV spectra shows peak at 590 cm^{-1} due to reaction of genipin with the amino acid that is present in agarose Polymeric matrix giving rise to blue color.

III.4.3.7 Morphological analysis

The optical micrographs of genipin-fixed agarose was taken with 70 X magnifications and compared with the parent agarose (Figures III.4.3a & b). Optical micrograph of genipin-fixed agarose was dissimilar in morphology and in color with those of the parent polysaccharide. These suggest that the cross linking reaction of genipin in agarose brought about transformation in the polysaccharide resulting in the changed morphology and color in the product.

SEM images of the non-modified and modified agarose have been presented in Figures III.4.3c & d. The SEM images also confirmed that morphology of the agarose polymer has been modified.

III.4.3.8 Elemental analysis and protein estimation

The percentage of C, H, and N were 35.58%, 6.44% and 0.0%. The CHN percentages remained unchanged in genipin-fixed agarose relative to unmodified agarose. The total nitrogen and protein values of agarose and genipin-fixed agarose were similar (0.07% and 0.4% respectively) before crosslinking and after crosslinking with genipin, presumably because of the very low quantity of genipin that was involved in the crosslinking process with agarose.

III.4.3.9 Rheological measurement

Variations of dynamic viscosity with shear rate are summarized in Figure III.4.10 with 1% sol of agarose and genipin-fixed agarose. It was observed that 1% sol of the genipin-fixed agarose hydrogel showed less gel-thinning behavior and high viscosity values than the sol of the parent agarose under applied shear rates. These values indicate enhanced network formation in the product facilitated by the crosslinker genipin. The stability of modulus during storage at 25°C was investigated and is depicted in Figure III.4.11. The storage modulus values of the 1% gels of agarose and genipin-fixed agarose hydrogel. It was observed that the G' and G'' values for both samples slightly increased with increment of time. This observation shows the stability of the samples under stress for long time. It was also observed that the G' values for genipin-fixed agarose hydrogel was slightly higher than that of agarose.

III.4.4 CONCLUSION

In this study the effect of genipin, a naturally occurring crosslinker, on the properties of agarose has been demonstrated, and a value added product was prepared. Genipin imparted thermal stability and enhanced swelling ability, crystallinity and lower gelation degree. The genipin-fixed agarose hydrogel exhibited superior absorbent property and stability in acidic solution at pH 1.2, lower degradation rate relative to the parent agarose. Thus, this naturally occurring crosslinking agent, which is less cytotoxic than the others [12] can be exploited to prepare crosslinked agarose, based materials for super absorbent and biomedical applications [23].

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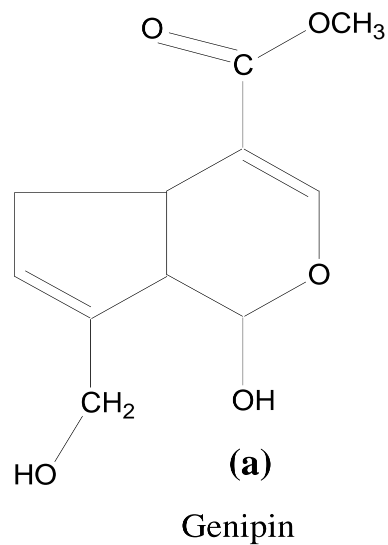
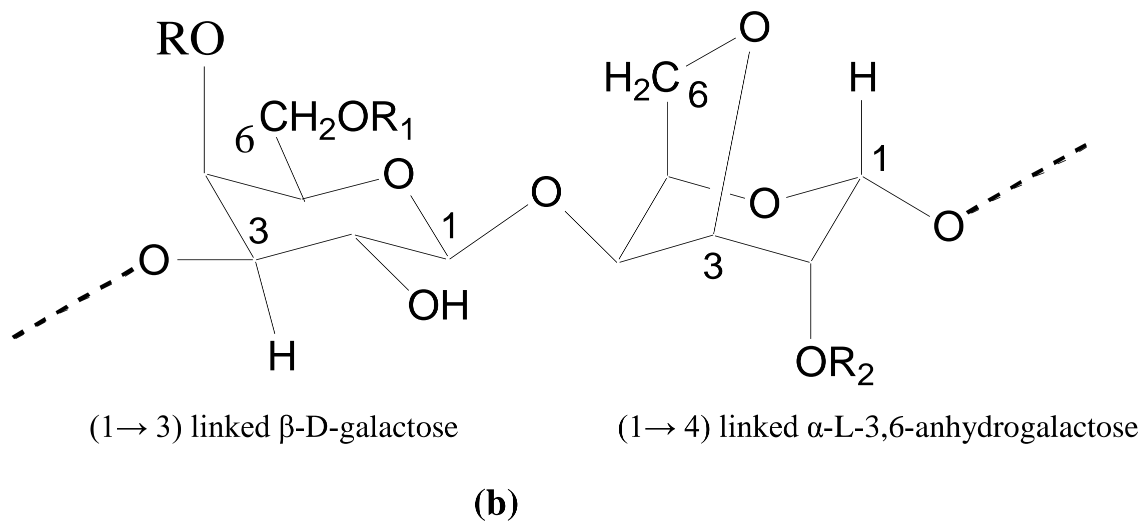


(a)

Genipin



(1→ 3) linked β-D-galactose

(1→ 4) linked α-L-3,6-anhydrogalactose

(b)

Figure III.4.1 Structure of genipin (a), and agarose (R = H or Me, R_1 = H or Me, R_2 = H or Me) (b)

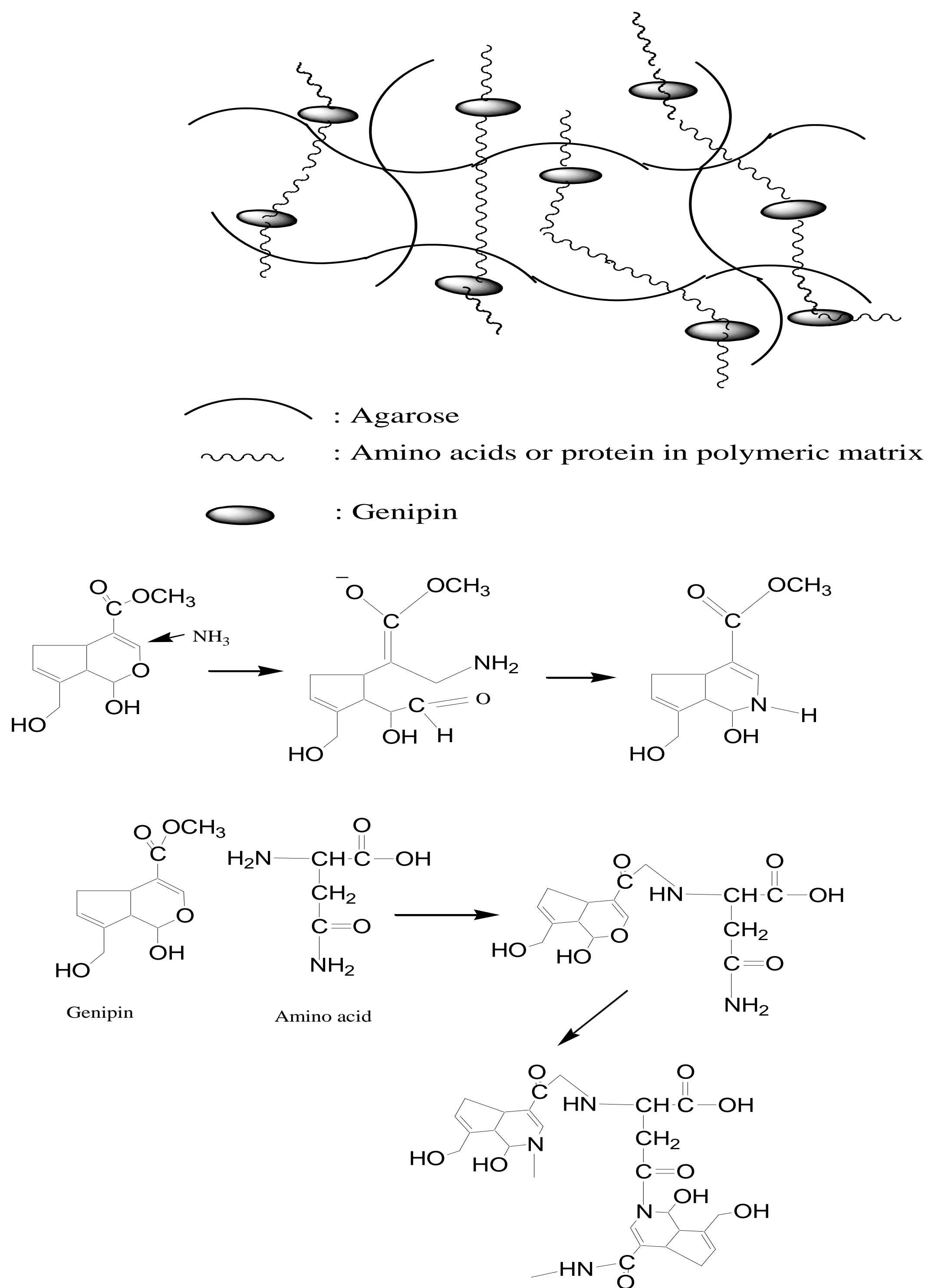


Figure III.4.2 Schematic illustration of the formation of genipin-fixed agarose.

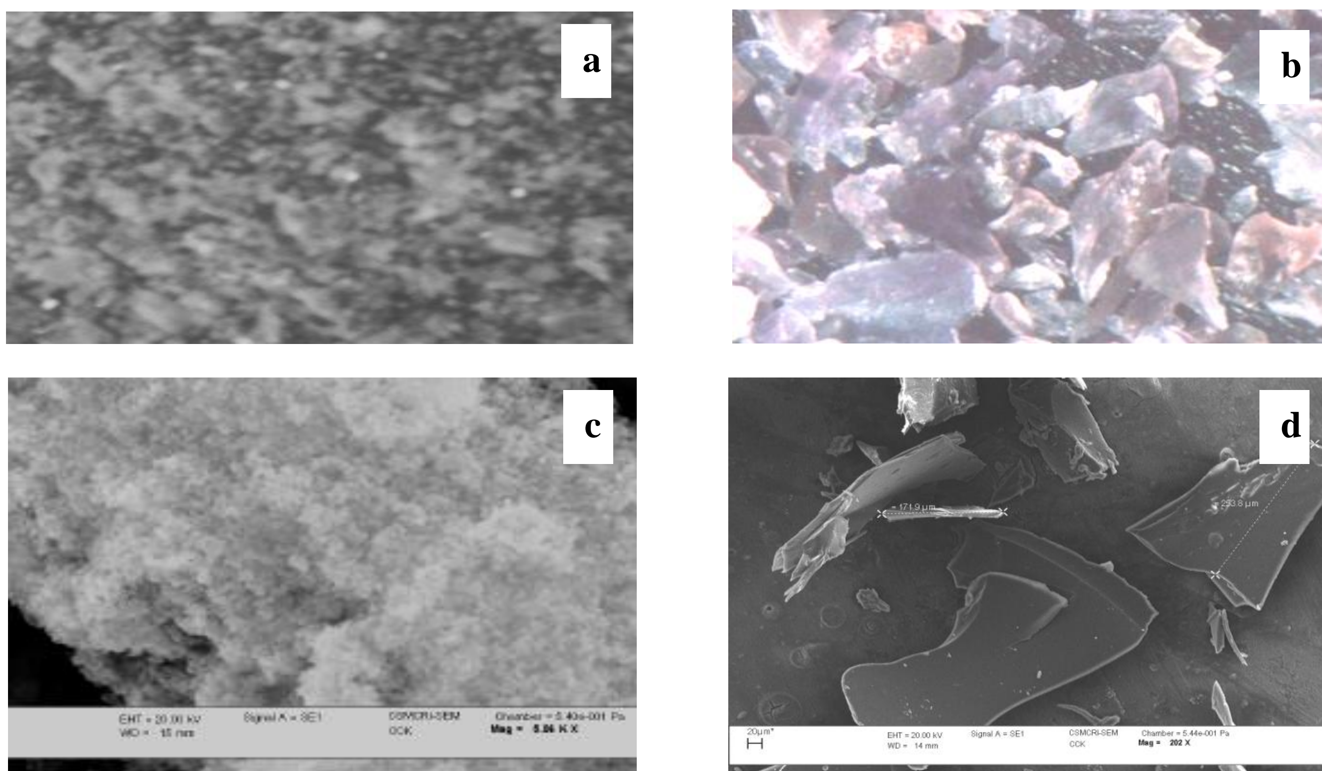


Figure III.4. 3a-d Optical micrographs of (a) agarose and (b) genipin-fixed agarose (with 0.8 wt% genipin), and SEM images of (c) agarose and (d) genipin-fixed agarose (with 0.8 wt% genipin).

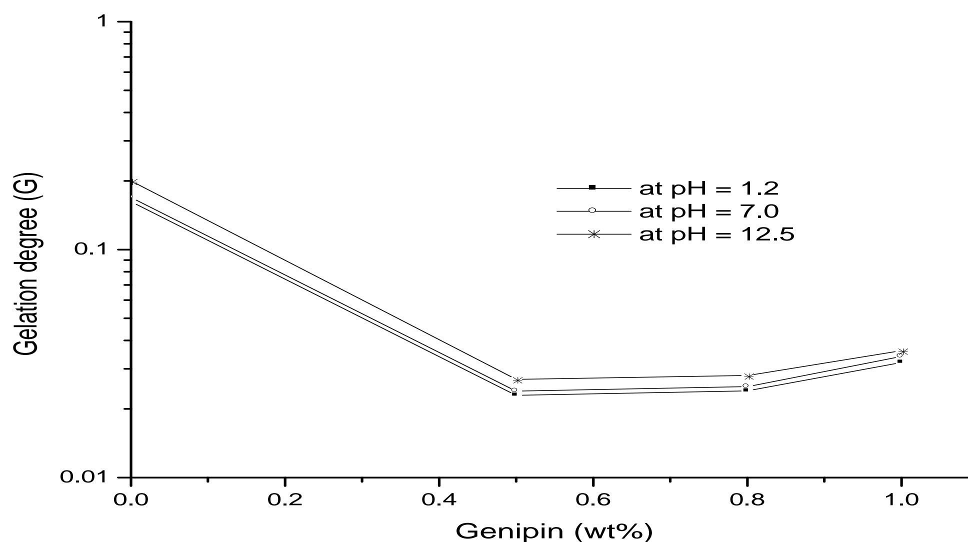


Figure III.4.4 Dependence of degree of gelation degree on concentration of genipin in the different pHs media.

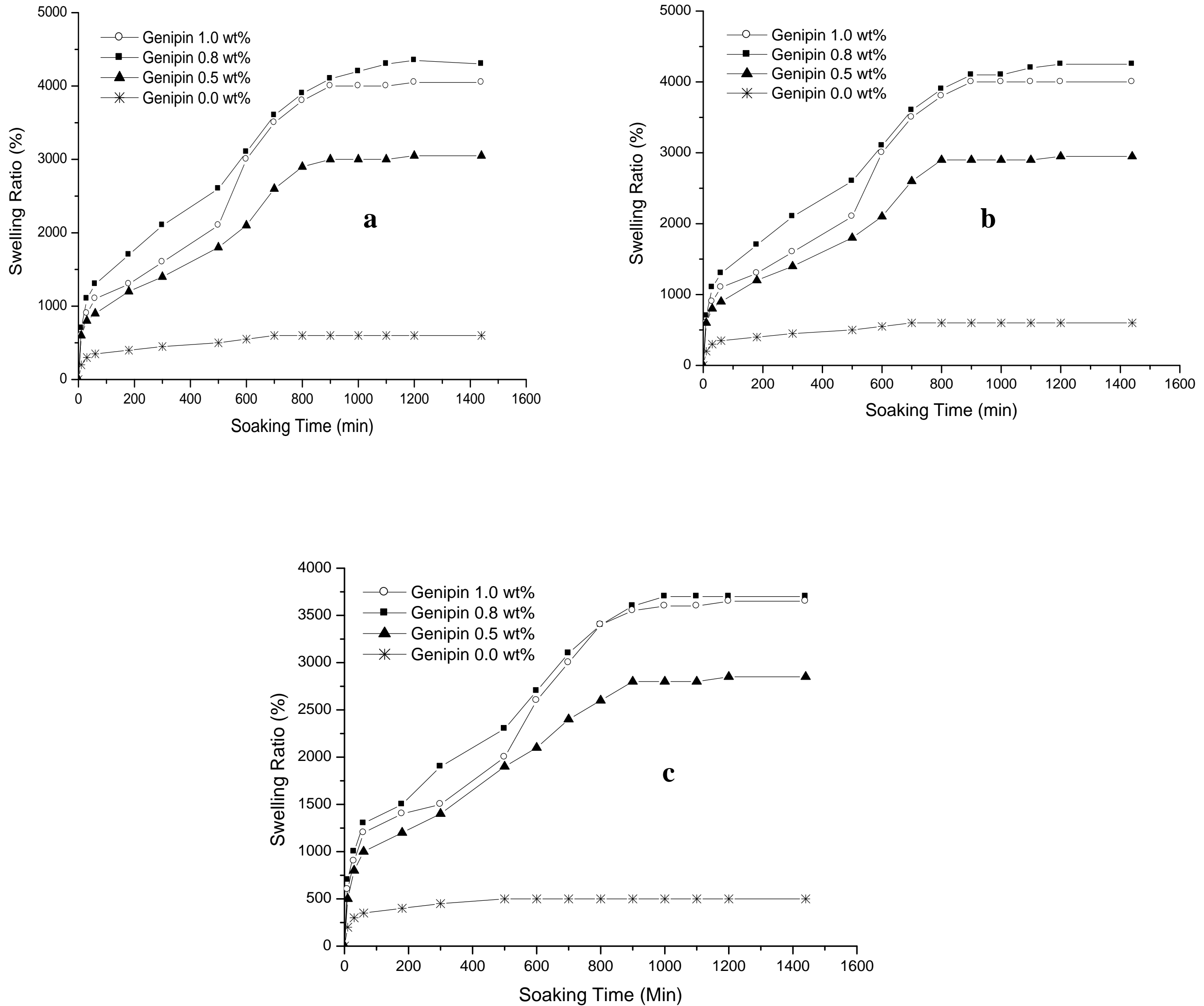


Figure III.4.5a-c Swelling ratios of agarose and the different genipin-fixed agarose obtained after 85 h cross-linking reaction; soaking at (a) pH 1.2; (b) pH 7.0, and (c) pH 12.5.

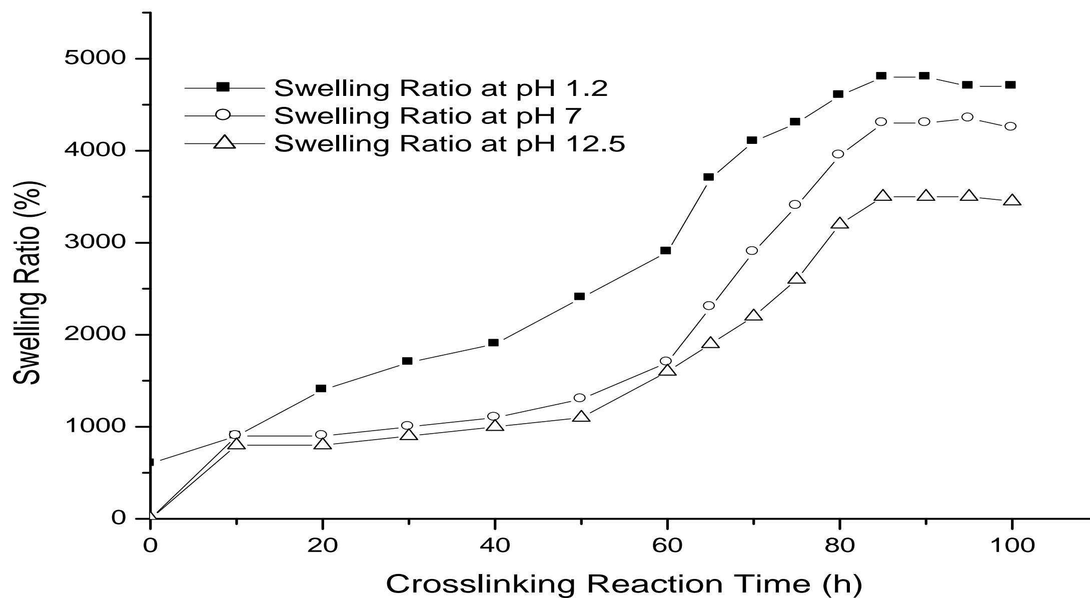


Figure III.4.6 Effect of cross-linking reaction time on the swelling ratio of genipin-fixed agarose (with 0.8 wt% genipin).

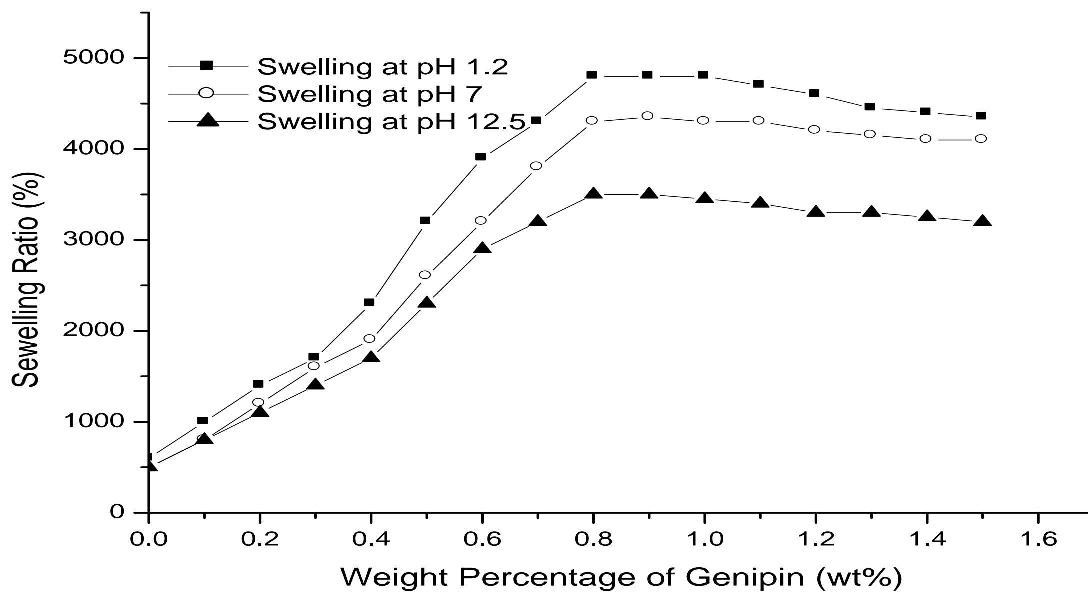


Figure III.4.7 Effect of weight percentage of genipin on the swelling ratio of genipin-fixed agarose, obtained after 85 h cross-linking reaction.

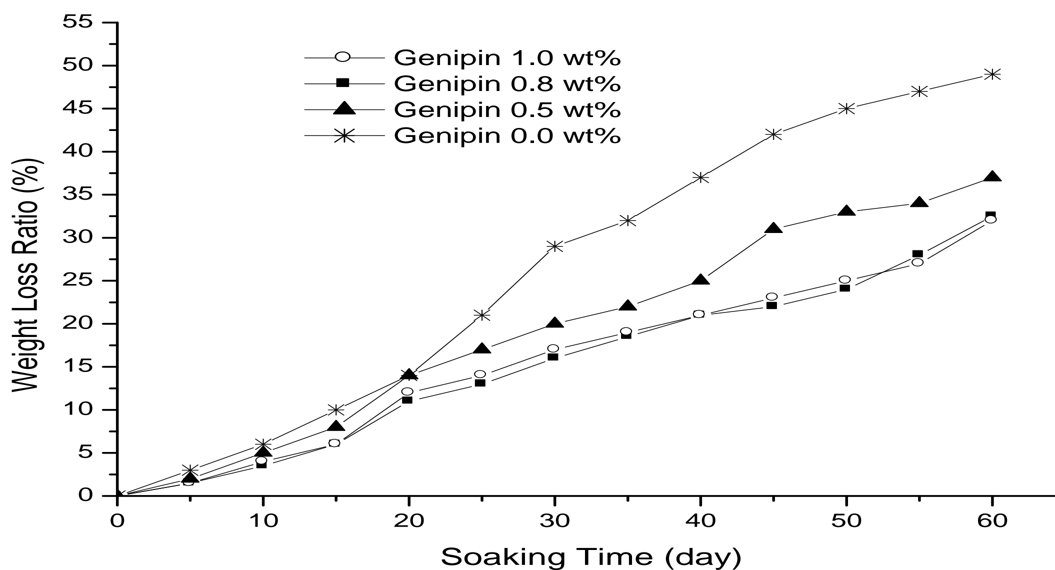


Figure III.4.8 Effect of weight percentage of genipin on the weight loss ratio of agarose and genipin-fixed agarose in Ringer's solution, obtained after 85 h cross-linking reaction.

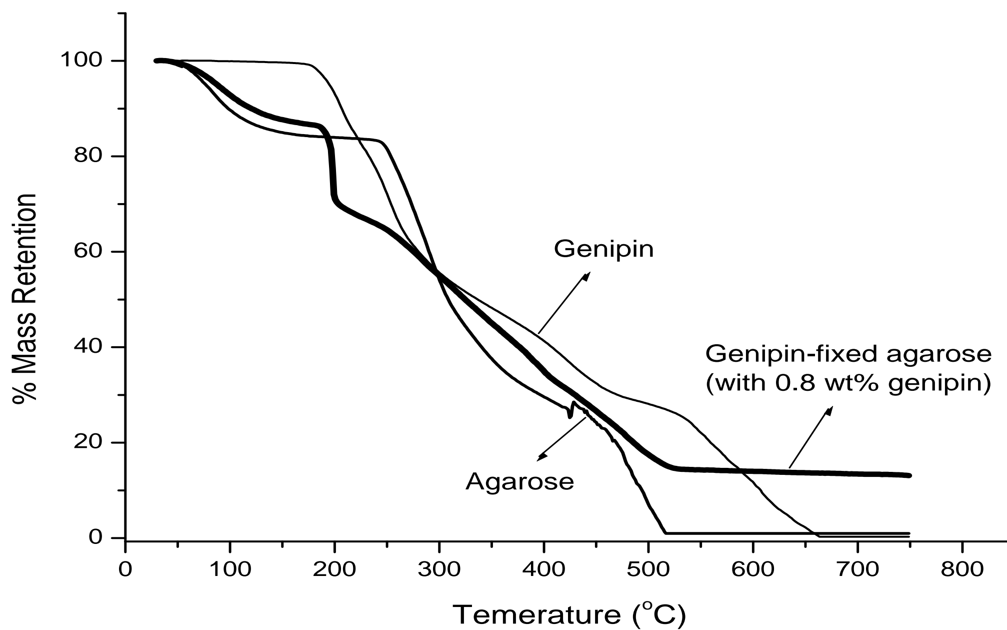


Figure III.4.9 Thermogram (TGA) of agarose, genipin and genipin-fixed agarose (with 0.8 wt% genipin).

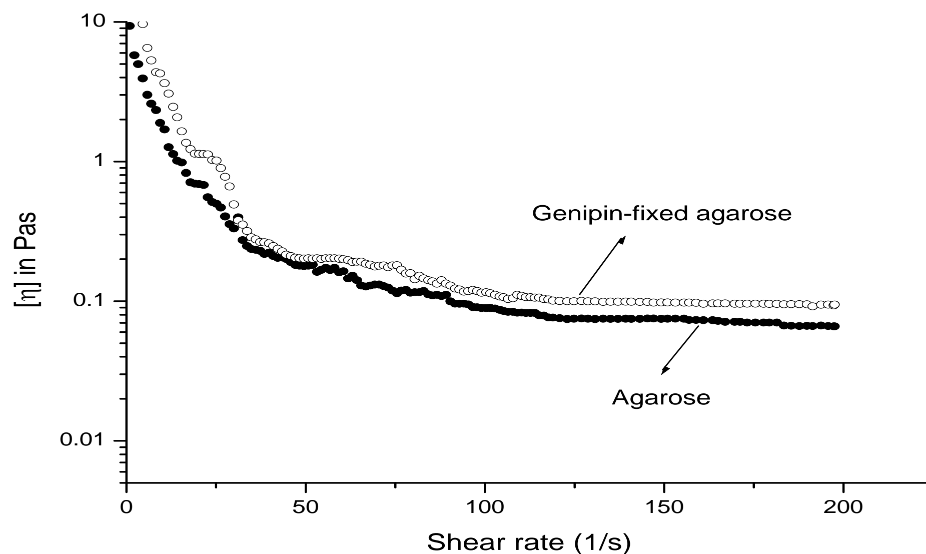


Figure III.4.10 Variations in shear viscosities of agarose and genipin-fixed agarose hydrogel (with 0.8 wt% genipin).

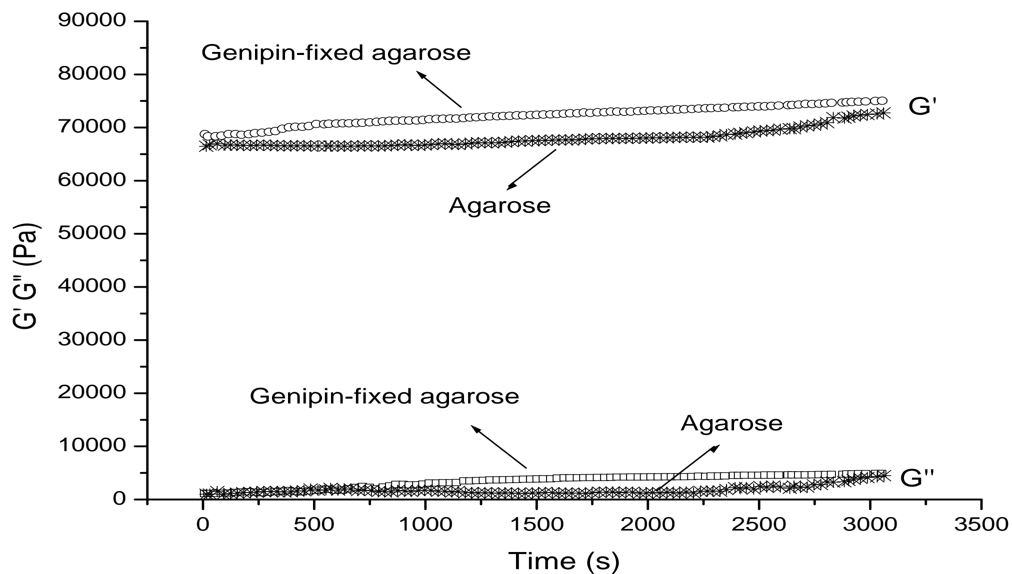


Figure III.4.11 Time dependence of modulus (G′G″) of agarose and genipin-fixed agarose hydrogel (with 0.8 wt% genipin)

CHAPTER III.5

PREPARATION AND PROPERTIES OF GENIPIN-FIXED *KAPPA*-CARAGEENAN

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III.5.1 INTRODUCTION

Hydrophilic gels are in increasing demand in the biomedical and pharmaceutical applications due to their biocompatibility [1-3]. Most of these applications in the past have made use of synthetic water soluble polymers (WSP) such as polyacrylic acids, polyacrylamides, polyethelene oxide, polyvinyl alcohols and polyvinyl pyrrolidones. Subsequently, these hydrogels were modified by blending with some natural polymers with WSP [4]. The resultant hydrogels exhibit different properties than those of the original polymers [5-6]. The improved properties of the hydrogels were harnessed either as super absorbents or as controlled delivery systems. The natural polymers that have been widely used for this purpose are cellulose, starch, chitin, *kappa*-carrageenan, agar and alginates [7]. Synthesis and characterization of *kappa*-carrageenan based superabsorbent hydrogels e.g., persulphate-induced graft copolymerization of acrylamide, acrylic acid and methacrylic acid onto *kappa*-carrageenan have been reported [8]. Genipin has been was widely used in herbal medicine [9], and the dark blue pigments obtained by its spontaneous reaction with amino acids or proteins used in the fabrication of food dyes [10]. It was reported that porcine pericardia crosslinked with genipin led to the formation of stable crosslinked products [11]. Gelatin-derived bioadhesives display higher biocompatibility and less cytotoxicity when crosslinked with genipin than with other agents, such as formaldehde, glutaraldehyde and epoxy compounds [12-13]. Although there is no nitrogen in the structure of *kappa*-carrageenan, proteins are present in kC matrices. The estimation of amino acid concentrations in various polymeric matrices including agar, agarose and *kappa*-carrageenan was described by Palace et al. [14] as well as by Selby & Selby [15].

In an ongoing program of our laboratory on modification and value addition of seaweed polysaccharides for preparing hydrogels with improved properties [16-22], we report herein preparation of crosslinked *kappa*-carrageenan hydrogel, using the naturally occurring crosslinker genipin. The crosslinked product revealed improved properties in comparison to the non-modified parent polysaccharide. In view of the unusual stability

and swelling behavior of the crosslinked hydrogel in acidic pH and in Ringer's solution, it may be useful in biomedical and food applications.

III.5.2 MATERIALS AND METHODS

III.5.2.1 Materials

The phycocolloid kC was extracted from the red seaweed *Kappaphycus alvarezii* (*Eucheuma cotonii*) collected from the Gulf of Mannar in the Bay of Bengal (09.15′N, 78.58′E). Genipin was purchased from Challenge Bioproducts Co., Ltd. Taiwan. Iso-propanol (Laboratory Reagent grade) was purchased from Ranbaxy Chemicals Ltd., Mohali (Punjab), India.

III.5.2.2 Preparation of kappa-carrageenan (kC)

The *kappa*-carrageenan (kC) used in this study was prepared from the dry red seaweed *Eucheuma cotonii* using the extraction procedure described by Craigie & Leigh [23], having a gel strength 450 ±5.5 g/cm^2 (1% gel in 1% KCl at 30°C), as determined by Hutardo-Ponce & Umezaki [24].

III.5.2.3 Preparation of genipin-fixed kC

In a typical batch, kC solution was prepared by dissolving 2 g of kC in 50 ml of distilled water at 100°C for 120 s in a microwave oven; the pH of the solution was 8.10. A stock solution (10%) of genipin was prepared in demineralised water. The aqueous solution of the kC was then mixed with different aliquots of the genipin stock solution at 40°C to obtain the final polymer (kC)-genipin mixture with weight percentages of genipin lying in the range of 0.001 to 1.5 wt%. The homogeneous viscous mixtures were kept at room temperature (30°C) and allowed to react for different time periods, up to 40 h, in eight different experiments. Each reaction product was obtained as follows. Isopropanol (IPA) was added to the reaction mixture (1:2, w/w) and the resultant mixture was allowed to stand for 24 h. The dehydrated product was then collected and washed twice with

acetone (1:1, w/w) to remove the unreacted genipin, if present. The product was air dried followed by drying in the oven at 50°C for 2 h.

III.5.2.4 CHARACTERIZATION

III.5.2.4.1 Bulk and True Density Measurements

To estimate the bulk density of blend polysaccharides before and after crosslinking, they were dried until a constant weight, and then transferred to a10 ml volumetric flask (W_1), filling the same up to the mark and was weighed (W_2). The bulk density (d_o) of the polymers was calculated, using Equation (1) [25].

$$d_0 = (W_2 - W_1) \div 10 \quad (1)$$

where, W_2 is the total weight of the polymer and flask, and W_1 is the weight of the flask.

To estimate the true density of dried genipin-fixed kC and non-modified kC, 0.35 g each of the sample, were first dried until a constant weight (W_o), were reached and then placed in a 10 ml volumetric flask of known weight at 20°C. To the flask were added 8 ml of cyclohexane and the mixture was kept at 20°C for 24 h. The flask was then filled with cyclohexane up to the mark and was weighed (W). The true densities (d) of the samples were calculated, using Equation (2) [25].

$$d = W_o \div [10 - \{(W - W_o) \div d_c\}] \quad (2)$$

where, W is the total weight of the polymer and the solvent, and W_o the weight of the dry polymer sample, d_c the density of the solvent ($d_{cyclohexane}$ = 0.778 g/ml).

III.5.2.4.2 Pore volume and porosity Measurements

The pore volume of the non-modified kC and genipin-fixed kC were measured by monitoring the weight gain of the products in cyclohexane [25]. The dried samples were placed into tubes with a porous glass bottom (sintered glass). The tubes were kept inside a flask filled with cyclohexane for 48 h at 20°C. The excess cyclohexane was removed by centrifugation at 1500 rpm for 1 min and weighed (W_1). The volume of cyclohexane absorbed by the polymers was used to estimate the porosity of the polymeric particles. The porosity (Ø) of the samples was determined by

$$Ø = V_p \div V_o \quad (3)$$

where, V_p is the pore volume in the polymeric particles

$$V_p = W_1 - W_o \quad (4)$$

and V_o is the true volume of polymeric particles

$$V_o = W_o \div d \quad (5)$$

where, W_o is the weight of dry polymeric particles, and d the true density of dried polymeric particles, determined according to Eq. (2).

III.5.2.4.3 Swelling ratio measurements

The *kappa*-carrageeenan (kC) and genipin-fixed kC with different percentages of genipin with respect to the polysaccharide namely of 0.75, 1.0 and 1.5% (w/w), were used. In the swelling ratio (%) measurements, the dry hydrogel was weighed (W_1) and immersed in different pH solutions (e.g. 1.2, 7.0 and 12.6) separately. After the designated soaking time had elapsed, the wet samples were wiped dry with filter paper to remove excess liquid, and weighed (W_2). The swelling ratio (%) was calculated using the following Equation 6. The results are the mean and standard deviation of 4 replicates.

$$\text{Swelling ratio (\%)} = [(W_2 - W_1) \div W_1] \times 100 \quad (6)$$

III.5.2.4.4 Degradation rate measurement

In vitro degradation studies were performed using Ringer's solution (8.6 mg ml^{-1} sodium chloride; 0.3 mg ml^{-1} potassium chloride and 0.33 mg ml^{-1}calcium chloride dihydrate), under aseptic conditions. *kappa*-carrageenan and genipin-fixed kC hydrogels with genipin percentages of 0.75, 1.0, and 1.5% (w/w) were used. The samples were weighed (Wo), introduced in capped plastic tubes filled with 25 ml of Ringer's solution, and incubated at 37°C for different time periods, up to 40 hours. At the end of each period the samples were drawn, dried, and weighed (Wt), the weight loss ratio ΔW (%) being calculated using Equation 7 [26]. The results are the mean and standard deviation of 4 replicates.

$$\Delta W (\%) = [(Wo - Wt) \div Wo] \times 100 \quad (7)$$

III.5.2.4.5 MS/MS analysis

The MS analyses were done in a Waters Q-Tof micro YA-260 mass selective detector using Waters MassLynx Version 4.0 software. Negative electron spray mode (ES) was used for the analyses. For Mass spectrum analysis the following parameters were used: Capillary voltage 2700 V, sample cone voltage: 30.0 V, extraction cone voltage: 0.5 V, desolvation temperature 150°C, source temperature: 80°C, syringe rate 0.5 μl/min, ion energy 2.0 V, collision energy 7.0 V.

For the MS analysis the genipin-fixed kC was hydrolyzed in methanolic HCl for one hour at 60°C. The resulting carbohydrate was precipitated in excess of methanol. The supernatant obtained by centrifugation was evaporated to dryness. The residue was dissolved in 5 ml HPLC grade methanol and subjected to MS analyses.

In an additional experiment to establish the fact that genipin was present in the kC matrix through cross-linking but not as a physical mixture, genipin-fixed kC was heated in HPLC grade methanol at 60°C for 1 h under stirring condition without adding acid. The supernatant methanol leaving behind the blue colored product was evaporated to dryness and the residue was redissolved in HPLC grade methanol and was subjected to MS analyses.

III.5.2.4.6 Other characterization of genipin-fixed kC

UV-Vis spectra were recorded on a Varian CARY 500 Scan UV-Vis-NIR spectrophotometer. Optical rotation was measured on a Rudolph Digi pol - 781 Polarimeter (Rudolph Instruments Inc, NJ, USA). X-ray diffractions were studied on a Philips analytical X-ray instrument (2θ = 10 to 60°). Optical microscopy was carried out on an Olympus model SZH 10, Japan with 70X magnification and thermal analysis (TGA were done on a TGA Toledo Mettler TGA System Switzerland. Apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072) using Spindle No.1 at rpm 60. Intrinsic viscosity [η] was determined at 30°C using an Ostwald viscometer with a flow time 120 Sec for 1 M NaCl. For this, sol samples of kC and genipin-fixed kC were prepared in 1 M NaCl at a concentration of 0.02% to 0.12% (w/v).

III.5.2.4.7 Scanning electron microscopy (SEM)

Vacuum oven dried samples of the powder of the non-modified and modified kC were mounted on a sample holder and coated with gold. The samples were examined with a scanning electron microscope (Model Carl-Zeiss Leo VP 1430) at an accelerating voltage of 20 kV and 202 X magnification.

III.5.2.4.8 Protein estimation

Total nitrogen was estimated by Kjeldahl method on a KEL PLUS-KES 20L Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN Equipments, Chennai, India). Crude protein content was calculated multiplying the nitrogen content by the approximate factor 6.25 [27]. The results are the mean and standard deviation of 4 replicates.

III.5.2.4.9 Rheological measurements

Dynamic rheological measurements were performed using a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany), and the Rheowin Pro 2.1. The measuring geometries selected for measurements were a cone/plate and plate/plate (60 mm diameter, 1° rad angle). Viscosities at varying shear rate were studied at 25°C. Oscillation measurements were carried out in controlled deformation mode with 0.05 % strain. Results are the average and standard deviation of three measurements, automatically generated by the software. During measurements, outer surface of the samples were covered with silicone oil to avoid losses due to evaporation.

III.5.2.4.10 Statistical analyses

Data were analyzed using analysis of variance (ANOVA). Results were considered statistically significant when $p < 0.001$. Calculations were performed using Origin Software, Version 6 (Microcal Software Inc. MA, USA).

III.5.3 RESULTS

III.5.3.1 Physical properties

The results of bulk and true density, pore volume and porosity measurements for genipin-fixed kC and non-modified kC are given in Table III.5.1. The crosslinked kC with genipin showed higher bulk density and lower true density, pore volume and porosity than those of the non-modified kC (Table III.5.1).

TableIII.5.1 Physical properties of the crosslinked and non-modified kC

Products	Bulk density (d_0) (g/ml)	True density (d) (g/ml)	Pore volume (V_p) (ml/g)	Porosity (ø)
Non-modified kC	0.564	1.014	0.503	1.458
Crosslinked kC	0.706	0.674	0.279	0.721

III.5.3.2 Swelling and degradation

The results of swelling experiments with genipin-fixed kC in acidic (pH 1.2), neutral (pH 7.0) and in alkaline (pH 12.5) media are presented in the Figures III.5.1a, b, c, respectively. The genipin-fixed kC hydrogel with 1 wt% genipin showed maximum swelling ratio (%), 4500 ± 32.56, 3750 ± 22.15 and 2400 ± 23.87 in acidic, water and alkali medium respectively. It is important to note that the genipin-fixed kC exhibited maximum swelling in all media over ca. 1100 min and was almost steady up to ca. 1500 min (ca. 25 h) beyond which the hydrogel sample began losing weight in the acidic medium (Figure III.5.1a). All the genipin-fixed kC revealed a statistically significant higher value of swelling ability in acidic pH, as compared to the neutral and alkali pH ($p < 0.001$). The crosslinked kC with 1.0 wt% genipin, showed significantly higher value of swelling ability in all pHs solution, as compared to the genipin-fixed kC with 0.75 wt% genipin ($p<0.001$). The analysis of variance also revealed that the values of swelling ability for genipin-fixed kC with 1.0 and 1.5 wt% genipin are not significantly different ($p> 0.001$).

The effect of cross-linking reaction time on the swelling ratio of genipin-fixed kC is presented in Figure III.5.2. The swelling ratio increased with increasing cross-linking reaction time up to 20 h beyond which there was no increase in the swelling ratio. Genipin-fixed kC, obtained after 20 h cross-linking reaction time revealed a statistically significant higher value of swelling ratio in all pHs, as compared to the non-modified kC ($p < 0.001$). The mass loss ratio of non-modified kC and genipin-fixed kC (with 1.0 wt% genipin) was measured in Ringer's solution, wherein 100% and 38% mass losses were observed respectively (Figure III.5.3).

III.5.3.3 Viscosity measurements

The progress of the cross-linking reaction of kC by genipin with time was evaluated by measuring intrinsic viscosity as a function of time at 30°C (Figure III.5.4). The non-modified polysaccharide (kC) revealed values of apparent and intrinsic viscosities of 65 ± 2.5 mPa s and 362 ± 2.39 ml/g at 80°C and 30°C, respectively. The intrinsic viscosity of genipin-fixed kC increased steadily with cross-linking reaction time, reaching a plateau after ca. 20 h (Figure III.5.4). The maximum values for apparent and intrinsic viscosities registered were of 95 ± 3.44 mPa s and 455.6±2.24 ml/g, respectively.

III.5.3.4 Nitrogen and protein contents

The non-modified kC revealed nitrogen and protein contents of 0.32 ± 0.003 % and 2.0 ± 0.002 % respectively, while the genipin-fixed kC revealed a nitrogen content of 0.30± 0.004.

III.5.3.5 Optical microscopy

The optical micrographs of kC and genipin-fixed kC (using 1.0 wt% genipin, after 20 h of cross-linking reaction) are presented in Figures III.5.5a-b. The kC and genipin were colorless before mixing. After 30 min of reaction, a light blue color appeared, the mixture becoming dark blue after 20 h of reaction time (Figure III.5.5b).

III.5.3.6 Scanning electron microscopy (SEM) analysis

The scanning electron micrographs of the modified and non-modified kC are depicted in Figure III.5.5c-d. The SEM images indicated significant influence on the particle size and

morphology of the kC after cross-linking with genipin. The non-modified kC was looking fibrous or thread-like and can be distinguished easily from the modified Kc (Figure III.5.5c), because, the morphology of modified kC looked very compact, cloudy and dense may be due to combination of many samall particles (Figure III.5.5d).

III.5.3.7 MS/MS analysis

The mass pattern of standard genipin is presented in Figure III.5.6a. The m/z (%): 225 (M^+-1, 65%), 207 (10%), 123 (100%), 101 (50%), 97 (8%) for standard genipin (Figure III.5.6a). The MS of the recovered genipin that was extracted with methanol from the acid hydrolysate of genipin-fixed kC produced the following major ion fragments besides other contamination peaks (Figure III.5.6b). The m/z (%): 225 (M^+-1, 35%), 207 (10%), 123 (100%), 101 (50%), 97 (15%). The corresponding MS pattern of the control experiment did not show the peaks at m/z 225, 207, 123, 101 and 97, that were observed in the MS of standard genipin.

III.5.3.8 UV and optical rotation

Non-modified kC does not have any maxima in the UV-Vis region. The genipin-fixed κC in aqueous solution (pH 7.45 at 30°C) showed λ_{max} 590 nm, whereas genipin revealed λ_{max} at 240 nm. The absorbance at 590 nm by the blue coloured crosslinked kC indicated binding of genipin with free amino acids or protein present in the polymeric matrix of kC [28]. The $[\alpha]_D$ values at 35°C for kC, genipin and genipin-fixed kC were +67.64 ± 1.7° (c 0.025, H_2O), +111.65 ± 1.2 ° (c 0.025, H_2O) and +78.61 ± 2.14° (c 0.025, H_2O), respectively.

III.5.3.9 Thermogravimetric analysis

The TGA curves for kC, genipin and genipin-fixed kC are shown in Figure III.5.7. Non-modified kC showed weight loss in three stages, the first one (8%) occurring in the range 30-140°C probably due to the loss of moisture. The subsequent weight losses were of 38% and 98%, and occurred in the temperature ranges of 250-270°C and 275-520°C respectively. Complete weight loss was observed at ca. 650°C for non-modified kC. In

case of genipin-fixed kC the TGA profile was similar to that of non-modified kC. However, at the maximum temperature investigated (750°C), only 84% mass loss was observed.

III.5.3.10 X-ray diffraction analysis

The X-ray diffraction pattern of kC and genipin-fixed kC hydrogel are presented in Figure III.5.8. The X-ray diffractograms of kC and genipin-fixed kC revealed amorphous compounds, with intense peaks at $2\theta = 20.62^{o}$ and 20.5^{o}, respectively. A closer examination of the XRD pattern of genipin-fixed κC revealed several sharp emerging peaks ca. $2\theta = 20.5^{o}$, as well as there was an enhancement of the weak peak at ca. $2\theta = 8.5^{o}$, when compared to that of kC.

III.5.3.11 Rheological measurements

Variation of dynamic viscosity with the shear rate is depicted in Figure III.5.9. The genipin-fixed kC showed lower gel-thinning and higher dynamic viscosity than those of the non-modified kC, under applied shear (Figure III.5.9). The stability of storage modulus (G') and loss modulus (G'') during storage at 25°C is depicted in Figure III.5.10. The storage modulus (G') and loss modulus (G'') values for both samples increased with time, the values for storage modulus being higher for the genipin-fixed κC.

III.5.4 DISCUSSION

In this study the naturally occurring cross-linker genipin reacts with kC in aqueous medium at ambient conditions to afford a stable hydrogel network. The cross-linking of genipin with kC imparted significant absorbent capacity and network stability of kC. The genipin-fixed kC showed greatest water absorbency in the acidic medium at pH 1.2 after ca. 1100 min and beyond this the absorbency remained virtually steady up to 1500 min (Figure III.5.1a). This result is noteworthy especially when viewed against the fact that non-modified kC is readily dispersible in water and it degrades rapidly in acidic pH. Relatively lower water absorbencies were encountered in alkaline and neutral pHs in the order: pH 12.5 < pH 7.0 < pH 1.2 (Figures III.5.1a-c). This unusual stability of the crosslinked kC hydrogel may be ascribed to its molecular architecture wherein the

glycosidic linkages were not readily available for solvation and hydrolytic reaction for a long duration till the optimum swelling was achieved, indicating rigidity of the network. The cross-linking reaction time was optimized (ca. 20 h) by measuring the swelling ratio and the intrinsic viscosity of the products at different time intervals (Figures III.5.2 and 4). The intrinsic viscosity and swelling ratio increased gradually with cross-linking reaction time, no significant differences being found after 20 h of cross-linking reaction time. This may have some positive correlation with the decrease in true density, pore volume and porosity of the genipin-fixed kC, while its bulk density increased. The relatively greater stability of the crosslinked kC was also demonstrated in the degradation studies in Ringer's Solution (Figure III.5.3). Optical micrographs (Figure III.5.5a-b) and SEM images (Figure III.5.5c-d) and X-ray diffraction pattern (Figure III.5.8) showed distinct differences in the morphology and molecular arrangements of genipin-fixed kC respectively, when compared to those of the non-modified kC, which can be ascribed to a crosslinked architechture.

Genipin is known to react with amino groups and proteins developing blue color [10]. Nitrogen and protein were estimated to be ca.0.32% and 2.0% respectively in the parent kC sample used in this study. Palace et al. estimated the amino acid concentration of glycine and amino acid (excluding glycine) in the polymeric matrix of kC to be 508 ng/mg (0.051%) and 4870 ng/mg (0.487%), respectively [14]. It is, therefore, apparent that genipin reacted with the protein present in the polymeric matrix of kC [14], affording the crosslinked product. The progress of reaction was evident from the change of color of reaction mixture from colorless to deep blue over 20 h. The MS pattern indicated that genipin got fixed chemically, not as a physical mixture in the *kappa*-carrageenan polymeric matrices to form genipin-fixed kC (Figures III.5.6a & b). The dynamic viscosity and storage modulus measurements showed lesser shear thinning and greater G' value, respectively, compared to the parent kC indicating the presence of stronger network in the genipin-fixed kC (Figures III.5.9 & 10). The increase in $[\alpha]_D$, UV absorbance at 590 nm [28-29], thermal stability (Figure III.5.7) of genipin-fixed kC compared to non-modified kC, also confirmed that genipin was involved in the carrageenan network.

III.5.5 CONCLUSIONS

In this study, genipin-fixed *kappa*-carrageenan (genipin-fixed kC) was prepared in an ambient conditions using genipin, a naturally occurring cross linker. The unusual stability and swelling capacity of *kappa*-carrageenan derived crosslinked polymer in pH 1.2 makes it an interesting material for biomedical applications, such as super absorbents and sustained delivery systems [30].

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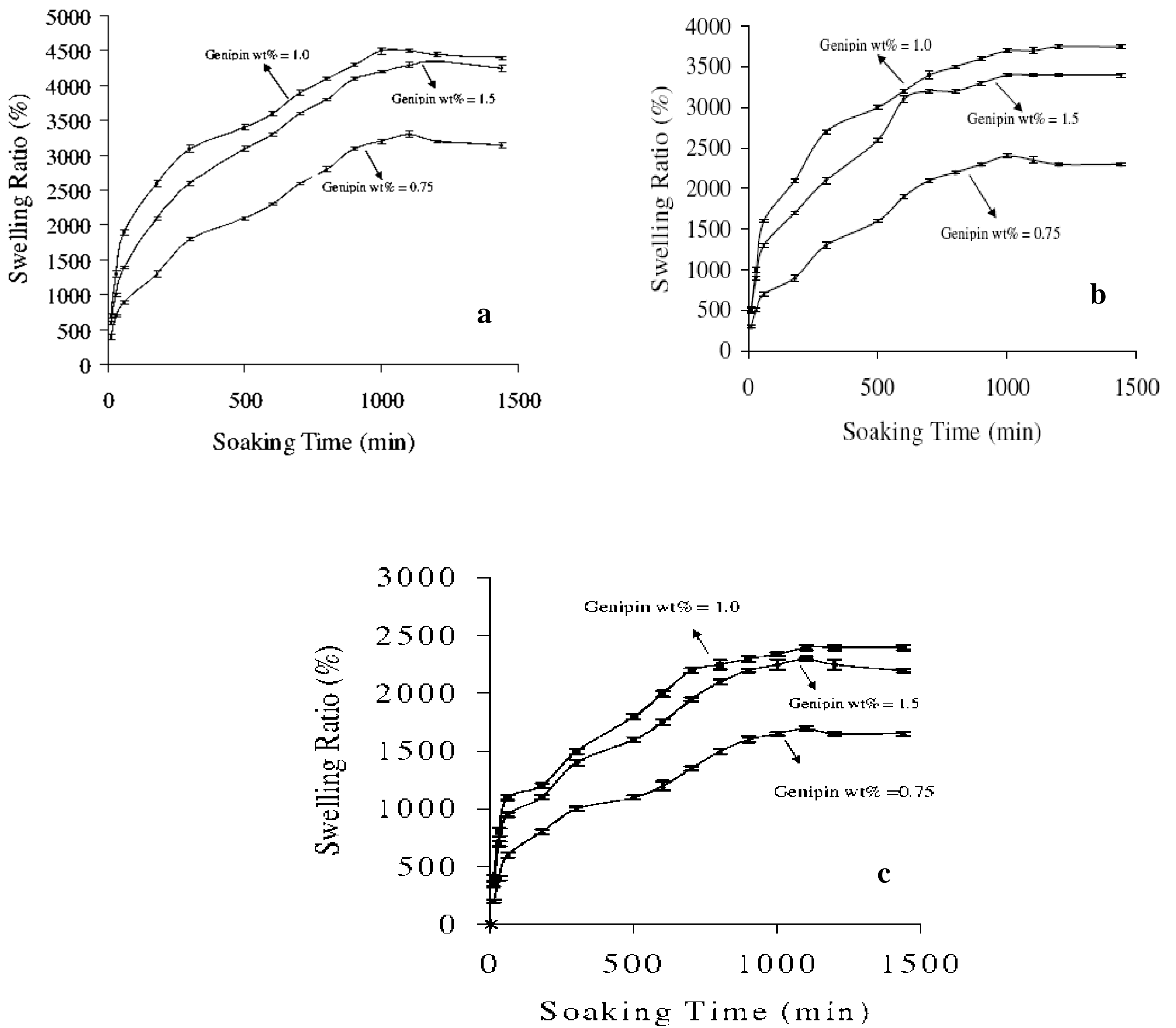

Figure III.5.1a-c Swelling ability of genipin-fixed κC with different weight percentages of genipin in solutions with different values of pH: **(a)** pH 1.2; **(b)** pH 7 and **(c)** pH 12.5. Data represent the mean ± standard deviation, $n = 4$.

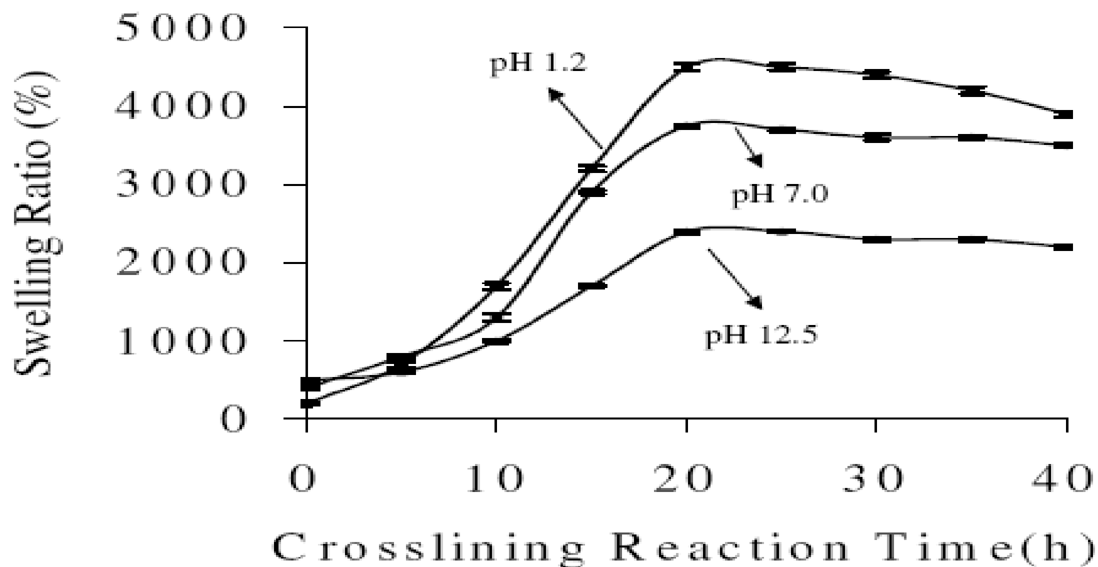

Figure III.5.2 Effect of cross-linking reaction time on the swelling ability of genipin-fixed kC. Data represent the mean ± standard deviation, $n = 4$.

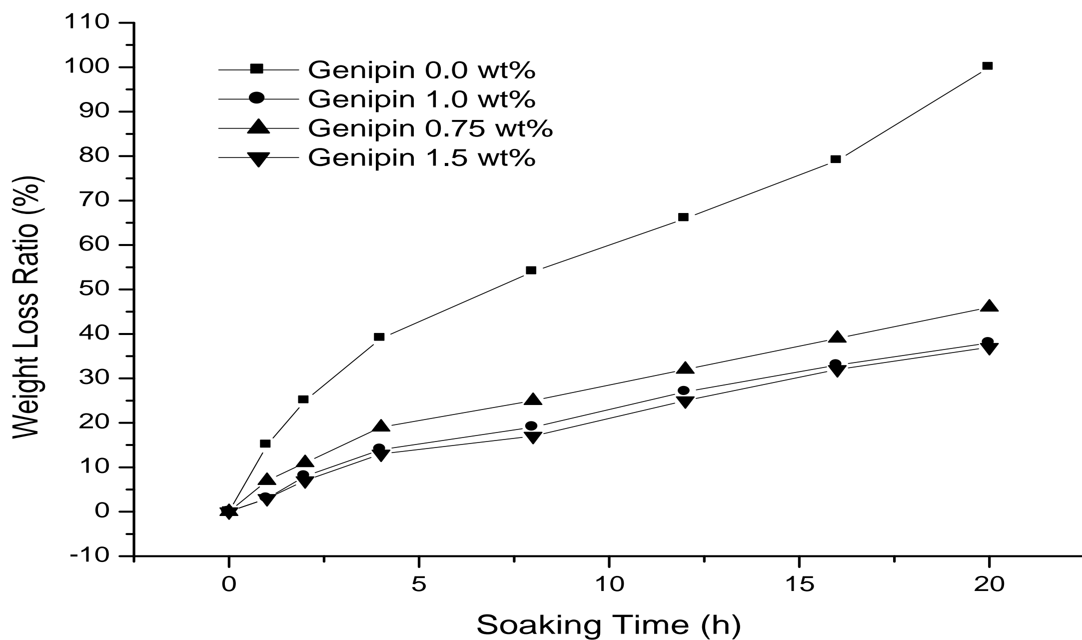

Figure III.5.3 Effect of weight percentages of genipin on the weight loss of genipin-fixed kC. Data represent the mean ± standard deviation, $n = 4$.

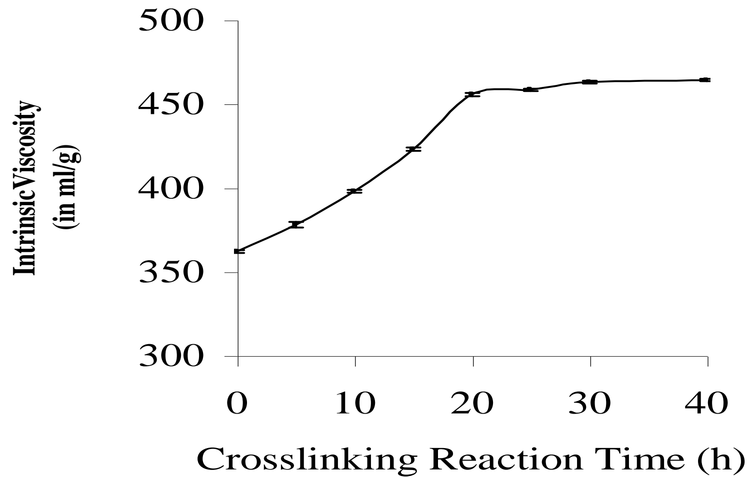

Figure III.5.4 Intrinsic viscosity of genipin-fixed kC as a function of cross-linking reaction time at 35°C in 1M NaCl. Data represent the mean ± standard deviation, n = 4.

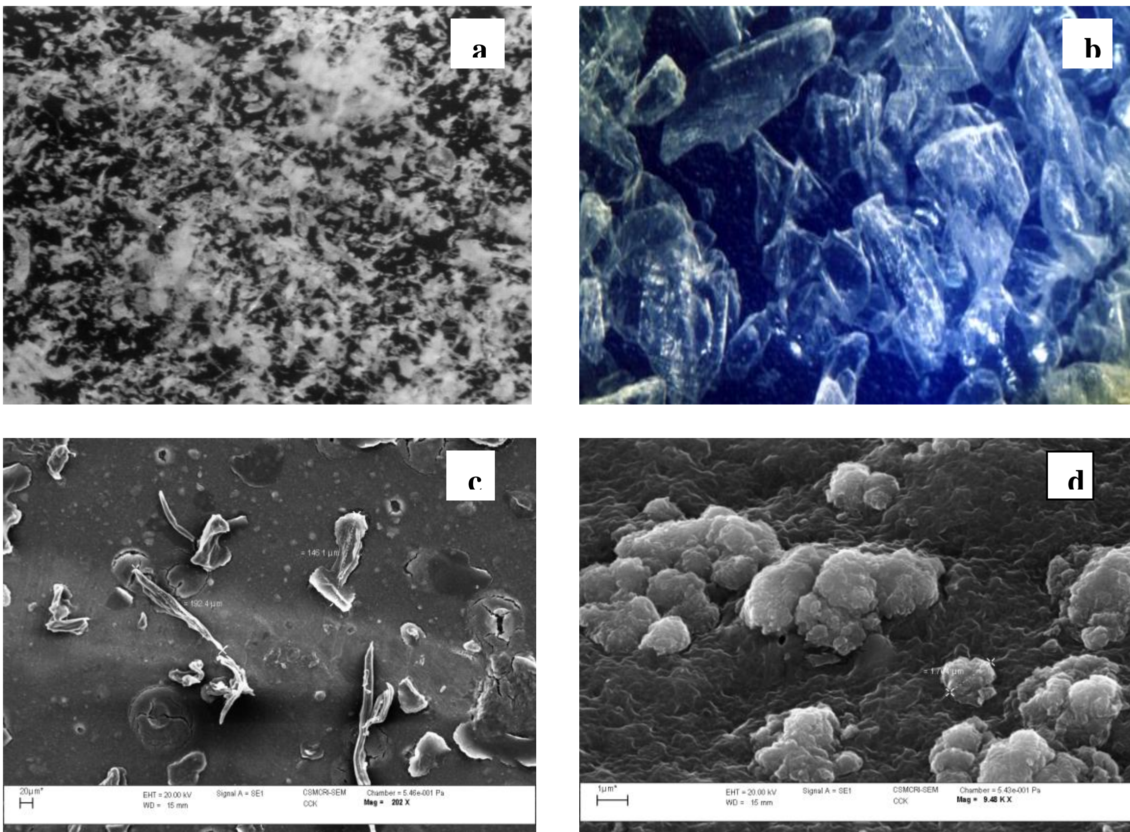

Figure III.5.5a-d Optical micrographs (**a**) *kappa*-carrageenan and (**b**) genipin-fixed kC at 70 X magnification; and scanning electron micrographs of (**c**) *kappa*-carrageenan and (**d**) gnipin-fixed kC, at 202 X magnification.

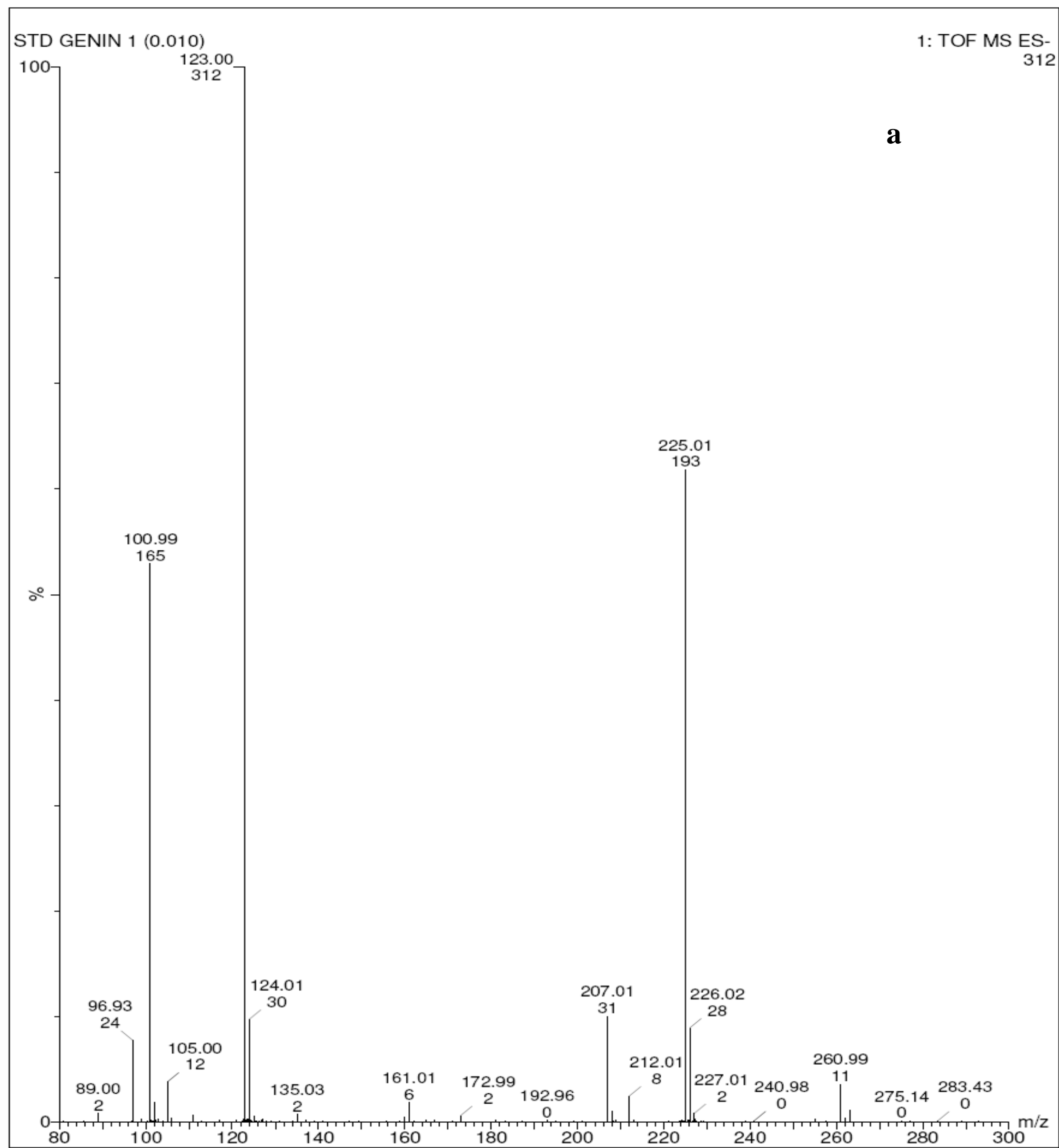

Figure III.5.6a Mass spectrum of standard genipin.

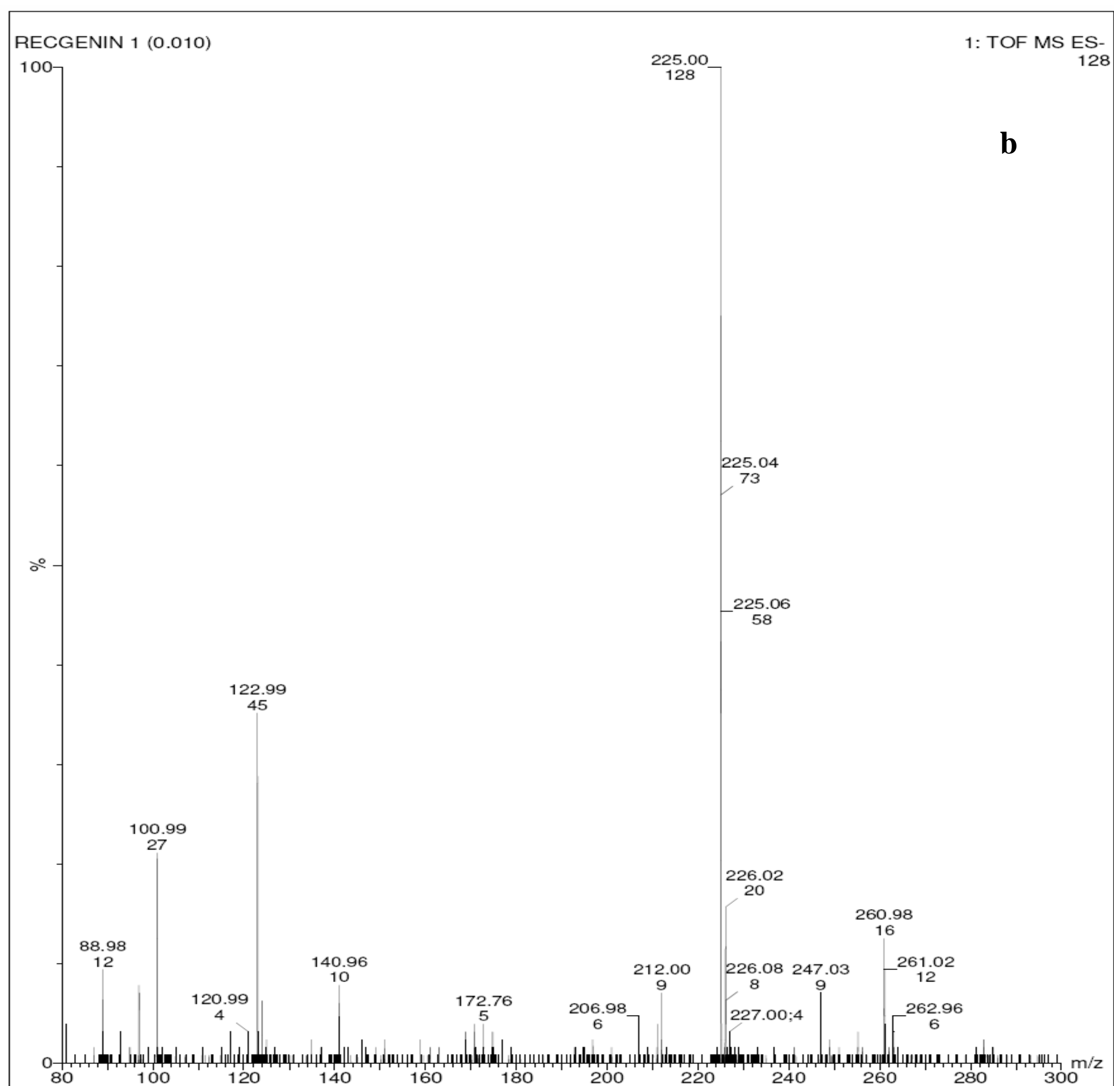

Figure III.5.6b Mass spectrum of "recovered genipin" extracted from acid hydrolysed genipin-fixed kC.

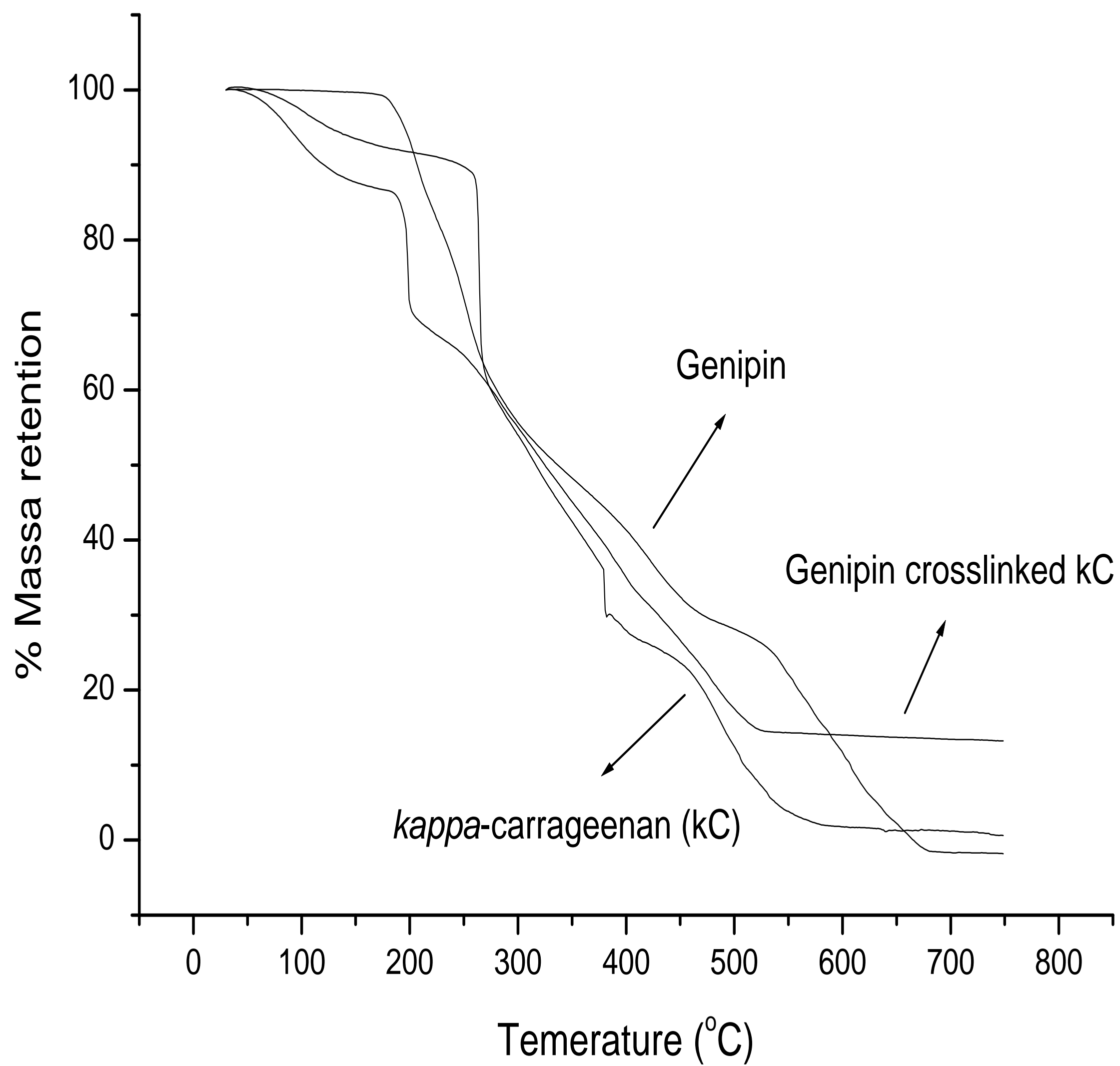

Figure III.5.7 Thermogram (TGA) of *kappa*-carrageenan, genipin and genipin-fixed kC.

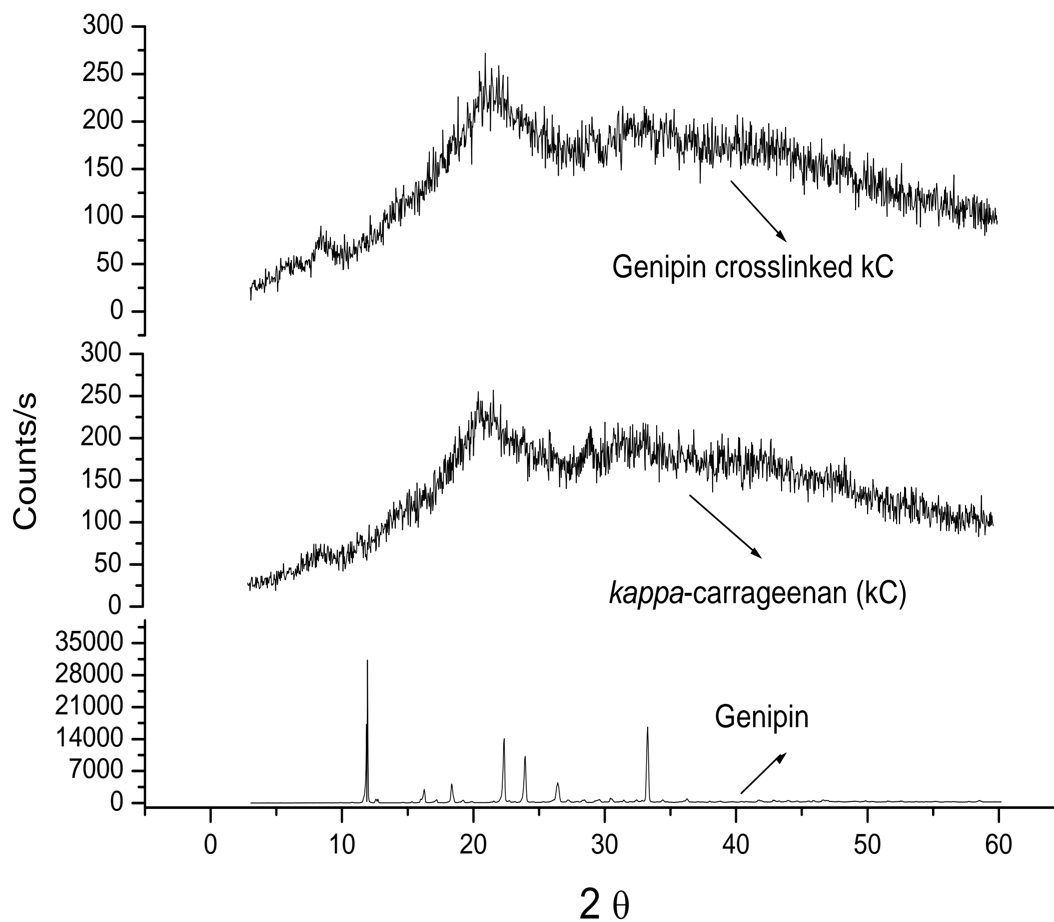

Figure III.5.8 X-ray diffraction pattern of *kappa*-carrageenan, genipin and genipin-fixed kC.

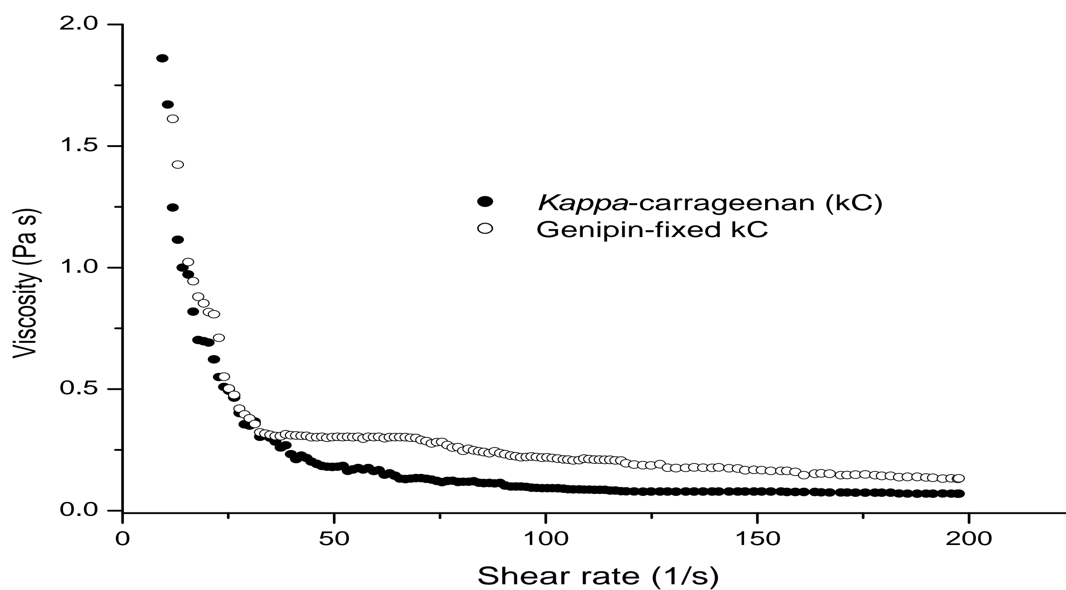

Figure III.5.9 Variations in shear viscosities of *kappa*-carrageenan and genipin-fixed kC.

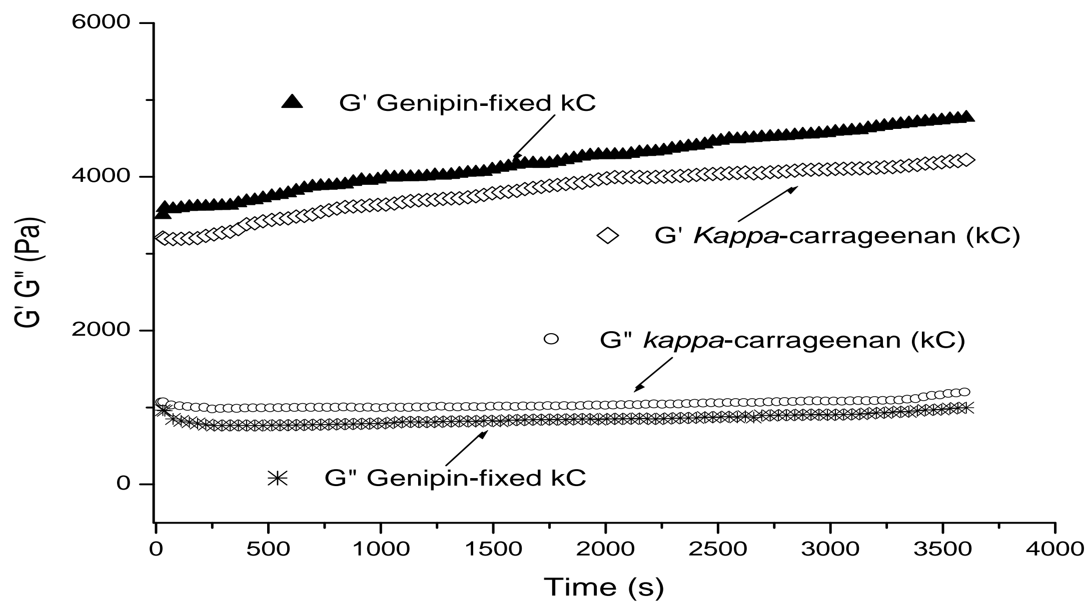

Figure III.5.10 Time dependence of moduli (G′G″) of *kappa*-carrageenan and genipin-fixed kC.

CHAPTER III.6

PREPARATION OF A STABLE HYDROGEL BASED ON GENIPIN-CROSS LINKED POLYSACCHARIDE BLEND

III.6.1 INTRODUCTION

III.6.2 MATERIALS AND METHODS

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III.6.3 RESULTS

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III.6.4 DISCUSSION

III.6.5 CONCLUSIONS

III.6.6 REFERENCES

III.6.1 INTRODUCTION

Several reports are available in the literature for preparing hydrogels by graft copolymerization of acrylamide, acrylic acid and methacrylic acid onto *kappa*-carrageenan and agar, using water soluble reagents e.g., persulphate [1, 2]. Many authors have reported on hydrogel technologies providing products suitable for applications in biomedical, personal care as well as in nano-sensor applications [3]. Bai and Li have recently reported specific food applications as well as carriers for immobilization for hydrophilic polymers [4].

Genipin (Figure III.6.1a) has been widely used in herbal medicine e.g. for its anti-inflammatory, diuretic, choleretic and haemostatic properties. But it has now emerged as a crosslinking agent in polymers [5, 3]. Genipin has also been approved for food medicine uses in Japan, Korea, Taiwan and in Southeastern Asia [3, 6]. The dark blue pigments obtained by its spontaneous reaction with amino acids or proteins have been used in the fabrication of food dyes [7]. The blend gelatin-maltodextrin and gelatin-derived bioadhesives display higher biocompatibility and less cytotoxicity when cross-linked with genipin than with other agents, such as formaldehyde, glutaraldehyde and epoxy compounds [8, 9].

In an ongoing program of modification and grafting of seaweed polysaccharides for preparing hydrogels with improved properties in our laboratory [10-17], we report herein preparation of a hydrogel by crosslinking of a blend of the food hydrocolloids agar (Figure III.6.1b) and kappa-carrageenan (Figure III.6.1c) with naturally occurring crosslinker genipin. The properties of cross-linked blend hydrogel have been studied and it holds the potential useful in specific food applications as well as polymeric carrier or support.

III.6.2 MATERIALS AND METHODS

III.6.2.1 Materials

Agar and *kappa*-carrageenan were extracted in our pilot plant from 1.5 kg *Gelideilla acerosa* and 0.5 kg *Eucheuma cottonii* of Indian waters following the methods described by Roleda et al. [18], and Craigie & Leigh [19], respectively. Laboratory grade isopropanol (IPA) was used as received from Ranbaxy Fine Chemicals Ltd., Mohali, India, without

further purification. Genipin was purchased from M/s. Challenge Bioproducts Co., Ltd. Taiwan.

III.6.2.2 Preparation of polysaccharide blend

Blend hydrogel samples were prepared as follows: the agar and kC sols in water were prepared at the autoclaving temperatures 120°C and 107°C for 20 min each, respectively. The two sols were then mixed in required proportions in a microwave oven at 70°C for 120 sec. Three blend hydrogel samples with total polysaccharide concentration 3 wt%, were prepared using the following ratios of agar and kappa-carrageenan: (I) Agar:kC =1:1 (w/w) or 50 wt% each; (II) Agar:kC=1:2 (w/w) or 33.33 wt% agar and 66.66 wt% kC; and (III) Agar:kC =1:3 (w/w) or 25 wt% agar and 75 wt% kC. The resulting sols were kept at room temperature to obtain soft to hard gels, which were then cut into small pieces and dehydrated with isoproapanol for 24 h. Dehydrated gels were dried in air followed oven drying at 50°C for 2-4 h.

III.6.2.3 Preparation of genipin-fixed agar/kC

The hydrogel blend with 25 wt% agar and 75 wt% *kappa*-carrageenan showed maximum swelling ability in all pH conditions. Therefore, this particular blend was selected for further investigations. The crosslinker genipin content was varied from 0.1 wt% to 1.2 wt% (w.r.t. the blend) to optimize the concentration of the crosslinker. The blend polymer (2 g) was dissolved in 100 ml of distilled water at 120°C for 20 min in an autoclave; the pH of the solution was 7.20. A stock solution aqueous solution of the blend polymer was (10%) of genipin was prepared in 10 ml of water. To the sol of the blend were added different aliquots of the genipin stock solution (to have 0.5 wt%, 0.8 wt% and 1.0 wt% genipin concentration with respect to the blend in the mixture) at 60°C in separate experiments, and mixed well. The homogeneous viscous solutions were kept at room temperature (30°C) for different durations from 5 h to 40 h, in different experiments. The growth of cross linking reaction was checked periodically by working up a certain reaction mixture at various time intervals and then measuring the swelling capacity and intrinsic viscosity. The work-up was done as follows: the reaction mixture was poured in isopropanol (1:2 w/w) and the mixture was kept at room temperature for 24 h. The hydrogel product was then isolated and was washed (x3) with methanol to remove unreacted crosslinker, then the hydrogel was air dried and then in an oven at 50°C for 2 h.

III.6.2.4 Estimation of bulk and true densities, pore volume and porosity

Bulk density, true density, pore volume, and porosity were estimated as reported in the literature [4] and method has been mentioned in the Chapter 5, under Sections *III.5.2.4.1* & *III.5.2.4.2.*

III.6.2.5 Swelling ratio and degradation rate measurements

Swelling ratio and degradation rate were measured as described in the literature [20]. The detailed methods have been mentioned in the Chapter 5, under Sections, *III.5.2.4.3* & *III.5.2.4.4*.

III.6.2.6 Syneresis index

The amount of water exuded from the gel samples after standing for a certain period of time was determined and quantified using a modified method [21]. The syneresis index values of the gel samples were taken as the difference between the initial weight of the gel and its final weight after 2 h. This value indicates the water holding capacity of the gel.

III.6.2.7 MS/MS analysis

The MS/MS analyses were done in a Waters Q-Tof micro YA-260 mass selective detector using Waters MassLynx Version 4.0 software. This was carried out as mentioned in Chapter 5, Under Section *III.5.2.4.5*.

III.6.2.8 Other characterizations of genipin-fixed agar/kC

UV-Vis spectra were recorded on a Varian CARY 500 Scan UV-Vis-NIR spectrophotometer. Optical microscopy was carried out on an Olympus model SZH 10, Japan with 70X magnification and thermal analysis (TGA were done on a TGA Toledo Mettler TGA System Switzerland. Apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072) using Spindle No.1 at rpm 60. Intrinsic viscosity [η] was determined at 30°C using an Ostwald viscometer with a flow time 116 Sec for 1 M NaCl. For this, samples of non-modified agar/kC blend and crosslinked blend were prepared in 1M NaCl at a concentration of 0.02% to 0.12% (w/v).

III.6.2.9 Protein estimation

Total nitrogen was estimated by Kjeldahl method [cf. 22], on a KEL PLUS-KES 20L Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN Equipments, Chennai, India). Crude protein content was calculated multiplying the nitrogen content by the approximate factor 6.25 [22]. The results are the mean and standard deviation of 4 replicates.

III.6.2.10 Rheological measurements

Dynamic rheological measurements were performed using a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany), and the Rheowin Pro 2.1 as mentioned in Chapter 5.

III.6.2.11 Statistical analyses

Data were analyzed using analysis of variance (ANOVA). Results were considered statistically significant when $p < 0.001$. Calculations were performed using Origin Software, Version 6 (Microcal Software Inc. MA, USA).

III.6.3 RESULTS

III.6.3.1 Physicochemical properties

The effect of crosslinking, of genipin with Agar/kC blend, on the bulk density, true density, pore volume, porosity, intrinsic viscosity, apparent viscosity, and syneresis are showed in Table III.6.1. The bulk density, true density, intrinsic viscosity, apparent viscosity and water holding capacity was increased after crolinking of genipin with blend polysaccharides (Table III.6.1). The genipin-fixed Agar/kC revealed a statistically significant higher values of bulk density, true density, intrinsic viscosity and apparent viscosity, as compared to the non-modified Agar/kC ($p < 0.001$). The pore volume and porosity of the non-modified blend decreased after crosslinking with genipin (Table III.6.1), showing a statistically significant lower values of pore volume, porosity and syneresis, as compared to the crosslinked blend ($p > 0.001$).

Table III.6.1 Physical properties of the non-modified and cross-linked blends

Properties	Non-modified blend	Crosslinked blend (with 0.8 wt% genipin)
Apparent viscosity (at 70°C, in cP s)	52±0.57	69±0.62
Syneresis index (%)	34±0.64	26±0.77
Intrinsic viscosity	241.53±0.58	435.85±0.97
Bulk density (g/ml)	0.4041±0.0.002	0.6123±0.0018
True density (g/ml)	1.030±0.007	1.022±0.006
Pore volume (Vp) (ml/g)	2.091±0.006	1.008±0.004
Porosity (**Ø**)	6.166±0.098	3.433±0.073
Protein (%)	1.81±0.075	1.80±0.073

III.6.3.2 Effect of crosslinking reaction time

The effect of cross-linking reaction time on the swelling ratio of genipin-fixed agar/kC is presented in Figure III.6.2. The swelling ratio increased with increasing cross-linking reaction time up to 30 h (p <0.001), beyond which there was no increment in the swelling ratio.

III.6.3.3 Swelling measurement

The results of swelling experiments of the Agar/kC blend and crosslinked blend (with 0.8 wt% genipin) in acidic (pH 1.2), neutral (pH 7.0) and in alkaline (pH 12.5) are presented in the Figures III.6.3a, b, c. The cross-linked blend hydrogel with 0.8 wt% genipin showed maximum swelling ratio (%), 8600 ± 32.56, 9380 ± 22.15 and 7000 ± 23.87 in acidic, water and alkali media, respectively. It is important to note that the crossslinked blend exhibited significantly higher swelling (p <0.001) in all media over ca. 18 h and was almost steady up to ca. 24 h (Figures III.6.3a, b, c).

III.6.3.4 Effect of crosslinker

The effect of crosslinker concentration on the swelling ratio of the genipin crosslinked Agar/kC blend is presented in Figure III.6.4. The swelling ratio increased with increasing crosslinker concentration up to 0.8 wt% (w.r.t. blend polysaccharides) beyond which there was no increase in the swelling ratio. Crosslinked blend, obtained with 0.8 wt%

crosslinker revealed a statistically significant higher value of swelling ratio in all pHs, as compared to the non-modified Agar/kC blend ($p < 0.001$).

III.6.3.5 Degradation rate measurement

The mass loss ratio of non-modified blend and genipin crosslinked blend (with 0.8 wt% genipin) was measured in Ringer's solution, wherein 41.9% and 19.9% mass losses ($p < 0.001$), respectively, were observed (Figure III.6.5).

III.6.3.6 Thermogravimetric analysis

The TGA curves for non-modified and crosslinked blends are shown in Figure III.6.6. The former showed weight loss in three stages, the first one (12 %) occurred in the range 50-110°C probably due to the loss of moisture. The subsequent weight losses were of 35% and 91%, and occurred in the temperature ranges of 150-240°C and 275-620°C, respectively. In the case of the crosslinked blend, the TGA profile was similar, showing mass losses in four stages. However, at the maximum temperature investigated (750°C), only 73% mass loss was observed in the crosslinked blend.

III.6.3.7 Optical Microscopy and Scanning Electron Microscopy (SEM)

The optical micrographs and SEM of the non-modified and crosslinked blends (with 0.8 wt% genipin) are presented in Figure III.6.7a-d. The genipin-fixed blend hydrogel was dark blue in appearance as a result of the cross-linking reaction over 30 h.

III.6.3.8 MS/MS analysis

The mass pattern of standard genipin is presented in Figure III.6.8a. The m/z (%): 225 (M^{+}-1, 65%), 207 (10%), 123 (100%), 101 (50%), 97 (8%) for standard genipin (Figure III.6.8a). The MS of the recovered genipin that was extracted with methanol from the acid hydrolysate of genipin-fixed kC produced the following major ion fragments besides other contamination peaks (Figure III.6.8b). The m/z (%): 225 (M^{+}-1, 35%), 207 (10%), 123 (100%), 101 (50%), 97 (15%). The corresponding MS pattern of the control experiment did not show the peaks at m/z 225, 207, 123, 101 and 97, that were observed in the MS of standard genipin.

III.6.3.9 Rheological measurements

The variation of dynamic viscosity with shear rate is depicted in Figure III.6.9. The crosslinked blend hydrogel showed lower gel-thinning behavior and higher dynamic viscosity values as compared to the non-modified blend under applied shear rates.

III.6.3.10 UV spectrum

The Agar/kC blend does not have any absorption maxima in the UV-Vis region. The crosslinked blend in aqueous solution (pH 7.45 at 30°C) showed λ_{max} 590 nm, whereas genipin exhibited λ_{max} at 240 nm. The absorbance at 590 nm by the blue coloured cross-linked agar/kC blend indicated binding of genipin with free amino acids or protein present in the polymeric matrix of blend self-assembly [23].

III.6.4 DISCUSSION

The naturally occurring cross linker genipin produced a blue colored Agar/kC blend in aqueous medium at ambient conditions to afford a stable hydrogel network, possessing significant absorbent capacity and network stability [cf. 24].

The genipin-fixed Agar/kC showed the greatest water absorbency in the neutral, acidic and alkaline medium at pH 7.0, 1.2 and 12.5 after ca. 18 h and beyond this the absorbency remained virtually steady (Figures III.6.3a-c). The crosslinked hydrogel showed superior swelling ratios ca. 8600% and ca. 9400% in pH 1.2 and 7.0, respectively (Figures III.6.3a,b). This result is significant especially when viewed against the fact that parent kC is readily dispersible in water and that it degrades rapidly in acidic pH. Relatively lower water absorbency was encountered in alkaline pH (Figure III.6.3c). This unusual stability of the crosslinked blend hydrogel may be ascribed to its molecular network assembly. The cross-linking reaction time was optimized (ca. 30 h) by measuring the swelling ratio of the crosslinked blend products at different time intervals (Figure III.6.2). The swelling ratio increased gradually with cross-linking reaction time, no significant differences being found after 30 h of cross-linking reaction time. The measurement of bulk density, true density, pore volume, porosity, apparent viscosity, and syneresis, also indicated crosslinking of genipin with Agar/kC blend (Table III.6.1). The bulk density, true density, apparent viscosity increased after crosslinking of genipin with blend polysaccharides (Table III.6.1). The crosslinked blend revealed a statistically

significant higher value of bulk density, true density and apparent viscosity, as compared to the non-modified blend (p <0.001). The pore volume, porosity and syneresis values of the non-modified blend decreased after crosslinking with genipin (Table III.6.1).

The relatively greater stability of the crosslinked Agar/kC was also demonstrated in the thermal analysis by TGA and degradation studies in Ringer's Solution (Figures III.6.5 & 6). SEM images and Optical micrographs (Figure III.6.7a-d) showed distinct differences in the morphology and colour of genipin-fixed Agar/kC when compared to that of Agar/kC composite, this may be due to the change in the molecular arrangements induced by genipin.

Genipin is known to react with amino groups and proteins developing blue color [7]. It must be mentioned that there is no nitrogen containing group in the agar and carrageenan structure. However, nitrogen and protein were estimated to be ca. 0.29% and 1.81% in the Agar/kC blend, respectively. It is, therefore, apparent that genipin reacted with the protein present in the polymeric matrix of Agar/kC blend [25], affording the crosslinked product. The MS and MS/MS data indicated that genipin got fixed chemically, not as a physical mixture, in the Agar/kC blend polymeric matrices to form genipin crosslinked Agar/kC polymer (Figure III.6.8a, b). The dynamic viscosity measurement showed more shear thinning of the non-modified blend hydrogel network than the cross-linked blend hydrogel (Figures III.6.9). The increase in apparent, bulk density, true density and UV absorbance at 590 nm [23, 26], and thermal stability (Figure III.6.6) of the crosslinked blend compared to the non-modified blend, also confirmed that genipin was involved in the crosslinked blend network assembly.

III.6.5 CONCLUSIONS

Blend of food hydrocolloids, viz. agar and *kappa*-carrageeenan (Agar/kC) was treated with the natural crosslinker genipin in aqueous medium to impart functional stability. The genipin-fixed blend exhibited remarkable stability in the wide range of pH 1-12, as well as in Ringer's solution. Compatibilities of unmodified blend (Agar/kC) and the crosslinked blend product in aqueous solution were studied by measuring apparent and intrinsic viscosities and UV absorptions. Properties of Agar/kC blend and the genipin cross-linked blend in solid state were evaluated by bulk and true density, pore volume, porosity, thermogravimetric analysis (TGA), swelling ability, X-ray diffraction,

degradation rate in vitro, optical microscopy and rheological measurements. The cross-linked blend, exhibited higher viscosity, thermal stability and swelling ability compared to those of the un-modified blend. The presence of genipin in the cross-linked polymer network was confirmed by MS/MS analysis. This genipin cross-linked blend presents an immense potential for food applications and in other pH-specific applications as well [27].

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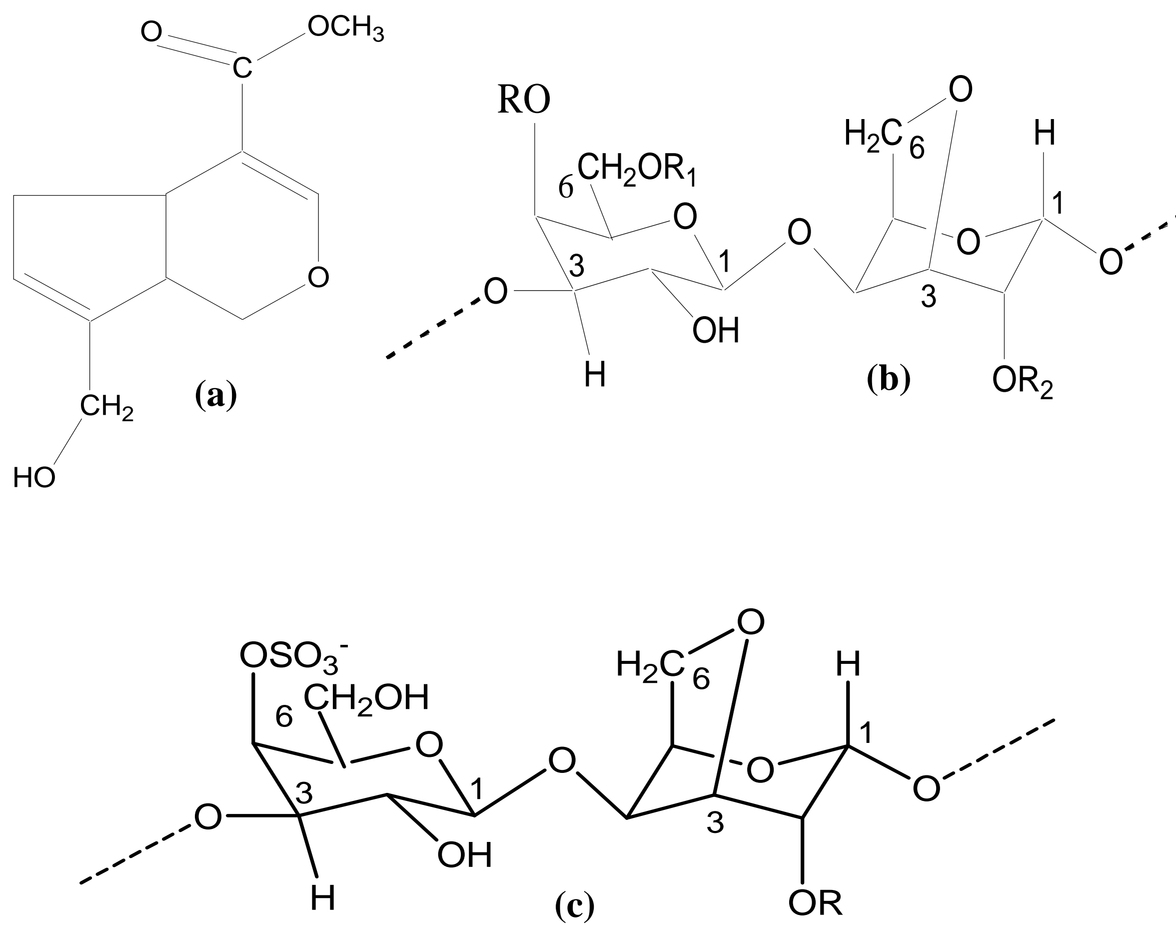

Figure III.6.1a-c Structure of genipin (a), agar (R = H or SO_3^-, R_1 = H or Me, R_2 = H or Me) (b), and *kappa*-carrageenan (c), used in the study.

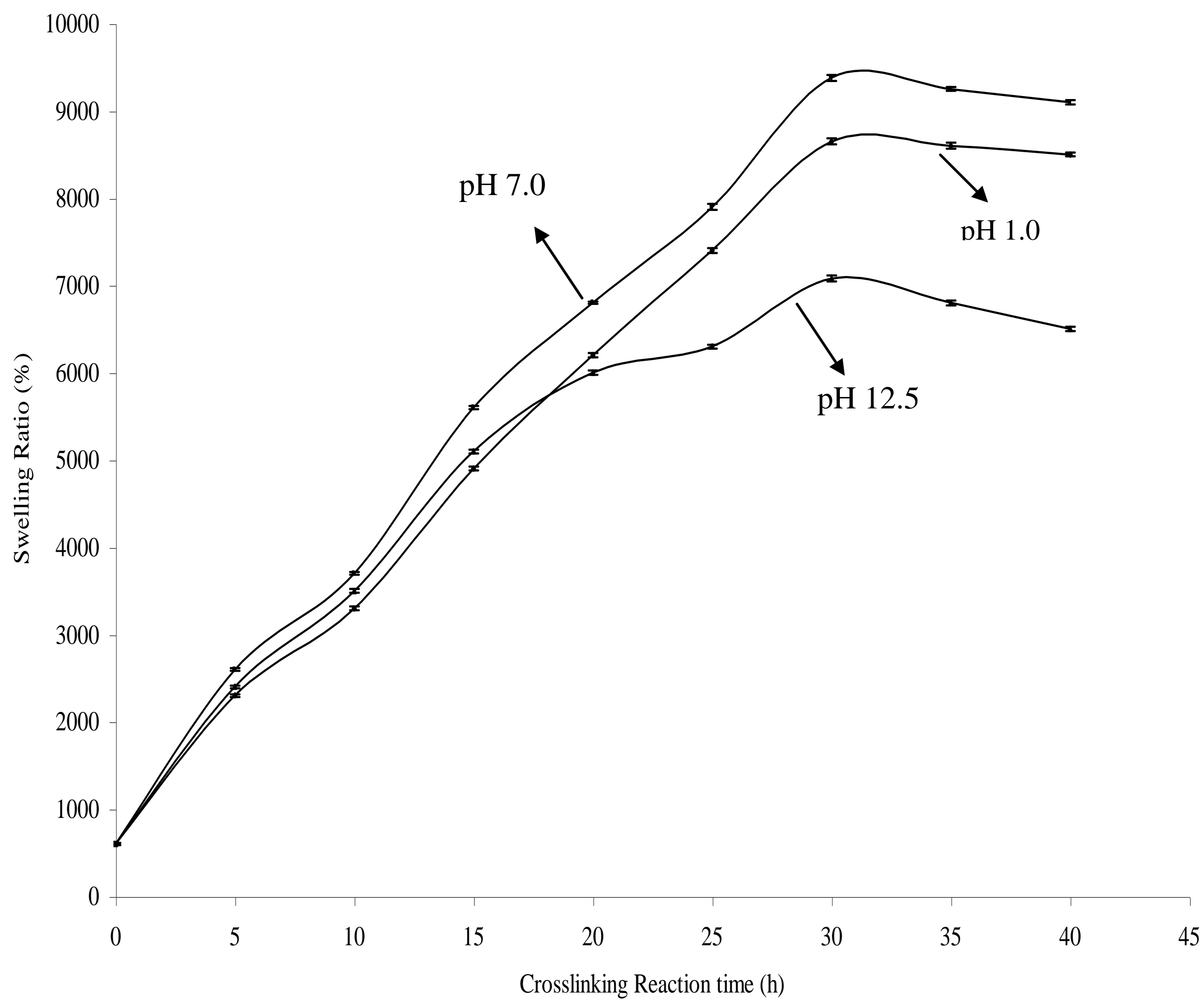

Figure III.6. 2 Effect of cross-linking reaction time on the swelling ability of crosslinked Agar/kC blend product. Data represent the mean ± standard deviation, $n = 4$.

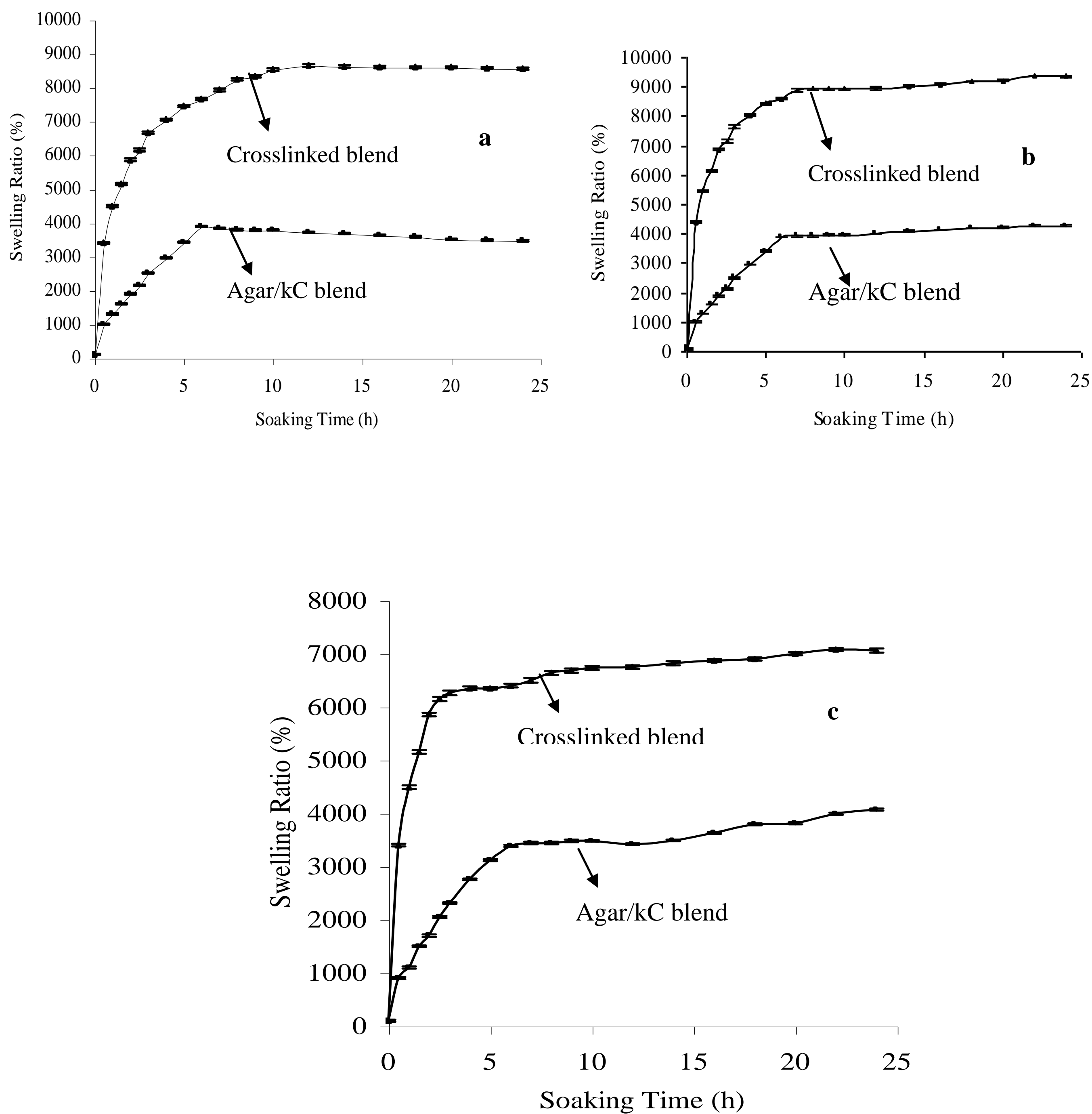

Figure III.6.3a-c Swelling ability of Agar/kC blend and crosslinked blend (with weight percentages of genipin = 0.8wt %) (a) at pH 1.2; (b) at pH 7.0; and (c) at pH 12.5. Data represent the mean ± standard deviation, *n* = 4).

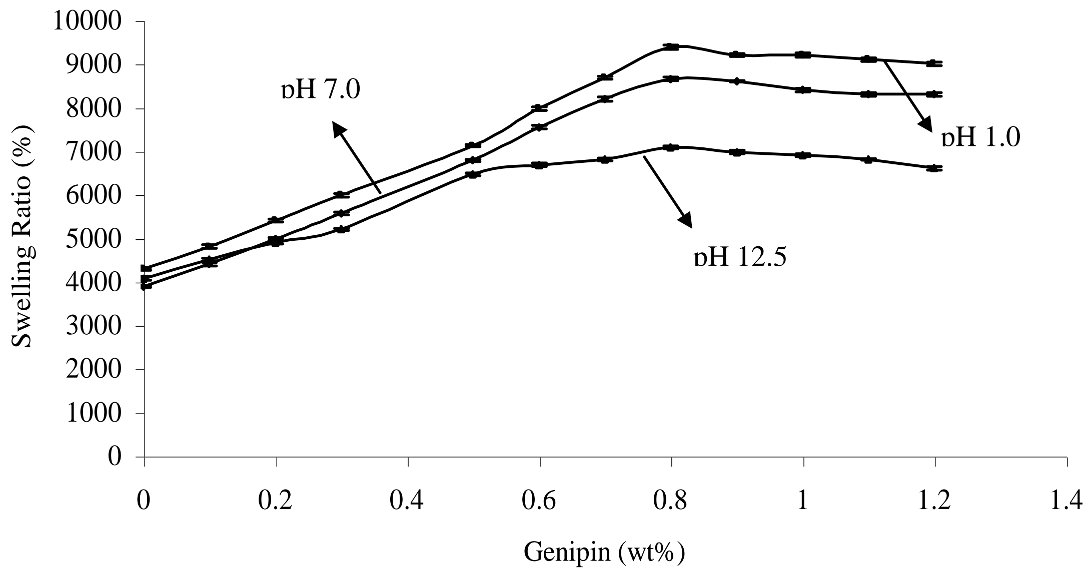

Figure III.6.4 Effect of weight percentages of genipin on the swelling ability of crosslinked Agar/kC blend in different pH media. Data represent the mean ± standard deviation, $n = 4$.

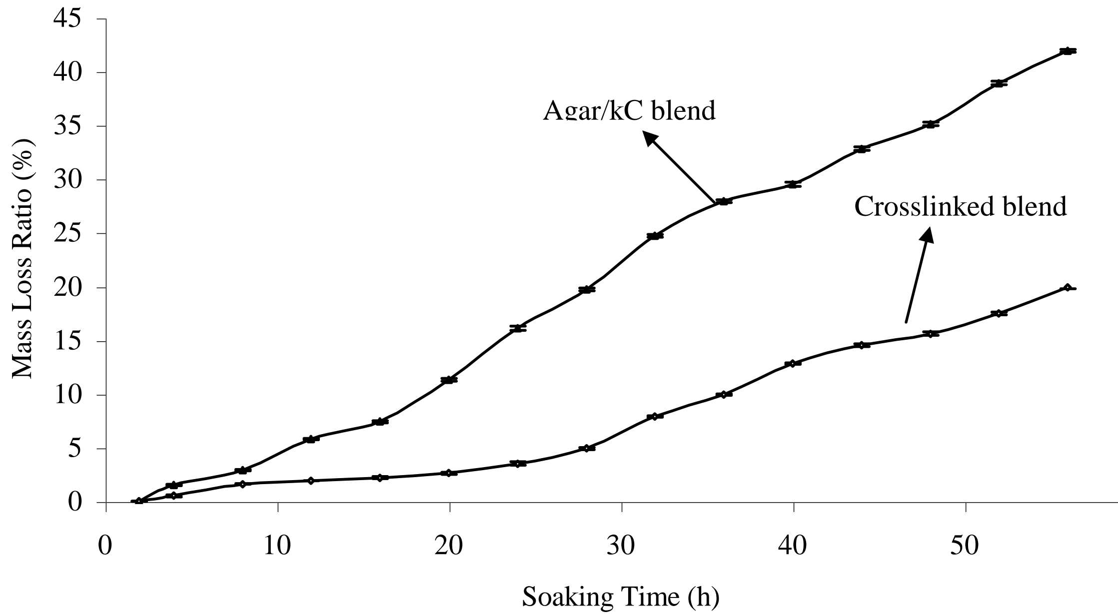

Figure III.6. 5 Effect of weight percentages of genipin on the mass loss ratio of non-modified Agar/kC blend and crosslinked Agar/kC blend. Data represent the mean ± standard deviation, $n = 4$.

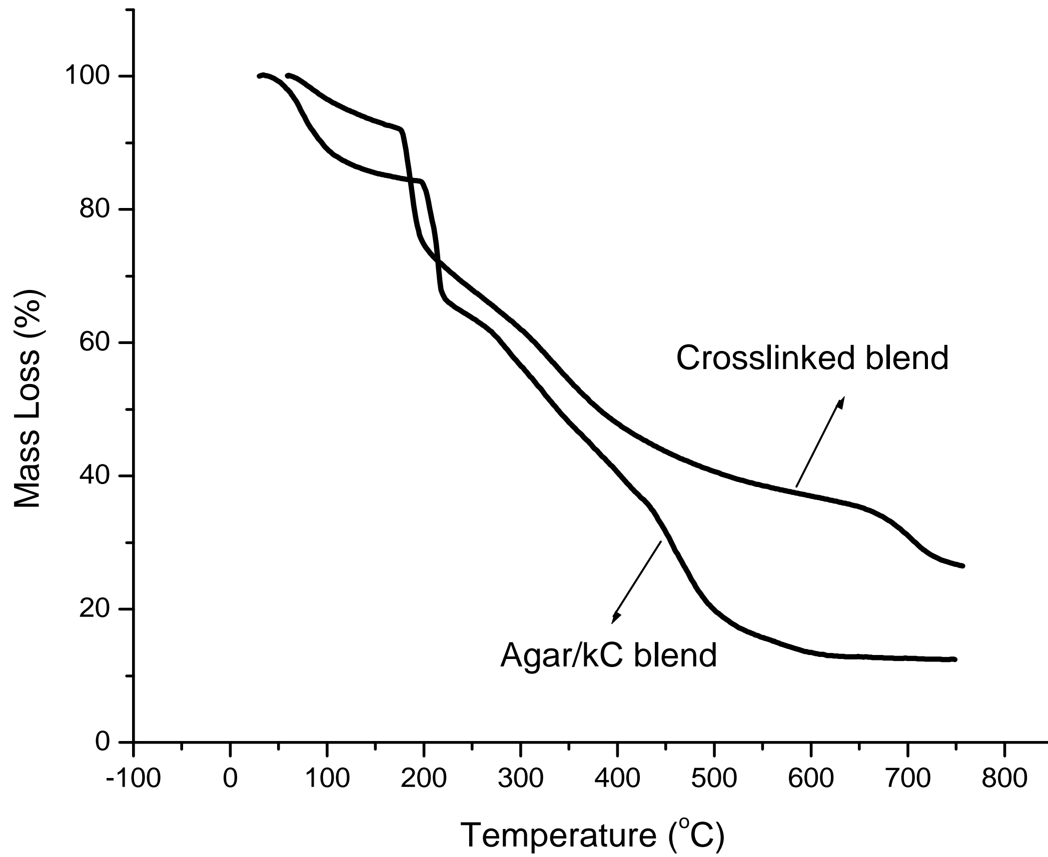

Figure III.6.6 Thermogram (TGA) of non-modified Agar/kC blend and crosslinked Agar/kC blend (with 0.8 wt% genipin).

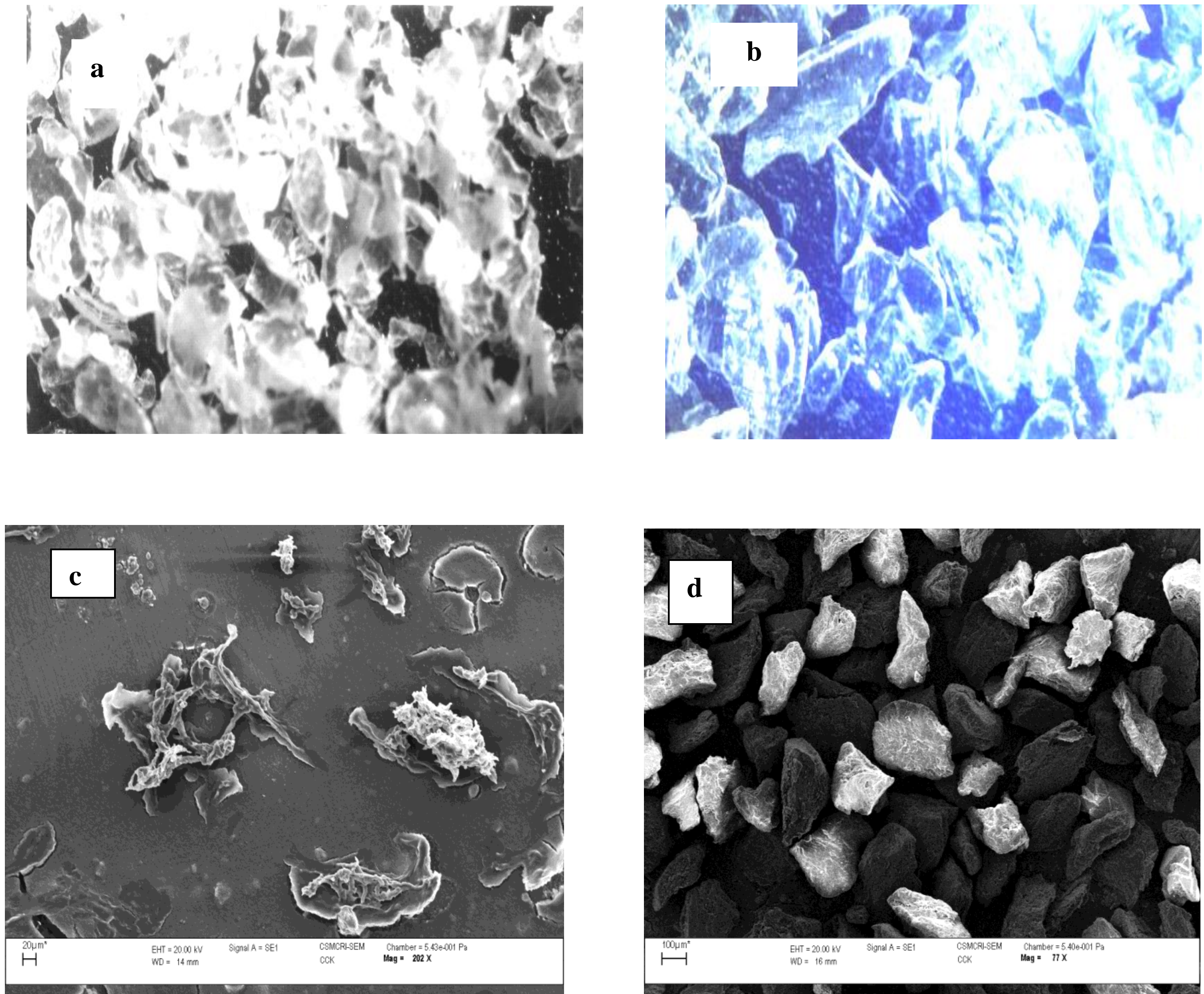

Figure III.6.7a-d Optical micrographs and SEM images of **(a, c)** non-modified Agar/kC blend and **(b, d)** crosslinked Agar/kC blend**,** at 70X and 77 X magnification, respectively.

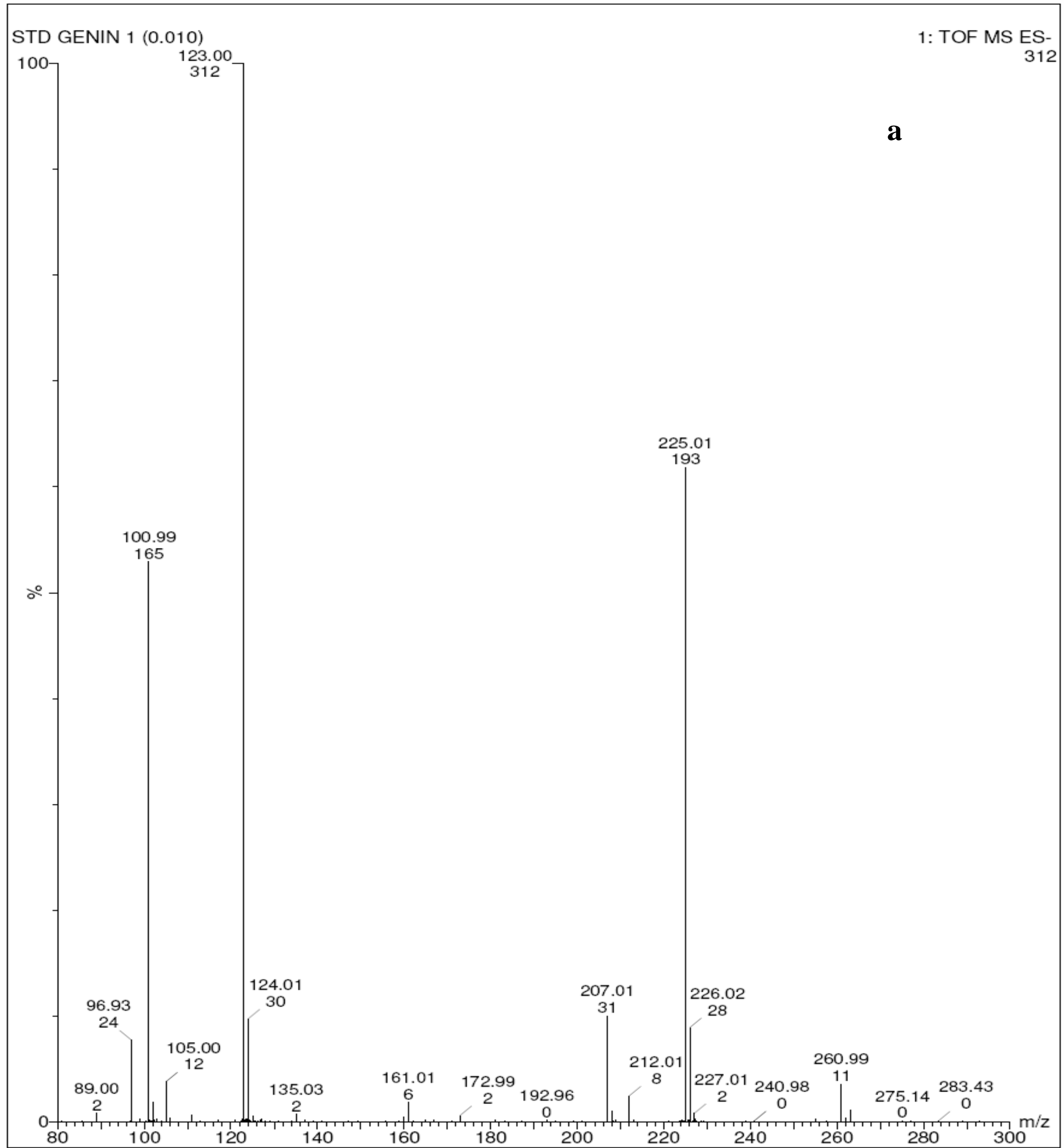

Figure III.6.8a Mass spectrum of standard genipin.

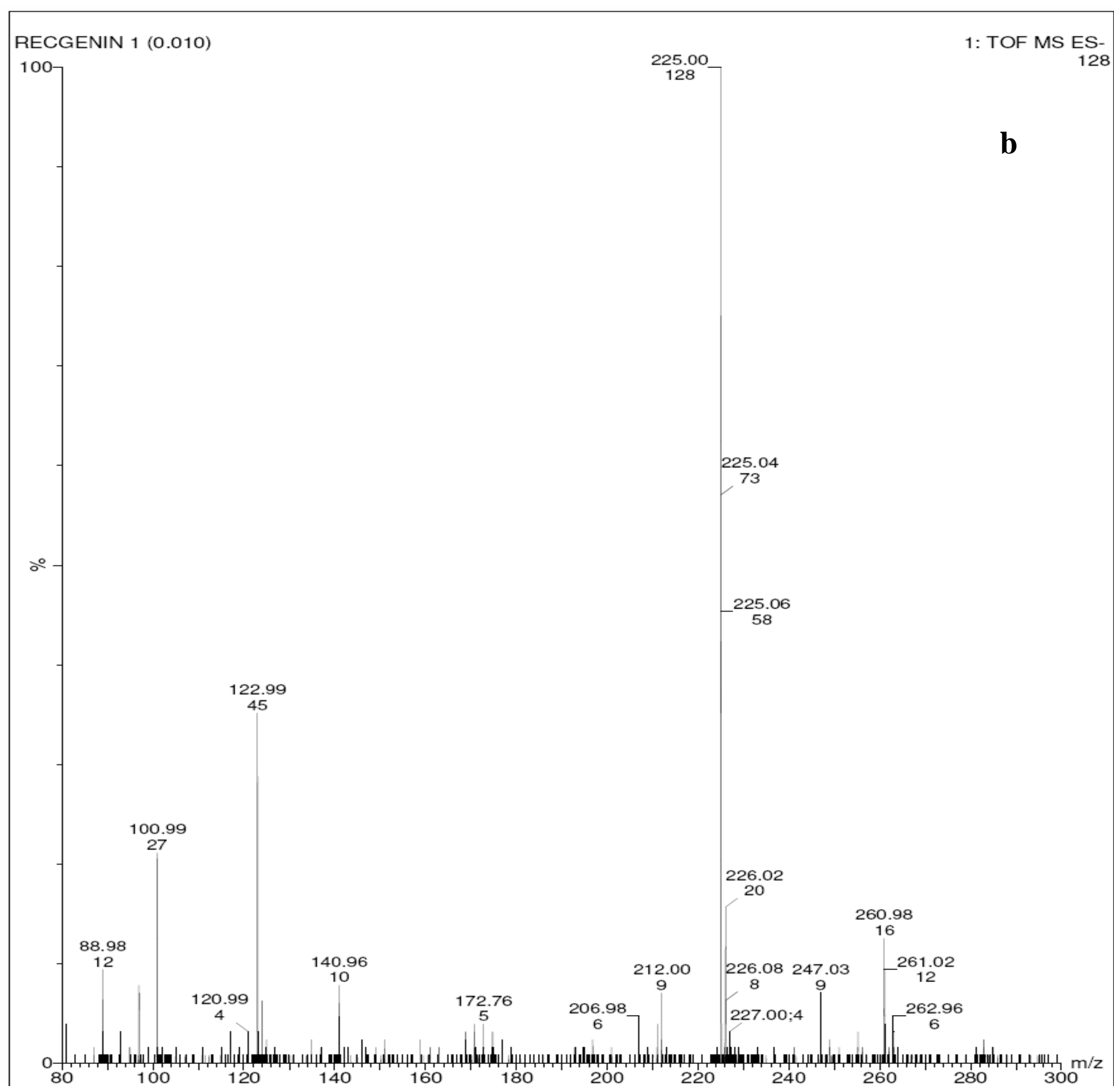

Figure III.6.8b Mass spectrum of "recovered genipin" extracted from acid hydrolysed genipin-fixed kC.

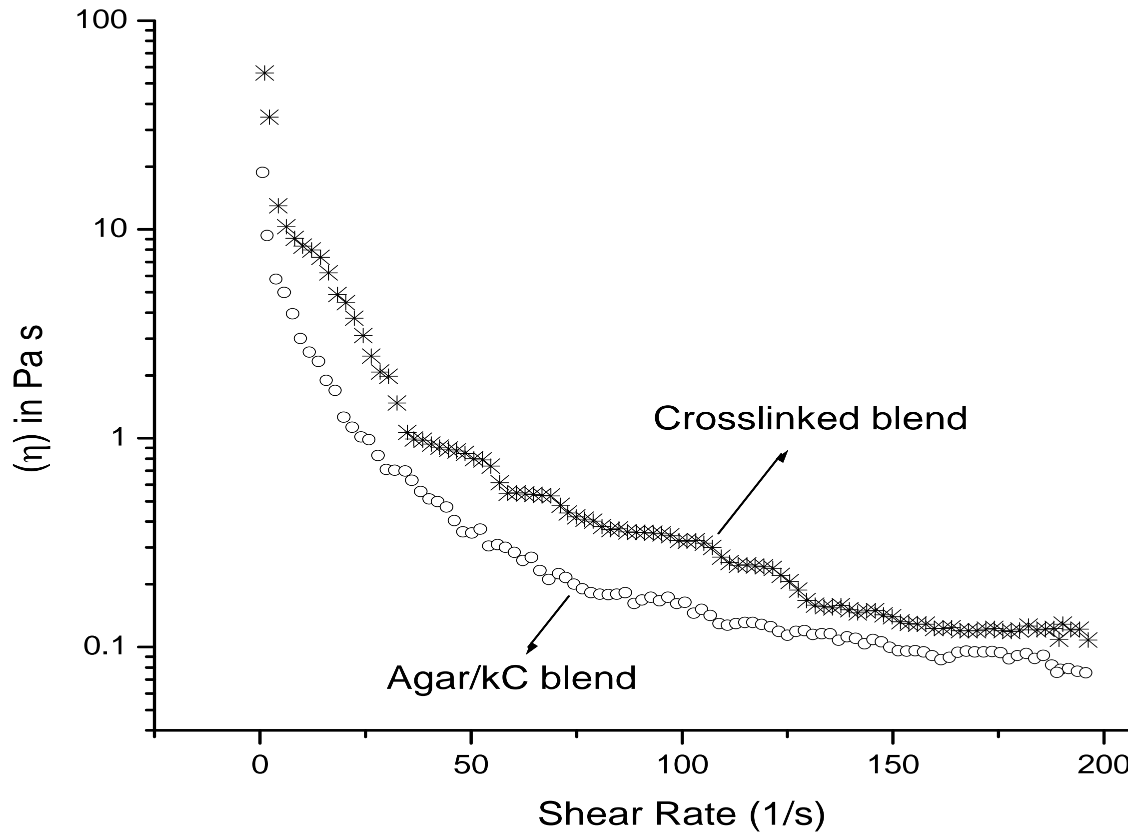

Figure III.6.9 Variations in shear viscosities of non-modified Agar/kC blend and crosslinked blend (with 0.8 wt% genipin).

APPENDIX

List of publications

1. **Ramavatar Meena**, Kamalesh Prasad and A.K Siddhanta. Studies on 'sugar reactivity' of agars extracted from some Indian agarophytes. ***Food Hydrocolloids**,* 20, 1206-1215, 2006.

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6. Arup Kumar Siddhanta; **Ramavatar Meena**; Kamalesh Prasad; Bharatkumar Kalidas Ramavat; Pushpito Kumar Ghosh; Karuppanan Eswaran; Sangalya Thiruppathi; Vaibhav Ajit Mantri. A cost-effective process for preparing agarose from gracilaria spp. UK patent: GB 2429209 A, February 21, 2007.

Technology Developed/Transferred

1. Developed a simple and cost-effective process for preparation of superior quality agars from Indian agarophytes in the lab and pilot plant scale, and this technology has been given to **CEFI, LRC- Port Blair,** based on our *USP: 2005/0267296 A1* [Arup Kumar Siddhanta, **Ramavatar Meena,** Kamalesh Prasad, Bharatkumar Kalidas Ramavat, Pushpito Kumar Ghosh, Karuppanan Eswaran, Sangaiya Thiruppathi, Vaibhav Ajit Mantri].

2. Developed a simple and cost-effective process for preparation of superior quality agarose from different Indian agarophytes in the lab and pilot plant scale, and this technology is ready for transfer to industry for commercial production of agarose.

Conferences/Symposia

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2. **Ramavatar Meena,** Kamalesh Prasad, Mahesh U. Chattbar and A.K. Siddhanta. Preparation of copolymer hydrogel of agar and sodium alginate blend. (*Presented in XX Carbohydrate Conference (CARBO XX) Lucknow University, Lucknow , India*, November 24-26, 2005). Abstract: OP- 10, p 50.

3. **Meena, Ramavatar,** K. Prasad & A. K. Siddhanta. PREPARATION OF SUPERIOR QUALITY PRODUCTS FROM INDIAN AGAROPHYTES. Presented *in* 7th Asia-Pacific Conference on Algal Biotechnology*, during December 1-4, 2009 at Delhi University-Delhi.*

4. Kamalesh Prasad, **Ramavatar Meena**, A.K Siddhanta. Microwave induced grafting of seaweed polysaccharides by methyl methacrylate. (*Presented at National Seminar on Polymer, Gels and Surfactants, M.S University, 2005, Vadodara, India, 11–13 th March 2005*. Abstract: OP- 03, p 31.

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