CHEMICAL AND RHEOLOGICAL STUDIES ON SEAWEED POLYSACCHARIDES

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By

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CERTIFICATE

This is to certify that the contents of this thesis entitled "Chemical and Rheological Studies on Seaweed Polysaccharides" is the original research work of MR KAMALESH PRASAD, carried out under our supervision.

We 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 therein, contribute in general to the advancement of knowledge in chemistry and in particular to the **Chemical and Rheological Studies on Seaweed Polysaccharides**.

Prasad

Kamalesh Prasad

Date : 5/06/2005

To

My Beloved Parents

&

'Happy'

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Bhavnagar **Kamalesh Prasad**
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ABBREVIATIONS

3,6-AG	3,6-Anhydro galactose
$CDCl_3$	Deuterated chloroform
CMC	Critical micelle concentration
C18	Octadecyl silica stationary phase column
CPC	Cetyl pyridinium chloride
cP	Centipoise
CTAB	Cetyl trimethyl ammonium bromide
DMSO	Dimethyl sulphoxide
DMF	N,N-dimethyl formamide
DSC	Differential scanning calorimetry
DS	Degree of substitution
$d\gamma/dt$	Shear rate
EEO	Electroendoosmosis
FT-IR	Fourier Transform Infra Red
FT-IR-ATR	Fourier Transform Infra Red-Attenuated total reflection.
f	Frequency
G'	Storage modulus
G''	Loss modulus
g	Graft
HLB	Hydrophile / lipophile balance
ICP	Inductively coupled plasma spectrometer
J	Joule
KPS	Potassium persulphate
LC-MS	Liquid chromatography-mass selection.
τ	Shear stress
MMA	Methyl methacrylate
MW	Molecular weight
mp	Melting point
NMR	Nuclear Magnetic Resonance
$[\eta]$	Dynamic viscosity
Pa	Pascal
PEG	Polyethylene glycol
PS	Polysaccharide
p	Probability
R_f	Retention factor
Rt	Retention time
rpm	Rotation per minute.

RP-HPLC	Reverse-phase-high performance liquid chromatography
Spp.	Species
SEM	Scanning electron microscope
SLS	Sodium lauryl sulphate
TLC	Thin Layer Chromatography
TGA	Thermogravimetric analysis
T_g	Glass transition temperature
T_{gel}	Gelling temperature
T_m	Melting temperature
UV-Vis	Ultra violet visible
WVP	Water vapour permeability

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Thesis Title : Chemical and Rheological Studies on Seaweed Polysaccharides

Part-I

Review on physical and chemical modification of polysaccharides with special reference to agar and carrageenan

This review compiles the information available in the literature related to modification of polysaccharides with special reference to agar and κ-carrageenan.

Polysaccharides have been proposed as the first biopolymers to have formed on Earth. After many years of oblivion, the area of polysaccharide chemistry is presently receiving renewed attention. This is due to the increase in the understanding of the role of biopolymers in various biological processes. Active research on the modification of the basic polysaccharides are under way in various labs to explore the possibilities of applications in newer fields such as in physicochemical, biochemical, biomedical and industrial applications. Yalpani has reviewed methods for selective modifications of polysaccharides. Numerous reactions have been reported in the literature describing methods to remove various labile groups like sulphate, pyruvate, acetyl from the polysaccharides.

Considerable numbers of chemical and enzymatic methods are now available for the selective transformations of many polysaccharides including the seaweed polysaccharides like agar and carrageenan. In view of the inadequacies of most of these techniques, in terms of their achievable degrees of selectivity and conversion efficiencies, further advancements are obviously required, particularly for seaweed polysaccharides are susceptible to acidic pH conditions. Suitable mild oxidizing, acylating, alkylating agents as well as suitable protecting agents are required to be developed for cost effective modification of polysaccharides. These developments would provide further understanding of the fundamental structure-property relationships of the polysaccharides, which would be useful in designing novel products for newer applications including the functional ones.

(**A Review article entitled 'Modification of naturally occurring Polysaccharides' is communicated to Progress in Polymer Sciece**).

Part- II

Physicochemical and rheological studies on agar extracted from *Gelidiella acerosa* collected from various locations of west and southeast coasts of India in different seasons

Chapter II.1

Agars of *Gelidiella acerosa* (Forsskal) J. Feldmann & G. Hamel (Rhodophyta, Gelidiales) of west and southeast coasts of India

In the course of our continuing studies on evaluation of agarophytes of Indian waters, it has been observed that the agarophyte *Gelidiella acerosa* produces very good quality of agar (with high gel strength in the range of 600-1400 g cm^{-2}) in 20-30% yield (with respect to bone dry seaweed), employing an improved process developed in our laboratory (unpublished results).

In the present study, it has been revealed that the gelling properties vary significantly with the sites of collection. In this investigation the physicochemical, rheological and thermal properties of agars extracted from 16 samples of *Gelidiella acerosa* collected from various places of the west coast of India (4 samples) were evaluated and compared the same with those occurring in the Gulf of Mannar region at the southeast coast (12 samples). Seasonal variation of the agar quality has been discussed in chapter II.3.

Due to the relatively higher natural biomass of *Gelidiella acerosa* in the west and southeast coasts of India, we have selected these two localities for comparison of the quality of agar in the present investigation. It was observed that the quality of agar extracted from *Gelidiella acerosa* occurring in the southeast coast of India is of superior quality.

In this investigation preparation of high quality agar from *Gelidiella acerosa* occurring in Indian waters, has been described which is suitable for bacteriological and molecular biology works. The rheological characterization of this agar are reported herein for the first time. The agar obtained from the *Gelidiella acerosa* of southeast coast has been found to be of superior quality than that obtained from the west coast of India. These results would be useful in bioprospecting of agarophytes occurring on the Indian coasts.

The agar gels investigated herein possessed all the required properties useful for their specific end applications. All agar gels of this study were found to be of good quality as revealed by their physicochemical properties e.g. low sulfate (1-1.7%) as well as relatively low gelling (37-41°C) and melting (79-87°C) temperatures (Table II.1.1a). Comparatively low 3,6-anhydrogalactose contents in the agars of west coast of Gujarat (27-32%) and high 3,6-anhydrogalactose contents in the agars obtained from southeast coast (35-44%) are manifested in the respective gel strengths of the agars studied (Table II.1.1a and 2a). The agar gels obtained from *Gelidiella acerosa* collected from Krusadai island was found to be the best. The agars obtained from Ervadi collections were found to be as good as that obtained from Krusadai island. In general the agars obtained from the seaweed samples of Gulf of Mannar region are better in quality than those obtained from the west coast of India (Table II.1.1a and 2a). These agar gels, obtained from *Gelidiella acerosa* of Gulf of Mannar region, exhibited the highest breaking strength, highest degree of cohesiveness, rigidity and thermal stability (Fig. II.1.3a,3b & 7). Stronger gels are of value because of their strength, resilience, elasticity, relative transparency, relative permanence and thermoreversibility. The agar extracted from *Gelidiella acerosa* collected from west coast of India produced gels of lower strength, cohesiveness, thermal stability and rigidity (Fig.II.3a, 3b, & 7).

The agars obtained from *Gelidiella acerosa* of Gulf of Mannar regions have been found to be suitable for bacteriological and molecular biology work (vide Chapter II.2). To our knowledge, this is the first comprehensive report of comparison of agars obtained from *Gelidiella acerosa* from southeast coast and west coast of India. These results will be useful in bioprospecting work on agarophytes of India. **(*Bioresource Technology,*** 2007, 98, 1907-1915***)***

Chapter II.2

Bacteriological agar from *Gelidiella acerosa* (Forsskal J. Feldmann & G. Hamel (Rhodophyta, Gelidiales) collected from Gulf of Mannar, Bay of Bengal

In this Chapter preparation of bacteriological agar from a specific sample of *Gelidiella acerosa* collected from Gulf of Mannar has been described. Detailed investigations on the physicochemical, spectroscopic and rheological characteristics of this bacteriological agar have been carried out. The suitability of the agar for bacteriological and molecular biology applications has been validated.

The agar that has been studied herein contains low ash (2.16%) i.e., low metal ion and high 3,6-anhydrogalactose contents in this agar are in consonance with its high gel strength (1.5% gel, 830 g/cm^2 at 20°C) and observed high viscosity values (40 cP at 80°C in 1.5% sol). High gel strength of this agar makes it economical, because of the lower usage level in various applications (Table II.2.1). The absence of absorbance maxima in the 200-800 nm range in UV-Vis spectrum, indicates that the agar is free from any coloured contaminants i.e. pigments. The sol of this agar is clear at high temperature and starts to appear cloudy at ca. 60°C while cooling. Rheological properties showed that this agar sample gels at 0.3% w/v concentration and does not undergo syneresis in concentrations more than 1% w/v. The bacteriological assays of this agar sample reveal that this agar is suitable for bacteriological and molecular biology works and compared well with HiMedia Agar CR 301.

To our knowledge this is the first report of preparation of superior quality bacteriological agar from *Gelidiella acerosa* of Indian waters. The results of this study described herein will be useful for bioprospecting of agarophytes for producing high quality agar.
(***Rheology part is communicated to Applied Rheology, 2006***)

Chapter II.3

Evaluation of agars of *Gelidiella acerosa* (forsskal) J. Feldmann & G. Hamel (Rhodophyta, Gelidiales) collected from the west coast of India

In a continuing programme of evaluating agarophytes of Indian waters 20 samples of *Gelidiella acerosa* of the Indian coasts were investigated for their agar contents. Although, there are a few reports on the agar of *Gelidiella acerosa* of southeast coast of India having gel strengths 300 g/cm^2 and 790 g/cm^2, yet no details of extraction procedure and other physicochemical and rheological data are available in these citations.

In this chapter quality of agar obtained from *Gelidiella acerosa* collected from the natural stock occurring at the west coast of India at various locations in different seasons is reported. Native agar samples were extracted from twenty *Gelidiella acerosa* samples collected systematically between December 1999 and June 2000, and the properties of these agars were studied. The results reported herein will be useful in bioprospecting of the agarophyte *Gelidiella acerosa* as a source of superior quality agar, which has so far been unknown from this region.

The agar gels tested in this investigation, possessed adequate properties that would meet the requirements of specific end applications. The agars obtained from *Gelidiella acerosa* of Okha and Diu in the month of April, June and May, June 2000 respectively were found to be of superior quality among all the collections in the lot, this is shown by their physicochemical properties e.g. relatively low sulfate (1-1.4%) as well as relatively low gelling (38-41°C) (Table II.2.1a). Lower ash contents of these agars indicated low metal contents. Comparatively low 3,6-anhydrogalactose contents in the agars of Dwarka, Veraval, Porbandar (31-35%) than those in the agars obtained from Okha and Diu (35-37%) are manifested in the higher gel strengths of the agars of the latter (Table II.2.1a). The agar gels obtained from *Gelidiella acerosa* collected from Okha in the month of April and June 2000 and Diu in the month of May and June 2000 were found to be the best. These agar gels exhibited the highest breaking strength, highest degree of cohesiveness, rigidity (Fig.II.2.2, Fig.II.2.4 and Fig. II.2.5). Stronger gels are of value because of their strength, resilience, elasticity, relative transparency, relative permanence and thermoreversibility. The agar extracted from *Gelidiella acerosa* collected from Dwarka, Veraval and Porbandar produced gels of lower strength, cohesiveness, and rigidity (Fig.II.2.2 and Fig.II.2.3). The usefulness of the latter agars is limited to the weaker gels that are used in the food industry. In the present study, it has been demonstrated that the gelling properties vary significantly with the sites of collection as well as with the seasons, and sources of superior quality agar from the west coast of India have been identified

To our knowledge, this constitutes the first report on the evaluation of *Gelidiella acerosa* of the west coast of India, as a source of superior quality agar. These results will be useful in bioprospecting work on the agarophytes of Indian waters.
(Indian Journal of Marine Science,March 2006, in press)

Part-III

Studies on the interaction of surfactants and fatty acids with agar gel

Chapter-III.1

On the properties of agar gel containing ionic and nonionic surfactants

In the course of our studies to develop a process of extraction of agar from the red seaweed *Gelidiella acerosa*, it was realized that the quantity of water required in the process is kept large to overcome problems of choke up during filtration of the agar sol. Such problems tend to be especially acute when the sol viscosity and gelling temperature of the agar are high. However, large usage of water greatly reduces throughput and increases energy cost. We conjectured that it may be possible to utilize suitable high HLB surfactants to influence the rheological properties of agar and undertook a systematic study in this direction. Additional insight was obtained from DSC-TGA investigations.

In the present study, it has been demonstrated that ionic surfactants enhance agar sol viscosity and also the stiffness of the gel. The gelling and melting temperatures are also raised upon incorporation of surfactant. The behaviour is similar with both cationic and anionic surfactants and the effects are most pronounced near or above the critical micelle concentrations of the surfactants. Nonionic surfactants, on the other hand, lower the viscosity of agar sol and the stiffness of the gel. Moreover, these surfactants also reduce the gelling and melting temperatures. Non-ionic surfactants show significant influence on gel strength even at 0.1% level whereas the trend with ionic surfactants is reasonably systematic in the concentration range of 0.1-0.4% (w/v). The gelling temperatures are less sensitive to variations in surfactant concentration beyond 0.1% concentration, indicating that the origins of the two effects are different. Gelling temperatures obtained from the intersection point of the storage and loss moduli curves ($T_{\tan\delta=1}$) recorded under controlled stress mode are found to be in good agreement with the more conventional visual method of measuring gelling temperature. The concentrations of surfactants employed were near or above the critical micelle concentrations in all cases, which suggests that micellar aggregates influence the gel network in a profound way. Differential scanning calorimetric studies provide clear evidence of two endotherms in the DSC profile of control agar gel. In presence of surfactants, the profiles are altered in a profound manner and the results are in reasonable accord with the observed melting temperatures.

SLS shows a different behaviour than other ionic surfactants in as much as the gelling and melting temperatures are lowered in its presence unlike with other ionic surfactants. On the other hand, it does not behave like nonionic surfactants either since, unlike with nonionic surfactants, this lowering in gelling and melting temperatures is not accompanied by a lowering of gel strength when the SLS concentration is above the cmc SLS could, therefore, be a useful additive for bacteriological agar formulation which demands high gel strength and low (35-37^{o}C) gelling temperature and which may not be otherwise achievable with certain agar types, e.g., the agar of the present study from *Gelidiella acerosa.* The remarkable ability of SLS to inhibit water loss from agar gel upon heating is yet another interesting result that may offer clues for design of systems to inhibit water loss.

For processing of agar extracts, the utility of nonionic surfactants is self-evident. These surfactants lower the sol viscosity, which would allow for easier filtration of concentrated extractives, thereby helping to substantially reduce water usage in the process. Moreover, the lowering of the gelling temperature minimizes the possibility of accidental gelling of hot extractive during work-up operations. When coupled with the freeze-thaw or pressure syneresis methods of purification of agar, the removal of the surfactant from the agar has been found to be feasible. This would help restore the pristine quality of the agar. Studies at the bench scale level have confirmed the feasibility of the scheme.

Additional efforts will be required to unravel the role of surfactants at a fundamental level. The useful effects described herein may provide the necessary motivation for such studies.
(***International Journal of Biological Macromolecules*** **35:133-144, 2005).**

Chapter-III.2

Surface tension studies of Agar-CTAB sols

In this work surface tension measurements have been utilized to characterize the interaction/binding between the nonionic agar and the cationic surfactant CTAB (Cetyl trimethyl ammonium bromide). We have already reported in Chapter III.1 the thermal and rheological studies of agar in presence of CTAB in the gel state. Therefore, it was considered appropriate to study the interaction of agar in presence CTAB in the sol state by surface tension measurements. The data generated in this study may be useful to explore newer applications of agar e.g in pharmaceutical (as micelling agent) and oil industries (oil spill removal). It was decided that the agar that was extracted in our laboratory from *Gelidiella acerosa*, which was characterized by IR, NMR studies, will be used in the present investigation. This decision was based on the following observations: the surface tension of double distilled water was measured to be 69.2 mN/m, while those of 0.01 % (w/v) Oxoid agar (Oxoid Agar No.1 (Oxoid, UK; Lot/Ch.-B: 810501-2) and Difco agar (Difco Laboratories, 0140-01, Detroit Michigan USA) were 54.8 mN/m and and 51.2 mN/m respectively. The agar that was used in this investigation (AS042901) had surface tension value (in 0.01 %, w/v) 65.1 mN/m, indicating thereby the latter being associated with minimum surface active contaminations.

The principal finding of this work is that CTAB binds with agar and the extent of binding can be determined by measuring surface tension. It has been estimated that at CMC level

concentration of CTAB in agar sol (at 27.1^{o}C), 218 mmol of CTAB was bound to each repeating unit (1,4-linked galactopyranose 3,6-anhydrogalactopyranose moiety) of agar polymer.
(***Indian Journal of Chemistry* 44A: 2445-2449,2005**).

Chapter-III.3

Physical modification of agar: rheological and HPLC analysis

The increased use of agar in various biological applications including biotechnology and molecular biology makes it more precious. It has been a constant endeavour of many research programmes to modify the physical and chemical properties of commercially important biopolymers. The extensive application of agar as gelling agent in many cosmetics and laxative formulations has made physical modification of agar all the more important. Preparation of low gel strength or creamy agar and use of it as skin moisture is patented by Kojima and coworkers. This prompted us to undertake studies on the physical modification of agar by using fatty acids. Rheological, thermal and viscoelastic properties of the agar fatty acid adduct were studied. The most obvious effect was found in lower gelling and enhanced dynamic flow behaviour of the agar sol. The quantity of fatty acid in the agar fatty acid complex was determined by HPLC and LC-MS analysis of the methanol extract of the adduct. To our knowledge, the studies of interaction of agar-fatty acids have not been reported in the literature. Results of the present study may be useful in exploring newer application areas for agar.

It was observed from above studies that fatty acids bind to agar resulting in an agar-fatty acid adducts. The adduct is more hydrophobic and has more fluidity than control agar in the gel state. This investigation provides a physical method of modification of agar. Such modified agars could be used in various cosmetic and health care product formulations, wherein both the gelling characteristics of agar as well as the merits of fatty acids could be beneficially exploited.
(Polymer Journal 37: 826-832,2005)

Part-IV

Preparation and properties of seaweed polysaccharide based thin films

Chapter IV.1.1

Preparation of seaweed polysaccharide (agar and refined κ-carrageenan) based thin films.

In the mid-1990s, the outbreak of Bovine Spongiform Encephalopathy (BSE) or "mad cow" disease in Europe drew global attention. Subsequently, it has brought about a major change in the safety perception in the areas of human, cattle and poultry health and welfare. Accordingly, the US Food and Drug Administration (FDA) advisory committee recommended that "gelatin should no longer be considered generally recognized as safe" (GRAS) for either food of pharmaceutical products. Consequent upon these developments, attempts have been under way to find suitable alternatives to gelatin for making soft capsules. This has prompted this investigator to undertake studies for developing seaweed polysaccharide based thin films which would have applications in pharma and food packaging industries. This chapter summarizes the work done in this aspect.

It has been demonstrated in the present investigation that it is possible to prepare thin films with agar and refined κ-carrageenan in presence of plasticizers (e.g. glycerol, ethylene glycol polyethylene glycol, mannitol and sorbitol). In the case of refined κ-carrageenan additives e.g. KCl and glycine were added for enhanced film strength (tensile strength 120 g/cm^2 vs. 58 g/cm^2 of agar film with glycerol). Thermal measurements revealed that refined κ-carrageenan films were more thermally stable than agar films. Scanning electron micrographs showed that refined κ-carrageenan films constituted of more ordered molecular association than agar films. It has been observed that κ-carrageenan produces superior films in presence of plasticizer and additives. Rheological measurements revealed that the film forming material containing refined κ-carrageenan possesses enhanced thixotropic properties compared to the film forming material containing agar. The carrageenan containing material required higher temperature (ca. 45^{o}C) while casting the films and in case of agar the casting of films could be done ca. 35^{o}C. Wettability and swelling measurements showed that agar films were more hydrophilic and have higher water absorbing capacity than its refined κ-carrageenan counterpart [Tables IV.1.6 and Fig.IV.1.3].

Various rheological models that have been reported in the literature were considered to find out the best one that suits the flow behaviour of the film forming materials containing agar and refined κ-carrageenan. It has been found that in the sol state the film forming material containing agar is best fitted to Ostwald de Waele model for stress, strain and viscosity parameters. On the other hand, Herschel-Bulkeley model fits well for the film forming material containing refined κ-carrageenan in sol state for stress and strain parameters, and its viscosity parameter suits well with the Ostwald de Waele model. These measurements would be useful for drawing out the operational conditions for preparing films from these polysaccharides. Technologically speaking, these results would be of help in predicting the operational parameters for unknown or little known systems. The seaweed polysaccharide systems studied herein would be useful for developing non-animal gelatin based formulations suitable for pharma, health care and food packaging industries.

(*A process for the preparation of biodegradable semirefined kappa carrageenan Based Films*. **PCT application No PCT/IB2004/003977; Dec 3, 2004, US application No. 11/003,250; Dec 3, 2004.**)

(***Rheology part is communicated to Applied Rheology, 2006***)

Chapter IV.2

Chemical modification of seaweed polysaccharides and thin films derived thereof

This chapter consists of Section A and Section B dealing with "Chemical modification of seaweed polysaccharides" and "Surface modification of κ-carrageenan based thin films" respectively.

SECTION A: Chemical modification of seaweed polysaccharides

In this section carbanilation reaction that was used to induce hydrophobicity on κ-carrageenan has been described. Carbanilates of polysaccharides are useful in various fields as they are soluble in organic solvents. Cellulose carbanilates are used as surface sorbents in the separation of enantiomers by chromatography. Carrageenan carbanilate derivative was prepared, which was characterized to be consisted predominantly of carrageenan dicarbanilate. To our knowledge this is first report of preparation of carrageennan carbanilate derivative.

Modification of polysaccharide by grafting is popular for the preparation of new polysaccharide derivatives. Many grafting reactions reported so far in the literature are time consuming and the conditions are harsh, which often lead to degradation of parent polysaccharides. In this section of this Chapter grafting reactions of acid sensitive seaweed polysaccharides with methyl methylacrylate (MMA) by microwave irradiation are described. This was found to be a rapid and simple method preparing agar and carrageenan copolymers. The microwave conditions were optimized to achieve polysaccharide copolymers with minimum degradation and highest degree of efficiency (E% 98 for agar-*graft*-PMMA and E% 99 for carrageenan-*graft*-PMMA). The products were characterized by FT-IR, X-ray diffraction, TGA and optical microscopy. In both the cases the copolymers were thermally more stable than the corresponding original polysaccharides. The crystallinity index of carrageenan copolymer was 0.2586, while that of carrageenan was found to be 0.0847.

(***Journal of applied polymer Science*, 2006, Published online (www.wileyinterscience.com)**)

(*Microwave induced grafting of seaweed polysaccharides by methyl methacrylate* **Oral presentation at National Seminar on Polymer, Gels and Surfactants, M.S University, 2005, Vadodara, India, 11–13 th March 2005). Abstract : OP- 03, p 31**.

SECTION B: Surface modification of κ-carrageenan based thin film

Water insoluble derivative of κ-carrageenan were prepared and characterized by modifying the polysaccharide with phenyl isocyanate. Grafting of natural seaweed polysaccharides agar and κ-carrageenan by microwave irradiation has been demonstrated. Most important advantage of this method is that the reaction conditions are very simple and duration of reaction is also very brief. Many chemical reactions leads to degradation of these types of polysaccharides due to their very high acid sensitivity but in this study it has been shown that microwave irradiation under controlled conditions can result in the formation of undegraded copolymer of polysaccharides. This work would promote microwave irradiation technique for graft copolymerisation reactions. Hydrophobized biopolymers have been reported to be of great potential in bone tissue engineering applications.

Thin films were prepared with grafted agar and κ-carrageenan. The hydrophobicity, swellability, FT-IR analysis of the films showed that the films thus prepared became hydrophobic compared to the control polysaccharides. This study thus provides a method of preparing hydrophobic thin films which would have wider applicability in comparison of the hydrophilic films prepared by the control polysaccharides. (**A manuscript entitled "** *Hydrophobisation of agar and carrageenan films : A new strategy*" **has been submitted to J of Applied Polymer Science, for publication**)

Thesis summary entitled "Chemical and Rheological studies on Seaweed Polysaccharides" is published in *Applied Rheology* 16:32-34,2006.

REVIEW ON PHYSICAL AND CHEMICAL MODIFICATION OF POLYSACCHARIDES WITH SPECIAL REFERENCE TO AGAR AND CARRAGEENAN

I.1 INTRODUCTION

Polysaccharides have been proposed as the first biopolymers to have formed on Earth [1]. After many years of oblivion, the area of polysaccharide chemistry is presently receiving renewed attention. This is due to the increase in the understanding of the role of biopolymers in various biological processes. Active research on the modification of the basic polysaccharides are under way in various labs to explore the possibilities of applications in newer fields such as in physicochemical, biochemical, biomedical and industrial applications [2-8]. Yalpani has reviewed methods for selective modifications of polysaccharides. Numerous reactions have been reported in the literature describing methods to remove various labile groups like sulphate, pyruvate, acetyl from the polysaccharides [10,11].

Importance of polysaccharide modification can be felt, as most of the literatures related therein are intellectual properties. Efforts have been made to convert hydrophilic polysaccharides to water insoluble derivatives as well as other oxidized products viz., nitrosyl derivatives [12]. Amphiphilic moieties have been introduced to polysaccharides in order to impart surface active properties [13]. Shibata et al. has reported etherified products for microorganism derived polysaccharide pullulan [14]. Due to the excellent swelling nature many of the polysaccharides they are being extensively modified to give adhesives useful for paper industry. Further incorporating alkyl groups in the polysaccharides have increased the functionality, which helps to provide strength, stiffness useful for applications in paper industry [15].

Natural polysaccharides are frequently modified with chemicals to give them new properties for broader applications. Sometimes the modifications can be made in steps, giving a range of new products having properties quite different from the parent material. In many instances, the modification can produce a very specific effect because of the novel properties of the new structure. It is sometimes possible to impart properties of one polysaccharide on to another, allowing the replacement of a high-priced gum with a lower-priced modified gum. Such chemistry is fairly well established, but focused on specialized food research or process chemistry, mainly concerning starch and cellulose [16]. Polysaccharides occurring in nature are highly hydroxylated, usually with three hydroxyl groups for each chain unit. These hydroxyl groups, in conjunction with the ring and glycosidic oxygen atoms, provide five loci on an average for hydrogen bonding on each chain unit. Consequently polysaccharides are very hydrophilic and tend to bind water very tenaciously. Polysaccharides like cellulose, mannans of ivory nut, and starch amylose, where formation of strong junction zones persists and all tend to be insoluble in water under normal conditions. Due to the use of cellulose and starch in the paper and pulp, food and allied industries they are the most popular polysaccharides, which have been modified extensively to explore newer areas of application with complete change in their properties of the derivatives [17-19].

Until recently the prevailing approach to polysaccharide modifications involved use of non-selective chemical reagents, which results randomly derivatised polysaccharides products, sometime most of them were of no interest. Now the trend is to selectively modify the polysaccharides. Various functional groups present in the polysaccharides are being modified to give derivatives having different properties [20]. Selective modification methods have eliminated formation of undesired side products. Typical products obtained from these methods include alkyl, hydroxyalkyl, carboxyalkyl, triphenyl ethers, halogen, nitrates, phosphates, xanthate esters, sulphonates, sulphates of many polysaccharides [21,22,23]. Selective modification has made the characterization much easier than that in random modification reactions [24,25]. Chemical modification of chitosan has been reported and the same has been used in the removal of mercury from polluted water [26]. Yang, J. and Du, Y., have reported Chinese lacquer polysaccharide from Lac tree *Rhus vernisifera* active against leukopenia induced by cyclophosphamide [27]. Dye present in water as pollutant (from textile industries) can also be absorbed by tertiary aminated cross linked polysaccharide derivatives [28]. Researchers from TNO Nutrition and Food Research, Netherlands have reported an alternative for their brand product Carbopol, which is crosslinked polyacrylates optionally or partially esterified with liphophilic alkyl group, is not biodegradable used in cosmetics, the new starch based modified polysaccharide which is 100 % biodegradable can replace the existing carbopol [29].

It is surprising to note the extensive change in the properties of neutral polysaccharides that takes place on introduction of a small degree of derivatisation. For example, adding one acetyl unit to 100 glucosyl unit in starch increases its freeze thaw stability and introduction of three carboxy methyl group per 10 glucosyl unit in cellulose make it highly viscous gum. Polysaccharides with branched side chains or charged units, which tend to be more soluble, are less altered by derivatisation. Such material may have commercial importance in providing a specific effect as for example in improving protein binding or surface activity [16]. Polysaccharides are modified by interaction with polyamino carbohydrates, this is advantageous to use in paper and pulp industry [30]. Amphiphilic and alkyl moieties has been introduced in polysaccharides like cellulose, gums, chitosan, alginates, Sugars etc. which are useful in paper industry [31]. Starch from various sources has been chemically modified to give starch derivatives of applications in diverse fields [32].

Extensive work has been done to impart hydrophobicity to polysaccharides which due to their hydrophilic nature are not applicable to aqueous systems, in one report, prolamin such as zein, a microbial polysaccharide, has been allowed to react with molded polysaccharides to increase its water resistance [33]. A process producing a foamed water-based adhesive is reported involving forming a foamable adhesive composition containing a polysaccharide modified with an alkyl succinic anhydride and then applying energy to said foamable adhesive composition to produce adhesive foam [34]. Chitosan is one among the natural polysaccharides which is widely modified for various applications, the chitosan-derived biomaterials are useful in myriad biomedical applications, including as an immunoadjuvant in laser/sensitizer assisted immunotherapy [35].

Preparation of fucose rich oligo- and polysaccharides and their effect on aging is reported by Pterszegi et al. [36]. Synthesis of amphiphillic amylase, starch, alginates and haluronates and their physicochemical properties are reported in the literature [37,38]. Natural polysaccharides like cellulose, chitin/chitosan are useful biopolymers, but due to their limited applications these biopolymers have been used to prepare novel nano composites expanding their field of applications [39].

Preparation of compositions for altering the structural, chemical, physical and mechanical properties of polysaccharide materials using biological crosslinking agents based on multimeric structures of polysaccharide binding domains fused or linked to a biological or chemical entity and to the resulting biological compositions. The invention is exemplified by the use of a cellulose binding domain (CBD) fusion-protein containing two cellulose binding domains - a cellulose binding domain-Protein A-Ab complex or a S-peptide-cellulose binding domain-S-protein fusion to enhance mechanical properties, such as wet strength of tissue paper, filter paper and cotton yarn [40].

Polysaccharides composed of pentoses or hexoses have two or three hydroxyl groups available for chemical reaction and modification. For the hexose containing polysaccharides there are only one primary and two secondary hydroxyl groups available for chemical reactions. The ultimate aim of the modifications is to produce new materials having different properties and newer applications. Most of the modifications performed are on starch, cellulose and B-512 F (dextrans from *Leuconostoc mesenteroides*)**;** if the degree of substitution is more than 1 then this can result in the formation of completely new derivative [41].

Most important derivatives of polysaccharides are found in the form of their ethers, esters, oxidized, grafted and cross-linked products. Starch and cellulose are among the polysaccharides, which were most commercially exploited, and most successfully derivatised.

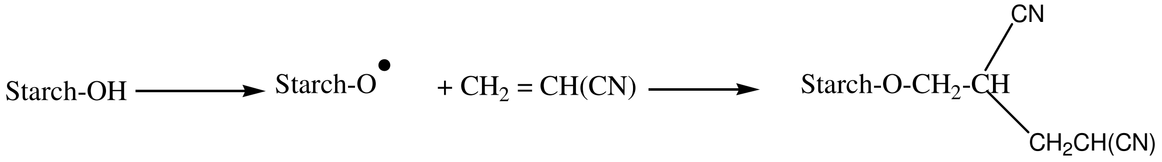
I.2 Formation of Polysaccharide Esters

Polysaccharides are polyols and hence readily form esters with a variety of esterifying reagents. Commercial esters are formed traditionally by reaction with acids and acid anhydrides. However, in the laboratory acetates can be prepared by using other reagents like ketene and vinyl acetate. Earliest esters includes cellulose acetates used to prepare plastics and fibers, prepared by treating cotton linters with acetic anhydride using acetic acid as solvent and sulphuric acid as catalyst. Starch acetates were prepared by following same method that with cellulose with slight alterations. The derivatives found to have improved freeze thaw stability and stabilizer in colloidal dispersions. Both organic carboxylic acid esters and inorganic esters have been prepared. Polysaccharides esters are generally soluble in organic solvents. Some of the most important esters are made of cellulose. Cellulose nitrate popularly known as guncotton a major constituent of smokeless powder, is the oldest inorganic salt of cellulose, which is obtained, by reacting cellulose with the mixture of nitric and sulphuric acid [42]. Starch nitrates are also used as explosives. Celluloid, which is an important commercial plastic, is a cellulose derivative of lower nitrate substitution. Polysaccharides react with carbon disulphide and sodium hydroxide to give polysaccharide xanthates [43].

I.3 Grafting

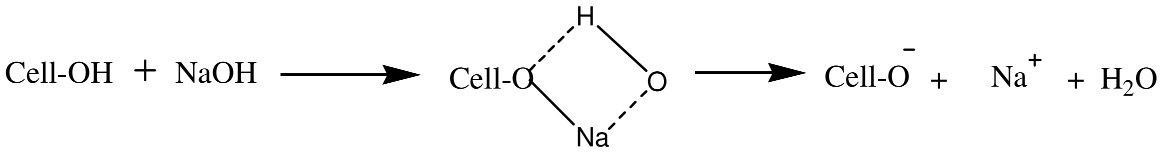
Grafting a synthetic polymer to a natural polysaccharide is a way of creating large molecules that have some of the characteristics of each individual polymer. Such works are extensively directed towards the preparation of water aborbants and biodegradable polymers that can be cast into sheets [44]. Grafting is initiated by generating one or more free radicals on the polysaccharide chain and allowing these free radicals to react with the polymerable monomers that will constitute the grafted chain. X-ray irradiation, UV-irradiation, beta irradiation etc. can be used to generate free radicals. Chemicals like ceric ammonium nitrate, ferrous ion, hydrogen peroxide etc. also are used as free radical generators [42]. Following is a pathway representing grafting on starch with acrylonitrile.

$$\text{Starch-OH} \longrightarrow \text{Starch-O}^{\bullet} + CH_2{=}CH(CN) \longrightarrow \text{Starch-O-}CH_2\text{-}\overset{\bullet}{C}H(CN)$$



I.4 Formation of Polysaccharide Ethers

Common industrial ethers are methyl, ethyl, hydroxyethyl, hydroxypropyl, carboxymethyl and aminoethyl. Mixed ethers are also available. Ethers can be formed with the alcohol groups of polysaccharides by reacting with alkyl halides or epoxides [45]. First ether prepared was methyl cellulose, prepared in 1905 by steeping of cellulose in 40-50% sodium hydroxide, followed by reaction with dimethyl sulphate. Due to carcinogenic nature of dimethyl sulphate, in modern methods the reaction is carried out by treating alkaline cellulose with methyl chloride in organic solvent [46]. Reaction follows the route of Williamson ether synthesis as shown below.

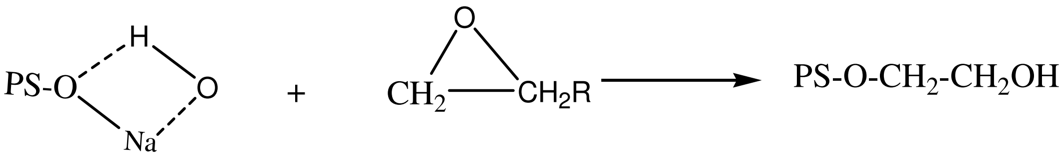


$$\text{Cell-}O^{-} + CH_3Cl \longrightarrow \text{Cell-}OCH_3 + Cl^{-}$$

Ether derivatives of polysaccharides are soluble in water. Many of the cellulose derivatives like DEAE cellulose, prepared by reaction of diethylaminoethyl cellulose with N,N-diethyl aminoethyl chloride, is used as cation exchange resin [47]. Hydroxylalkyl derivatives of starch and cellulose can be prepared by reacting starch and cellulose with epoxides and alkylene oxides [48]. Hydroxyethyl starches are used primarily as binders for pigmented coatings and surface sizing agents in paper industry.

Hydroxypropyl starches are of importance in food applications, where they are used as an edible, water soluble film coating. Formation of hydroxyalkyl derivatives for cellulose increases its water solubility. Hydroxypropyl derivatives of starch prevent retrogradation of starch chains [49]. Better water solubility of cellulose can be obtained by increasing DS. Methyl and ethyl derivatives of starches are prepared the same way of their cellulose counterpart, except first starch granules are heated with mineral acid at 100^{O}C, then alkali and methyl chloride is added. Another type of ether substitution, carboxymethyl is substituted successfully in number of polysaccharides, and carboxymethyl cellulose is one of the most important commercially important derivative.

Hydroxyalkyl substituted gums represent the largest group of polysaccharide ether derivatives, they are often prepared by the attack of poly-anion on a oxirane ring to produce the corresponding hydroxyalkyl derivative. The route of reaction is shown below.



I.5 Formation of Cross-linked Polysaccharide Ethers

The reaction of alkaline polysaccharides with epichlorohydrin results in the formation of ethers and the cross-linking polysaccharides chains. The reaction has been most successfully done to B-512 dextran to give a molecular sieve or gel filtration product [50]. The B-512F dextran cross-linked gels were commercialized by Pharmacia AB (Uppsala, Sweden) in 1959 as a gel matrix called Sephadex for GPC (Gel permeation Chromatography). By reaction of this with polyvinyl acetate it gives beads of different molecular weight cutoffs [51].

I.6 Polysaccharide Phosphates

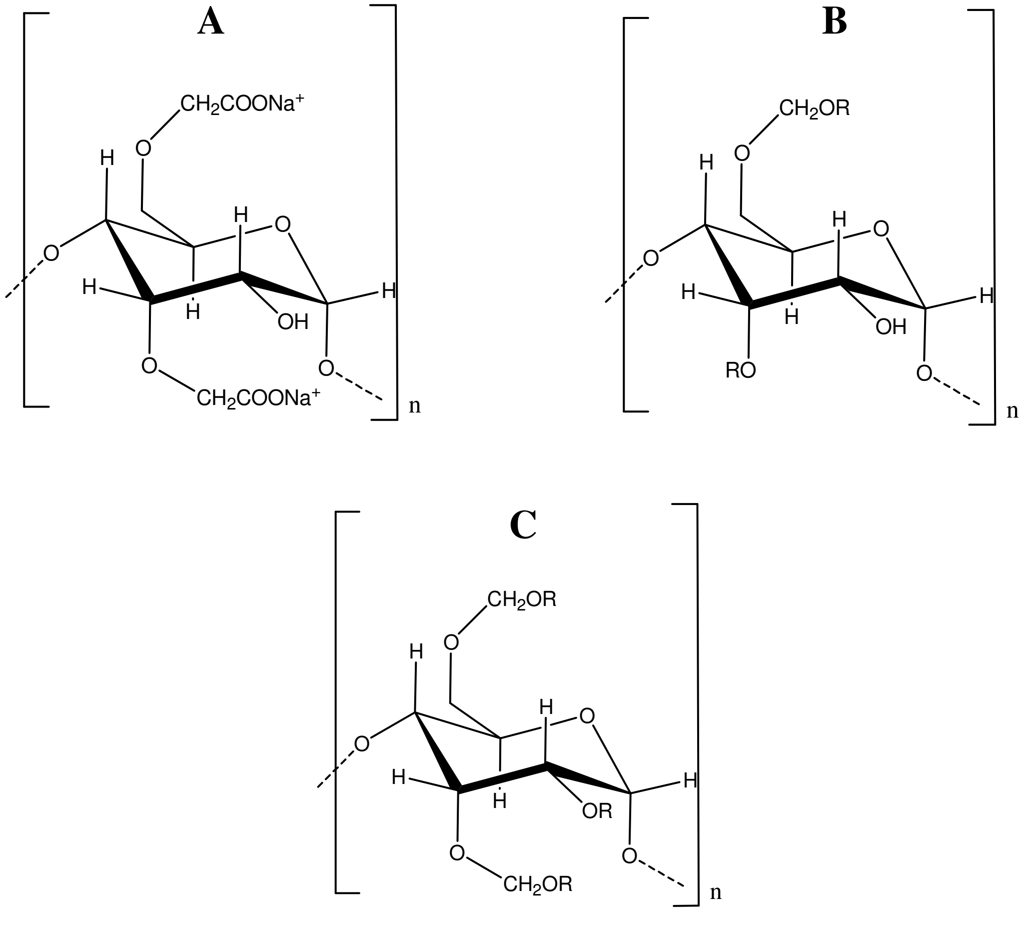
The hydroxyl groups of polysaccharides can be phosphorylated by reaction with tri-polyphosphate at pH 5-6.5 [52]. Phosphate oxychloride, tri-metaphosphate can also be used as phosphorous inducing agents [53].

I.7 Polysaccharide Sulphates

Most common reagent to give sulphate derivatives for polysaccharides is trimethylamine sulphur trioxide in N, N-dimethyl formamide. Other sulphating agents include dimethyl sulphoxide/sulphur trioxide. N,N-dimethyl formamide/sulphur trioxides are used for sulphating alginic and pectic acid [54].

Dextran sulphate seems to specific affinity for binding nucleic acids and is a potent inhibitor for ribonuclease [55]. Dextran sulphate also inhibits the replication of some viruses [56] including HIV [57], and has been considered as a possible drug in the treatment of AIDS.

Dragan et al. have described novel chemical reactions leading to formation of salphonamide derivative of dextran [58]. Gums like cassia tora, xanthan, guar also been chemically modified to impart more functional properties [59]. Attempts have been made to modify chitosan in the gel state by adding mono- or dibasic phosphate salt [60]. Fig.I.1 depicts structures of some important modified polysaccharides.



R = $CH_2CHOHCH_3$

Fig.I.1: Structure of some modified polysaccharides. (A) Carboxymethyl cellulose; (B) Methyl or ethyl cellulose; (C) Hydroxypropylmethyl cellulose

I.8 Chemically Modified Starch

The three hydroxy groups in each glucose residue of starch can be chemically modified in several ways in order to modify the properties of starch [61]. Low degrees of substitution have major effect on properties of starch pastes. Chemical modification decreases the tendency of starch molecules to self-associate and thus improves solubility in water and lessens the tendency towards phase separation and gelation on cooling. Chemically modified starches are used in a variety of value-added products and account for perhaps 1/6 of all starch sold [62]. Granular starches are reacted with small quantities of chemical crosslinking agents in order to make granules more resistant towards heat and shear forces during processing [36]. Examples are the reaction of aqueous starch suspensions with phosphorous oxychloride or adipic acid anhydride. Cross-linked starches have stable paste viscosity over long periods at high temperature and are used in many food applications.

Starch graft polymers (SGP) have long synthetic or natural polymer chains which are chemically attached to the scratched backbone. They typically contain 10-60% by weight of grafted polymer. SGPs are most often prepared by generating a free radical on the starch by redox reaction or radiation then attached the polymerization of an unsaturated monomer. Some examples are starch-g-polyacronitrile (SPAN), starch-g-polymethylacrylate (SPMA). The property of SPAN depends highly on the properties of the copolymer grafted and hence can be tailored to suit many properties. For example hydrolysed SPAN is highly water absorbent and SPAN can be processed to give tough plastics [63]. Substitution of starch to an average of 0.05-0.10 of a hydroxyalkyl per D-glucopyronosyl unit results in a drastic alteration of paste and film properties. The gelatinization temperature decreases by as much as 18^{o}C, and thus starch granules readily get integrated when heated in water. On cooling the hydrosols are very coherent, translucent pastes which can be preserved for long time.

Another common type of etherification reaction is reverse Michael addition, involving the alkali catalysed addition of an activated vinyl group. Best example is reaction of acrylonitrile with polysaccharides to yield the O-(2-cyanoethyl) derivative as shown [64].

$$\text{PS-O}^{-} + \text{CH}_2 = \text{CH-C} = \text{N} \quad + \quad \text{HOH} \rightleftharpoons \text{PS-O-CH}_2\text{CH}_2\text{-C} = \text{N} + \text{OH}^{-}$$

Starch can be substituted in this manner to DS levels approaching 3.0. Depending on the reaction conditions, highly water insoluble products are produced that can withstand steam sterilization.

I.9 Chemical modification of agar and carrageenan

Researchers are trying to substitute agar by kappa carrageenan in microbiological applications. Carrageenan has been suceesfully used as microbiological media [65]. A Japan based multinational company TAKARA BIO INC, has developed and is marketing an agar derived natural oligosaccharides under the trade name *Takara Agaoligo.* The company has reported anti oxidant and anti-inflammatory effects of these agar-derived oligosaccharides [66].

There are also reports of chemical modification of agar, agarose by inducing hydrophobic groups like alkyl, acetyl, hydroxy alkyl, to decrease gelling and melting temperature which is very crucial for bacterial growth [67]. Preparation and structural characterization of O-acyl agarose with low degree of substitution has been described by Garcia et al. [68]. Preparation of low gel strength agar gel has been reported, which are prepared by reacting high gel strength agar with salts of weak acids viz., citric acid, ascorbic acid. They are mainly used as massage gels, skin moisturizers, or as active carriers for pharmaceuticals which have to be applied through skins [69,70]. Hjerten has reported a product of agar prepared by cross liking divinyl sulphone with agar. This was further used in chromatography for separation [71]. Agarose having low electroendosmosis (EEO) value is used in isoelectric focusing, which is also mixed with a gum to achieve zero EEO [72].

A modified agarose or agar containing alkylated, alkenylated, acylated or hydroxyalkylated agarose in which the alkyl, alkenyl, and acyl groups contain from 1 to 3 carbon atoms and the hydroxyalkyl groups contain from 1 to 4 carbon atoms, which modified product has a gelling temperature at least $1^{O}C$ lower than the gelling temperature of the corresponding unmodified agarose or agar. The modified material also has a lowered melting point and a higher clarity than the unmodified. The compounds are useful as thickeners, for electrophoresis and for diffusive Interactions [73]. Sun Y Park et al. has describes biopolymer composite film preparation and their properties based on kappa carrageenan and chitosan [74]. A novel biopolymer based super absorbent hydrogel was synthesized through chemical crosslinking graft polymerization of acrylic acid on to kappa carrageenan in presence of crosslinking agent (N,N’ methylene bis acrylamide) and an initiator (Ammonium persulphate) is reported by Pourjavadi et al. [75]. Structural properties of agarose in presence of 50:50 sucrose and glucose syrup is studied [76]. Prasad et al. has described microwaved induced graft copolymerisation reactions for agar and κ-carrageenan. In this connection they have prepared agar-g-MMA, κ-carrageenan-g-MMA, agar-g-PVP, κ-carrageenan-g-PVP, the graft copolymers found to have different physicochemical and thermal properties in comparison to the control [77].

In view of the unusual properties of polysaccharides containing 3,6-anhydrogalactose ring e.g., agarose, carrageenan, a number of workers have developed synthetic methods for introduction of 3,6-anhydro residues [78]. The preparation of 3,6-anhydroamylose has been accomplished via internal nucleophilic displacement of good leaving group in a five step reaction involving tritylation, acetylation, detritylation, p-toluenesulphonylation and saponification [79].

Agar is been used as culture media for bacteria and in molecular biological works. Agar has been blended with various other ingredients to give other physically modified products having different commercial applications. Important agar blends are Trypton Glucose extract of agar is used in determining the standard plate count of milk and other dairy products. This is prepared by blending agar with tryptone and glucose [80]. Violet red bile agar, prepared by mixing agar with yeast, peptone, bile salts, lactose, sodium chloride, neutral red and crystal violet, is used for the direct plate count of coliform bacteria in water, milk, dairy and other food products. Desoxycholate agar is used for direct enumeration of coliform bacteria in dairy products. It is also can be used as non selective primary plating medium for the isolation of enteric pathogens. This contains is prepared by mixing agar with sodium desoxycholate, peptone, lactose and sodium citrate. Malt agar is prepared by mixing malt extract with agar in 2:1 ratio and used for the detection and isolation of yeast and molds from dairy products. Table I.1 summarises the various agar formulations and their applications

Galbraikh and coworkers have prepared 3,6-anhydrocellulose derivatives with 50-70 mol of anhydro substitution by saponification of 6-O-tosyl-2,3-di-O-acetylcellulose derivatives [81].

In an attempt to prepare analogues of agarose, Misaki and Tsummuraya have used a dimethyl sulphoxide-sulphur trioxide treatment to obtain O-6 sulfation. The partially sulphated glucan was converted into a 3,6-anhydro derivative using alkali treatment at 80°C. The modification resulted reduction of molecular weight (300000 to 200000) and intrinsic viscosity (1.86 to 0.029) of the products [82]. Rees has similarly introduced 3,6-anhydro residues into the galactosyl units of kappa carrageenan via alkali treatment, the 2-O-sulphate group performed a stabilizing effect in the product formation [83].

Table I.1 Important agar formulations and their applications

Type of agar	Application
Brilliant Green Bile agar	In determination of relative density of coliform bacteria in water.
Trypton glucose extract agar	Determining standard plate count of milk and other dairy products.
Violet red bile agar	Direct plate count of coliform bacteia in water and dairy products.
Desoxycholate agar	direct enumeration of coliform bacteria in dairy products and as non selective primary plating medium for the isolation of enteric pathogens
Potato dextrose agar	Determination of yeasts and moldsin butter.
Malt agar	Detection and isolation of yeasts and molds from dairy products.
Dextrose tryptone agar	Determination of thermophilic “flate sour” organisms associated with the spoilage of food products.

Gorin and Spencer have eliminated the α-D-glucopyranosyl (and other) side chains of a *Ceratocystis brunnea* glucomannan by means of selective introduction of 3,6-anhydro rings in the branch units. This was accomplished in a six step modification process, involving tritylation, acetylation, detritylation, p-toluenesulphonylation and successive treatments with sodium methoxide and an acid [84].

Carrageenan products comprise at least about 65% by weight of carrageenan and at least about 2% by weight of acid insoluble material and such products useful as components in food products, such as, dairy products, meats, and dessert gels as well as non-food products, such as, toothpaste formulations, cosmetics, and paints has been reported by Tsai et al. [85].

I.10 Polysaccharides modification in the gel state

The applications of the water-swellable gel in the medical field include wound dressings, adhesion-preventing materials, dialysis membranes, hemostatic materials, adhesive materials, injectable bulking materials for cosmetic and functional restorations, sealants, contact lenses, gene delivery vehicles, film forming creams; membranes, super absorbents, conductive electrode gels; lubricious coatings, hydrophilic coatings, materials for tissue regeneration, microcapsule materials and drug delivery systems. Due to the expanding applications of polysaccharides in gel form, processes are being developed to prepare new products based on polysaccharides [86]. Preparation of hydrogels by cross linking polysaccharides with polyfunctuional cross linking agent applicable to plastic and cosmetic surgery is also reported [87]. Massia et al. has described preparation of a hydrogel consists of acrylyol-modified dextran molecules and acrylyol-modified hyaluronan products cross-linked to form a hydrogel conjugate. Such hydrogels are useful in medical applications including the prevention of surgical adhesions, controlled drug delivery, tissue coatings, tissue adherence, and tissue supporting structures, and the coating of medical devices and related articles prior to placement within a patient [88].

Use of an agar gel in topical dressing for coating a burn area or other area of skin impairment by mixing with alginate, diehylene glycol and water is reported by Pellico [89]. E.M. Hassan describes a non-staining, pharmaceutically useful composition of ionic complexes made between the cationic polymer chitosan or its derivatives and the small anionic iodine-iodide complex (ICIC's). This was found to have better skin biocompatibility than povidone iodine and effectively promoted wound healing [90].

I.11 Polysaccharides based film preparation and surface modification

Preparation of polysaccharides thin films for multiple applications is an important mode of modification of polysaccharides. Patent literatures are available for the preparation of thin films based on starch and iota carrageenan [91]. Polysaccharides based biodegradable thermoplastic materials are also been patented. Surface modifications of polysaccharides are also reported by exposing the surface to actinic radiation [92]. Transparent, stable artificial extracellular protein films prepared by isocyanate cross linking is also reported [93]. Manufacture and uses for low molecular weight agar and agaroids reported by acid hydrolysis of high gel strength agar, such agars are useful for applications as skin moisturizers [91].

Patent applications are also available, which describes an invention of a medical hydrogel, made from polymerized polysaccharide macromers. The macromers are preferably polysaccharides decorated with polymerizable groups, for example, methacrylates. The macromers may also be made into polymers of at least two macromers polymerized together [94]. Semirefined κ-carrageenan based thin films in presence of various additives has been prepared by Ghosh et al. [95].

Surface modification of polysaccharides are also reported by contacting the surface with a selected organic modifying agent while irradiating the surface with ultraviolet and/or visible light that can be absorbed by the modifier. Surfaces are also can be modified by reacting with diazo compounds. Polymers with modified surfaces are useful for absorbing biologically active molecules, composites, printing plates and electronics [96]. A super absorbent polysaccharide based thin film can be prepared by cross linking a polysaccharides or derivative there of with a flexible spacer having long chain length and a terminal activated group [97]. Invention also describes preparation of a biodegradable thermoplastic material comprising a natural polysaccharides, plasticizer and a clay [98, 99].

Gennadios used κ-carrageenan along with dextrin to form a gelled composition (usually a film) enclosing active ingredients, which is water-soluble, they defined it as “Non-gelatin substitutes for oral delivery capsules, their composition and process of manufacture” [100].Sadaji Yamashita (Kanagawa-ken, JP) and Seiki Harada (Kanagawa-ken, JP) described the production of sustained release capsule and method for preparing the same, using seaweed polysaccharides (alginate and carrageenan) besides other gums, produced from terrestrial plants along with polyhydric alcohols [101]. Ninomiya et al. have reported “Edible film and method of making same” wherein they prepared heat-sealable edible films comprising at least a film layer containing a water-soluble polysaccharide (carrageenan along with polyhydric alcohol) as the principal component. These edible films are useful in sealing or packaging powdery foods, granular foods, dry solid foods, oily foods and the like [102].

Konjac (glucomannan), a gelling and thickening agent, being a powerful film former - both alone and in combinations with other gums such as carrageenan has been investigated [103]. Edward Zbygniew Nowak have described production of capsules comprising thermoplastic film of foamed modified cellulose material, preferably hydroxypropylmethyl cellulose. The foamed material dissolves rapidly in the mouth of consumer, releasing the capsule contents into the consumer's mouth [104]. Sun Y. Park et al. have reported production of biopolymer composite films based on κ-carrageenan and chitosan in presence of various organic hydroxy acids (e.g. citric, ascorbic and malic acids) as well as a plasticizer, and studied the properties of the films [105]. Dong Su Cha et al. have described the preparation of antimicrobial films based on Na-alginate and κ-carrageenan. They studied the antimicrobial activity, tensile strength and elongation properties of the films [106]. K.Nakamura et al. have reported the thermal properties (DSC) of water insoluble alginate films in presence of di- and trivalent cations (e.g. Cu (II), Al (III) and Fe (III)) which were used to replace the sodium (I) ion in sodium alginate [107]. Mention may be made of polyethylene glycol alginate based microcapsules for oral delivery of hirudin by Chandy et al. [108]. Microcapsules derived from polyelectrolyte complexes of sodium alginate with chitosan have been described by K. Y. Lee et al. [109].

I.12 Physical Modification of Polysaccharides

Attempts have been made to modify physically various polysaccharides, in this attempts various interaction of various substrates with polysaccharides are being studied [110]. Physicochemical and rheological studies on the interactions between polysaccharides and surfactants/fatty acids are also reported in the literature [111]. Interaction between hydroxymethyl cellulose and anionic surfactants in hexane, octane medium by dynamic surface tension measurements reported by Avranas et al. [112]. Interaction of kappa carrageenan with proteins is being widely studied due to the increase application of carrageenan in foods, viz. in beverages, as stabilizer and structure provider [113]. Interaction of alginic acid with surfactants are being studied by fluorescence [114]. Interaction of carrageenan with surfactants are also been reported [115]. Due to the increase consciousness for environment, polysaccharides are too exploited for the derivatives which can give directly surfactant properties, in this respect sea kelp

(A*hnfeltia concinna*), is also being used as ingredient for commercial hair and body wash [116]. Prasad et al. has reported interaction of agar gel in presence of ionic and nonionic surfactant, where decrease in the gelling temperature of agar gel in presence of SLS is demonstrated. The concept of decrement of sol viscosity in presence of nonionic surfactant was utilized in the extraction of agar from agarophytes where usage level of process water was reduced considerably [117].

I.13 Enzymatic Modifications

Considerable efforts are currently being directed in industry at the development and improvement of enzymic processes for the production of various microbial exopolysaccharides, e.g., curdlan, dextran, pullunan and xanthan gum [118]. The conversion of polysaccharides into fuels, solvents and other useful chemicals such as sucrose and the converse transformation of simple chemicals like methanol to polysaccharides for microbial oil recovery as well as isolation of some new type of lipo polysaccharides with emulsifying properties are also reported [119]. Debranching enzymes can selectively remove intact branch residues, such as 1-6 linked branches of (1-4), (1-6) α-D-glucans. The activity of synthetic enzymes may involve a) the transfer of oligosaccharides b) the cyclisation of oligosaccharide sections c) the modification of functional groups, such as oxidation of hydroxyl groups d) The transfer of non carbohydrate moieties such as sulphates. Important enzyme used for this type of modifications are Galactose oxidase which oxidises pro-hydrogen to aldehydes. Carbohydrate transferases (exo-glycosidases) which proceed according the reaction

$$\underset{\text{donor}}{\text{R-O-R'}} + \underset{\text{acceptor}}{\text{R''OH}} \rightleftharpoons \underset{\text{product}}{\text{R-O-R''}} + \underset{\text{by-product}}{\text{R'OH}}$$

The transfer may involve any acceptor hydroxyl function, but for the case of aldohexoses, the primary hydroxyl group is preferred [120]. A Japanese patent describes the preparation of a-glucosylated pullunan derivative using cyclodextrin glycosyltransferase or iso amylase [121].

I.14 Use and Potential Applications of Modified Polysaccharides

Concomitant with the expanding commercial utilization of polysaccharides, there is a growing demand for synthetic methods which facilitate selective structural modifications in order to effect, or ideally tailor, product properties such as viscosity [122], hydrophobicity/hydrophilicity [123,124], polyelectrolyte characteristics, gelation and metal chelating capacity [125,126,127]. One of the most important applications of selective modifications is the synthesis of analogues of natural polysaccharides, such as dextran, heparin or Xanthan Gum for purposes of reducing polymer cost, improving physical properties or ultimately structural proof for the native polymer [128,129,130,131]. Polysaccharides have also been modified for antitumor, blood anticoagulant activities for biomedical applications [20]. Preparation of conjugates of polysaccharides and biological substrates (proteins, enzymes), which involves minimum cross linking and extraneous chemical reactions may be used for affinity partition and immunology [20,132,133,134,135]. Selective modifications of polysaccharides were used for the preparation of permeable membranes with higher selectivity [136], matrices for drug delivery [137] and controlled release [138] formulations as well as for various processes relating to recovery of biological materials such as preparation of substrates for affinity chromatography [139], partitioning [140], electrophoresis [125], enzyme and cell immobilization [141].

Modification of polysaccharides have considerable importance in the synthesis of branched polysaccharides, where the branch type and length, stereochemistry is easily controllable and would help in the studies of structure-property relationship [142-146]. Carbamate derivatives of some polysaccharides have been reported to be useful for separation applications in paper industries [147]. Table I.2 summarizes the important polysaccharide derivatives and their applications.

I.15 SUMMARY

Considerable numbers of chemical and enzymatic methods are now available for the selective transformations of many polysaccharides including the seaweed polysaccharides like agar and carrageenan. In view of the inadequacies of most of these techniques, in terms of their achievable degrees of selectivity and conversion efficiencies, further advancements are obviously required, particularly for seaweed polysaccharides are susceptible to acidic pH conditions. Suitable mild oxidizing, acylating, alkylating agents as well as suitable protecting agents are required to be developed for cost effective modification of polysaccharides. These developments would provide further understanding of the fundamental structure-property relationships of the polysaccharides, which would be useful in designing novel products for newer applications including the functional ones.

Table I.2 Important polysaccharide derivatives and their applications.

Polysaccharides Derivative	Main application
Sodium carboxy methyl cellulose/CMC	In textiles industries, In detergents, in food applications as thickeners, In pharmaceuticals as tablet coaters and in cosmetics as stabilizers for hand lotions, as drilling fluids, in paper industries and as adhesives, as protective coatings, in agriculture.
Ethyl Cellulose	As toughness provider in lacquers, screen process inks, in gravures and flexographic inks, and adhesives, as hot melt plastics in combination with waxes and resins, as wrapping material.
Hydroxy propyl cellulose	As secondary stabilizer in suspension polymerization of vinyl chloride, as thickener in solvent based adhesives, alcohol based hair dressings, grooming aids, perfumes, colognes, inks, methyl chloride based paint removers, in electrical insulations.
Hydroxyethylcellulose	Thickeners in latex paint, as protective colloid in the production of vinyl acetate homopolymer latexes, in acrylic emulsions, in hydraulic systems based on Portland cement .
Cellulose triacetate	Mostly in membranes.
Alkyl, Acetyl derivatives of agar	As microbial or bacterial growth media.
Divinyl sulphone derivative of agar	For chromatographic separations.
Low gel strength agar or cream agar	In skin care lotions, as a moisturizers, massage gels, hair fixers, active carriers of pharmaceuticals applied through skins.
Potassium modified carrageenan	As pet foods, viscosity enhancer, stabilizer in ice creams, in shoe polish.
Hydroxy ethyl Starch	In paper industry as binder, as water soluble films for packaging.
Hydroxy propyl ethers	As food starches, as thickener for both water and milk based products, viscosity stabilizers.
Cationic Starch	As wet end additive in paper industry, as surface size for papers, to flocculate suspensions of negatively charged organic and inorganic particles.
Starch Phosphate	As wet end additive (with alum) in paper industry, in the preparation of amphoteric starches for wet end applications, in the preparation of adhesives and in foods.
Starch Acetate	To retard retrogadation, for surface sizing of papers, in food manufacture, in warp sizing in textiles.

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PART – II

PHYSICOCHEMICAL AND RHEOLOGICAL STUDIES ON AGAR EXTRACTED FROM *GELIDIELLA ACEROSA* COLLECTED FROM VARIOUS LOCATIONS OF WEST AND SOUTHEAST COASTS OF INDIA IN DIFFERENT SEASONS

CHAPTER II.1

AGARS OF *GELIDIELLA ACEROSA* (FORSSKAL) J. FELDMANN & G. HAMEL (RHODOPHYTA, GELIDIALES) OF WEST AND SOUTHEAST COASTS OF INDIA

II.1.1 INTRODUCTION

Carbohydrate polymers extracted from seaweeds are currently used for biological and industrial applications. The biopolymers that are extracted from seaweeds are increasingly exploited in diverse application areas including biotechnology. To fulfill the requirements for such applications, the purity as well as characterization followed by statutory evaluations of the product is of prime importance [1]. Among the polysaccharides extracted from algae, agar is the one that is widely used for biotechnological purposes [2,3]. Agar is the phycocolloid of most ancient origin. In Japan, agar is considered to have been discovered by Minoya Tarozaemon in 1658 and a monument is Shimizu-mura commemorates the first time it was manufactured. Originally, and even in the present times, it was made and sold as an extract in solution (hot) or in gel form (cold), to be used promptly in areas near the factories; the product was then known as *tokoroten.* Its industrialization as a dry and stable product started at the beginning of the 18th century and it has since been called *kanten*.

The name agar originated in Indonesia and referred to red seaweed of the genus *Eucheuma* though it really refers to the phycocolloid of these seaweeds [4]. Agar eventually became known for its use as a gelling agent and medium for culturing microbes. In 1882, Koch was the first to use agar in microbiology. Agar was traditionally used in Europe for preparing jams and jellies. These efforts led to the discovery of many agar-bearing seaweeds and to the development of commercial industries for agar production in Europe, Africa and North and South America [1]. By the early 1900s, agar became a gelling agent 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 production by modern industrial freezing techniques was initiated in 1921 in California, U. S. A. by a Japanese 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 artificial freezing process in all seasons because the natural winter freezing process is not possible in India.

DESCRIPTION OF AGAR

In 1945 Dr. Tseng defined agar as "the dried amorphous, gelatin-like, non-nitrogenous extract from *Gelidium* and other agarophytes, being the sulfuric acid ester of a linear galactan, insoluble in cold but soluble in hot water, a one per cent neutral solution of which sets at 35°C to 50°C to a firm gel, melting at 80°C to 100°C."

The United States Pharmacopoeia (1980) defined agar as "the dried hydrophilic colloid extract obtained from *Gelidium cartilagineum*, *Gracilaria confervoides*, and related algae of the class Rhodophyceae".

Araki (1966) referred to agar as a gel-forming substance obtainable from certain species of red seaweeds called "agarophytes" composed of neutral gelling molecules, agarose, and to a lesser extent acidic non-gelling molecule, agaropectin. He also noted that agaropectin is closely related to agarose as it has a similar backbone structure. Araki's definition of agar involved the chemical structure of the polymers, but it was an over simplification of the complex continuum between neutral and highly charged polymers existing in algae [5].

CHEMISTRY OF AGAR

The chemical components of agar had been analysed from mid 1800s to mid 1900s by many researchers, who verified that it is consisted of D-galactose, 3,6-anhydro-L-galactose and sulphate. Subsequently, the substituted galactose such as methylated, sulphated and pyruvated galactoses were also proved to be the constituents of the agar molecule. Araki offered the evidence proving the heterogeneity of the agar by separating the agar into two different polysaccharides named agarose and agaropectin using the acetylation method [5]. The agarose is virtually a neutral polymer, while the agaropectin is an acidic polymer. Later, Araki et al. and other researchers analysed agar by acid hydrolysis and enzymatic degradation and isolated agarobiose and neoagarobiose, respectively. This revealed that agarose is composed of agarobiose repeating disaccharide units alternating with 1,3-linked-α-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactopyranose. The agaropectin seems to have the same backbone as the agarose, but contains considerable amount of acid groups such as sulphate, pyruvate and glucuronate groups.

During the period 1960s to 1980s, application of the new techniques in the study of agar such as fractionation, ion exchange chromatography, enzymatic degradation and especially ^{13}C-NMR spectroscopy permitted a more precise study of the basic chemical structure and distribution of the repeating units in various agars.

Recent fractionation studies by Yaphe et al. on DEAE-Sephadex A-50 column indicated that agar is not made up only of one neutral and one charged polysaccharide but is composed of a complex series of related polysaccharides which range from a virtually neutral molecule to a highly charged (sulphated) galactan [6]. The neutral polysaccharide has gelling ability and approaches the structure of an ideal agarose, which still contains a trace of sulphate (0.1 to 0.5%) and pyruvic acid (0.02%). More recently, ^{13}C-NMR spectra of the carbon atoms in agarose contents in agars isolated from different species of *Gracilaria*, the structural feature of various forms of alternating disaccharides has been ascertained [7].

^{13}C-NMR spectroscopic studies of agars from *Gelidiella*, *Laurencia* and *Porphyra spp*. are reported. The former two algae were found to contain agar composed mainly of 6-OCH_3-agarobiose with high content of methoxyl group and good gel strength, while *Porphyra spp*. has a higher content of biogenetic precursor of agarose, 1,3-linked-2-D-galactopyranose alternating with 1,4-linked-β-L-galactopyranose-6-sulphate, which has no gelling ability [7]. But after alkali modification the spectrum of the polymer exhibits the signals of 12 carbons typical to agarose, possessing high gel strength. Infrared spectroscopy has been employed in determining the structure of agar [8].

All the above analyses indicate that agar is made up of family of low sulphated galactans constituting the cell walls of marine algae that belong to the genera *Gelidium*, *Gracilaria*, *Ahnfeltia* and *Pterocladia* [9-12]. A 1.5% solution of agar is clear and when it is cooled to 34-43°C it forms a firm gel, which does not melt again below 85°C. It is a mixture of polysaccharides whose basic monomer is galactose. These polysaccharides can be sulphated in variable degrees but to a lesser degree than in carrageenan. For this reason the ash content is below those of carrageenan, furcelleran (Danish agar) and others. Backbone of agar is shown in Fig. II.1.1.

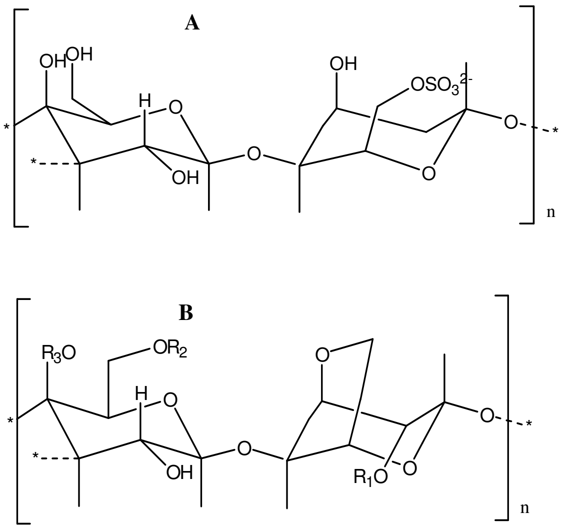

Fig. II.1.1 Disaccharide repeating units of agar. (A) Biogenetic precursor of agarobiose (*Porphyra haitanensis* and *Gracilaria blodgettii*); (B) Various agarobioses.

1: $R_1=R_2=R_3=H$	agarobiose (Alkali-treated *Gracilaria asiatica*, *Porphyra haitanensis*)
2: $R_1=R_3=H$, $R_2=CH_3$	6-OCH_3-agarobiose (*Gracilaria verrucosa*)
3: $R_1=CH_3$, $R_2=R_3=H$	2-OCH_3-agarobiose (*Gracilaria eucheumoides*)
4: $R_1=R_2=CH_3$, $R_3=H$	2, 6-di-OCH_3-agarobiose (*G. eucheumoides*)
5: $R_1=R_2=H$, $R_3=SO^-_3$	4-CSO_3-agarobiose (*G. eucheumoides*)
6: $R_1=CH_3$, $R_2=H$, $R_3=SO^-_3$	2-OCH_3-4-OSO_3-agarobiose (*G. eucheumoides*)
7: $R_1=R_3=H$, $R_2=SO^-_3$	6-CSO_3-agarobiose (*G. textorii*, *G. damaecornis*, *Gloiopeltis furcata*)

SOURCE

Agarophytes, the red seaweeds used as the raw material for manufacturing agar, mainly belong to the genera *Gelidium* (*Gelidiaceae*), *Gracilaria* (*Gracilariaceae*), *Pterocladia* (*Gelidiaceae*), and *Ahnfeltia* (*Phyllophoraceae*) in different countries. *Gelidium* yields the best quality of agar, but its cultivation is difficult and its natural resource is less than *Gracilaria*, which is being cultivated in several countries and regions in commercial scale. *Pterocladia* and *Ahnfeltia* grow only in a few regions and are utilized only in New

Zealand and Russia, respectively. *Gracilaria*, the most abundant and promising resource of agar production, has probably more than 150 species, distributed mainly in the temperate and subtropical zones. Some of them are cultivated on large scales in Chile, Taiwan, Vietnam, and to some extent in Thailand [13].

Armisen has reported that the estimated production of dried agarophytes in India is 600 t dry weight of *Gracilaria* and 300 t dry weight of *Gelidiella*, while the production of *Gracilaria* in South Korea is 50 t dry weights. Chile has the highest production (6800 t dry weight) of *Gracilaria* [13].

Gelidiella acerosa (Forsskal) Feldmann *et* Hamel is a warm water tropical alga. It occurs in Indian, Pacific and Atlantic Oceans and is one of the main sources of raw material for the manufacture of agar in the Philippines [14]. Moreover, limited information is available in the primary literature on the characteristic of agar from *Gelidiella acerosa* [15]. The world's first source of agar was *Gelidium* from Japan in the beginning of the seventeenth century, but at the turn of the twentieth century the increasing demand for phycocolloid exceeded the supply of this alga. Since then *Gracilaria* has played an important role in the production of agar [16].

INDIAN RESOURCES

Gelidiella acerosa and *Gracilaria edulis* are the two principal agarophytes utilized by the Indian agar industry. Various species of *Gracilaria* have been exploited in India to produce food grade agar. The most important problem faced by the seaweed industry, viz. paucity of raw material. The most notable reports are those of Verma et al. [17], who estimated about 335 of wet *Gracilaria*, about 19 tons of wet *Gelidiella* that can be harvested from the reef areas around Pamban. Desai estimated that about 5 tons of wet *Gelidiella* and 20 tons of dry *Gracilaria* could be harvested from the Gulf of Kutch [18]. He also reported that an estimated 300 tons of dry harvestable *Gelidiella* and about 3000 tons of dry harvestable *Gracilaria* from the Gulf of Mannar.

The agar obtained from these seaweeds especially members of *Gelidiaceae* is of superior quality and widely used in a number of preparations in biomedical, food, cosmetics and pharmaceutical industries [13]. But due to the poor quality of agar obtained from *Gracilaria* in India researchers concentrated on the extraction of agar from other varieties of seaweeds of family Rhodophyceae. *Gelidiella acerosa* is one of the red seaweeds, which has been reported to be a source of good quality agar [13]. Agars are industrially important for their excellent thickening or gelling abilities [19, 20]. The physical properties and texture of agar gel are significant factors in determining its application, especially in food industry. In India *Gelidiella acerosa* and *Gracilaria edulis* are the two principal agarophytes utilized by the Indian agar industry. *Gelidiella* is emerging as the preferred source as it produces superior quality of agar with high gel strength of 600-850 g cm^{-2} (unpublished results). According to one estimate during 1979 to 1995 the average seaweed landings in India were as follows: *Gelidiella acerosa*, 270 tonnes dry wt, and *Gracilaria edulis,* 320 tonnes dry wt [21]. Another report indicates a total standing biomass of *Gelidiella acerosa* and *Gracilaria edulis* in India to be 5000 tonnes wet weight per year [22]. Many authors have reported on the extraction of agar from *Gelidiella acerosa* of Indian waters, which has, however, been sketchy and there exists no report on the systematic studies on *Gelidiella acerosa* for their agar contents [23-25]. In this connection mention may be made to superior quality agar from Indian red seaweed by Deoki from CSIR [26] wherein preparation of agar is reported from *Gelidiella acerosa*. Y.A Doshi has reported agarose preparation from agar wherein it is described that agarose is prepared by soaking agar from *Gelidiella acerosa* seaweed with *Hypnea*, and agar like highly sulfated polysaccharide from *Hypnea musciformis* seaweed, [27]. Siddhanta et al. have prepared a very high grade agarose from *Gracilaria* spp. [28].

RHEOLOGICAL PROPERTIES

Rheology is defined as the science of deformation and flow of matter in response to the application of mechanical force. It generally includes materials that show both deformation and flow under the influence of mechanical force. It too includes materials such as solid emulsion paints or thermoplastic polymers, which become liquid under the influence of stress and increased temperature, respectively. Polysaccharides such as cellulose, starch, alginates, chitosan etc. have been widely studied for their rheological behaviours as these materials exhibit viscoelasticity; i.e., both irreversible (energy dissipative processes, viscous flow) and reversible (energy storage processes, elasticity) changes [29].

Rheology of polysaccharides has been widely investigated. Selva et al. has reported viscoelastic behaviour of galactomannans on pectin/calcium network [30]. Hargreaves et al. has done some study on aggressive, viscoelastic property of gluten from some generic variation of bread wheat [31]. Rheological study of casein-guar mixtures has been reported by Lefevre et al. [32]. Rheological and thermal properties of agar fractions from various agarophytes is also reported by Lii and Lai [33]. Falshaw and Furneaux have reviewed various studies on rheological properties of carrageenan along with other polysaccharides [34].

For hot water soluble polysaccharides like agar, carrageenan., rheological studies can provide important information on the stability of gels under applied stress and strain conditions, dynamic viscosities, variation of loss and storage moduli with frequency, time and temperature. Rheological properties of thermoreversible gels were correlated with the molecular characteristics of junction zones formed by ordered helices [35, 36]. For example, the storage modulus (G′) of a gel is proportional to the average number of junction zones within the gel network. Clark showed that the dependence of the modulus on polysaccharide concentration increased with the average number of double helices participating in a junction zone [37]. Recently, changes in dynamic storage and loss moduli (G′ and G″) accompanying the development of a gel structure have been examined to clarify the sol-gel transition mechanism of the gel [37,38]. Hurtardo-Ponce and Umezaki have reported rheological properties of agars from *Gracilaria* spp. occurring in the Philippines. They were also successful in producing gels with best rheological properties among the species tested [39]. The variation of G' as a function of agarose concentration and temperature were studied by Watase et al. [40]. Tobitani et al. has optimized rheological parameters for carrageenan gel in skimmed milk [41].

Viscosity. The phycocolloids are viscous in nature and the type of raw material and processing conditions affect the viscosity of agar and other phycocolloid dispersions. The viscosity of agar dispersion at 45^{o}C is relatively constant from pH 4.5 to pH 9.0 and is not greatly affected by age or ionic strength within the limits of pH 6.0-8.0 [16]. Once gelation begins, however, viscosity at constant temperature increases with time as well as with decreasing temperature. Viscosity change (decrease) with shear rate shows the gel thinning property.

Moduli (G' and G"). It is clear, that the G' (storage) and G" (loss) depend on temperature, frequency and shear rate. G' decreases with increasing temperature while G" increases with increasing temperature. There exists a linear relationship between the changes in time and frequency with those of G′ and G″, while G’ always remains higher than G″.

APPLICATION/USES OF AGAR

Food industry. 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, meringues, icings, cookies; cream shells etc. Agar is useful in low-calorie breads or biscuits since it is non-nutritive, because it acts as a bulking agent [16].

Microbiology. Agar is 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^{o}C, and melting temperature of 75-120^{o}C [20].

Miscellaneous. Agar is used as impression materials (in prosthetic dentistry, criminology, tool making and other fields), Medicine and pharmaceuticals, electrophoresis, photographic stripping films, cosmetics, lotions, papers, biodegradable thin films for wide uses and laboratory applications etc [19].

Formulations. Agar is very useful in preparing strong water gel systems for forming molds, or for creating molds themselves. For emulsification, agar is generally useful in difficult to handle products, as well as where a very thick emulsion is desired. Because agar is a polysaccharide having high molecular weight, it is useful for the flocculation of large molecular weight proteins and other polysaccharides. Its typical uses are in icing stabilizers; dental molds; culture media; nutrient agar; desoxycholate agar; endo agar etc.

In the course of our continuing studies on evaluation of agarophytes of Indian waters, it has been observed that *Gelidiella* produces very good quality of agar (with high gel strength in the range of 600-1400 g cm^{-2}) in 20-30% yield (with respect to bone dry seaweed), employing an improved process developed in our laboratory (unpublished results).

In the course of our studies it has been observed that the quality of agar extracted from *Gelidiella acerosa* occurring in the southeast coast of India is of superior quality [42]. Due to the relatively higher natural biomass of *Gelidiella acerosa* in the west and southeast coasts of India, we have selected these two localities for comparison of the quality of agar in the present investigation.

In this investigation isolation of high quality agar from *Gelidiella acerosa* occurring in Indian waters, which is suitable for bacteriological and molecular biology works as well as its rheological characterization are reported for the first time. The agar obtained from the *Gelidiella acerosa* of southeast coast has been found to be of superior quality than that obtained from the west coast of India. These results would be useful in bioprospecting of agarophytes occurring on the Indian coasts.

II.I.2 MATERIALS AND METHODS

II.I.2.1 Collection

Gelidiella acerosa was collected from the following places on the West coast: Okha (22°28′N, 69°05′E), Dwarka (22°27′N, 69°12′E), Veraval (20°54′N, 70°22′E), Porbandar (21°38′N, 69°37′E) and Diu (20°45′ N, 70°58′E); Southeast coast : Pudumadam (9°16′N, 9°19′E), Krusadai island (9°16′N, 79°19′E), Ervadi (9°15′N, 78°58′E), Sullimunthal (9°15′N, 78°58′E), Kilakarai (9°16′N, 79°19′E) in the month February to April 2001. Specimen of each sample (AS-IV-79-11, AS-IV-79-13, AS-IV-83-10, AS-IV-83-12, AS-IV-84-1, AS-IV-84-2, AS-IV-84-3, AS-IV-143-07, AS-IV-143-08, AS-IV-144-01, AS-IV-144-02, AS-IV-144-04, AS-IV-144-05, AS-IV-142-09, AS-IV-148-02, AS-IV-143-06) has been deposited with the CSMCRI Herbarium for future reference.

II.I.2.2 Extraction and isolation

Dried material of each sample was subjected to extraction adapting the method described by Roleda et al. [43] with modification. For extraction, 10 g of dry seaweed as received was soaked in 300 ml of water for two hours followed by acid treatment for one hour. This was then washed with tap water to remove all the adhering acids, further soaked for another half an hour to make it free from all leached acids. Further autoclaved by adding 300 ml distilled water for 90 min at 120 °C under 22 psi pressure. The mixture was homogenized by grinding and 300 ml more water was added to make the extractive filterable, celite powder was added and filtered on a celite bed and what Mann filter paper

No 541 in hot condition. The filtrate was gelled at room temperature. The agar was purified by three consecutive freeze-thaw cycles.

II.I.2.3 Physicochemical analyses

Total Sugar. Total sugar in the agar samples were measured by colorimetric method following the method of Dubois using glucose as standard [44].
Range of the method: 10-100 μg.
Sample preparation: 10-20 mg sample was solubilised in 10 ml of distilled water
Reagents: Std. Glucose solution: 50 μg AR grade glucose/ml distilled water.
AR Grade sulphuric acid
5% phenol solution : 5 g AR grade phenol in 100 ml distilled water.

Method For standard chart, 0.1-1.0 ml aliquots of glucose were taken. Volume was made up to 2 ml with distilled water. 1.0 ml 5 % phenol was added to each of the test tubes. Tubes were kept in ice bath. 5 ml conc sulphuric acid was added in one shot to each of the test tubes. Tubes were cooled. Further the tubes were incubated at room temperature for 30 min. UV absorbance were taken at 485 nm.

3,6-anhydrogalactose. 3,6-anhydrogalactose content of the agar samples were estimated by turbidimetric method described by Yaphe and Arsenault using fructose as standard [45].
Range of the method 8μg-32μg.
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 warned to dissolve benzoic acid).
Working Solution: 3.0 ml of stock solution to 100 ml with distilled water.
2. Acetal Solution : Stock solution : 82 mg acetal (100 μl) in 10 ml distilled water.
Working solution : I ml of stock solution 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 a icebath. 10 ml of resorcinol - acetal reagent was added to each of the tubes. Kept at 20°C for 4 min. These tubes were transferred to water bath at 80°C for 10 min. Further the tubes were cooled in icebath. 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.

Sulphate. Sulphate content of the agar samples was estimated by turbidimetric method using potassium sulphate as standard [46].

Range of the method 20µg-200µg .Agar samples were hydrolysed in 1.0N HCl at 110°C for 17 h in a sealed tube, hydrolysate was centrifuged at 5000 rpm for 20 min and supernatant was used for analysis.

Reagents

1. Stock solution : 360 mg Potassium sulphate in 100ml distilled water (working solution: 1:2 dilution of stock solution gives approximately 99µg of sulphate per 0.1ml).
2. Barium chloride- Gelatin solution: 100 ml of 0.5% gelatin solution was prepared and kept at 4°C overnight and 0.5 gm of Barium chloride was added to that and allowed to stand for 3-4 h before use.
3. 4% tricholoroacetic acid (TCA) in distilled water.

Method Aliquots of potassium sulphate working solution were taken upto 0.2ml 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 with 0.2ml 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 solution was prepared with a particular aliquot of agar sample and volume was made upto 0.2ml with 1N HCl and TCA (3.8 ml) was added followed by gelatin-barium chloride solution (1ml). The UV absorbance was measured (E) at 360 nm against a blank solution containing 1N HCl (0.2 ml), TCA (3.8 ml) and gelatin-barium chloride solution (1ml).

In the second set of experiment, each experimental solution was prepared with same sample aliquots volume which was used for set "A" experiment and volume was made upto 0.2 ml with 1N-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 1N-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 hydrolysed agar sample is other than sulphate.

Gel strength measurements

A 1.5% solution (50 ml) of agar was prepared in an autoclave at 100°C. After the formation of gel at room temperature the gel 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). The gelling and melting temperatures were measured according the method described by Craigie et al. [47]. For measurement of gelling temperature, 10 ml sol of agar was allowed to cool gradually and a thermometer was emerged in the sol. The temperature at which the thermometer was fixed to the gel was noted. For melting temperature the gel was heated on a water bath and one iron ball (ca 1g of weight) was placed on the surface of the gel. The temperature at which the ball touched the bottom of the tube was noted.

Apparent viscosity. Apparent Viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No.1 at 60 rpm was used for measuring apparent viscosities of agar samples (1.5% in de ionised water) at 80°C.

Foreign starch, foreign insoluble matter, hot and cold water solubility, water absorption were done using standard methods [48]. The methods of analyses are described below.

Methods

Foreign starch. 0.2 % (w/v) of agar samples were prepared in distilled water by boiling. Then 0.1% iodine in ethanol was added at room temperature. Appearance of blue color indicate presence of starch.

Foreign insoluble matter. 0.2% (w/v) of agar samples were dissolved in distilled water by boiling. Which was further filtered through Whatmann filter paper No. 541 of known weight. This is then dried in oven at 50-60°C for 3 h. The difference in weight gave the amount of foreign insoluble matter.

Cold and hot water solubility. A known amount of agar samples were soaked in cold water under constant stirring for 2-3 h, then filtered carefully and the agar was dried and weighed to know the amount of agar dissolved, alternatively TDS of the filtered water was determined to know the amount of agar dissolved. Similarly a known amount of agar sample was dissolved in hot water by boiling and filtered carefully. Further the filtrate was dried and amount of agar filtered or dissolved was determined.

Water absorption. A known amount of agar was soaked in water for 2-3 h. Weight gained by the sample after press dry indicated the amount of water absorbed by the sample.

Weight average molecular weight of the samples were determined, by using the Mark–Houwink equation for agarose as described by Rochas and Lahaye, 1989 [49].
$[\eta] = 0.07\ M^{0.72}$, where $[\eta]$ is ml/g.,

II.I.2.4 Rheological measurements

Samples of sol and gel were prepared by dissolving agar in de mineralised water by heating. 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 and 70°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 applying 0.05% strain, the temperature of gel being maintained at 25°C using the DC50 water circulator. Measurements were carried out immediately after placing gel sample on the plate/plate. To avoid loss due to evaporation at higher temperature the outer surface of the samples were covered with silicon oil. All experiments were carried out in triplicates and slippage of gel due to applied stress was carefully avoided by selecting appropriate operation parameters.

II.I.2.5 Thermal and spectral analyses

Thermo gravimetric measurements (TGA) were carried on a Mettler Toledo TGA system, Switzerland, machine with agar gels (10-11mg) using a temperature programme (40°C to 180°C at a heating rate 5°C min^{-1}) in an air atmosphere.

IR Analysis. 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. All spectra were the average of two counts with 10 scans each and a resolution of 5 cm^{-1}.

II.I.3 RESULTS AND DISCUSSION

II.I.3.1 Physicochemical properties

Agar yields expressed as percentage (on the basis of bone dry seaweed as received from the field) ranged from 19-24%. Among the Agar samples from Gujarat coast the highest agar yields were obtained from *Gelidiella acerosa* collected from Veraval followed by those from Okha, Dwarka and Porbandar. High viscosity value (45 ± 2.5 cp at 80°C) was obtained for the 1.5% agar solution extracted from the *Gelidiella acerosa* collected from Krusadai island in the month of February 2001. On the other hand the product from Porbandar had the lowest viscosity (15 ± 2.5cp at 80°C). Gelling and melting temperature of the all agar products ranges between 37-42°C and 79-86°C respectively. Among the samples of west coast of India, the agar sample from Okha gave highest gel strength (400±25 g/cm^2) and from Porbandar gave lowest gel strength (225±25 g/cm^2) at 20°C in 1.5% gel. On the other hand, among the Gulf of Mannar samples the product from Krusadai gave highest gel strength (845±25 g/cm^2) and from Kilakarai gave the lowest gel strength (500 ±25) (Table II.1.2a). On comparison it was found that the among the 2001 collections, agar obtained from the *Gelidiella acerosa* of Okha was the best among the west coast samples and that extracted from the *Gelidiella acerosa* of Krusadai was the best among all and quality of agar from Ervadi was comparable to that with Krusadai Island. (Table II.1.1a & 2a). Agar samples have been extracted from *Gelidiella acerosa* collected from various locations in the Gulf of Mannar region during March 2002 to June 2002 and the quality of these agars were found to be better than those extracted from any other collections of *Gelidiella acerosa* of various locations in the west coast of India. The physicochemical properties of the various agar samples collected from various places in the Gulf of Mannar are collectively presented in Table II.1.2a and Table II.1.2b. The scrutiny of tables 1b and 2b reveal that less threshold gel concentration, more hot and cold water solubility, less ash content, less foreign insoluble material content in south east coast agar samples in comparison to that with west coast agar samples makes the former more suitable for applications in microbiology and biotechnology.

Table II.1.3 consists the intrinsic viscosity values for the agars which shows more value for southeast coast agar samples and obviously the molecular weight of these agar samples (Ervadi and Krusaidai Island) were higher in comparison to west coast agar samples. Weight average molecular weight for agar samples from south east coast were in the range (1.79-2.72) x 10^5D and that of west coast were (2.11-2.55) x 10^5D.

Table II.1.1a Physicochemical analysis of agar samples extracted from *Gelidiella acerosa* collected from west coast of India

Agar	Place/Date of collection	Yield[a] (%)	Gel Strength (± 25 g/cm^2)[b]	Total Sugar (%)	3,6-AG (%)	Sulphate (%)	Gelling T(± 2^0C)	MeltingT (± 2^0C)	Apparent Viscosity at 80^0 C (± 2.5 cp)
AS047805	Porbandar April 2001	21.0	225	31	27.0	1.7	37	79	15
AS047911	Okha April 2001	23.0	400	39	32.0	1.0	39	87	30
AS047915	Veraval April 2001	24.0	310	34	28.0	1.1	38	82	22
AS047913	Dwarka April 2001	23.0	300	36	28.0	1.0	39	80	19

Table. II. 1. 1b Physicochemical analysis of agar samples extracted from *Gelidiella acerosa* collected from west coast of India

Agar	Place/Date of collection	Thresh hold gel conc. (%)	Foreign insoluble matter (%)	Water absorption (Times of its weight)	Cold water solubility (wt%)	Hot water solubility	Ash (± 0.2) %	Weight average MW (x 10^5) D
AS047805	Porbandar April 2001	0.5	0.82	12	1.6	99.1	3.5	2.55
AS047911	Okha April 2001	0.3	0.69	16	2.3	99.3	3.0	2.11
AS047915	Veraval April 2001	0.5	0.75	14	1.6	99.25	3.8	2.6
AS047913	Dwarka April 2001	0.4	0.79	12	2.0	99.2	3.6	2.41

[a] Yields were determined on the basis of bone dry as received seaweed

[b] Gel strength was measured in1.5% aqueous gel at 20°C.

Table II. 1. 2a Physicochemical analysis of agar samples extracted from *Gelidiella acerosa* collected from southeast coast of India

Agar	Place/Date of collection	Yield[a] (%)	Gel Strength (± 25 g/cm^2)[b]	Total Sugar (%)	3,6-AG (%)	Sulphate (%)	Gelling T (± 2^0C)	Melting T(± 2^0C)	Apparent Viscosity (1.5%) at 80^0 C (± 2.5 cp)
AS04 8312	Krusadai Island February 2001	22	845	42	44	1.4	41	86	45
AS04 14307	Krusadai island March 2002	26	800	39	41	1.1	39	81	42
AS04 14802	Krusadai Island June 2002	28	600	37	35	1.2	40	84	36
AS04 8403	Ervadi February 2001	19	800	37	36	1.0	39	86	33
AS04 14308	Ervadi March 2002	28	730	42	43	1.0	38	84	34
AS04 14401	Ervadi April 2002	25	700	38	41	1.0	39	82	37
AS04 14402	Ervadi June 2002	20	720	40	40	1.1	40	86	33
AS04 8401	Sullimunthal February 2001	21	600	40	35	1.2	40	81	34
AS04 14404	Sullimunthal June 2002	29	450	35	39	1.2	38	84	34
AS04 8402	Kilakarai February 2001	21	500	37	35	1.0	41	84	38
AS04 14209	Kilakarai June 2002	26	465	38	41	1.2	37	81	30
AS04 14306	Pudumadam June 2002	26	600	38	41	1.2	37	83	32

[a] Yields were determined on the basis of bone dry as received seaweed

[b] Gel strength was measured in 1.5% aqueous gel at 20°C

Table II. 1. 2b Physicochemical analysis of agar samples extracted from *Gelidiella acerosa* collected from southeast coast of India

Agar	Place/Date of collection	Thresh hold gel conc. (%)	Foreign insoluble matter (%)	Water absorption (Times of its weight)	Cold water solubility (wt%)	Hot water solubility	Ash (± 0.2) %	Weight average (x 10^5) D
AS04 8312	Krusadai Island February 2001	0.21	0.19	24	6.0	99.8	1.7	2.72
AS04 14307	Krusadai island March 2002	0.25	0.20	22	5.4	99.8	2.0	2.64
AS04 14802	Krusadai Island June 2002	0.31	0.26	18	5.3	99.69	2.3	2.55
AS04 8403	Ervadi February 2001	0.25	0.26	18	5.4	99.75	2.0	2.31
AS04 14308	Ervadi April 2002	0.32	0.29	20	6.0	99.68	2.0	2.64
AS04 14401	Ervadi May 2002	0.3	0.2	20	6.0	99.7	1.8	2.61
AS04 14402	Ervadi June 2002	0.3	0.2	19	5.1	99.7	1.9	2.4
AS04 8401	Sullimunthal February 2001	0.33	0.35	20	5.0	99.77	2.5	2.1
AS04 14404	Sullimunthal June 2002	0.5	0.4	14	5.1	99.5	3.1	1.79
AS04 8402	Kilakarai February 2001	0.39	0.45	16	5.2	99.6	3.1	1.85
AS04 14209	Kilakarai June 2002	0.4	0.45	14	4.2	99.6	3.0	1.96
AS04 14306	Pudumadam June 2002	0.45	0.5	18	4.0	99.55	2.8	2.0

[a] Yields were determined on the basis of bone dry as received seaweed

[b] Gel strength was measured in 1.5% aqueous gel at 20°C

Table. II. 1.3 Intrinsic viscosities of better agar products of west coast and southeast coast of India

Agar	Place/Date of collection	Concentration (%)	Reduced Viscosity (ml/g)	Intrinsic Viscosity at 32 °C (ml/g)
AS047911	Okha April 2001	0.02	481	477.09
		0.04	485	
		0.06	483	
		0.08	491.39	
		0.12	495	
		0.13	500	
AS048312	Krusadai Is February 2001	0.02	574.93	572.73
		0.04	576	
		0.06	581.13	
		0.08	582.8	
		0.12	581.97	
		0.13	590	
AS048403	Ervadi February 2001	0.02	517.6	509.03
		0.04	520	
		0.06	522.5	
		0.08	536	
		0.12	540	
		0.13	555	

II.I.3.2 Dynamic rheological properties

Flow properties. The comparison of the steady rheological characterization of the two best samples of agar (from Okha and Krusadai island of 2001 collections) as well as three other samples from the west coast is shown in Fig. II.1.2. It may be noted that the variations in rheological properties of the all the agar samples obtained from the Gulf of Mannar region (Krusadai Is, Ervadi, Sullimunthal, Kilakarai and Pudumadam) were not significant. The dynamic shear viscosity (η) decreased with increasing shear rate (γ), corresponding to a shear-thinning behavior in agar samples as expected. The effect of shear was pronounced on agar from Okha in comparison to the agar from Krusadai island (Fig.II.1.2a and Fig.II.1.2b). This indicates that the agar extracted from *Gelidiella acerosa* collected from Krusadai Island is less susceptible to gel thinning than those of Okha and the other agar samples obtained from the west coast. Dynamic shear viscosity was measured at two different temperatures 70°C and 45°C (semisolid state). For experiments conducted at 70°C gel thinning behaviour was observed for all the agar sols. Newtonian behaviour was observed in low shear rates for all the agar gel samples. But agar from *Gelidiella acerosa* collected from Krusadai Island shows less gel thinning behaviour, in the other words these agar gels were more viscous than the other agar samples. Similar behaviour was observed at 45°C. Gel thinning behaviour was observed at higher shear rates for agar sols at 45°C, on the other hand at 70°C shear thinning was predominant through out whole range of applied shear rate.

Oscillatory rheological analysis. Fig. II.1.3a and Fig. II.1.3b shows the thermal variation in the small deformation moduli observed on cooling of 1.5% (w/v) various agar matrices. On cooling both G′ and G″ increased rapidly around 48-50°C. It has been observed that the increment in G′ and G″ in more for the agar gel samples from Krusadai Island and Ervadi, followed by Okha, Veraval, Dwarka and Porbandar, this indicates less flexibility or higher elasticity of these agar gels in comparison to that of Okha, veraval, dwarka and Porbandar. The increment is the lowest for Porbandar indicating weaker gel structure for this agar. A subsequent time sweep at 25°C (Fig.II.1.4) shows relatively weak modulus with no time dependence. It has been observed that there is no syneresis for the agar gel samples from Krusaidai Is, Ervadi and Okha (evident from steady G′ values with increasing time) and syneresis for the agar gel samples from Dwarka, Veraval and Porbandar (evident from decreasing G′ values with increasing time). Fig.II.1.5a and Fig.II.1.5b illustrate the response of the same samples to increasing the small angular frequency (ω) at 25°C. A gel like response (G′ subsequently higher than G″) is obtained with only slight frequency dependency of the moduli for the agars from Krusadai, Ervadi and Okha. But for agars from Dwarka, Veraval and Porbandar, the moduli is more frequency dependent indicating relatively weaker gels to the former. It was observed that the tan δ values were less than unity at different frequency values (1-10 Hz) this indicates that the frequency changes do not have any effect on the nature of agar gel samples. The tan δ values (G″/ G′) for agar obtained from Okha is more than that of agars from Krusadai as well as Ervadi indicating the presence of more viscous components in the former. At higher frequencies (more than 20 Hz) there is a change in the material's response showing noisy signals, indicating disintegration of the initial structure for all the agar gel samples studied.

II.I.3.3 Spectral analysis

FT-IR is an excellent, non destructive and easily accessible technique which provides important information for the chemical structure, specially location of the sulphated groups [50-53]. FT-IR spectra were recorded for the agar samples obtained from west coast and south east coast and are shown in Fig.II.1.6a and 6b. All the agar samples from west and southeast coast of India showed characteristic bands in the finger print region 500-1500 cm^{-1} (Fig.II.1.6). Both show characteristic bands at 740, 773, 930 cm^{-1} due to β-galactose skeletal bending. The band at 890 cm^{-1} is specific for agar and may be attributed to anomeric C-H stretching of β-galactose residues. The weak band at 895-900 cm^{-1} is due to β-anomeric-CH-deformation [54, 55]. Other bands observed were at IR (ν_{max} , cm^{-1}): 3416-3415 (s, O-H stretching), corresponds for the total sugar present [3], 1647-1619 (s, CO-NH-stretching), 1418-1460 (sh, C(6)-H of local symmetry), 1376-1422 (sh, C(6)-O local symmetry), 1380 (m) and 1154 (sh) (S=O stretching), 1076-1068 (s), C-O-C stretching, 932 (m), 3,6-anhydrogalactose ring vibration. 893 (m, β-anomeric-CH- deformation), 773 (w, β-galactose skeletal bending), 740-700 (w, β-galactose skeletal bending), 714 (w, β-galactose skeletal bending). Difference in IR bands observed between this two agar samples were the shape of OH band for agars from west coast is not as broad as that of southeast coast. As this band corresponds to the total sugar present in the sample, it indicate lower sugar content in west coast agar in comparison to south east coast, which is further evidenced from determination of total sugar (Table II.1.1a). Absence of band at 844 cm^{-1} indicates that C-4 sulphate is absent in both the agar samples. [Abbreviations for intensity of the IR bands: s = strong; sh= shoulder; m=medium; w=weak].

II.I.3.4 Thermal analysis

From TGA measurements it has been observed that the 1.5% (w/v) agar gel extracted from *Gelidiella acerosa* collected from west coast of India show 80 % mass loss at temperature ranges 90-110°C and those from Krusadai island and Ervadi at 120 –130°C. These show that the agar gels from Gulf of Mannar are thermally more stable than those from west coast of India. (Fig. II.1.7).

II.I.4 SUMMARY

Each of the agar gels investigated possessed properties that are required to be known for its specific end applications. All agar gels of this study were found to be of good quality as revealed by their physicochemical properties e.g. low sulfate (1-1.7%) as well as relatively low gelling (37-41°C) and melting (79-87°C) temperatures (Table II.1. 1a). Comparatively low 3,6-anhydrogalactose contents in the agars of west coast of Gujarat

(27-32%) and high 3,6-anhydrogalactose contents in the agars obtained from southeast coast (35-44%) are manifested in the gel strengths of the agars studied (Table II.1.1a and 2a). The agar gels obtained from *Gelidiella acerosa* collected from Krusadai island was found to be the best. The agars obtained from Ervadi collections were found to be as good as that obtained from Krusadai island. In general the agars obtained from the collections of Gulf of Mannar region are better in quality than those obtained from the west coast of India (Table II.1.1a and 2a). These agar gels, obtained from *G. acerosa* of Gulf of Mannar region, exhibited the highest breaking strength, highest degree of cohesiveness, rigidity and thermal stability (Fig. II.1.3a, Fig. II.1.3b & Fig. II.1.7). Stronger gels are of value because of their strength, resilience, elasticity, relative transparency, relative permanence and thermoreversibility [56]. Higher gel strength agars have been reported to be due to the presence of strongly gelling agarose fraction [5]. The agar extracted from *Gelidiella acerosa* collected from west coast of India produced gels of lower strength, cohesiveness, thermal stability and rigidity (Fig.II.3a, 3b, & 7). The usefulness of latter agars is limited to the weaker gels that are required by the food industry. Food grade agars have lower gel strength and are reported to be containing more agarose molecules that are substituted with sulfate or pyruvate [9].

Rheological properties of agar gels are difficult to evaluate especially when there is a lack of uniformity of measurement due to difference in the in the geometry of the instruments employed, methodology of gel preparation and testing [57]. Numerous reports have shown that the yield and physical properties of agar preparations depend on the source of agarophytes, environmental, physiological factors as well as the extraction and recovery procedures [58]. In the present study, it has been revealed that the gelling properties vary significantly with the sites of collection. In this investigation the physicochemical, rheological and thermal properties of agars extracted from 16 samples of *Gelidiella acerosa* collected from various places of the west coast of India (4 samples) were evaluated and compared the same with those occurring in the Gulf of Mannar region at the southeast coast (12 samples). Seasonal variation of the agar quality has been discussed in chapter II.3. The agars obtained from *Gelidiella acerosa* of Gulf of Mannar regions have been found to be suitable for bacteriological and molecular biology work (vide Chapter II.2).

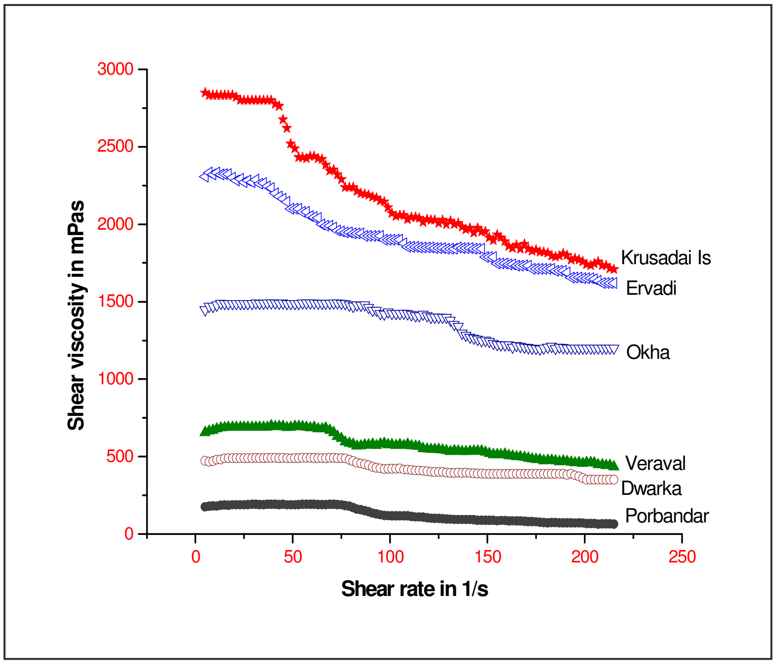

Fig. II.1.2a Gel thinning behaviour of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places at 45^{o}C

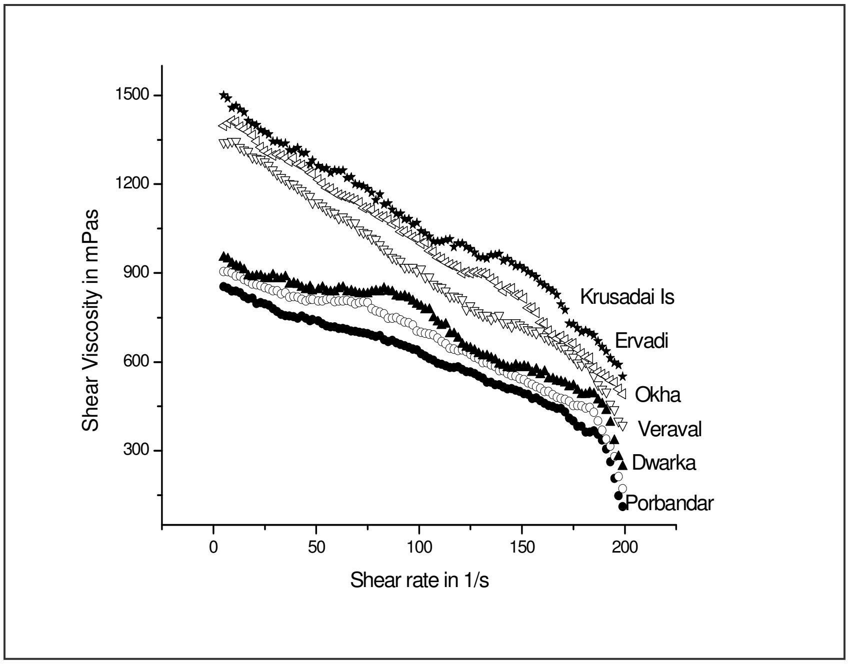

Fig. II.1.2b Gel thinning behaviour of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places at 70^{o}C

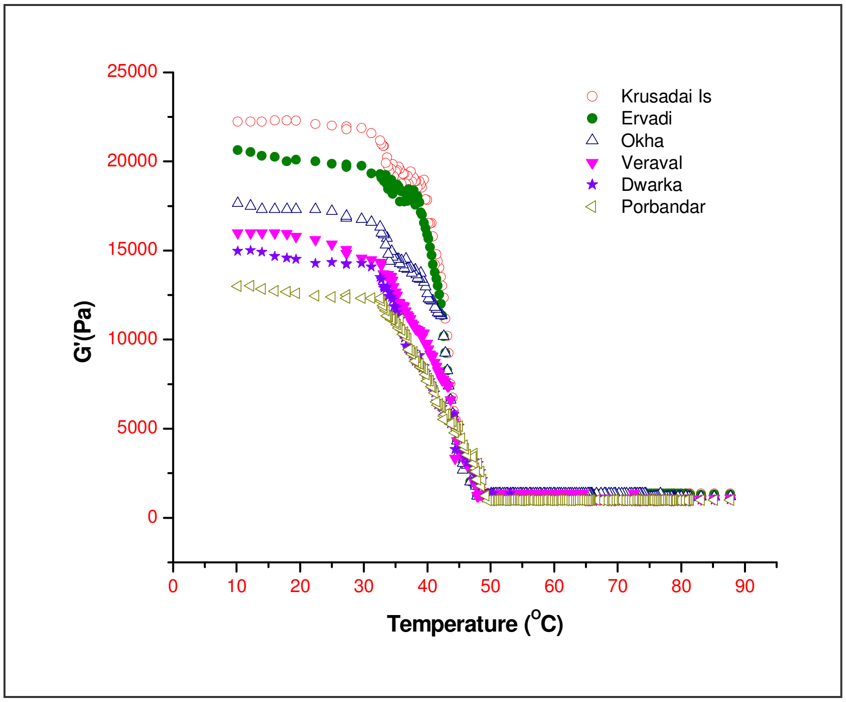

Fig. II.1.3a Temperature dependency of G' of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places. Controlled Deformation mode, Strain 0.05%; Frequency 1Hz; Cooling rate 1°C/min; Geometry used cone/plate

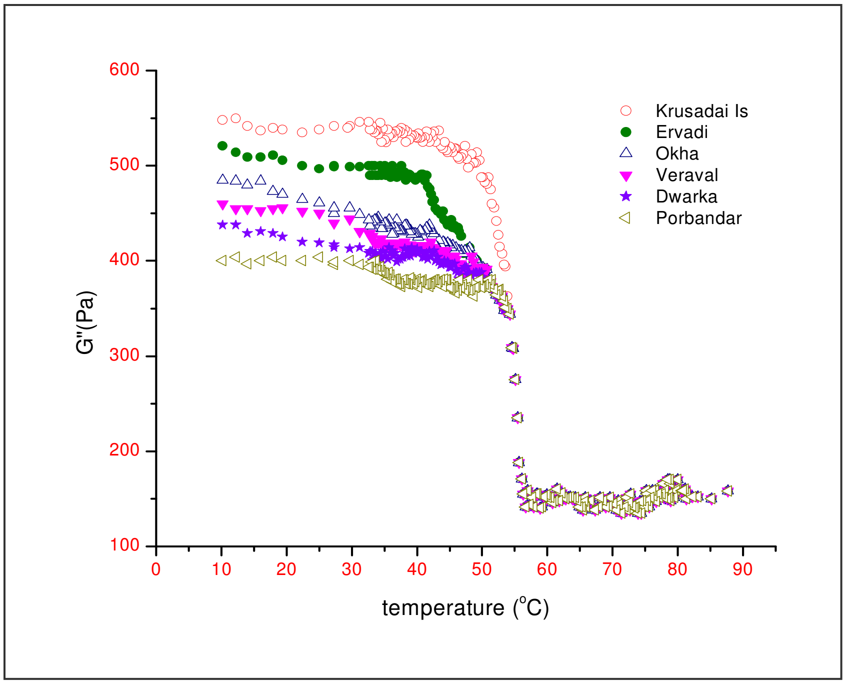

Fig. II.1.3b Temperature dependency of G" of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places. Controlled Deformation mode, Strain 0.05%, Frequency 1Hz; Cooling rate 1°C/min; Geometry used cone/plate

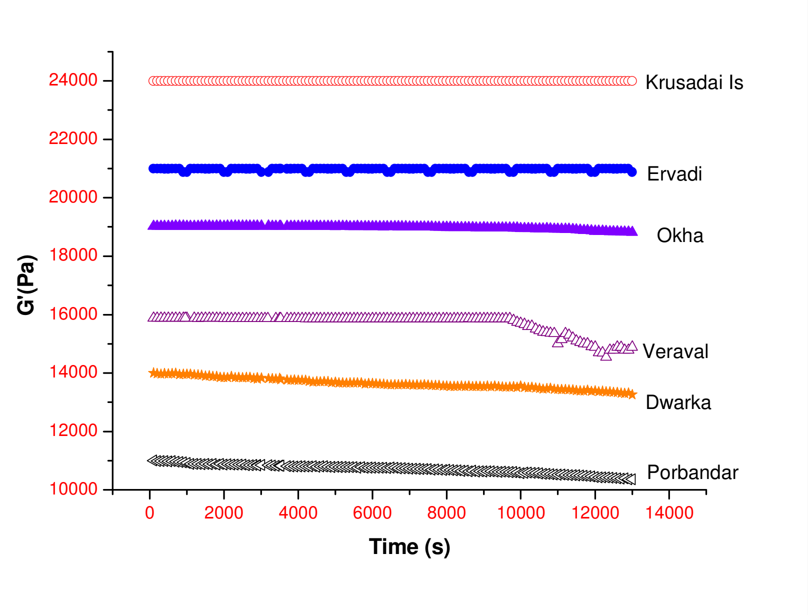

Fig. II.1.4 Time dependency of G′ of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places. Controlled Deformation mode, Strain 0.05%, Frequency 1Hz; Geometry used plate/plate

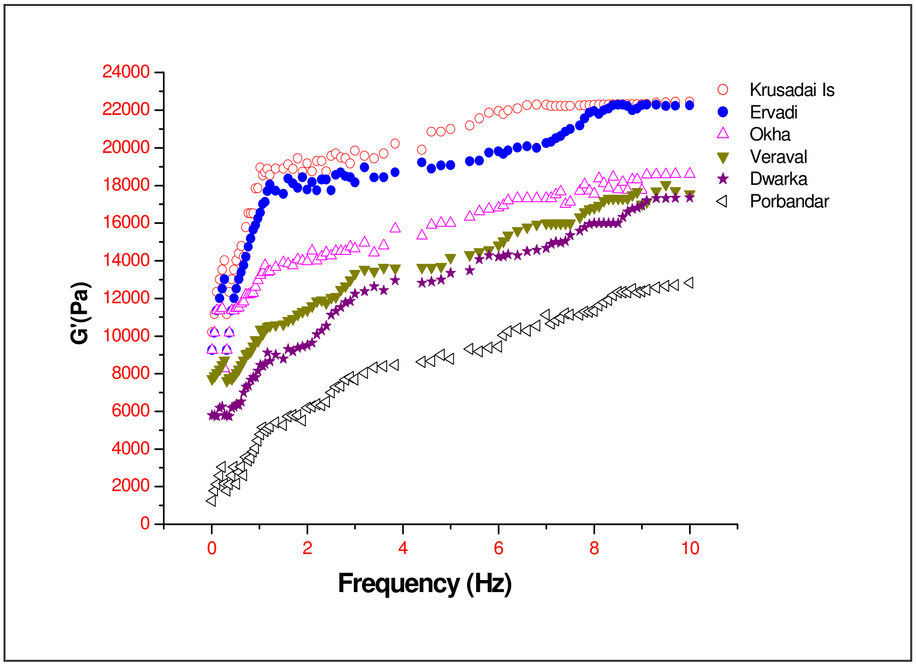

Fig. II.1.5a Frequency dependency of G′ of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places. Controlled Deformation mode, Strain 0.05%, Frequency 0.01-10Hz; Geometry used plate/plate

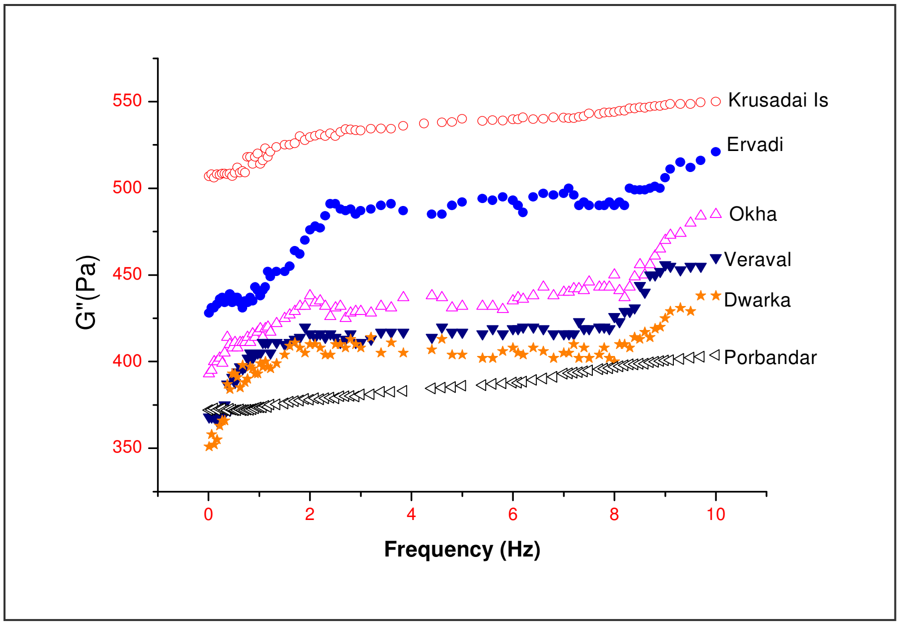

Fig. II.1.5b Frequency dependency of G″ of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places. Controlled Deformation mode, Strain 0.05%, Frequency 0.01-10Hz; Geometry used plate/plate

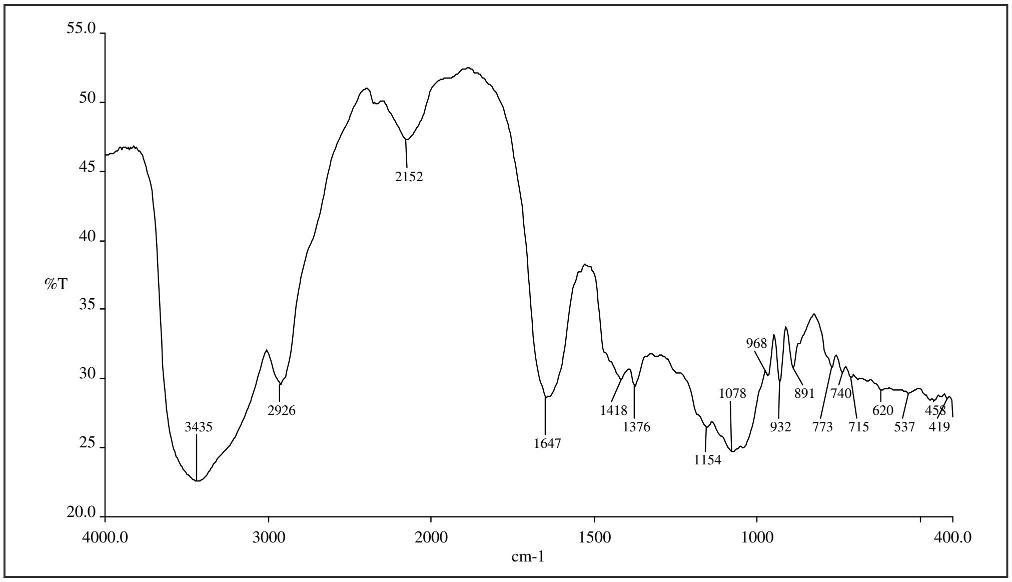

Fig. II.1.6a FT-IR spectra of agar extracted from *Gelidiella acerosa* collected from southeast coast of India

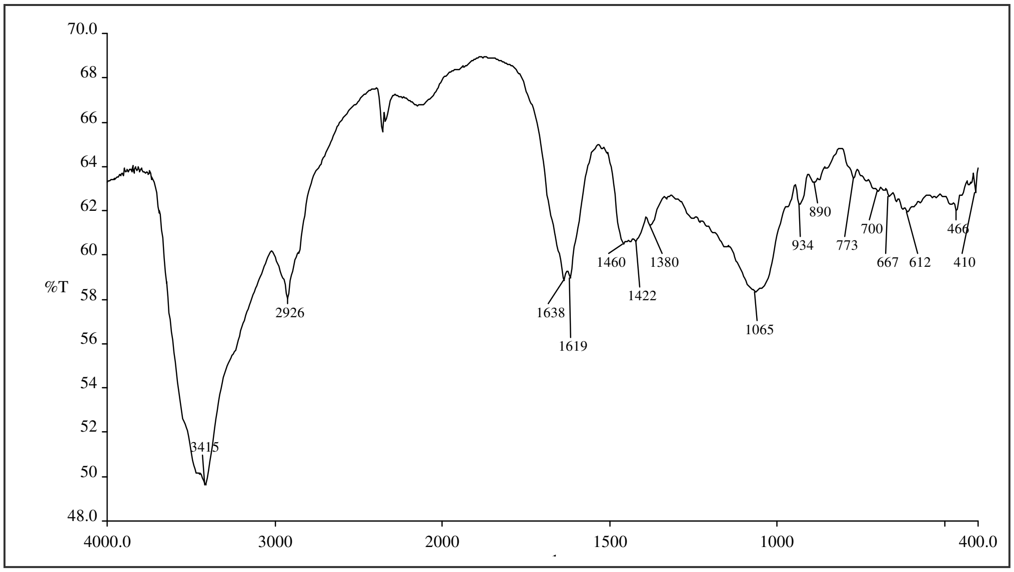

Fig. II.1.6b FT-IR spectra of agar extracted from *Gelidiella acerosa* collected from the west coast of India

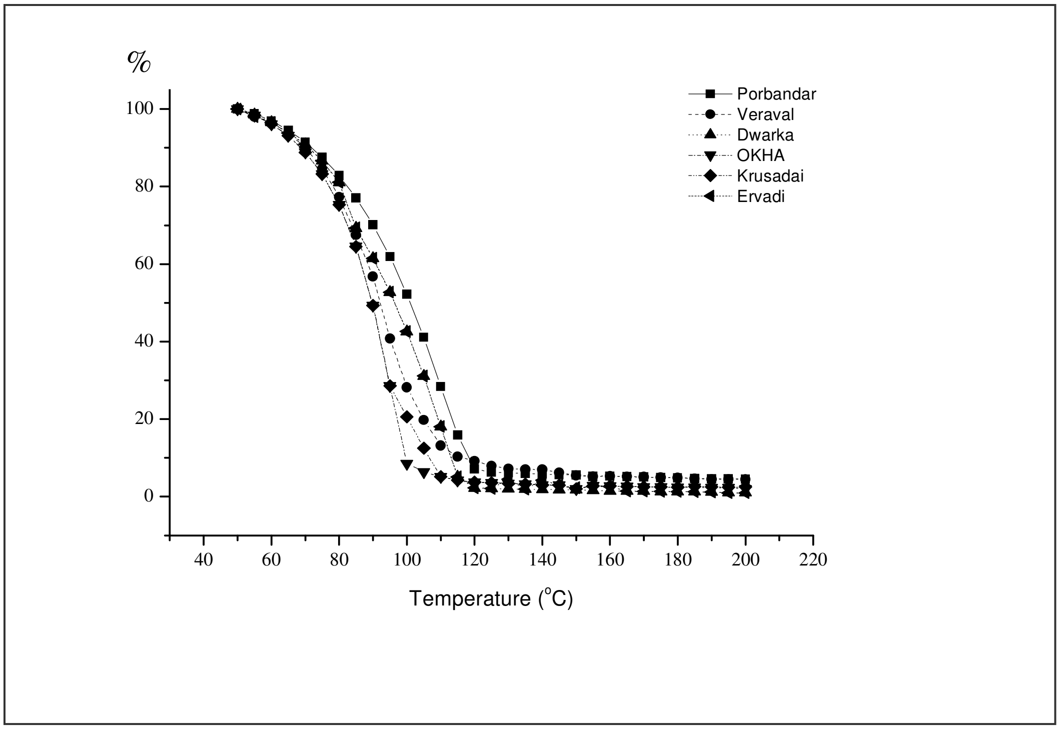

Fig. II.1.7 TGA profiles of various agar gels (1.5% gel) obtained from *Gelidiella acerosa* collected from different places.

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CHAPTER II.2

BACTERIOLOGICAL AGAR FROM *GELIDIELLA ACEROSA* (FORSSKAL) J. FELDMANN & G. HAMEL (RHODOPHYTA, GELIDIALES) COLLECTED FROM GULF OF MANNAR, BAY OF BENGAL

II.2.1. INTRODUCTION

In this Chapter preparation of bacteriological agar from a specific sample of *Gelidiella acerosa* collected from Gulf of Mannar has been described. Detailed investigations on the physicochemical, spectroscopic and rheological characteristics of this bacteriological agar have been carried out. The suitability of the agar for bacteriological and molecular biology applications has been validated.

The results of this study described herein will be useful for bioprospecting of agarophytes for producing high quality agar.

II.2.2. MATERIALS AND METHODS

II.2.2.1 Collection

Gelidiella acerosa was collected from the natural habitat in the Gulf of Mannar at Ervadi (9°15′N, 78°58′E) in the southeast coast in Tamil Nadu, India, in March 2000 (Code No. AS0402909). Specimen of the seaweed (AS-IV-29-9) has been deposited with the CSMCRI Herberium for reference.

II.2.2.2 Agar extraction and isolation

Seaweed sample (10 g) was immersed in 500 ml distilled water for 2 h then acetic acid pretreatment was given for 1 h and heated in an autoclave at 120°C for 1.5 h. The extract was homogenized and filtered under pressure through celite bed. The filtrate was allowed to gel at room temperature and kept overnight at –20°C. Frozen gel was thawed, washed with distilled water and air dried for 24 h followed by oven drying at 60°C for 1 h [1].

II.2.2.3 Physicochemical analyses

The gel strength (g cm^{-2}) was measured by using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). The measurements were performed on a 1.5% w/v agar gel having kept overnight at 10°C, using a solid cylindrical plunger 1 cm in diameter [2].

Dynamic rheological measurements for the agar sol and gel were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany). The measuring geometries selected were a

cone/plate (60 mm diameter, 1^o rad angle and 1.00 mm gap) for measurements of flow properties at 45^oC in solutions, taking 5 ml agar sol on to the rheometer. Plate/plate (35 mm diameter and 1.00 mm gap) geometries were used for measurements in agar gel at 25^oC [3]. The outer surface of the sample was covered with silicon oil to prevent losses due to evaporation for the measurements done at higher temperatures.

Frequency sweep measurements of storage modulus G′ and loss modulus G″ were performed at 25^oC at a strain (γ) value 0.01. Time dependence of of moduli during storage at 20^oC was monitored at frequency 1 Hz and a strain value of 0.05. Dynamic viscosities at varying shear rate were studied at 45^oC. The temperature was maintained using the water circulator DC50.

Gelling and melting temperatures of agar gel was measured as described by Craigie *et al.* [4].

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

3,6-Anhydrogalactose was estimated by improved phenol-resorcinol reagent using fructose as standard [5]. Sulphate content in the agar samples was estimated by turbidimetric method as described by Dodgson *et al.* using K_2SO_4 as standard [6]. Detailed Method has been described in previously in Chapter II.1.

Total Sugar: Total sugar of the agar samples were measured by colorimetric method following the method of Dubois using glucose as standard [7]. Method has been described in previously in Chapter II.1.

Elemental Analyses were performed on a Perkin-Elmer CHNS/O Analyser, Series II, A2400, Perkin-Elmer USA, using around 5 mg of the sample.

Foreign starch, foreign insoluble matter, hot and cold water solubility were done using standard methods [8].

ICP analyses of agar were carried out with 0.1078% agar sol in double distilled water, on an Inductively Coupled Plasma Atomic Emission spectrometer (Spectroflame Modula FTM08), Spectroanalytical Instruments, Germany.

Whiteness Index was measured on a Whiteness Index measurement instrument using $MgCO_3$ as standard, employing a galvanometer to measure the reflected light intensity.

Weight average molecular weight. Intrinsic viscosities [η] were determined at 32°C using an Ostwald viscometer with a flow time 2.11 min for 1 M NaCl. For this, sols of agar were prepared in 1 M NaCl at a concentration 0.02% to 0.14% of agar (w/v).

II.2.2.3 Spectral analyses

Infrared spectroscopy was 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}.

^{13}C NMR spectrum (noise-decoupled) was recorded on a Bruker Avance DPX 200 Spectrometer at 50 MHz. Samples (50 mg/ml) was dissolved in D_2O and spectrum was recorded at 70^oC with 9279 accumulations, pulse 5.9 µs, acquisition time 1.245 s and relaxation delay 6 µs using DMSO (δ39.5) as internal standard.

A 0.2% solution (at 80^oC) of agar was scanned by UV-visible spectrophotometer in the wave length region 200-800 nm of light. UV-Vis spectrum was recorded on a Shimadzu 160A UV-Vis Spectrophotometer, Shimadzu Corporation, Kyoto, Japan.

II.2.3 RESULTS AND DISCUSSION

II.2.3.1 Physicochemical analyses

Table II.2.1 summarizes the results of physicochemical analysis of the agar sample. The yield is expressed as percentage (on the basis of bone dry seaweed as received from the field) ranged from 35-37%.

Viscosity values (47 ± 2.5 cP) were measured at 75°C in 1.5% agar sol. The gel strength of the agar sample obtained was (830±25 g cm^{-2}). Gelling and melting temperatures of the agar was measured to be 40°C and 90-93°C respectively. Repeat extractions of agar using the same sample of seaweed resulted in similar yield and quality of agar after storing the air dried seaweed in a plastic bag for more than two years. 3,6-Anhydrogalactose, sulphate was found to be 40% and 1.6% respectively. Elemental analyses showed the presence of carbon (41.40%), hydrogen (4.42%), nitrogen (0.21%), oxygen (50.07%).

Table II.2.1 Physicochemical characteristics of the agar extracted from *Gelidiella acerosa*

Characteristics	Agar (AS-IV-64-3)
Yield (%)	35 (± 2)
Color	Pale yellowish
Whiteness index (%)	65.13
Moisture (%)	8.0
Gelling temperature (°C)	40±1
Melting temperature (°C)	90±3
Threshold gel concentration (%)	0.20
Foreign insoluble matter[a] (%)	0.19
Foreign starch	Not found
Water absorption (times of its weight)	20.0
Cold water solubility (wt%)	5.4
Hot water solubility (wt%)	99.8

Gel Strength (1.5% gel at 20°C) (g cm^{-2})	830±25
Viscosity (1.5%, at 75°C)	47±2.5 cP
3,6-anhydrogalactose (%)	40±2
pH (1.5% sol at 70°C)	6.89
Ash (%)	2.0±0.2
Sulphate (%)	1.5±0.1
Sodium [a] (%)	0.1365
Potassium [a] (%)	0.0279
Calcium [a] (%)	0.4175
Magnesium [a] (%)	0.1434
Sulphur [a] (%)	0.5400
Phosphorus [a] (%)	0.0050
Weight average molecular weight [b]	2.74 $x10^5$ Dalton.

[a] Analysed on ICP

[b] Determined by using Mark-Hauwink Equation.

II.2.3.2 Viscosity average molecular weight

Determination of viscosity average molecular weight from intrinsic viscosity. At first specific viscosity, reduce viscosity and intrinsic viscosities were determined using the following equations :

$\eta-\eta_{0}/\eta_{0} = t - t_{0}/ t_{0} = \eta_{sp}$

$\eta_{red} = \eta_{sp} / c$

$[\eta] = \eta_{sp} / c$, where c *tends to* zero.

Where c is the concentration in g/100ml, t is the time of flow for sample and t_0 is the time of flow for blank (solvent in which samples is prepared, In this case 1 M NaCl).

Intrinsic viscosity $[\eta]$ was calculated by linearly extrapolating the reduced viscosity to zero concentration from concentrations of 0.02% to 0.14% w/v, The value was found to be 575.51 ml/g (Fig.II.2.1). Weight average molecular weight of the sample was measured, in triplicate by using the Mark–Houwink equation for agarose as described by Rochas and Lahaye, 1989 [9].

$[\eta] = 0.07\ M^{0.72}$, where $[\eta]$ is ml/g.

The molecular weight was found to be 2.74 x10^5 Dalton . The molecular weight of this agar sample (2.74 x 10^5 Dalton) is in good agreement with those reported for agar by other researchers determined by light scattering techniques [10,11,12].

In the UV-Vis spectrum no absorption maxima of 0.20% agar sol at 80°C was obtained in the range 200-800 nm. This indicates that the agar sample is free from color contaminants or pigments. Low ash content (≤ 2%) indicates very low metal ion contents in the agar.

II.2.3.3 Spectral analysis

The FT-IR spectrum of the agar of *Gelidiella acerosa* (AS-IV-64-3) is similar to that of the agar sample (Difco Laboratories, 0140-01, Detroit Michigan USA) in the finger print region 500-1500 cm^{-1} (Fig.II.2.2). Both show characteristic bands at 740, 773, 930 cm^{-1} due to β-galactose skeletal bending. The band at 890 cm^{-1} is specific for agar and may be attributed to anomeric C-H stretching of β-galactose residues. The weak band at 895-900 cm^{-1} is due to β-anomeric-CH-deformation [13,14]. Other bands observed were at IR (ν_{max} , cm^{-1}): 3435 (s, O-H stretching), 1638 (s, CO-NH-stretching), 1457 (sh, C(6)-H of local symmetry), 1420 (sh, C(6)-O local symmetry), 1377 (m) and 1153 (sh) (S=O stretching), 1076 (s, C-O-C stretching, 932 (m,3,6-anhydrogalactose ring vibration), 893 (m,β-anomeric-CH- deformation), 773 (w, β-galactose skeletal bending), 740 (w, β-galactose skeletal bending), 714 (w, β-galactose skeletal bending). The reason for appearance of a strong broad band at ca.1635 cm^{-1} in this agar as well Difco agar (Fig. II.2.2) is not clear. Main difference in FT-IR spectra of between this two agars is the appearance of a band at 847 cm^{-1} which is due to C-4 sulphate [14] in Difco agar. Absence of this band in the agar sample investigated here indicates absence of C-4 sulphate. [Abbreviations for intensity of the IR bands: s = strong; sh= shoulder; m=medium; w=weak].

Carbon-13 NMR spectroscopy of agar has been reported by several authors [15, 16]. Fig.II.2.3 depicts the ^{13}C NMR spectrum of the agar of *Gelidiella acerosa*, the carbon resonances having been ascertained by comparison with those reported earlier in the literature. The ^{13}C NMR signals are: δ 69.8 (anomeric carbon); 61.4 (C-6 galactose unit);

68.7 (C-4 galactose unit); 69.4 (attributed due to C-6 of 3,6-anhydrogalactose unit); 75.3 (C-5 galactose unit); 77.3 (C-5 of 3,6-anhydrogalactose unit); 80.1 (C-3 of 3,6-anhydrogalactose unit); 82.2 (C-3 galactose unit); 98.2 (C-1 of 3,6-anhydro-α-L galactose residue); 102.4 (C-1 of D-galactose).

II.2.3.4 Dynamic rheological properties

Fig.II.2.4 shows the frequency dependence of G' and G'', of these agar gels of different concentrations measured at 25°C. The 0.3 % w/v agar dispersion can be judged as a semi-dilute polymer solution based on the predominant G' over G'' at low frequencies and a crossover of G' and G'' in the higher frequency range (ca. 20 Hz). The concentration was further increased to 1.5% (w/v) and compared with 0.5% (w/v). Both samples exhibited typical gel type mechanical spectra i.e., G'>G'' in the entire frequency range examined. The moduli of the true gels is almost frequency independent with tan δ (=G''/G') values less than 0.1 at all examined frequencies, suggesting that the gel was predominantly elastic. The moduli of the dispersion at lower concentrations are slightly dependent on frequency and tan δ values were ca 0.33. Slightly frequency dependent moduli and tan δ values over 0.1 are typical rheological signatures of a weak gel system [16]. (Fig.II.2.5)

The 0.5% (w/v) agar dispersion exhibited a gel-like spectrum that is G'>G'' at all examined frequencies. We have found that there exists little thermal hysteresis between coil-to-helix and helix-to-coil transition temperatures for agar gel in lower concentrations but the reverse is true (40°-90°C) for agar gel in higher concentrations.

The stability of moduli during storage at 25°C was investigated and is depicted in Fig.II.2.6. The storage modulus values of the true gel formed at the concentration 1% w/v were larger than those of dispersions of lower concentration of agar. However, the moduli of the true gel gradually decreased with time up to 1% gel, which may be due to the gel network that was gradually shrinking and undergoing syneresis (releasing water) as a result of continuing slow aggregation of helices even after gelation. For agar dispersions at higher concentrations i.e., >1% (w/v) it has been observed that the moduli did not change significantly when stored at 25°C (Fig.II.2.6) indicating the absence of syneresis in 1.5 % (w/v) agar gel [cf. 16].

Temperature dependencies of G′ and G″ for various concentrations w/v agar gel (0.5-1.5%) are summarized in Fig.II.2.7a and 7b. This experiment was conducted by decreasing temperature from 90°C to 20°C. As expected, it was observed that with decrement of temperature G″ decreases and reverse trend was observed for G′ values. At ca.40°C which is the transition temperature of sol to gel. At this temperature there is a change in the values of G′ and G″, increasing suddenly. Further, with decrement of temperature the latter values either increases or level off [cf. 12]. The observation is in good agreement with the gelling temperature determined by the method described by Craigie et al. [4]. No such behaviour was observed for gels of concentrations lower than 0.5% (w/v).

The linear viscoelastic strain region of a weak gel is usually considered to be narrower than a true strong gel [16]. In Fig.II.2.8, a comparison of large deformation properties between a true gel and a weak gel constituting the dispersion of non-aggregating helices is made at comparable moduli values. Since the width of linear strain region is known to be dependent on the concentration of polymer [16], the linear viscoelastic region of the true gel (1% and 1.5% w/v) was observed up to a strain value of ca. 0.1, the gel got fractured at a strain value ca.0.2. But for agar dispersion in low concentration, the linear viscoelastic region extended up to a value of 0.2 beyond which the spectra remained linear showing no sign of fracture of gel network (Fig.II.2.8). This indicates that the gel like mechanical characteristics, i.e. G'>G", of the agar gel at lower concentration (0.5% w/v) remained unchanged even on the application of strain much larger than that causes breakdown of a true gel network.

Fig.II.2.9 shows the comparison of a steady state dynamic viscosities of a dispersion of non-aggregating agar helices (agar gel at lower concentrations) with those of agar gel of higher concentration. Under the steady shear flow, a Newtonian flow behavior was observed at the low limiting shear rate region and a strong shear thinning at higher shear rate for agar dispersions at lower concentrations. But for agar at higher concentartion i.e. for true gels a non-Newtonian or pseuodoplastic behaviour was observed both in low limiting shear rate as well as higher shear rate regions (Fig.II.2.9). Both show gel-thinning behaviour [cf.17].

Rheological measurements show that the dispersion of agar exhibits gel behaviour in 0.5% (w/v) concentration and above, while below 0.5% (w/v) it does not exhibit gel behaviour. This is in consonance with the observations obtained manually (Table II.2.1).

II.2.3.5 Rheological Model

Attempts have been made to fit the rheological characteristics described above in some rheological models reported in the literature. Validity of various rheological models for 0.5 %, 1 % and 1.5 % agar sols at 45^{o}C, will be discussed.

Ostwald de Waele model Logarithmic plots of shear stress vs shear rate for upward curves were used to calculate the consistency coefficient (m) and the flow behaviour index (n) for agar solutions of 0.5% , 1 % and 1.5 % (w/v) agar sols at 45 ^{o}C, concentration was calculated by using following equation.

$$\tau = m\dot{\gamma}^{n} \text{ -------------------------------- Eq.II.1.1}$$

τ is shear stress in Pa, $\dot{\gamma}$ is shear rate in 1/s.

Herschel-Buckley Model Following equation was used to evaluate Herschel-Buckley model consistency coefficient m' and flow behaviour index, n' values for agar sols of different concentrations at 25 ^{o}C.

$$\tau = \tau_{o} + m'\dot{\gamma}^{n'} \text{ -------------------------------- Eq.II.1.2}$$

τ_{o} is yield stress.

As reported by Marcott et al. [18] shear rate corrections do not have much effect on the flow behaviour of hydrocolloids, hence the shear rate corrections were not done for both type of models. The values of flow and consistency indices are illustrated in Table II.2.2. From Table II.2.2 and Table II.2.3 it is seen that, for Ostwald de Waele model, the flow index is increasing from 0.5 to 1.5% at 45°C and stiffness indices decreases. This means the fluidity increasing with increase in concentration i.e., more rupture in the structure for higher concentration under applied shear. Since 1.5% agar sol shows some yield stress hence the Herschel-Buckley model is more suitable for 1.5% agar sol, the determination factor (r^2) is also 0.995 i.e., near 1. On the other hand for 0.5% agar sol the consistency index in Herschel Buckley model is more, which is not true, hence this model is not applicable to 0.5% agar sol. Similarly since 1% agar sol do not possess yield stress hence Herschel-Buckley model can not be fitted for the behaviour, determination factor in all cases tends towards unity. It can be concluded that for lower concentration of agar sols at higher temperature Oswald de Waele or the power law model is more applicable and for sols having higher concentration (i.e., with yield stress) the Hershel-Buckley model is applicable. Fig.II.2.10, Fig.II.2.11, Fig.II.2.12, Fig.II.2.13, Fig.II.2.14 and Fig.II.2.15 depicts the shear stress-shear rate curves and the validity of above two models.

Table II.2.2 Values of 'n' and 'm' for Ostwald de Waele model for different agar concentrations at 45 °C

Concentration of agar (%)	m (Pa s^n)	Uncertainty	n	Uncertainty	r^2
0.5	0.6464	25	0.6580	8.2	0.9712
1.0	0.003385	15.24	0.7510	4.089	0.99
1.5	0.002795	13.73	0.9175	3.73	0.995

Table II.2.3 Values of 'n′' and 'm′' for Herschel-Buckley model for different agar concentrations at 45°C

Concentration of agar (%)	m′ (Pa s^n)	Uncertainty	n′	Uncertainty	Yield Stress	r^2
0.5	4.594	76	0.3438	33	-8.809	0.9864
1.0	0.00799	36.22	0.6086	9.89	-0.02219	0.99
1.5	0.002213	36.44	0.9585	6.854	0.007203	0.995

II.2.3.6 Bacteriological and molecular biological assays

Very dilute solutions of saturated culture was streaked on agar plates containing antibiotic. Plates were kept overnight at 37°C. After 17 hours incubation it was found no significant difference in the number of colonies in the plates containing agar and HiMedia Agar. Both normal and transformed were applied on agar plate and the plasmid

DNA was isolated and treated with different restriction enzymes. It was found that this agar is suitable for molecular biology work for there was no degradation of DNA and intact band could be resolved. Due to its high gel strength at 1.5% concentration, 1% agar can be used easily for plating and streaking purposes. In preparing the plates and growing the bacterial colonies, no difference was observed when compared with commercially available agar (Product code CR 301) supplied by HiMedia Laboratories Pvt Ltd., Mumbai, India. This evaluation was done in Indian Institute of Chemical Biology, Kolkata.

II.2.4 SUMMARY

High quality bacteriological grade agar may be extracted from selected seaweeds that give the agar a gelling temperature of 34°-35°C, which enables one to add heat sensitive antibiotics to the agar gel used in microbiological studies. The cooler agar is easier to handle and condensation in a petri dish occurs without much problem. Gel strength over 800 g/cm^2, presence of divalent cation less than 1000 ppm are the general characteristics of good bacteriological grade agar [19].

The agar that has been studied herein contains low ash (2.16%) i.e., less metal ion and high 3,6-anhydrogalactose contents in this agar are in consonance with its high gel strength and observed high viscosity values. High gel strength of this agar makes it economical, because of lower usage level in various applications (Table II.2.1). The absence of absorbance maxima in the 200-800 nm range in UV-Vis spectrum, indicates that the agar is free from any coloured contaminants i.e. pigments. The sol of this agar is clear at high temperature and starts to appear cloudy at ca. 60^{o}C while cooling. Rheological properties showed that this agar sample gels at 0.3% w/v concentration and does not undergo syneresis in concentrations more than 1% w/v. The bacteriological assays of this agar sample reveal that this agar is suitable for bacteriological and molecular biology works and compared well with HiMedia Agar CR 301.

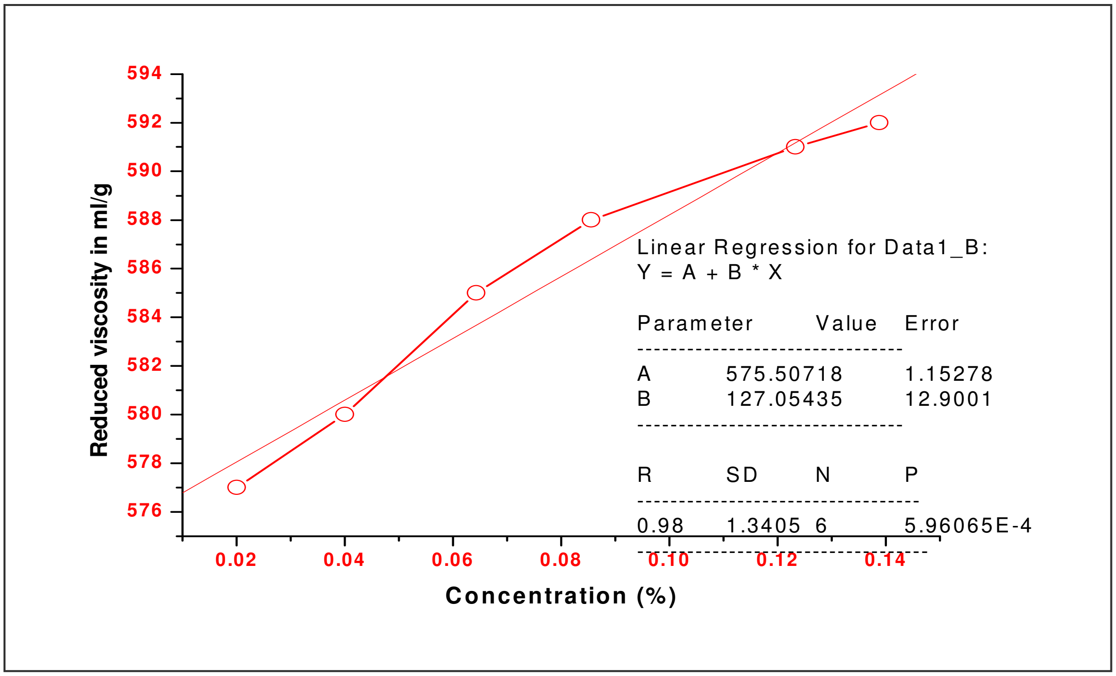


Fig.II.2.1 Extrapolation of reduced viscosity : Determination of Instrinsic viscosity for the agar sample.

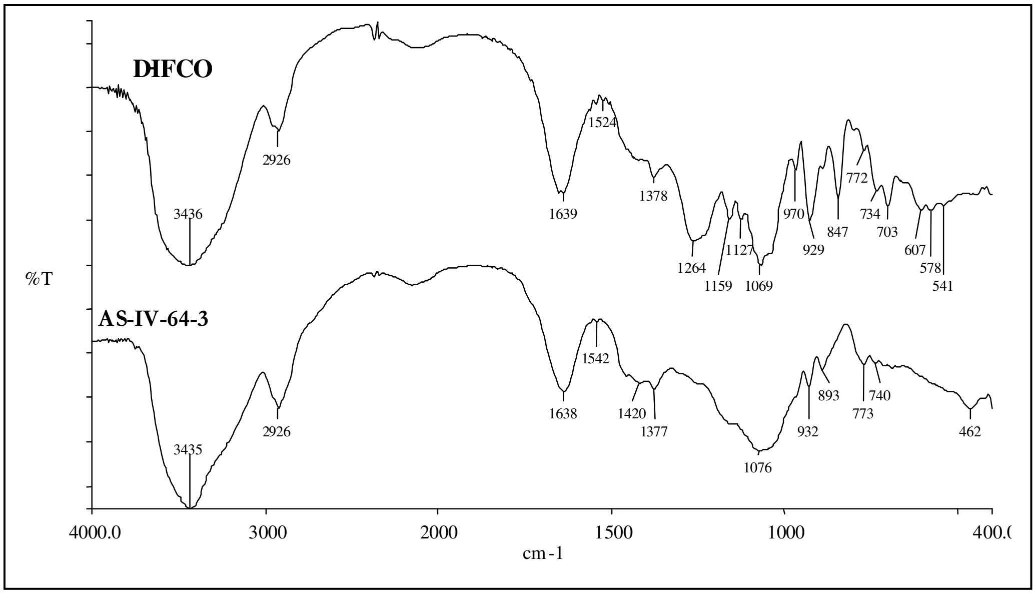
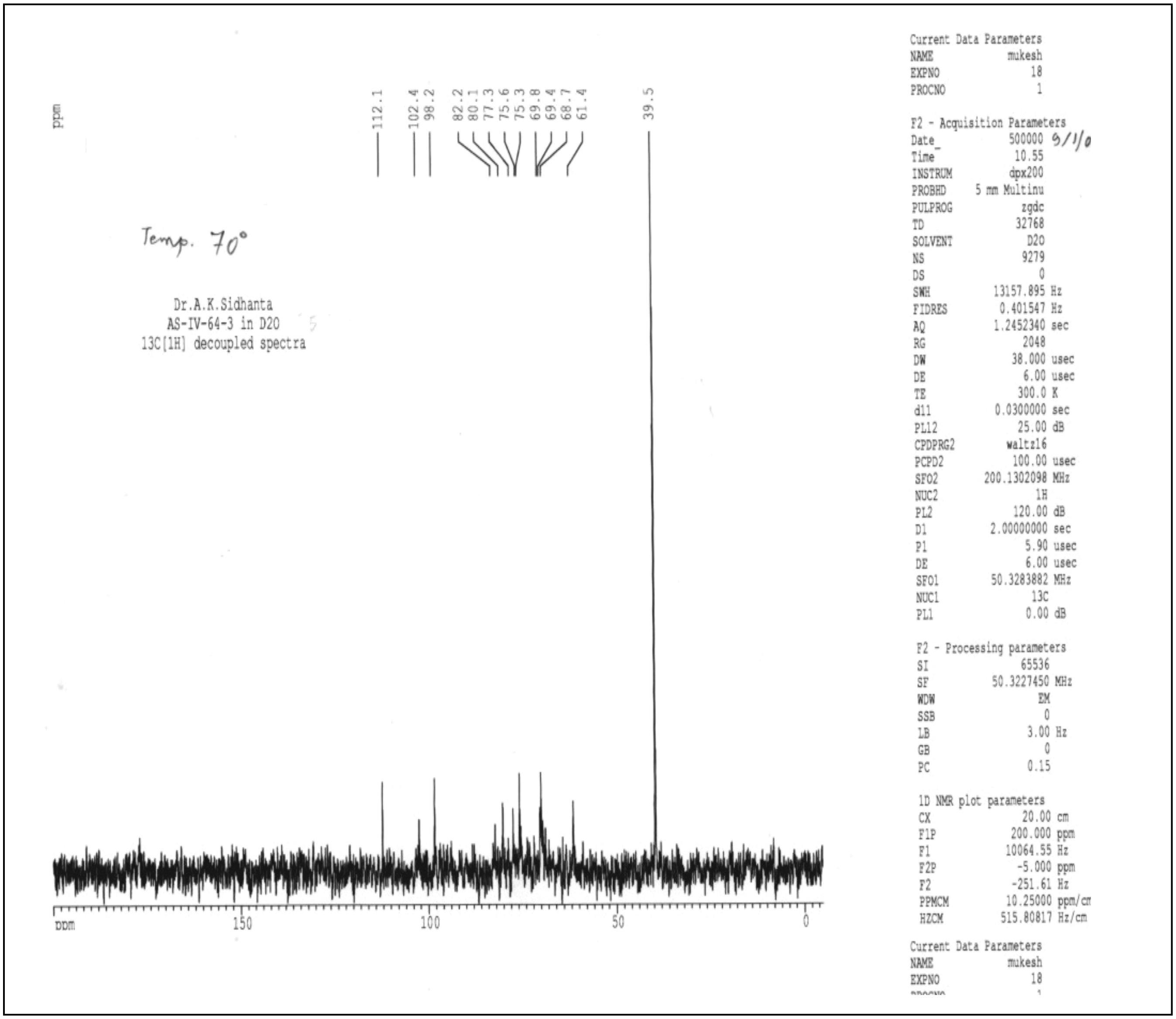


Fig. II.2.2 Infrared spectra of a) Difco agar b) Agar (AS-IV-64-3) extracted from *Gelidiella acerosa*.



Temp. 70°

Dr.A.K.Sidhanta
AS-IV-64-3 in D20
13C[1H] decoupled spectra

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Current Data Parameters
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EXPNO                18
PROCNO                1

F2 - Acquisition Parameters
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Time              10.55
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PULPROG            zgdc
TD                32768
SOLVENT             D20
NS                 9279
DS                    0
SWH           13157.895 Hz
FIDRES         0.401547 Hz
AQ            1.2452340 sec
RG                 2048
DW               38.000 usec
DE                 6.00 usec
TE                300.0 K
d11           0.0300000 sec
PL12              25.00 dB
CPDPRG2        waltz16
PCPD2            100.00 usec
SFO2        200.1302098 MHz
NUC2                 1H
PL2              120.00 dB
D1           2.00000000 sec
P1                 5.90 usec
DE                 6.00 usec
SFO1         50.3283882 MHz
NUC1                13C
PL1                0.00 dB

F2 - Processing parameters
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SF           50.3227450 MHz
WDW                  EM
SSB                   0
LB                 3.00 Hz
GB                    0
PC                 0.15

1D NMR plot parameters
CX                20.00 cm
F1P             200.000 ppm
F1             10064.55 Hz
F2P              -5.000 ppm
F2              -251.61 Hz
PPMCM          10.25000 ppm/cm
HZCM          515.80817 Hz/cm

Current Data Parameters
NAME             mukesh
EXPNO                18
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Fig.II.2.3 ^{13}C NMR spectra for agar

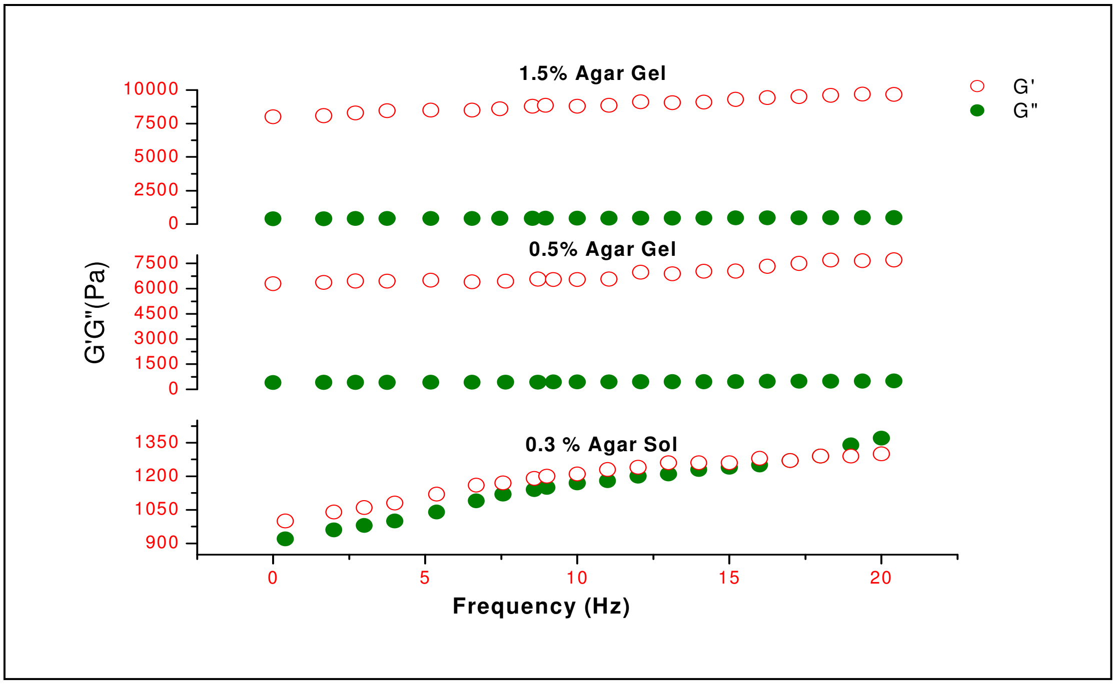

Fig. II.2.4 Frequency dependency of G′and G″ for 0.1%, 0.5% and 1.5% agar matrices. Controlled Deformation mode, Strain 0.05%, Frequency 1-20Hz; Geometry used plate/plate

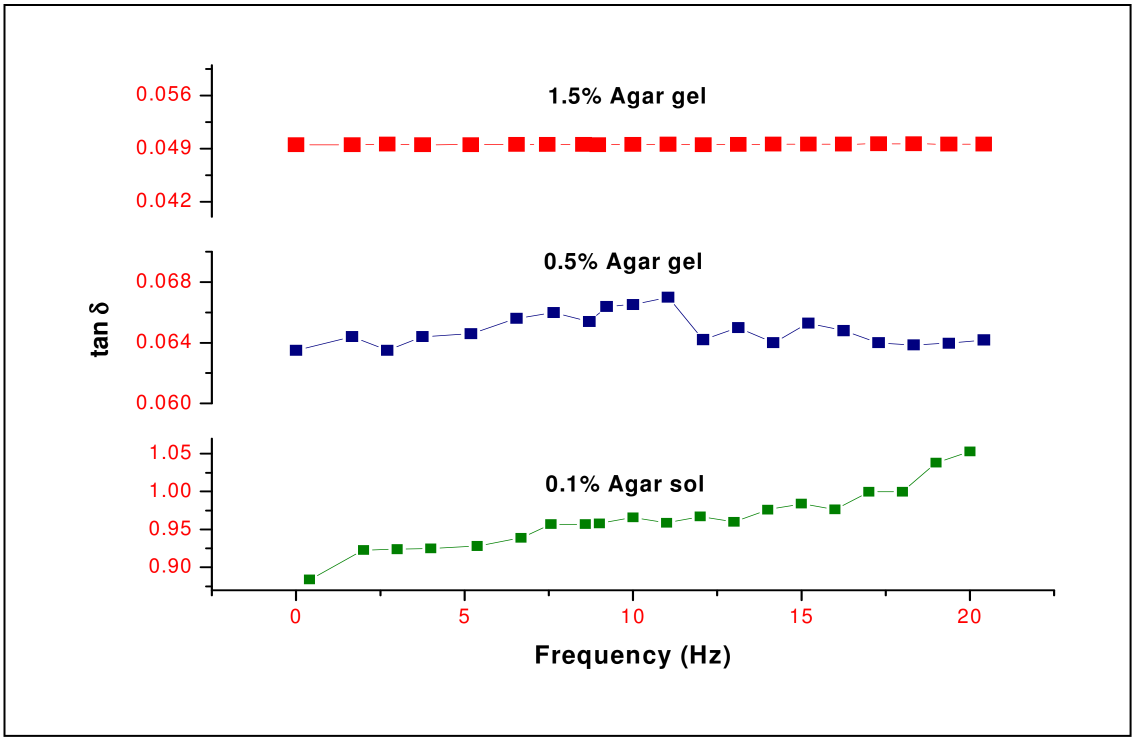

Fig. II.2.5 tan δ vs frequency for 0.1%, 0.5% and 1.5% agar matrices. Controlled Deformation mode, Strain 0.05%, Frequency 1-20Hz; Geometry used plate/plate.

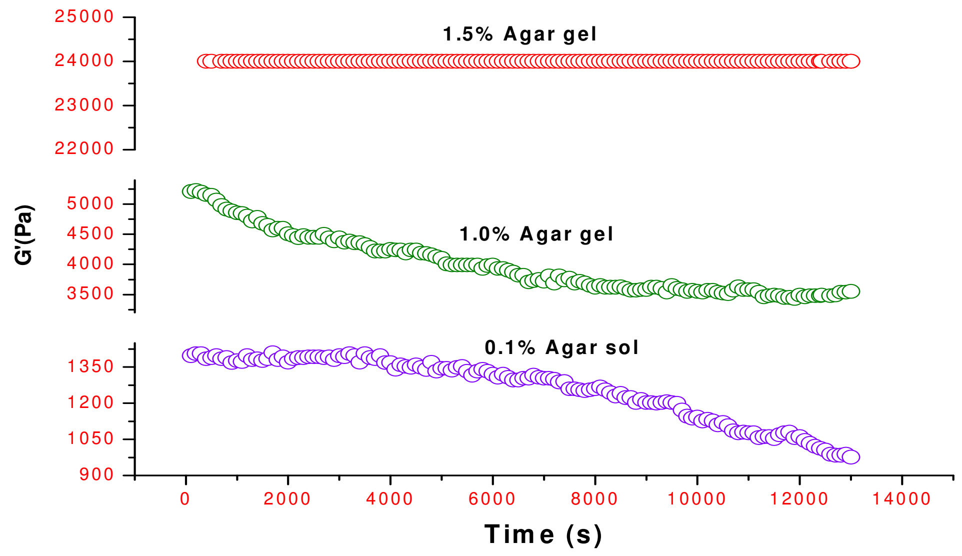

Fig. II.2.6 Time dependency of G′ for 0.1%, 0.5% and 1.5% agar matrices. Controlled Deformation mode, Strain 0.05%, Frequency 1Hz; Geometry used plate/plate (for 1% and 1.5%) cone/plate ; (for 0.1%).

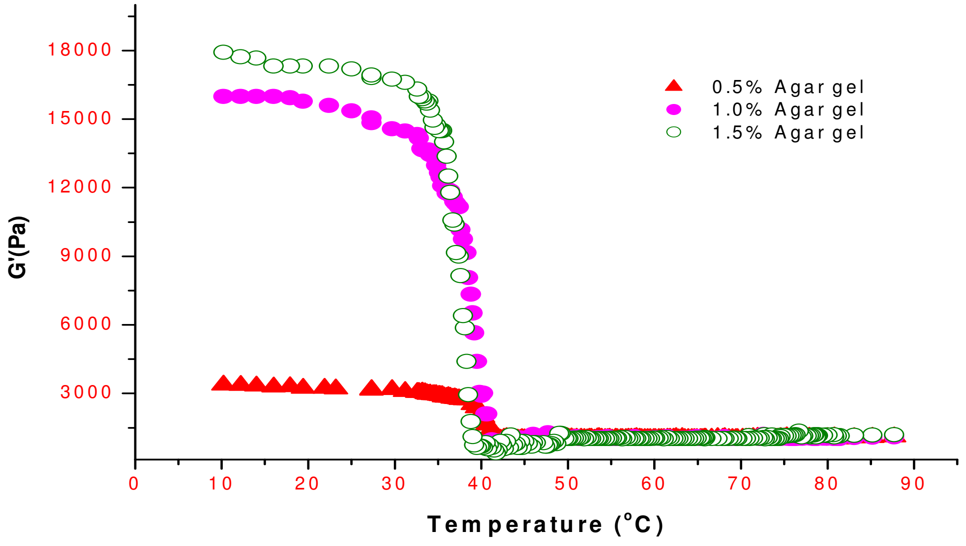

Fig.II.2.7a Temperature dependency of G′ of various agar gels Controlled deformation mode, Strain 0.05%, Frequency 1Hz; Cooling rate 1^{o}C/min; Geometry used cone/plate

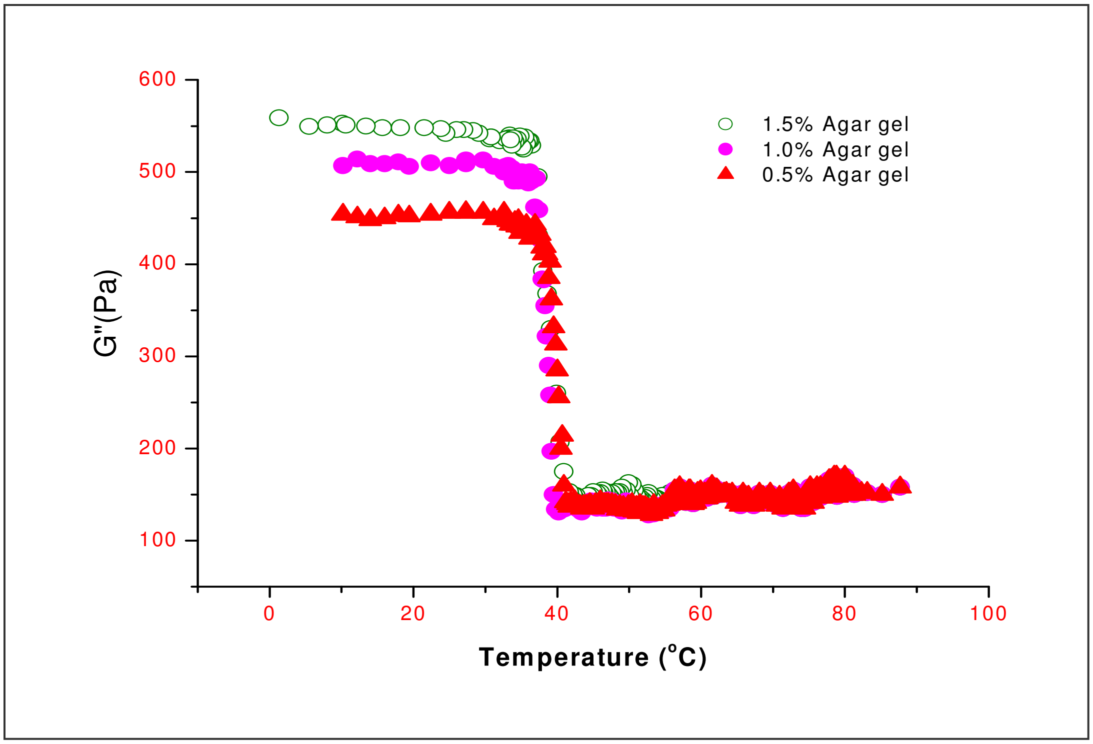

Fig.II.2.7b Temperature dependency of G″ of various agar gels.Controlled Deformation mode, Strain 0.05%, Frequency 1Hz; Cooling rate 1°C/min; Geometry used cone/plate

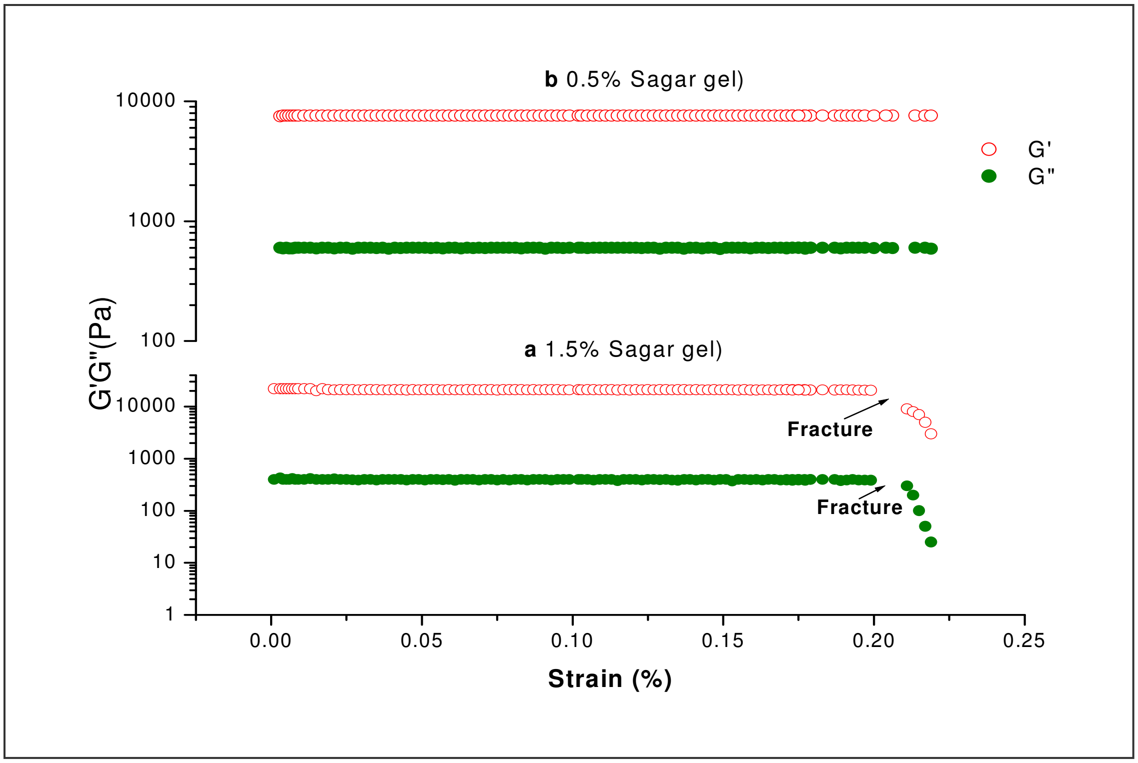

Fig. II.2.8 Strain dependence of G′ (blank circle) and G″ (solid circle) for a) 1.5% w/v agar gel b) 0.5% w/v agar gel.

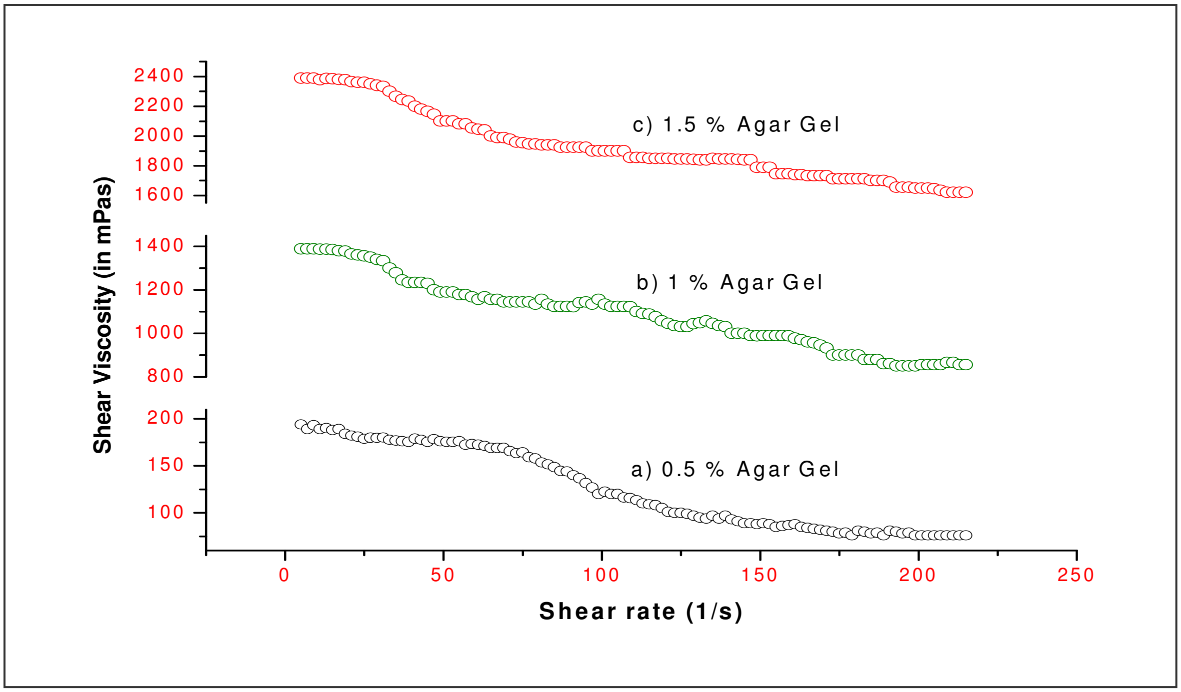

Fig.II.2.9 Shear strain dependence of steady shear viscosity of a) 0.5%% w/v agar gel b) 1% w/v agar gel c) 1.5% agar gel

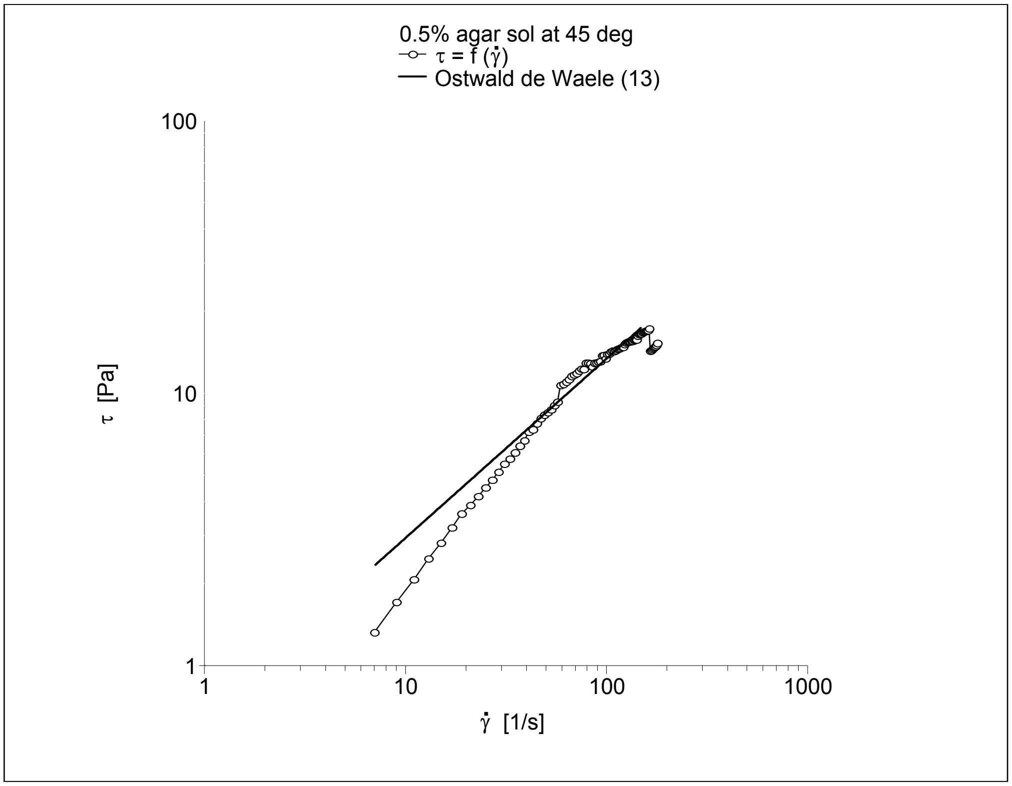

Fig.II.2.11 Logarithmic plots of shear rate vs shear stress and regression analysis according Herschel-Buckley model for 0.5 % agar sol at 45 ^{o}C.

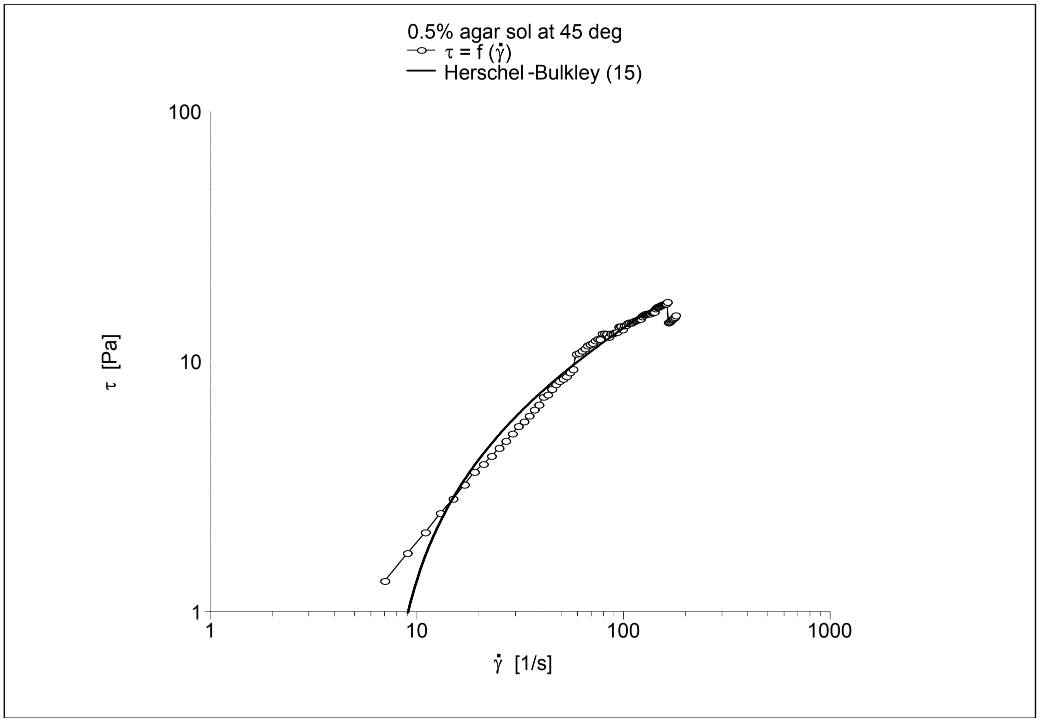

Fig.II.2.10 Logarithmic plots of shear rate vs shear stress and regression analysis according Ostwald de Waele model for 0.5 % agar sol at 45^{o}C

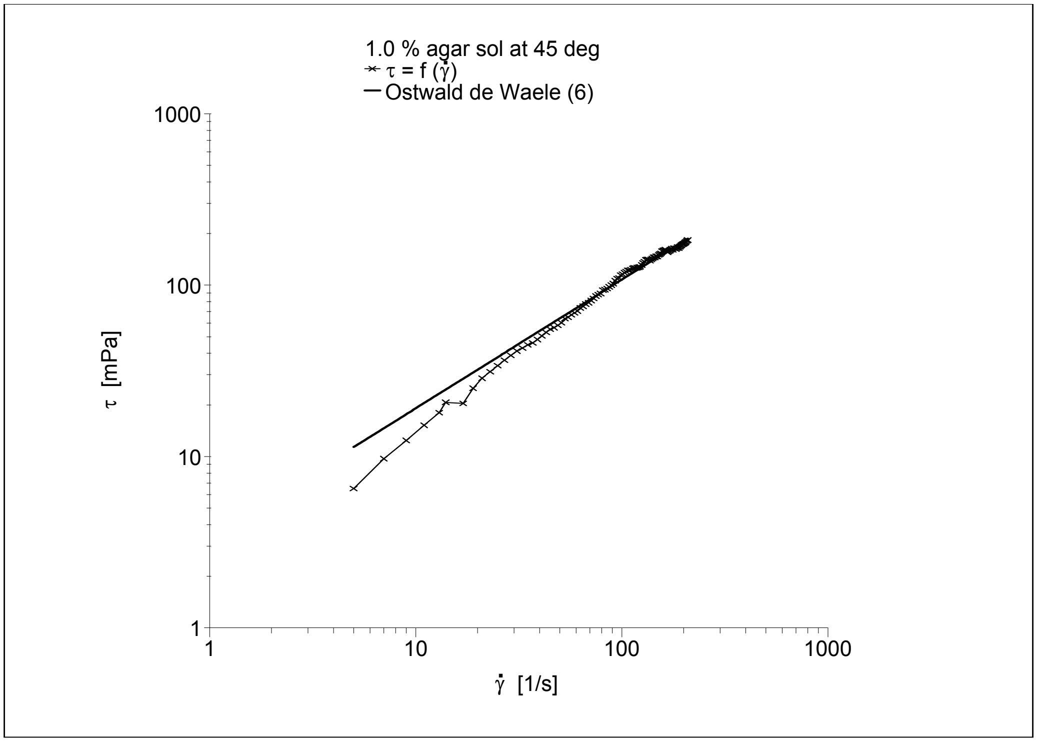

Fig.II.2.12 Logarithmic plots of shear rate vs shear stress and regression analysis according Ostwald de Waele model for 1 % agar sol at 45 ^{o}C

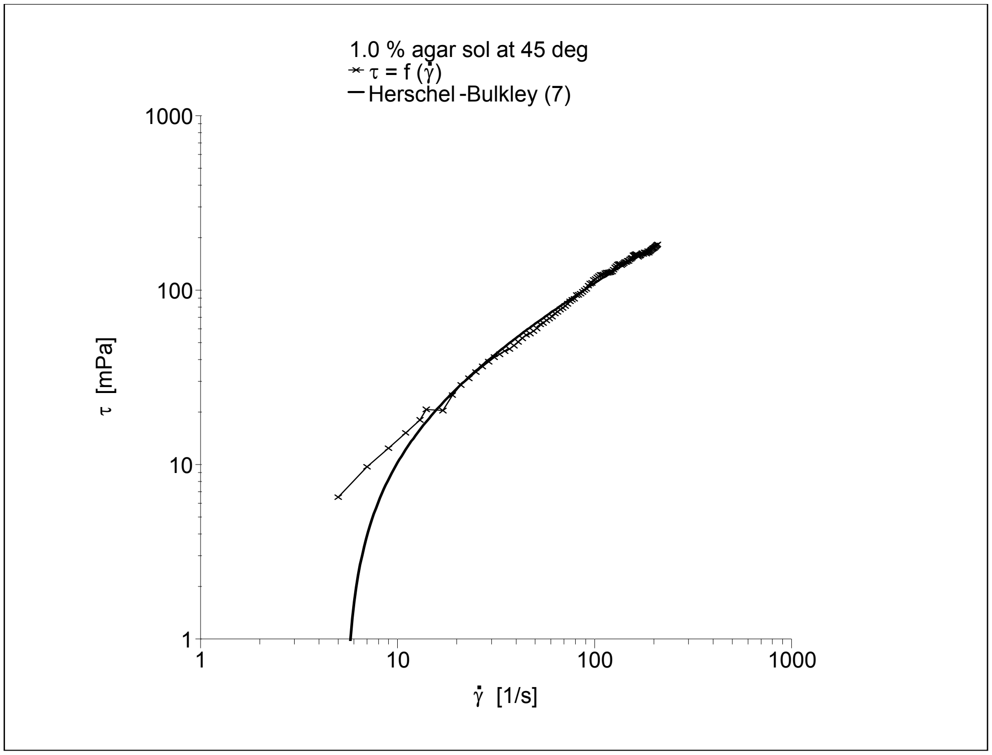

Fig.II.2.13 Logarithmic plots of shear rate vs shear stress and regression analysis according Herschel-Buckley model for 1 % agar sol at 45 ^{o}C

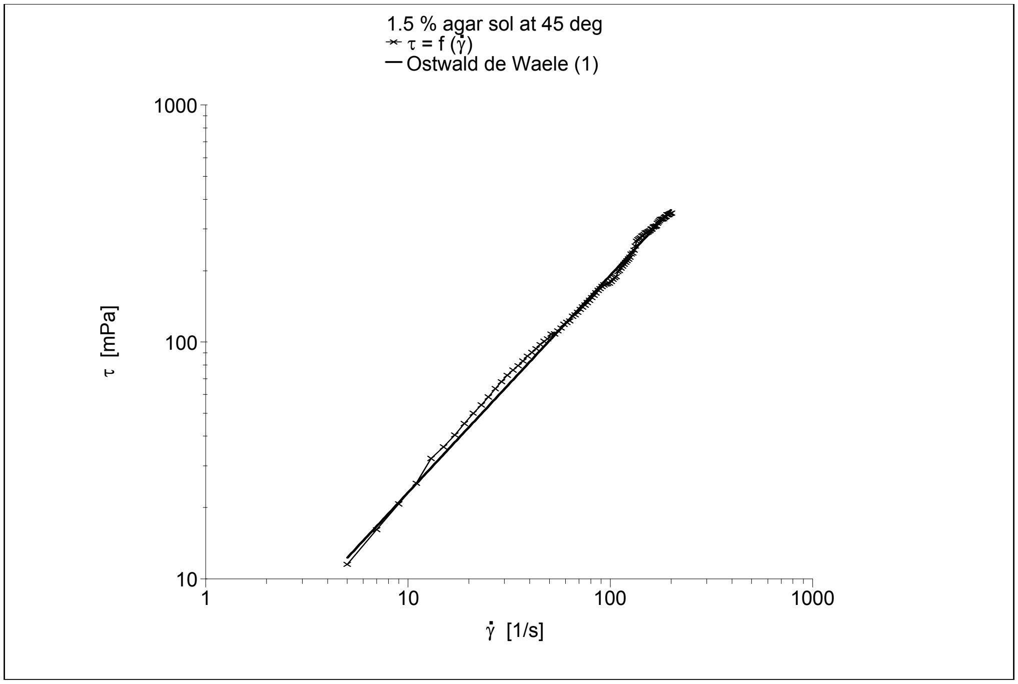

Fig.II.2.14 Logarithmic plots of shear rate vs shear stress and regression analysis according Ostwald de Waele model for 1.5 % agar sol at 45 ^{o}C

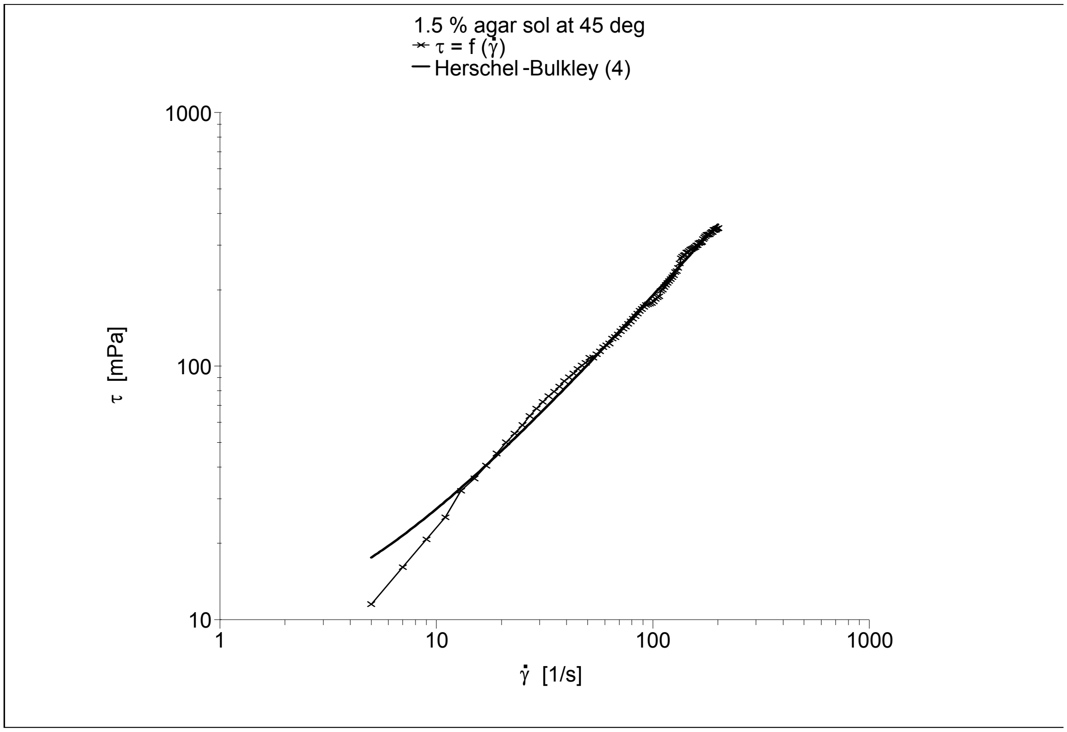

Fig.II.2.15 Logarithmic plots of shear rate vs shear stress and regression analysis according Herschel-Buckley model for 1.5 % agar sol at 45 °C

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CHAPTER II.3

EVALUATION OF AGARS OF *GELIDIELLA ACEROSA* (FORSSKAL) J. FELDMANN & G. HAMEL (RHODOPHYTA, GELIDIALES) COLLECTED FROM THE WEST COAST OF INDIA

II.3.1 INTRODUCTION

In a continuing programme of evaluating agarophytes of Indian waters 20 samples of *Gelidiella acerosa* of the Indian coasts were investigated for their agar contents. Although, there are a few reports on the agar of *Gelidiella acerosa* of southeast coast of India having gel strengths 300 g/cm^2 and 790 g/cm^2 [1-3], yet no details of extraction procedure and other physicochemical and rheological data are available in these citations.

In this chapter quality of agar obtained from *Gelidiella acerosa* collected from the natural stock occurring at the west coast of India at various locations in different seasons is reported. Native agar samples were extracted from twenty *Gelidiella acerosa* samples collected systematically between December 1999 and June 2000, and the properties of these agars were studied. The results reported herein will be useful in bioprospecting of the agarophyte *Gelidiella acerosa* as a source of superior quality agar, which has so far been unknown from this region.

To our knowledge, this constitutes the first report of on the evaluation of *Gelidiella acerosa* of the west coast of India, as a source of superior quality agar.

II.3.2 MATERIALS AND METHODS

II.3.2.1 Collection

Twenty samples of *Gelidiella acerosa* were collected once in a month from the following places of west coast of India between December 1999 and June 2000, Okha (22^{o}28′N, 69^{o}05′E), Dwarka (22^{o}27′N, 69^{o}12′E), Veraval (20^{o}54′N, 70^{o}22′E), Porbandar (21^{o}38′N, 69^{o}37′E) and Diu (20^{o}45′ N, 70^{o}58′E). Specimen of each sample (AS-IV-15-8, AS-IV-19-7, AS-IV-26-3, AS-IV-38-1, AS-IV-2-18, AS-IV-13-1, AS-IV-25-10, AS-IV-35-6, AS-IV-29-5, AS-IV-38-3, AS-IV-39-1, AS-IV-16-4, AS-IV-19-12, AS-IV-35-5, AS-IV-19-1, AS-IV-25-9, AS-IV-35-7, AS-IV-33-12, AS-IV-78-5, AS-IV-79-15) has been deposited with the CSMCRI Herbarium for future reference.

II.3.2.2 Agar extraction and isolation

Dried samples (10 g) were immersed in 500 ml distilled water for 2 h then acetic acid pretreatment was given for 1 h and heated in an autoclave at 120^{o}C for 1.5 h. The extract was homogenized and filtered under pressure through celite bed and Whatman filter paper No 541. The 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 [cf. 4].

II.3.2.3 Physicochemical properties

The gel strength (gcm^{-2}) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). The measurements were performed on a 1.5% w/v agar solution (Previously cured overnight at 10°C) [5], using a solid cylindrical plunger of 1 cm in diameter.

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.

Total Sugar: Total sugar of the agar samples were measured by colorimetric method following the method of Dubois using glucose as standard [6].

3,6-Anhydrogalactose was estimated by improved phenol-resorcinol reagent following the method by Yaphe and Arsenault using fructose as standard [7].

Sulphate content in the agar samples was estimated by turbidimetric method as described by Dodgson *et al.* [8] using K_2SO_4 as standard.

Foreign starch, foreign insoluble matter, hot and cold water solubility, water absorption were done using standard methods [9].

Details of the above all the methods are mentioned in Chapter II.1.

II.3.2.4 Dynamic rheological measurements

Agar samples in sol and gel state were prepared and dynamic rheological measurements of these samples were carried out on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany) using cone (1^o) and plate geometry of 60 mm dia and 0.052 mm gap for dynamic viscosity measurements of sol at 40^oC, with 1ml sample volume. 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' [10] was observed for the systems measured. Further details of the method are described in Chapter II.1. Gelling and melting temperatures of agar gel was measured as described by Craigie and Leigh [11]. The oscillation measurements (frequency and time dependencies of G' and G'') were carried out at 25^oC.

II.3.2.5 Weight average molecular weight

Intrinsic viscosities [η] were determined at 32°C using an Ostwald viscometer with a flow time 2.11 min for 1M NaCl. For this, solutions of agar were prepared in 1M NaCl at a concentration range 0.02% to 0.14% (w/v). Viscosity average molecular weights were calculated from the intrinsic viscosity using the Mark–Houwink equation for agarose,

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

where [η] is ml/gm as described by Rochas and Lahaye , 1989 [12].

Details of the above methods have been described in Chapter II.1.

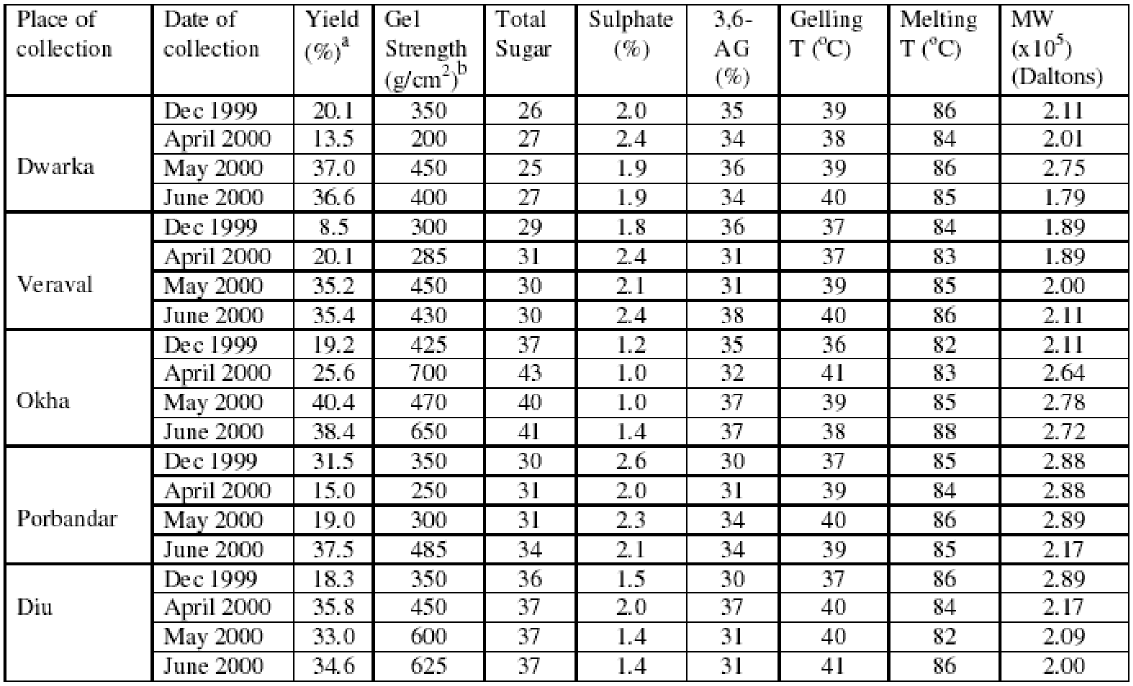
II.3.3 RESULTS AND DISCUSSION

The agar extracted from the *Gelidiella acerosa* samples of this study showed seasonal variation in their yield, physicochemical and rheological properties. The agar yield expressed as a percentage of bone-dry seaweed ranged from 13.5 –40.4 %. Total sugar, Sulphate and 3,6-anhydrogalactose contents were found to be in the ranges 25-43 %, 1.4-2.4% and 31-37% respectively. Among all samples yield was maximum for Okha agar in the month of May 2000 and lowest for Veraval in the month of march 1999. For sulphate content lowest was observed for Okha agar in the month of may and June 2000 and highest for Dwarka and Veraval agar in the month of April, June 2000 respectively. Similarly for total sugar and 3,6 –anhydrgalactose content highest observed for Diu and Okha agar in the month of April, June 2000 and May, June 2000 respectively. All this results shows better agar quality for Okha and Diu in the month of April and June 2000. All the results are furnished in Table II.3.1a for comparison.

II.3.3.1 Physicochemical properties

High viscosity values for 1.5 % (w/v) agar sols in double distilled water (35-45 cp at 80°C) were obtained from *Gelidiella acerosa* collected from Okha and Diu in the months of April and June 2000 respectively, while the lowest viscosity values (21-32 cp at 80°C) were with those extracted from the *Gelidiella acerosa* collected from Dwarka, Porbandar

Table. II.3.1a Characteristics of agars extracted from *Gelidiella acerosa* harvested at different times from different locations of west coast of India



Place of collection	Date of collection	Yield (%)[a]	Gel Strength (g/cm^2)[b]	Total Sugar	Sulphate (%)	3,6-AG (%)	Gelling T (°C)	Melting T (°C)	MW (x10^5) (Daltons)
Dwarka	Dec 1999	20.1	350	26	2.0	35	39	86	2.11
	April 2000	13.5	200	27	2.4	34	38	84	2.01
	May 2000	37.0	450	25	1.9	36	39	86	2.75
	June 2000	36.6	400	27	1.9	34	40	85	1.79
Veraval	Dec 1999	8.5	300	29	1.8	36	37	84	1.89
	April 2000	20.1	285	31	2.4	31	37	83	1.89
	May 2000	35.2	450	30	2.1	31	39	85	2.00
	June 2000	35.4	430	30	2.4	38	40	86	2.11
Okha	Dec 1999	19.2	425	37	1.2	35	36	82	2.11
	April 2000	25.6	700	43	1.0	32	41	83	2.64
	May 2000	40.4	470	40	1.0	37	39	85	2.78
	June 2000	38.4	650	41	1.4	37	38	88	2.72
Porbandar	Dec 1999	31.5	350	30	2.6	30	37	85	2.88
	April 2000	15.0	250	31	2.0	31	39	84	2.88
	May 2000	19.0	300	31	2.3	34	40	86	2.89
	June 2000	37.5	485	34	2.1	34	39	85	2.17
Diu	Dec 1999	18.3	350	36	1.5	30	37	86	2.89
	April 2000	35.8	450	37	2.0	37	40	84	2.17
	May 2000	33.0	600	37	1.4	31	40	82	2.09
	June 2000	34.6	625	37	1.4	31	41	86	2.00

[a] Yield is expressed on the basis of as received seaweed.

[b] Gel strength is expreseed at 20 °C.

Table. II. 3.1b Physicochemical analysis of agar samples extracted from *Gelidiella* acerosa collected from west coast of India

Place of collection	Date of collection	Thresh hold gel concentration (%)	Foreign insoluble matter (%)	Water absorption (Times of its weight)	Cold water solubility (wt%)	Hot water solubility (%)	Ash (± 0.2) %
Dwarka	Dec 1999	0.65	0.42	12	3.8	99.6	4.6
	April 2000	0.80	0.62	12	3.8	99.4	4.8
	May 2000	0.45	0.40	14	4.0	99.6	5.0
	June 2000	0.45	0.45	13	3.0	99.5	4.2
Veraval	Dec 1999	0.70	0.35	11	3.8	99.7	4.0
	April 2000	0.66	0.35	13	3.0	99.7	3.9
	May 2000	0.52	0.40	12	3.1	99.6	3.6
	June 2000	0.50	0.40	10	4.4	99.6	3.6
Okha	Dec 1999	0.50	0.29	19	4.9	99.7	2.3
	April 2000	0.35	0.22	21	5.8	99.8	2.3
	May 2000	0.45	0.29	20	4.4	99.7	3.2
	June 2000	0.35	0.32	18	3.4	99.6	3.0
Porbandar	Dec 1999	0.52	0.45	12	3.6	99.5	5.1
	April 2000	0.75	0.45	11	3.0	99.3	5.0
	May 2000	0.75	0.33	12	3.3	99.3	4.6
	June 2000	0.58	0.30	10	3.1	99.4	4.2
Diu	Dec 1999	0.50	0.30	15	4.0	99.5	3.0
	April 2000	0.54	0.35	17	4.5	99.5	3.2
	May 2000	0.38	0.30	17	5.4	99.6	2.14
	June 2000	0.37	0.30	18	5.4	99.7	2.14

and Veraval in the month of December 1999 and January 2000, at the same concentration (Table II.3.1c).

All the seaweed samples collected from various places in different seasons showed gelling and melting temperature ranging between 36-42°C and 82-88°C respectively. The lowest gelling temperature was recorded for the agar obtained from *Gelidiella acerosa* collected from Okha in the Month of April 2000 and Diu in the month of May 2000. The highest gelling and melting temperatures were recorded for the agar obtained from *Gelidiella acerosa* from Veraval in the month of May 2000 and from Diu in June 2000. Scrutiny of Table II.3.1a revealed that there is no positive correlation between the gel strength of agar and molecular weight, for instance, higher molecular weight is not necessarily associated with high gel strength and *vice versa* [cf. 13]. The agars obtained from Okha and Diu in the month of April 2000 and June 2000 respectively showed the highest gel strength in the range 600-700 gcm^{-2} and those from Dwarka, Porbandar, Veraval collected in the month of December 1999 and April 2000 had lowest gel strength in the 230- 350 gcm^{-2} at 20°C (Table II.3.1a). Therefore the corresponding seaweed samples can be treated as sources of superior quality agar.

Intrinsic viscosity [η] was calculated by linearly extrapolating the reduced viscosity values (in the concentration range 0.02% to 0.14% w/v) to zero concentration, molecular weight having been determined by using Mark–Houwink equation as described in Chapter II.2.

Table. II. 3.1c Intrinsic Viscosities for various agar samples in 1 M NaCl

Place of collection	Date of collection	Aparent Viscosity (cP)	[η] in ml/g
Dwarka	Dec 1999	21	477.0
	April 2000	20	460.67
	May 2000	24	577.32
	June 2000	27	423.79
Veraval	Dec 1999	21	440.7
	April 2000	26	440.7
	May 2000	19	459.02
	June 2000	29	477.0
Okha	Dec 1999	36	477.0
	April 2000	45	565.17
	May 2000	35	581.84
	June 2000	42	529.68
Porbandar	Dec 1999	32	596.84
	April 2000	31	596.84
	May 2000	32	598.33
	June 2000	32	486.79
Diu	Dec 1999	35	598.33
	April 2000	36	486.79
	May 2000	36	473.8
	June 2000	35	459.02

Weight average molecular weight of the samples were determined and plot of reduced viscosities vs concentration are shown in Fig.II.3.1 for the best agars from Okha and Diu. All experiments were carried out in triplicates. Intrinsic viscosities obtained for all the agar samples are listed in Table II.3.1c. Weight average molecular weight of agar from Okha was found to be in the range of 1.89-2.89 X 10^5 and Diu 2.00-2.89 X 10^5D (Table II.3.1a).

No starch was found in any of the agar samples. Agars having high viscosity and gel strength have lower threshold gel concentration and *vice versa*. For these agar samples high water absorption, low foreign insoluble matter content, high cold and hot water solubility were observed (Table II.3.1b).

II.3.3.2 Dynamic rheological properties

The agar samples of Okha and Diu i.e., agars obtained from *Gelideialla acerosa* samples collected from Okha in April 2000 and from Diu in June 2000 respectively, were selected for rheological measurements. Observing the similar rheological behaviour for the low gel strength agar samples i.e. the one obtained from *G. acerosa* collected from Dwarka in April 2000 was chosen as a representative of all these type of low gel strength agars, which presented in all the figures for purpose of comparison.

Fig.II.3.2 shows the rheological spectra of the frequency dependence of G' and G'', of agar gels of different places studied at different concentrations. The dispersion of agar (0.3% w/v) from Dwarka, Veraval and Porbandar can be judged as a semi-dilute polysaccharide solution based on a crossover of G' and G'' in the highest frequency range examined, this fact is also supported by the fact that the threshold gel concentration of all the agar samples from these places is above 0.3 %(w/v) in all collections (Table II.3.1b).

On the other hand, the agar from Okha and Diu in the month of April, June 2000 and May, June 2000 respectively at the same concentration showed a gel type spectra with predominant G′ over G″ in the entire frequency range examined at 25^{o}C. The moduli of the true gels (1.5% w/v agar gel) are almost frequency independent with tanδ (= G''/G') values less than 0.1 at all frequencies examined, suggesting that the gel was predominantly elastic for all agar gel samples. The moduli of the dispersion at lower concentrations are slightly dependent on frequency and tanδ values were ca 0.9. For 0.5 % agar dispersion from Okha and Diu in the month of April, 2000 is 0.15-0.2. Slightly frequency dependent moduli and tanδ values over 0.1 are typical rheological signatures of a weak gel system [cf.14] (Fig.II.2.2 Fig.II.3.3)

The 1 % w/v agar dispersion of all the agar samples exhibited a gel-like spectrum that is G'>G'' at all examined frequencies irrespective of place and time of collection. It was observed that there existed no thermal hysteresis between coil-to-helix and helix-to-coil transition temperatures for agar gel in lower concentrations (<0.3% w/v) but the reverse was true in the higher concentrations (>0.5% w/v).

The stability of moduli for agar obtained from all the places during storage at 25^{o}C and under strain 0.01% were measured and depicted in Fig.II.3.4. The storage modulus (G′) values of the true gel formed at the concentration 1% w/v for agar from Okha and Diu were higher than those from rest of the places. However, the moduli of the true gel (in

1.0% and 1.5% concentrations) from Dwarka, Porbandar and Veraval gradually decreased with time, which can be explained only if the gel network was gradually shrinking and undergoing syneresis due to continuing slow aggregation of helices even after gelation. For agar dispersions at higher concentrations from Okha and Diu, it has been observed that the moduli did not change significantly when stored at 25°C (Fig.II.3.4) this indicates that there was no syneresis for these agars.

Temperature dependencies of G′ and G″ for 1.5% w/v agar gel from all the places are summarised in Fig.II.2.4a and Fig.II.2.4b. The experiment was conducted by decreasing temperature from 90°C to 20°C in controlled deformation mode with strain 0.05%. It has been observed that with decrement of temperature G″ decreases and reverse trend was observed for G′ values. A 2-3 fold increase was observed in G′ values over G″ [cf.13]. The 1.5 % agar gels obtained from the selected *Gelidiella acerosa* samples of Okha and Diu showed more variation and the transition took place near the gelling temperatures (Fig.II.3.5a, Fig.II.2.4a and Fig.II.3.5b).

Shear viscosity was measured with changing shear rate at 45°C. Under the steady shear flow, a Newtonian flow behavior was observed at the low limiting shear rate region and a strong shear thinning at higher shear rate for agar sols at lower concentrations. (Fig.II.3.6). More gel thining behaviour was observed for agar samples obtained Gelidiella acerosa samples of Dwarka, Porbandar and Veraval. Conversely, agar gel samples obtained from *Gelidiella acerosa* samples of Diu and Okha showed lesser shear thinning indicating the coherence of the gel.

II.3.4 SUMMARY

The main three grades of agar that are available for various applications are: bacteriological (microbiological), sugar-reactive, and food-grade (industrial-grade). Food-grade (technical grade) is the lowest priced one being the least refined. It is used in foods as a standard thickener.

Although the quality of agar is usually judged in terms of its technical performance as a gelling agent and gel strength being one of the important characteristics of agar. Agars from different sources give different response during gel formation. This may be ascribed to the difference in molecular weight, molecular distribution, and the proportion of 3,6-anhydrogalactose units present in the polysaccharide, which are manifested in the physicochemical properties such as gel strength, melting and gelling temperatures [15,16].

In industries, different rheological parameters are used for benchmarking. The rheological parameters are directly influenced by the presence of various substituted groups in agar backbone [17].

Each of the agar gels tested in this investigation, possessed adequate properties that would meet the requirements of specific end applications. The agars obtained from *Gelidiella acerosa* of Okha and Diu in the month of April, June and May, June 2000 respectively were found to be of superior quality among all the collections in the lot, this is shown by their physicochemical properties e.g. relatively low sulfate (1-1.4%) as well as relatively low gelling (38-41^{o}C) (Table II.2.1a). Lower ash contents of these agars indicated low metal contents. Comparatively low 3,6-anhydrogalactose contents in the agars of Dwarka, Veraval, Porbandar (31-35%) than those in the agars obtained from Okha and Diu (35-37%) are manifested in the higher gel strengths of the agars of the latter (Table II.2.1a). The agar gels obtained from *Gelidiella acerosa* collected from Okha in the month of April and June 2000 and Diu in the month of May and June 2000 were found to be the best. These agar gels exhibited the highest breaking strength, highest degree of cohesiveness, rigidity (Fig.II.2.2, Fig.II.2.4 and Fig. II.2.5). Stronger gels are of value because of their strength, resilience, elasticity, relative transparency, relative permanence and thermoreversibility [18]. The agar extracted from *Gelidiella acerosa* collected from Dwarka, Veraval and Porbandar produced gels of lower strength, cohesiveness, and rigidity (Fig.II.2.2 and Fig.II.2.3). The usefulness of the latter agars is limited to the weaker gels that are used in the food industry [19].

It is known that the yield and physical properties of agar preparations depend on the source of agarophytes, environmental, physiological factors as well as the extraction and recovery procedures [15]. In the present study, it has been demonstrated that the gelling properties vary significantly with the sites of collection as well as with the seasons, and sources of superior quality agar from the west coast of India have been identified.

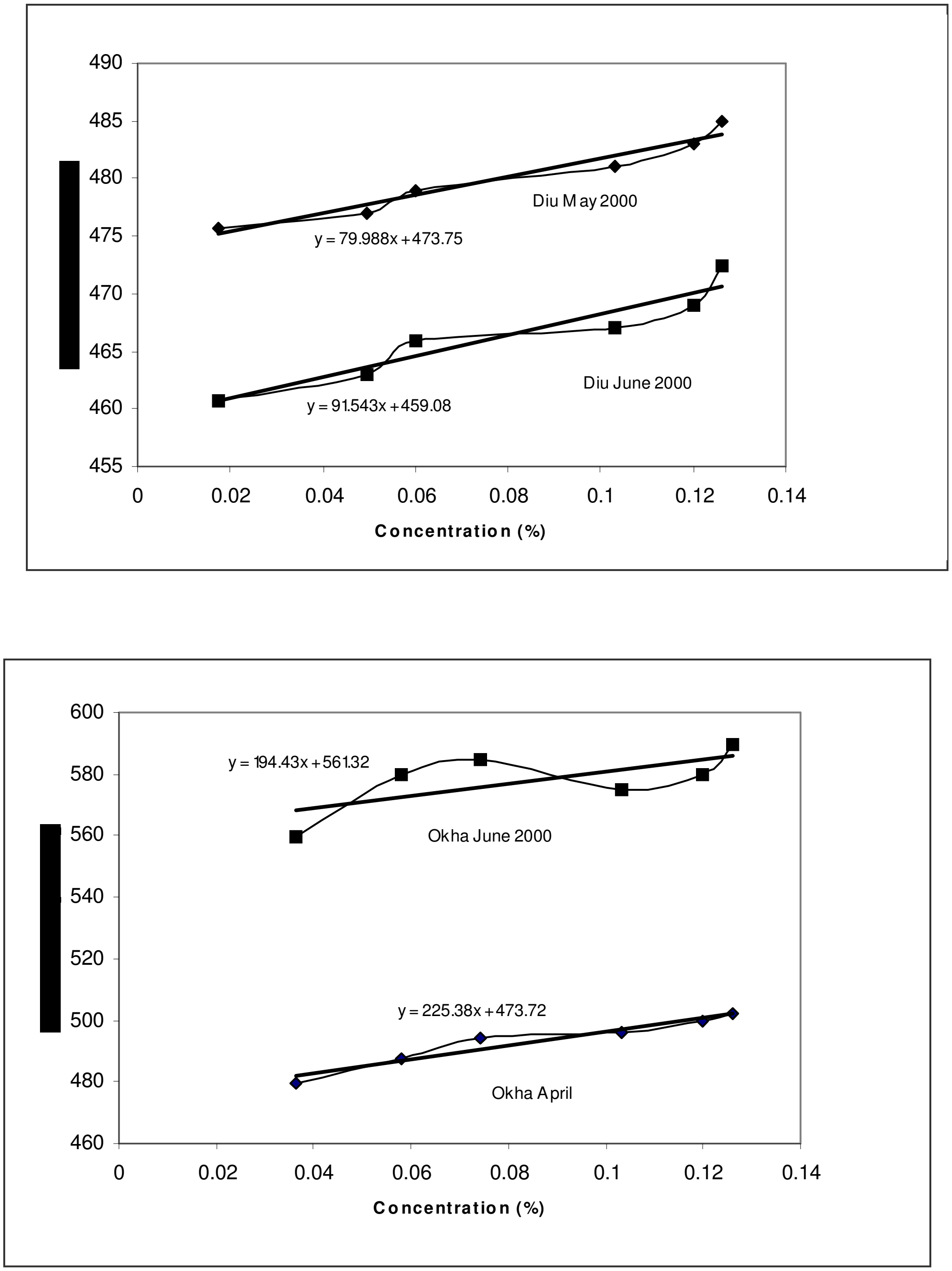


Fig.II.3.1a and 1b Determination of intrinsic viscosity for Diu and Okha agar at 32 ^{o}C

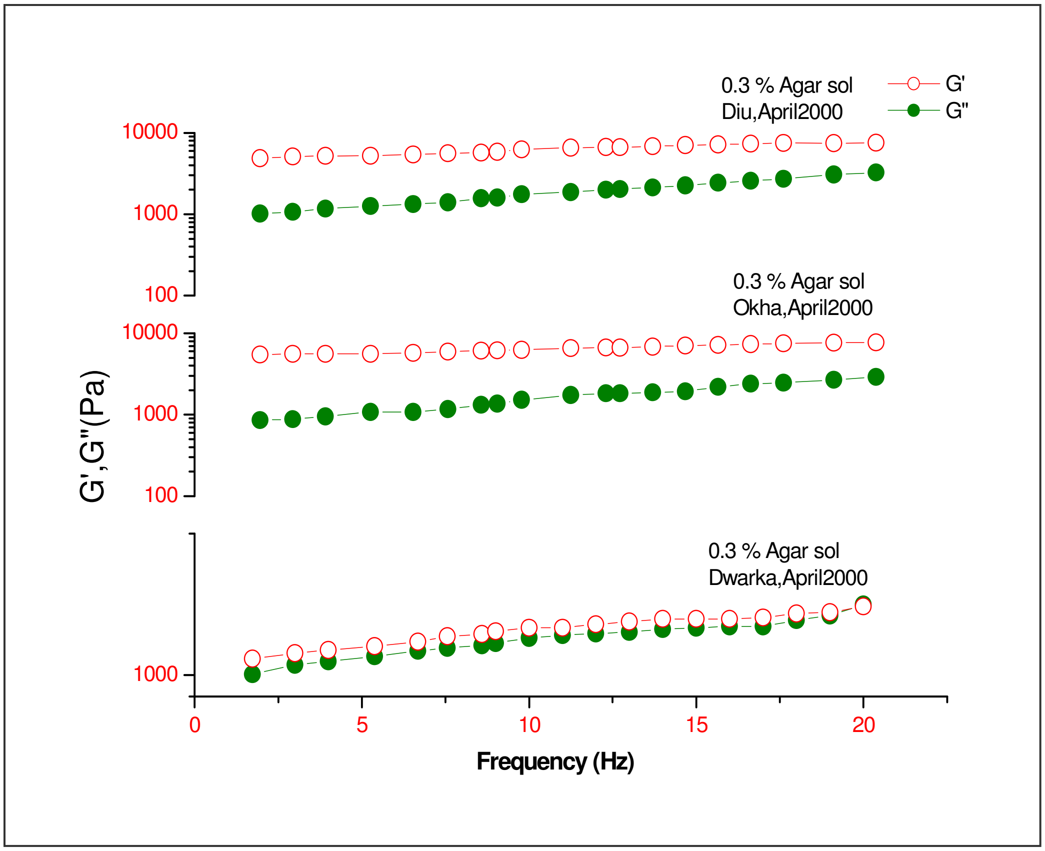


Fig. II.3.2 Frequency dependency profile of 0.3% agar sol samples

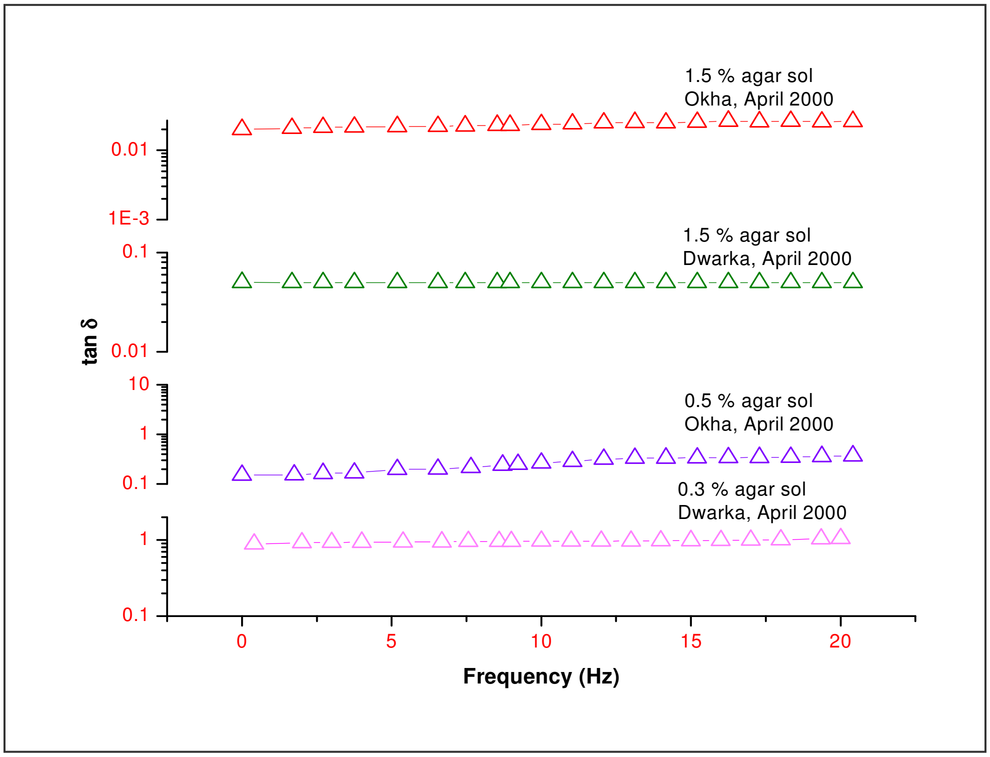


Fig.II.3.3 Variation of tan δ values with frequency

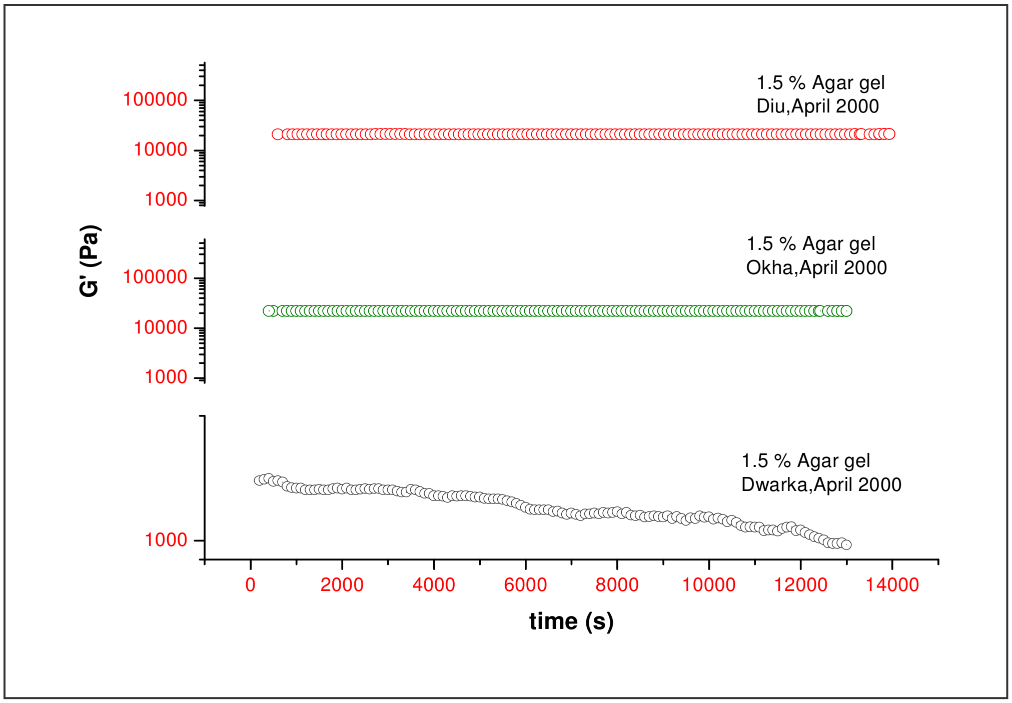


Fig. II.3.4 Time dependency profile for 1.5 % agar gel of different seasons and places.

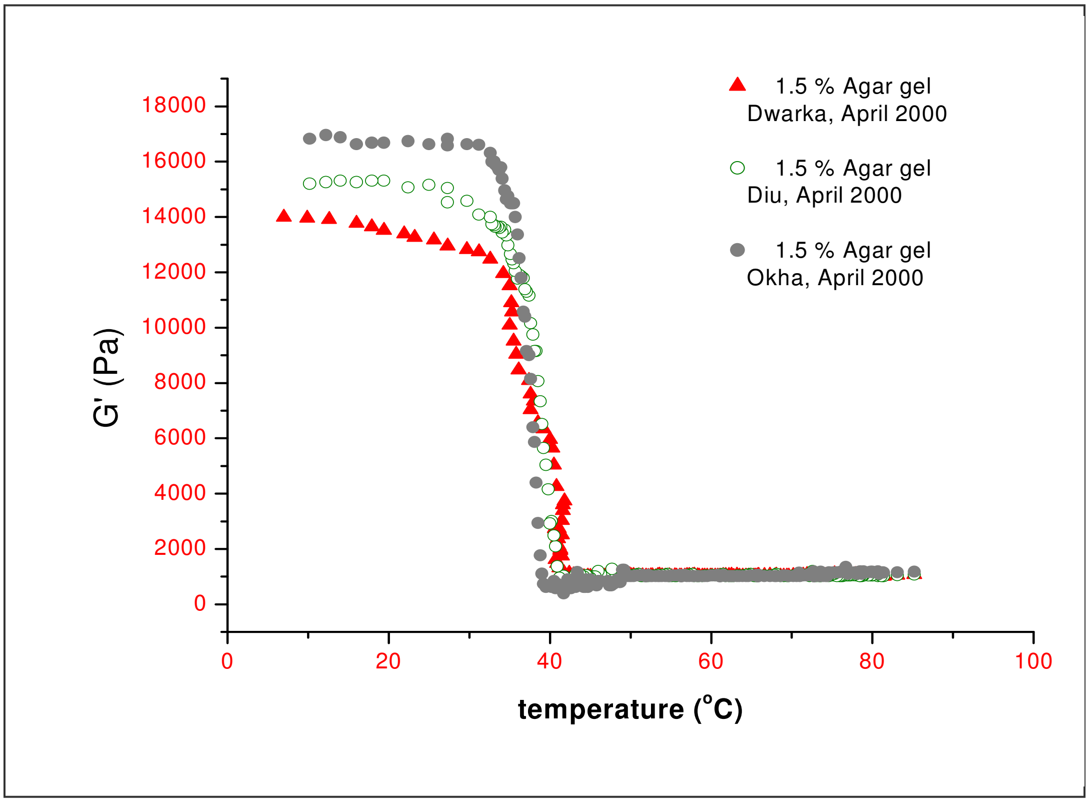


Fig.II.3.5a Cooling curve for G′ of 1.5 % agar gel samples

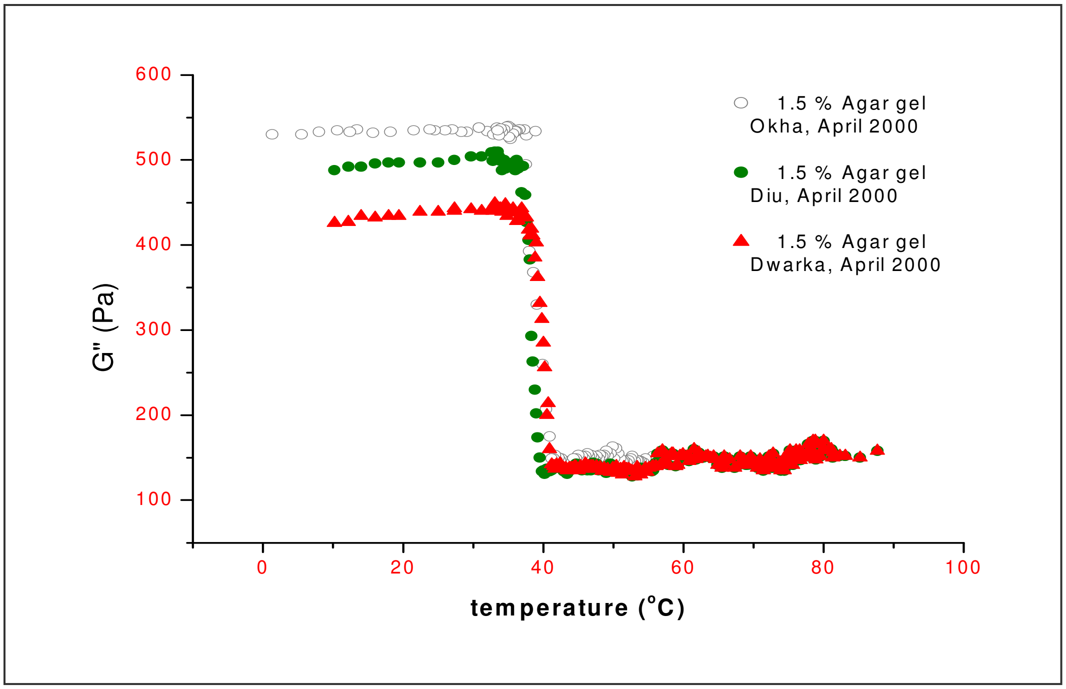


Fig. II.3.5b Cooling curve for G″ of 1.5 % agar gel samples

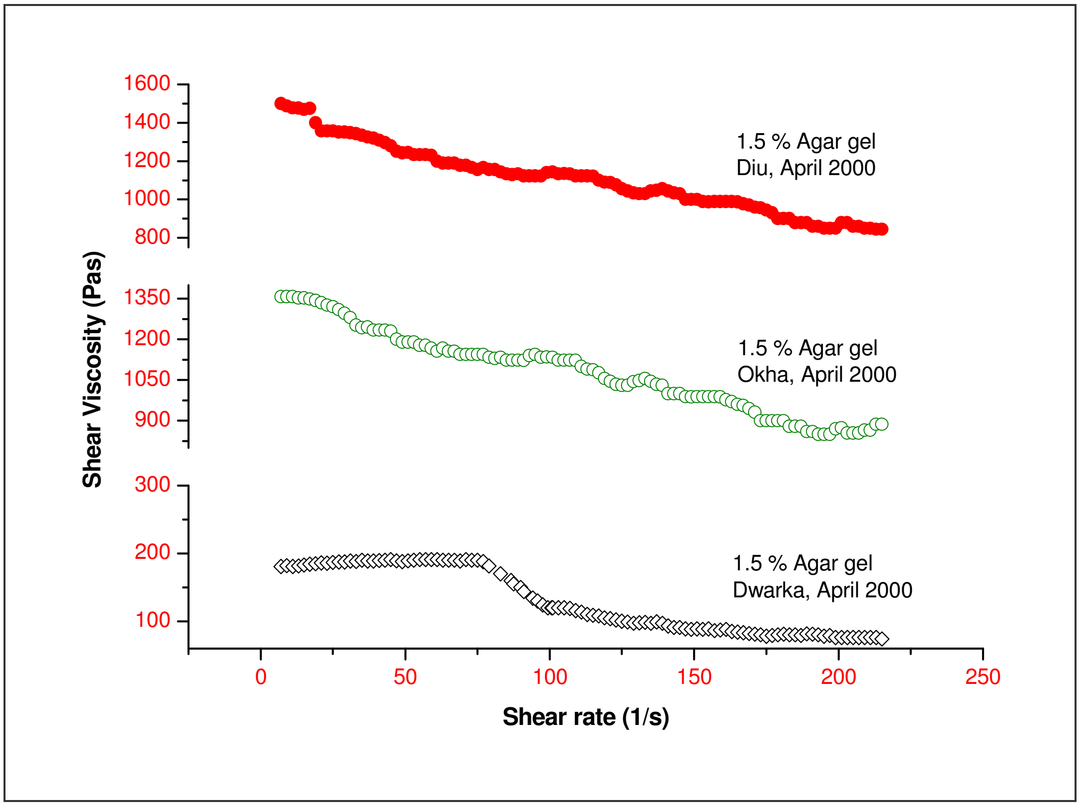


Fig. II.3.6 Gel thinning behaviour of agar gel samples of different seasons.

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PART – III

STUDIES ON THE INTERACTION OF SURFACTANTS AND FATTY ACIDS WITH AGAR

CHAPTER III.1

ON THE PROPERTIES OF AGAR GEL CONTAINING IONIC AND NONIONIC SURFACTANTS

III.1.1 INTRODUCTION

Biopolymers are of growing interest as they are used in industry to provide structure stability [1]. Agar and carrageenans, the phycocolloids obtained from many species of red seaweed (Rhodophyta), are industrially important for their excellent thickening or gelling abilities [2,3]. Both agar and carrageenan form hydrogels that are constituted of three-dimensional networks of polymer chains. The backbone of these phycocolloids is made up of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose. Gelation of polysaccharides in presence of inorganic salts as well as their crosslinking properties have been reported [4,5]. Gelation of many polysaccharides is effected by various additives. Effect of quality of solvent and ions on the rheology and gelation of κ-carrageenan have also been reported [4]. Tanaka et al. have reported the surfactant-mediated thermo-reversible gelation with multiple crosslinking junctions in associating polymer hydrogels [5-9]. Interaction of polymers and surfactants in aqueous solution has been studied by many researchers to explore the potential applications in chemical, petrochemical and pharmaceutical industries [10]. Existence of a limited range in the multiplicity of the network junctions results in high sensitivity of their rheological properties to external perturbation [11,12]. Higher levels of organization and, in particular, intermolecular networks, can also be proved by rheological studies of sols or gels. Polysaccharide solutions, gels, and biological assemblies show a behaviour intermediate between that of perfect solid and perfect liquid and resolution of induced stress into in-phase and out-of-phase components gives a measure of solid like and liquid like nature of the material, characterised by the rigidity modulus G′ and loss modulus G″, respectively. For true gels G′ is much larger than G″ and both show negligible frequency dependence [13]. Rheological properties of polysaccharide thickeners (guar gum and substituted guar gum) at different concentrations and temperatures and their interation with nonionic surfactants have been studied by Oblonsek et al. [14]. It is also reported by Lundqvist et al that interaction between amylose and ligands, such as iodide, lipids or surfactants involves formation of inclusion complexes [15].

It has been widely reported that surfactants can influence gelling characteristics of polymers in solution. Ermeneko et al. reported the interaction of κ-carrageenan gel with an ionic surfactant that leads to the formation of an ordered structure. The ordering results from hydrophobic and electrostatic interactions in the polymer network/surfactant system [16]. It is also reported that surfactants play an important role in promoting gelation of associating polymers [17]. The effect of adding lipids or surfactants to starch has been extensively studied in recent years. Lipids and surfactants have been shown to change the properties of ageing both in model systems [18].

In the course of our studies to develop a process of extraction of agar from the red seaweed *Gelidiella acerosa*, it was realized that the quantity of water required in the process is kept large to overcome problems of choke up during filtration of the agar sol. Such problems tend to be especially acute when the sol viscosity and gelling temperature of the agar are high. However, large usage of water greatly reduces throughput and increases energy cost. We conjectured that it may be possible to utilize suitable high HLB surfactants to influence the rheological properties of agar and undertook a systematic study in this direction. Additional insight was obtained from DSC-TGA investigations. Watanabe has recently reported results on slowly soluble detergent compositions comprising carrageenan and nonionic surfactants [19] but, to our knowledge, there exists no previous report on the interaction of agar gels with surfactant.

III.1.2 MATERIALS AND METHODS

III.1.2.1 Materials

Bacteriological agar was extracted from *Gelidiella acerosa* collected from Gulf of Mannar, at Ervadi ($9^o15'$N, $78^o58'$E) located in south east coast in Tamil Nadu, India, in March 2000, by following the method of Roleda et al. [20]. The same agar sample was used for all experiments. The effects of various surfactants on agar sol were studied. SLS, (Sodium lauryl sulfate; anionic), Brij 35 (Polyoxyethylene lauryl ether; non-ionic), Triton X-100 (polyoxyethylene octyl phenyl ether ($C_{34}H_{62}0_{11}$); nonionic), Cetyl pyridinium chloride (CPC; cationic), Cetyl trimethyl ammonium bromide (CTAB; cationic), Glycine, Alanine, Glycerol, Mannitol were procured as LR Grade surfactants from S.D. Fine Chemicals, Mumbai, India. Polyethylene 10-lauryl ether ($C_{12}E_{10}$; nonionic) was procured as AR Grade surfactant from Aldrich while the commercial surfactants SC893 (nonionic), SC896 (anionic), SC229 (anionic), and Crexlox3485 (nonionic) were procured from Uniqema, India. The surfactants were used as received without further purification.

III.1.2.2 Extraction of agar

Agar was extracted from *Gelidiella acerosa* through the conventional method of pressure extraction. Laboratory experiments were conducted with 10 g of seaweed while bench scale extraction was undertaken with 1.5 kg of seaweed. The ratio of seaweed to water taken for extraction was 1:30 in all cases. The hot extractive was then diluted with an equal quantity of hot water to thin out the solution and then filtered under vacuum. Based on the results of surfactant effect on sol viscosity *(vide infra)*, wherein Brij35 and Triton X-100 were found to have maximum effect in lowering sol viscosity, the latter surfactant was selected for further studies aimed at demonstrating its utility in reducing water usage in the process of preparing agar from the seaweed. Unlike in the conventional procedure described above, wherein the hot extractive is diluted with equal volume of hot water prior to filtration, non-ionic surfactant was incorporated into the hot extractive (0.4% w/v) and filtration attempted without any dilution. The filtrate was then subjected to freeze-thaw cycles. Agar yield was measured with respect to bone-dry seaweed while gel strength was estimated as described below. Studies were conducted both at laboratory scale and at bench scale.

III.1.2.3 Gel strength measurements

50 ml of 1.5% agar sol was prepared in a 50 ml beaker and the surfactant was added into the hot sol, stirred well and boiled to ensure complete dissolution. Maximum water loss during boiling was 4% and this loss was compensated by adding required quantity of hot water into the sol with stirring. The beaker was then covered and allowed to cool to room temperature to form a gel. After the formation of gel at room temperature, the gel was kept overnight at 10^{o}C. Gel strength was measured at 20^{o}C using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan).

III.1.2.4 Rheological measurements

Samples of sol and gel were prepared as described in Section III.1.2.3 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^{o} rad angle) geometry was selected for dynamic viscosity measurements of sol at 45^{o}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 0.5%

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 minute 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.

III.1.2.5 Physical properties

Measurement of pH was carried out using a model No. 535 pH meter from Systronics Scientific Instruments, India. Gelling and melting temperatures of agar gel in presence of various surfactants were measured in the mixtures as described by Craigie et al. [21]. Apparent viscosity was measured at different temperatures (80° to 50°C) using a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No.1 was used for apparent viscosities less than 100 cp and Spindle No.2 was used for measuring viscosities > 100 cp. The spindle speed was 60 rpm.

III.1.2.6 Thermal measurements

DSC/TGA measurements were carried out on a Toledo Mettler system from Switzerland. For this purpose, ca. 10 mg gel was taken in an aluminium crucible weighing 70μg and the measurements were done using a temperature programme of 30°C to 110°C at 2°C min^{-1} heating rate in an air atmosphere. TGA measurements were carried out using the temperature programme 40°C to 110°C at a heating rate of 2°C per minute in an argon atmosphere, using sample mass of ca. 10 mg.

III.1.2.7 Surface tension measurements

The surface tensions of aqueous solutions with and without surfactants were measured at 27.1°C (±0.5°C), by the static Wilhelmy plate method using a Dynamic Contact Angle Tensiometer (DCAT 21, DataPhysics, Germany). Measurements were carried out in triplicate. To ensure removal of surface-active contaminants, all glassware in contact with the sample were cleaned in acid and rinsed with double distilled water. The platinum plate was washed in double distilled water, heated in a Bunsen flame and left to cool at room temperature. The solutions were prepared with double distilled water.

III.1.3 RESULTS AND DISCUSSION

III.1.3.1. Physical properties

Several cationic, anionic and non-ionic surfactants were studied for their influence on agar sol and gel properties and the results are shown in Table III.1.1. As can be seen from the Table, ionic surfactants increased the melting temperature, gelling temperature and gel strength of the agar. The extent of deviation was, however, not the same with all ionic surfactants. Enhancements were most pronounced with SC229. Unlike in the case of other ionic surfactants, the gelling temperature was found to decrease markedly with SLS whereas the gel strength showed a decline for [SLS] $< 0.2\%$ and a rise in comparison to control agar gel for concentrations $\geq 0.3\%$ (w/v). In contrast to the behaviour of ionic surfactants, non-ionic surfactants reduced the melting temperature, gelling temperature and gel strength of the agar. The deviations were most pronounced with Triton X-100 and Brij 35 (Table III.1.1). Interestingly, whereas gel strength was sensitive to the application level of the surfactants, the gelling temperature was less sensitive to variations in surfactant concentration for most of the surfactants studied. Evidently, the factors that govern gel strength are different from those that control gelling temperature.

Although an in-depth study of the phenomenon in relation to surfactant properties is required, the results suggest that micellar aggregates play an important role and the dominant interactions occur between surfactant and water. Interactions, if any, between surfactant and the polysaccharide are weak in nature since the surfactant can be readily removed from the system in the course of the freeze-thaw cycles. Indeed, this has been exploited advantageously in designing a bench scale process wherein Triton X-100 was incorporated into the hot extractive prior to filtration, which resulted in a dip of viscosity from 35 cp to 22 cp at 80^{o}C. The surfactant was subsequently removed through successive freeze-thaw cycles. Progressive removal of surfactant through this process was evident from surface tension measurement of the thawed liquid after each freeze-thaw cycle; the value after the fourth cycle was 69 mN m^{-1} at 25^{o}C vs. 71 mN m^{-1} for deionised water. The agar gel strength increased steadily with removal of surfactant, and gel strength of ca. 600 gcm^{-2} was achieved after the fourth freeze-thaw cycle, which was the same as for the control agar gel (Table III.1.1). The yield of agar obtained through this process in the lab scale was 33% compared to 34 % for the conventional process. In the bench scale experiment, the corresponding yields were 28% and 31%, respectively (yields are expressed with respect to bone dry material). Effect of additives like Glycine, Alanine, Glycerol and Mannitol were marginal on physical, rheological and thermal properties of agar gel and hence the additives which effected considerably on these properties were selected for detailed rheological and thermal analysis.

III.1.3.2 Rheological properties

The viscosity of agar sol was sensitive to temperature and the concentration of surfactant (Table III.1.2). Deviations from the values obtained without surfactant were positive (higher sol viscosity) in case of ionic surfactants and negative (lower sol viscosity) in case of non-ionic surfactants. In the case of ionic surfactants, the extent of deviation increased gradually with increase in surfactant concentration up to ca. 0.4 % concentration (w/v); no substantial change in sol viscosity was observed beyond this value (Table III.1.2). Maximum increase in viscosity for the ionic surfactants studied was observed with SC229. The increase in sol viscosity was accompanied by a corresponding increase in gel strength following sol-gel transition, suggesting that the origin of these effects must be similar for sol and gel. Since the increase was found both with anionic and cationic surfactants, the sulphate residues in the polysaccharide presumably play no significant role in the observed phenomenon as otherwise differences could have been expected between anionic and cationic surfactants. Cationic surfactants, however, induced minor flocculation in the agar sol (presumably due to the presence of the sulphate in the agar polymer) but upon centrifugation to remove the flocs, the resulting mass continued to show higher sol viscosity while upon cooling of the sol, the resultant gel too exhibited substantially increased gel strength, in a manner akin to that found with anionic surfactants (Tables III.1. 1 & 2). Presumably, ion-dipole interactions between the surfactant and water molecules interfere with the junction zones in the gel network, although the manner in which such changes actually occur remains to be understood. The concentrations of surfactants taken were near or above the critical micelle concentration (cmc) in all cases (Table III.1.3) and the micellar nature of surfactant aggregates could have influenced the overall structure of the networks, particularly formation of new tertiary structures, which in turn, could affect T_{gel} and T_m.

Similarly, why the non-ionic surfactants studied in this work lower the viscosity and gel strength is also not clear. In accordance with the lower cmc values of the non-ionic surfactants, the viscosity decrease was drastic even at the lowest concentration of surfactant used, namely 0.1% (w/v) (Tables III.1.2 and 3). Thereafter, the decrease was more gradual. For example, in the case of Brij35, the viscosity data at 50^{0}C shows that the value drops from 127 cp to 78 cp when 0.1% (w/v) Brij35 is added into the hot extractive, and thereafter there is a steady decrease from 78 cp to 41 cp with increase in surfactant concentration from 0.1% (w/v) to 0.7% (w/v). At the higher concentrations of surfactant, the viscosity was reduced by around 65% at 50^{o}C. As observed with ionic surfactants, the sol viscosity and gel strength showed a direct correlation, i.e., the decrease in sol viscosity was accompanied by a corresponding decrease in gel strength following sol-gel transition, suggesting that here again the origin of the rheological effects must be similar for sol and gel.

Gel strength of agar was also measured in the presence of mixed ionic/non-ionic surfactant combinations. Data of the SC229/Triton X-100 system are given in Table III.1.4. As can be seen from the table, the gel strength did not change substantially in presence of 1:1 mixture presumably because of the opposite and equal nature of the effects. The effect of unequal proportions of surfactants in the mixture were evident, as might be expected, and gel strength was enhanced with increasing proportion of SC229 in the mixture of surfactants while it was lowered with increasing proportion of Triton X-100. Thus, the overall behaviour follows the average of individual effects and there is no evidence of any synergistic interplay between the ionic and non-ionic surfactant molecules.

We have attempted to study the rheological behaviour of the 1.5% agar matrix at 45^{o}C, with and without surfactant. This temperature is slightly above the gelling temperature of the matrix. As shown in Fig.III.1.1, the behaviour was non-Newtonian, with increase of shear thinning with shear rate [20]. The effect of shear thinning was most pronounced with nonionic surfactants (Triton X-100 and Brij 35). Non-ionic surfactants lose hydrogen bonds at their cloud point and when the matrix is cooled, the surfactants would tend to re-form the hydrogen bonds with water, and this process can lead to greater instability and, consequently, higher degree of shear thinning. Ionic surfactants, on the other hand, show less of an effect and the behaviour of the matrix in their presence is more similar to that of the control agar matrix.

Viscoelastic behaviour of 1.5% agar gels in absence and presence of 0.4% surfactant was studied by oscillatory measurements. As expected, all the samples showed gel-like behaviour, i.e., $G' >> G''$, when measurements were conducted at 25^{o}C in controlled deformation (CD) mode with a strain value of 0.5%. For all the gels containing surfactants, the variation of G′ with ω was fairly flat whereas for the control agar gel, the variation of G′ with ω was non-linear, with steeper rise for ω > 50 rad/s (Fig.III.1.2).

Phase transition temperatures of agar gels in presence of surfactants

G′ and G″ were plotted as a function of temperature (87.7°C to 10°C) for all of the gels studied. Figures 3A shows the plots for agar gel without surfactant and for gels containing CTAB, Triton X-100, Brij 35 and SLS. The data were recorded at 1 Hz frequency in controlled stress (CS) mode with 1 Pa stress rate and cooling rate of 0.38°C/min. Fig.III.1. 3B shows plots for the same system recorded at 1 Hz in controlled deformation (CD) mode with 0.5% strain and 1.0°C/min cooling rate. It can be seen from Fig.III.1. 3A that G′ and G″ follow similar profiles at higher temperatures whereas at lower temperatures G′ rises more steeply, as a result of which the curves intersect ($T_{\tan \delta=1}$). The data of $T_{\tan\delta=1}$ are given in Table III.1.1. It can be seen that the values match closely with T_m values measured by Craigie method [20,21]. Fig.III.1.3B shows the plots of the same systems recorded in controlled deformation (CD) mode. There is a 1.4 order of magnitude variation in tanδ over the temperature range of 30-50°C. Beyond this temperature range, the curves plateau at both ends. The results of Figure 3B are consistent with the data in Figure 2 and tally well with the observations reported by Lai & Lee [23] and Mohammed et al. [24]. The pronounced differences between the plots of Figures 3A and 3B arise largely from the differences in geometries and shear stress/shear strain values employed and are in accord with the earlier observations of Whyte et al. [25].

III.1.3.3 Thermal properties

To study the thermal stability of agar gels in presence of surfactants, DSC/TGA measurements were carried out on 1.5% agar gels with (0.4% w/v) and without different ionic and non-ionic surfactants. Fig.III.1.4 shows the DSC heating curves of ca. 10 mg of (a) control agar gel, and agar gel containing (b) CTAB, (c) SLS, (d) Triton X-100 and (e) Brij 35 recorded with water as reference. It can be seen from the figures that the control agar gel exhibits two endotherms with peaks at 81°C and 91°C. Kawasaki et al. have reported similar two-step transition for an N-isopolyacrylamide aqueous gel system [19]. A plots similar to that of control agar gel is seen with the non-ionic surfactant, Brij 35. In the case of Triton X 100, the endotherm at lower temperature is more prominent. In the case of SLS, this endotherm becomes dominant and the higher temperature endotherm is not seen. In the case of CTAB, the lower temperature it appears that the lower temperature endotherm shifts to ca. 89^0C and the higher temperature endotherm appears only as a shoulder. The DSC results for the gels containing surfactants are in reasonable accord with the corresponding values of T_m in Table III.1.1. For the control agar gel, the value of 86^0C for T_m is higher than what might have been predicted based on the DSC result.

Fig.III.1.5 shows the TGA profiles of agar gels with and without surfactants. As can be seen from the plots, the gels containing the ionic surfactants, SLS and CTAB, hold on to water most tenaciously whereas gels containing the non-ionic surfactants, Brij35 and Triton X-100, lose water most easily. The behaviour of the gel without surfactant is in between the above. These results correlate well with the DSC plots of Figure 4 and with the T_m values in Table III.1.1, in as much as evaporation of water would be facilitated once the gel undergoes a phase transition and melts. The only exception is SLS which holds on to water most tenaciously among all the systems studied even though the value of T_m is 77^0C at the 0.4% surfactant level employed, i.e., a value much lower than gel containing CTAB, and even gel without surfactants.

III.1.4 SUMMARY

It has long been known that certain surfactants cause gel inhibition in the gelling material resulting in a lowering of viscosity. In the present study, it has been demonstrated that ionic surfactants enhance agar sol viscosity and also the stiffness of the gel. The gelling and melting temperatures are also raised upon incorporation of surfactant. The behaviour is similar with both cationic and anionic surfactants and the effects are most pronounced near or above the critical micelle concentrations of the surfactants. Nonionic surfactants, on the other hand, lower the viscosity of agar sol and the stiffness of the gel. Moreover, these surfactants also reduce the gelling and melting temperatures. Non-ionic surfactants show significant influence on gel strength even at 0.1% level whereas the trend with ionic surfactants is reasonably systematic in the concentration range of 0.1-0.4% (w/v). The gelling temperatures are less sensitive to variations in surfactant concentration beyond 0.1% concentration, indicating that the origins of the two effects are different. Gelling temperatures obtained from the intersection point of the storage and loss moduli curves ($T_{\tan\delta=1}$) recorded under controlled stress mode are found to be in good agreement with the more conventional visual method of measuring gelling temperature. The concentrations of surfactants employed were near or above the critical micelle concentrations in all cases, which suggests that micellar aggregates influence the gel network in a profound way. Differential scanning calorimetric studies provide clear evidence of two endotherms in the DSC profile of control agar gel. In presence of surfactants, the profiles are altered in a profound manner and the results are in reasonable accord with the observed melting temperatures.

SLS shows a different behaviour than other ionic surfactants in as much as the gelling and melting temperatures are lowered in its presence unlike with other ionic surfactants. On the other hand, it does not behave like nonionic surfactants either since, unlike with nonionic surfactants, this lowering in gelling and melting temperatures is not accompanied by a lowering of gel strength when the SLS concentration is above the cmc SLS could, therefore, be a useful additive for bacteriological agar formulation which demands high gel strength and low (35-37°C) gelling temperature and which may not be otherwise achievable with certain agar types, e.g., the agar of the present study from *Gelidiella acerosa.* The remarkable ability of SLS to inhibit water loss from agar gel upon heating is yet another interesting result that may offer clues for design of systems to inhibit water loss. For processing of agar extracts, the utility of nonionic surfactants is self-evident. These surfactants lower the sol viscosity, which would allow for easier filtration of concentrated extractives, thereby helping to substantially reduce water usage in the process. Moreover, the lowering of the gelling temperature minimizes the possibility of accidental gelling of hot extractive during work-up operations. When coupled with the freeze-thaw or pressure syneresis methods of purification of agar, the removal of the surfactant from the agar has been found to be feasible. This would help restore the pristine quality of the agar. Studies at the bench scale level have confirmed the feasibility of the scheme.

Additional efforts will be required to unravel the role of surfactants at a fundamental level. The useful effects described herein may provide the necessary motivation for such studies.

Table III.1.1 Effect of surfactants on the physical properties of agar gel

Surfactant	Nature	Concentration of surfactant[a] (%)	Gel strength[b] (g/cm^2)	pH (at 80°C)	T_{gel} (Craigie et al.) (°C)[c]	T_m (°C)[e]
Nil	Control agar	---	600	6.8	41 (41.0)[d]	86
SLS	Anionic	0.1	509	7.4	36	75
		0.2	532	7.6	37	76
		0.3	669	7.6	37	75
		0.4	690	7.6	36 (36.4)[d]	77
SC896	Anionic	0.1	660	7.4	38	80
		0.2	680	7.8	39	80
		0.3	689	7.8	40	80
		0.4	700	7.8	42	82
SC229	Anionic	0.1	785	7.3	43	86
		0.2	857	7.5	44	86
		0.3	975	7.6	44	87
		0.4	979	7.6	44	88
CTAB	Cationic	0.1	798	8.3	45	84
		0.2	865	8.5	43	84
		0.3	891	8.6	44	87
		0.4	931	8.6	44 (43.1)[d]	88
CPC	Cationic	0.1	665	7.4	44	86
		0.2	732	7.5	43	84
		0.3	758	7.8	46	89
		0.4	800	8.2	47	90
Triton X-100	Nonionic	0.1	532	8.1	39	79
		0.2	492	8.3	37	74
		0.3	400	8.4	37	76
		0.4	400	8.4	37 (37.4)[d]	75
Brij35	Nonionic	0.1	445	7.1	37	75
		0.2	435	7.3	36	76
		0.3	430	7.3	37	78
		0.4	410	7.4	35 (37.3)[d]	73
$C_{12}E_{10}$	Nonionic	0.1	530	7.4	40	78
		0.2	520	6.9	42	80
		0.3	500	7.1	38	80
		0.4	495	7.4	38	78

Surfactant	Nature	Concentration of surfactant[a] (%)	Gel strength[b] (g/cm^2)	pH (at 80^{o}C)	T_{gel} (Craigie et al.) (^{o}C)[c]	T_m (^{o}C)[e]
SC893	Nonionic	0.1	475	7.4	37	78
		0.2	400	7.6	32	69
		0.3	415	7.4	34	70
		0.4	424	7.3	35	74
Crexlox3485	Nonionic	0.1	465	7.0	35	70
		0.2	460	7.3	37	73
		0.3	440	7.3	34	75
		0.4	450	7.5	35	78
Glycine	Amino acid	0.1	545	6.3	40	80
		0.2	530	6.6	40	78
		0.3	520	6.5	38	80
		0.4	530	6.4	39	79
Alanine	Amino acid	0.1	560	6.3	40	80
		0.2	550	6.6	40	78
		0.3	559	6.5	38	80
		0.4	570	6.4	39	79
Glycerol	Trihydric alcohol	0.1	600	7.3	45	86
		0.2	615	7.6	43	85
		0.3	640	7.4	44	87
		0.4	645	7.8	41	80
Mannitol	Hexa hydroxy alcohol	0.1	625	7.3	42	81
		0.2	635	7.5	44	83
		0.3	645	7.5	46	87
		0.4	645	7.7	46	90

[a] In all cases concentration of surfactant is with respect to the volume of gel of 1.5% agar; SLS : Sodium lauryl sulfate; CTAB : Cetyl trimethyl ammonium bromide; CPC : Cetyl pyridinium chloride; [b] Gel strength was measured in 1.5% gel at 20^{o}C. [C] Variation in T_{gel} (Craigie's method) was ± 1^{o}C ; [d] Values in the parentheses obtained from Fig.3A; [e] Variation in T_m (Craigie's method) was ± 1^{o}C.

Table III.1.2 Effect of various surfactants on the apparent viscosity of agar at different temperatures

Surfactant (% w/v)[a]	Nature	Viscosity[b] (±5) in cp			
		80°C	70°C	60°C	50°C
Nil[c]	-	51	64	89	127
SLS (0.1)	Anionic	40	49	81	117
” (0.2)	Anionic	60	84	124	150
” (0.3)	Anionic	75	92	145	170
” (0.4)	Anionic	87	100	156	190
” (0.5)	Anionic	88	106	159	193
” (0.6)	Anionic	88	106	160	194
” 0.7)	Anionic	87	106	166	190
SC896 (0.1)	Anionic	85	97	140	184
” (0.2)	Anionic	90	97	150	190
” (0.3)	Anionic	93	99	160	200
” (0.4)	Anionic	97	101	180	230
” (0.5)	Anionic	99	104	180	230
” (0.6)	Anionic	100	101	182	235
” (0.7)	Anionic	100	101	180	236
SC229 (0.1)	Anionic	124	134	200	240
” (0.2)	Anionic	129	149	230	260
” (0.3)	Anionic	150	179	249	280
” (0.4)	Anionic	169	200	240	290
” (0.5)	Anionic	170	207	242	291
” (0.6)	Anionic	169	204	247	292
” (0.7)	Anionic	169	203	241	291
CTAB (0.1)	Cationic	84	96	134	178
” (0.2)	Cationic	89	92	130	180
” (0.3)	Cationic	90	95	150	200
” (0.4)	Cationic	110	134	171	200
” (0.5)	Cationic	114	135	170	201
” (0.6)	Cationic	110	134	173	204
” (0.7)	Cationic	111	135	172	200
CPC (0.1)	Cationic	89	97	130	160
” (0.2)	Cationic	92	99	140	190
” (0.3)	Cationic	120	141	170	190
” (0.4)	Cationic	120	143	167	197
” (0.5)	Cationic	120	143	167	197
” (0.6)	Cationic	122	142	165	196
” (0.7)	Cationic	120	141	164	194

Contd Table III.1.2

Surfactant (% w/v)[a]	Nature	Viscosity[b] (±5) in cp			
		80°C	70°C	60°C	50°C
Triton X-100 (0.1)	Nonionic	36	40	50	63
” (0.2)	Nonionic	30	36	40	69
” (0.3)	Nonionic	30	37	39	43
” (0.4)	Nonionic	22	29	33	40
” (0.5)	Nonionic	21	29	34	41
” (0.6)	Nonionic	21	29	33	40
” (0.7)	Nonionic	22	29	33	41
Brij 35 (0.1)	Nonionic	46	55	69	78
” (0.2)	Nonionic	40	50	55	71
” (0.3)	Nonionic	36	44	48	66
” (0.4)	Nonionic	36	44	41	40
” (0.5)	Nonionic	36	43	40	40
” (0.6)	Nonionic	35	44	38	42
” (0.7)	Nonionic	36	44	39	41
Glycine (0.1)	Amino acid	45	64	79	126
” (0.2)	Amino acid	47	66	79	128
” (0.3)	Amino acid	48	67	82	129
” (0.4)	Amino acid	49	68	79	128
” (0.5)	Amino acid	45	64	79	126
” (0.6)	Amino acid	45	64	79	126
” (0.7)	Amino acid	45	64	79	126

Contd Table III.1.2

” (0.3)	Amino acid	44	64	88	126
” 0.4)	Amino acid	50	54	69	129
” (0.5)	Amino acid	54	66	89	130
” 0.6)	Amino acid	50	66	89	128
” (0.7)	Amino acid	50	67	91	120
Glycerol (0.1)	Trihydric alcohol	45	64	89	126
” (0.2)	Trihydric alcohol	35	54	79	126
” (0.3)	Trihydric alcohol	55	74	89	129
” (0.4)	Trihydric alcohol	40	60	69	133
” (0.5)	Trihydric alcohol	40	50	76	123
” (0.6)	Trihydric alcohol	39	54	79	126
” (0.7)	Trihydric alcohol	35	59	81	127
Mannitol (0.1)	Hexahydroxy alcohol	55	64	89	120
” (0.2)	Hexahydroxy alcohol	55	60	69	120
” (0.3)	Hexahydroxy alcohol	45	54	79	129
” (0.4)	Hexahydroxy alcohol	46	65	79	126
” (0.5)	Hexahydroxy alcohol	60	70	79	120
” (0.6)	Hexahydroxy alcohol	51	64	86	130
” (0.7)	Hexahydroxy alcohol	53	60	79	127

[a] Percent concentration of surfactants with respect to 1.5% agar sol-gel volume; SLS : Sodium lauryl sulfate; SC896 and SC229 were obtained from Uniqema India; CTAB : Cetyl trimethyl ammonium bromide; CPC : Cetyl pyridinium chloride; [b] Viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072), using Spindle No.1 for apparent viscosities less than 100 cP and Spindle No.2 for measuring viscosities >100 cP, and 60 rpm; [c]Values for 1.5% control agar gel (in absence of surfactants).

Table III.1.3 Critical micellar concentration (CMC)[a] values of some ionic and nonionic surfactants studied

Surfactants	Temperature (°C)	CMC (g/L)
Sodium Lauryl Sulphate[b]	50	2.26
Brij 35	27	0.03
Cetyl pyridinium chloride (CPC)	20	0.60
Cetyl trimethyl ammonium bromide (CTAB)	27	0.30
Triton X-100	27	0.19
Polyethylene 10-lauryl ether ($C_{12}E_{10}$)	20	0.02

[a] Kosswig K. SURFACTANTS-Relationship between structure and properties of surfactants. Ullmann's Encyclopedia of Industrial Chemistry, Sixth Edition, 2002, Electronic Release; [b] Stokes R J andEvans, D F, Fundamentals of Interfacial Engineering. Ch. 5; Wiley-Verlag, New York, 1996; p.212

Table III.1.4 Studies on the properties of agar gel in presence of mixture of surfactants

Surfactants	Nature	Concentration of surfactants[a] (%)	Gel strength[b] (±50 g/cm^2)	pH (at 80^0C)	T_{gel} (±1.0°C)	T_m (±1.0° C)
SC229 : Triton X-100 (1:4)	Nonionic : nonionic	0.1	552	8.1	39	79
		0.2	500	8.3	37	74
		0.3	400	8.4	35	76
		0.4	450	8.4	35	75
SC229 : Triton X-100 (1:1)	anionic : nonionic	0.1	680	8.0	40	81
		0.2	675	8.1	39	80
		0.3	640	8.1	39	79
		0.4	680	8.1	38	80
SC229: Triton X-100 (4:1)	Nonionic : nonionic	0.1	765	7.3	43	86
		0.2	847	7.5	44	86
		0.3	925	7.6	44	87
		0.4	937	7.6	44	88

[a] In all cases concentration of surfactant is with respect to the volume of gel of 1.5% agar; SLS : Sodium lauryl sulfate; [b] Gel strength was measured in 1.5% gel at 20°C.

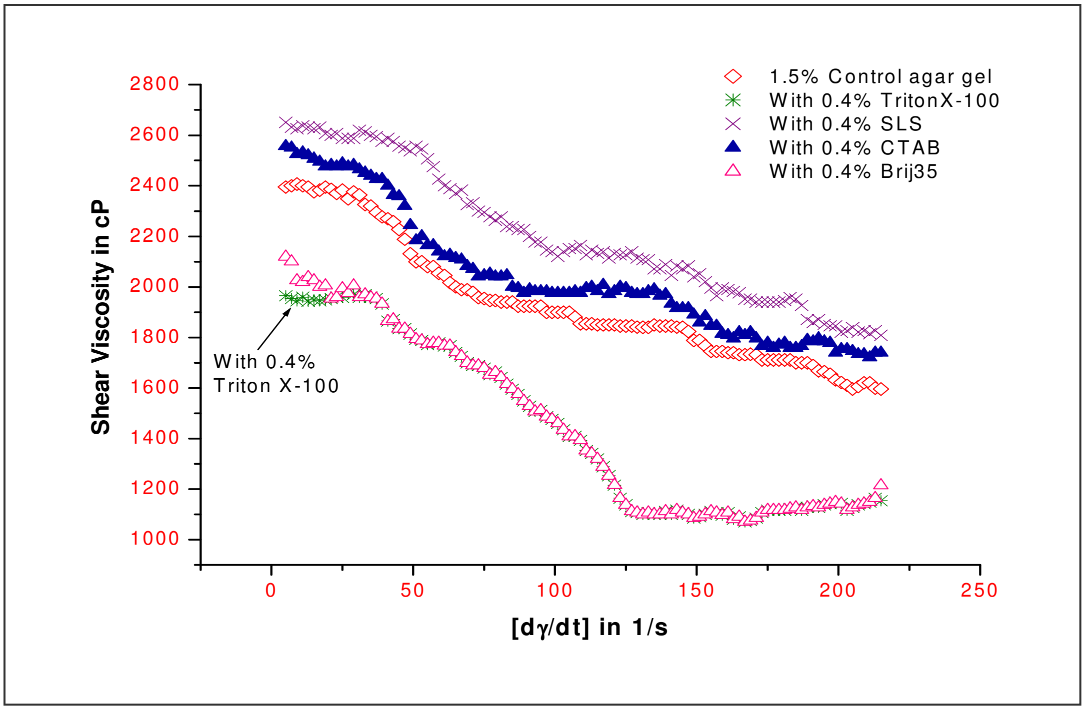

Fig.III.1.1 Gel thinning of agar gel (1.5% gel) in presence of ionic and nonionic surfactants

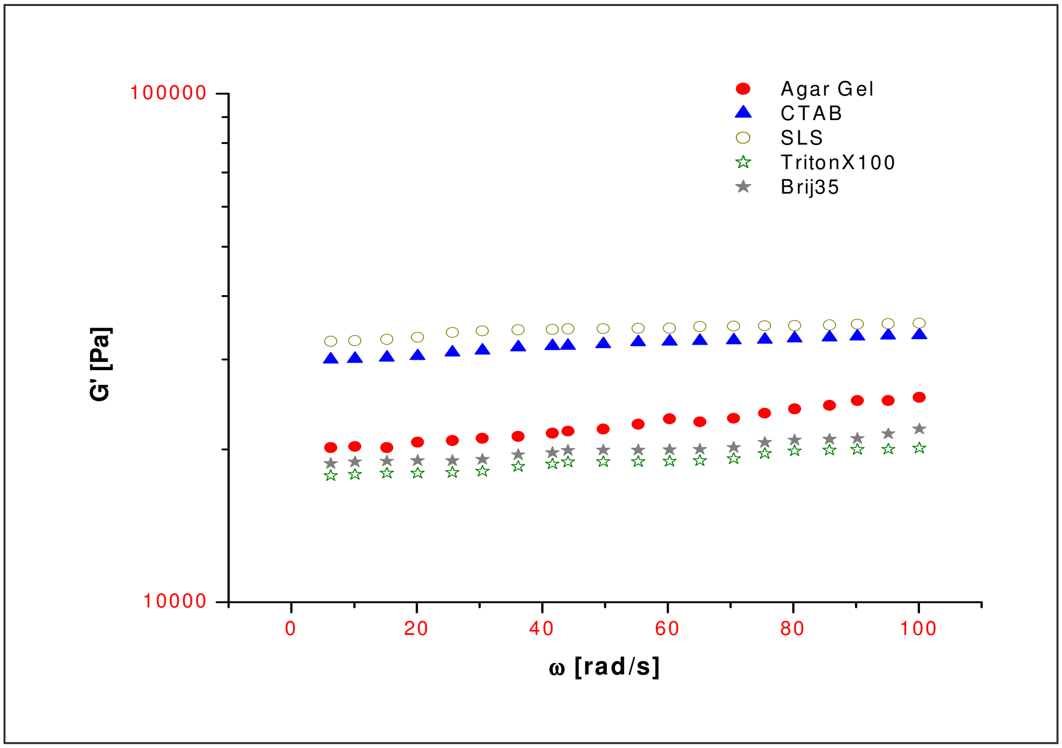

Fig.III.1.2 Frequency dependency of G' for agar gels (1.5% gel) in presence of ionic and nonionic surfactants (Controlled Deformation mode, PP 35 mm, 0.5% strain)

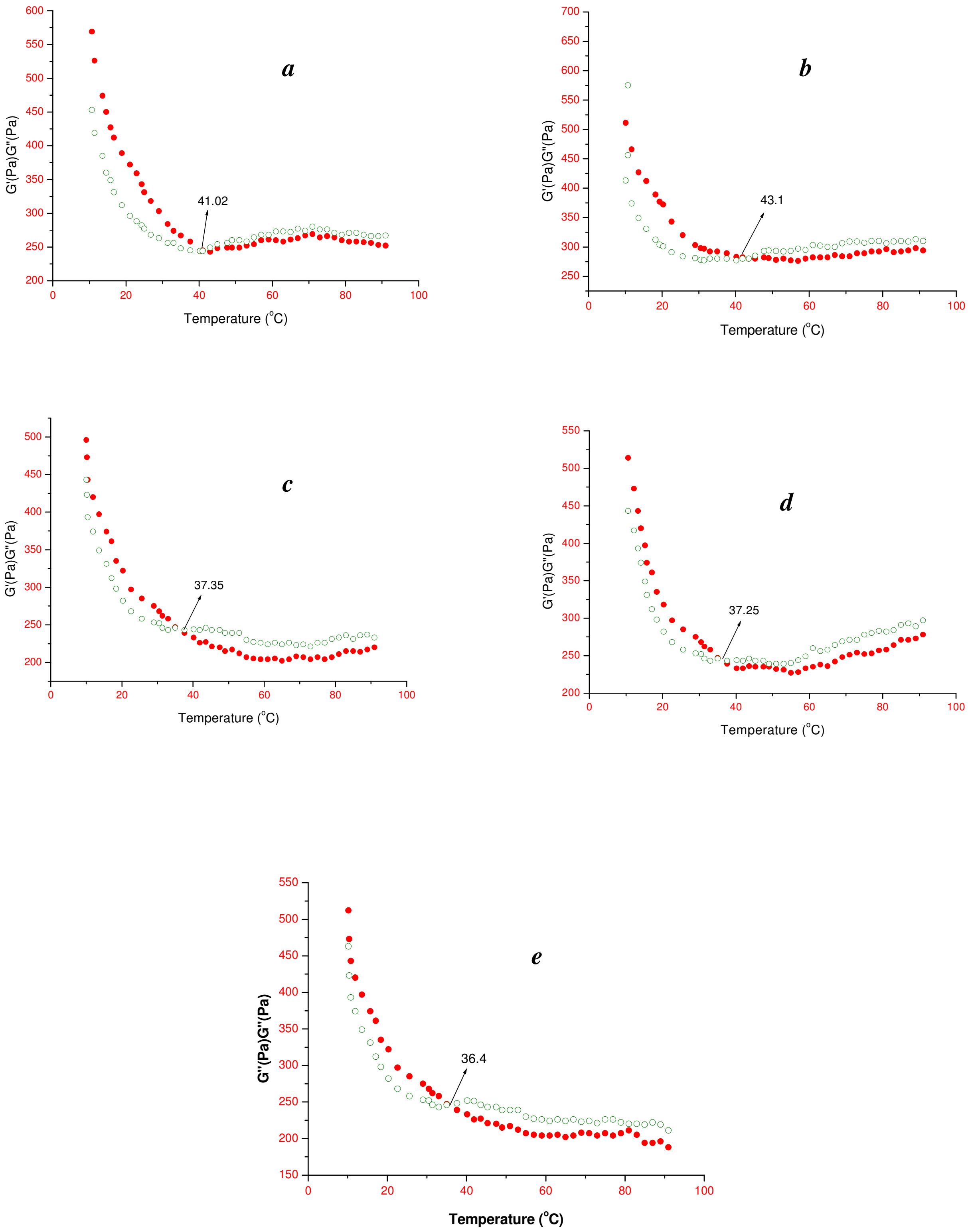

Fig.III.1.3A Temperature dependency of G'G'' of agar gel (1.5% gel) and of agar gels (1.5%) in presence of CTAB; Triton X-100; SLS and Brij35 (Controlled Stress mode, Stress: 1 Pa, Frequency 1Hz; Cooling rate 0.38°C/min; Geometry used cone/plate). ***a***: Agar gel; ***b***:Agar gel + CTAB; ***c:*** Agar gel + TritonX-100; ***d:*** Agar gel + Brij35; ***e:*** Agar gel + SLS.

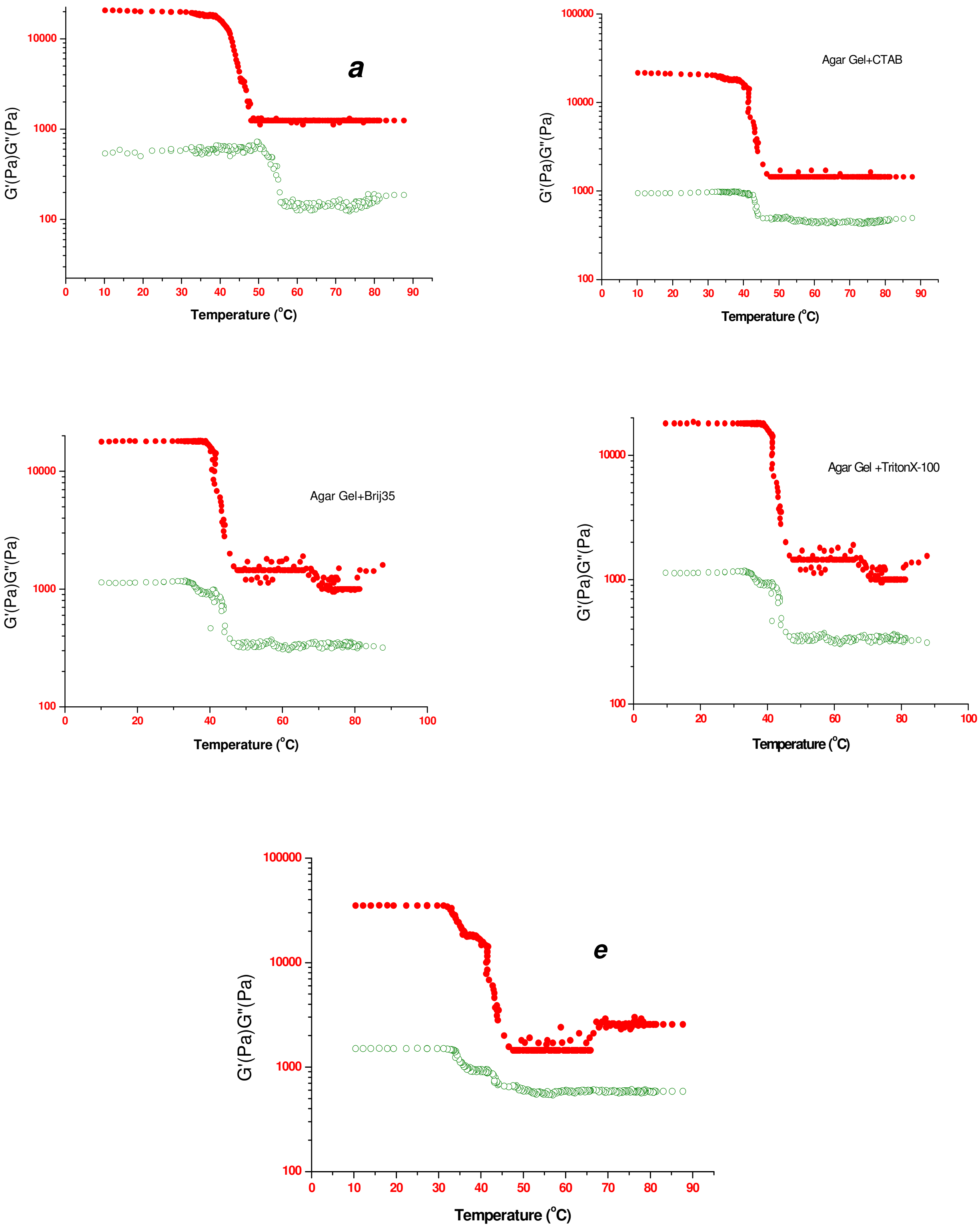

Fig.III.1.3B Temperature dependency of G'G" of agar gel (1.5% gel) and of agar gels (1.5%) in presence of CTAB, Triton X-100, SLS and Brij35 (Controlled Deformation mode, Strain 0.5%, Frequency 1Hz; Cooling rate 1°C/min; Geometry used cone/plate). ***a:*** Agar gel; ***b:*** Agar gel + CTAB; ***c:*** Agar gel + Brij35; ***d:*** Agar gel + TritonX-100; ***e:*** Agar gel + SLS. ● : G′ ; O : G″

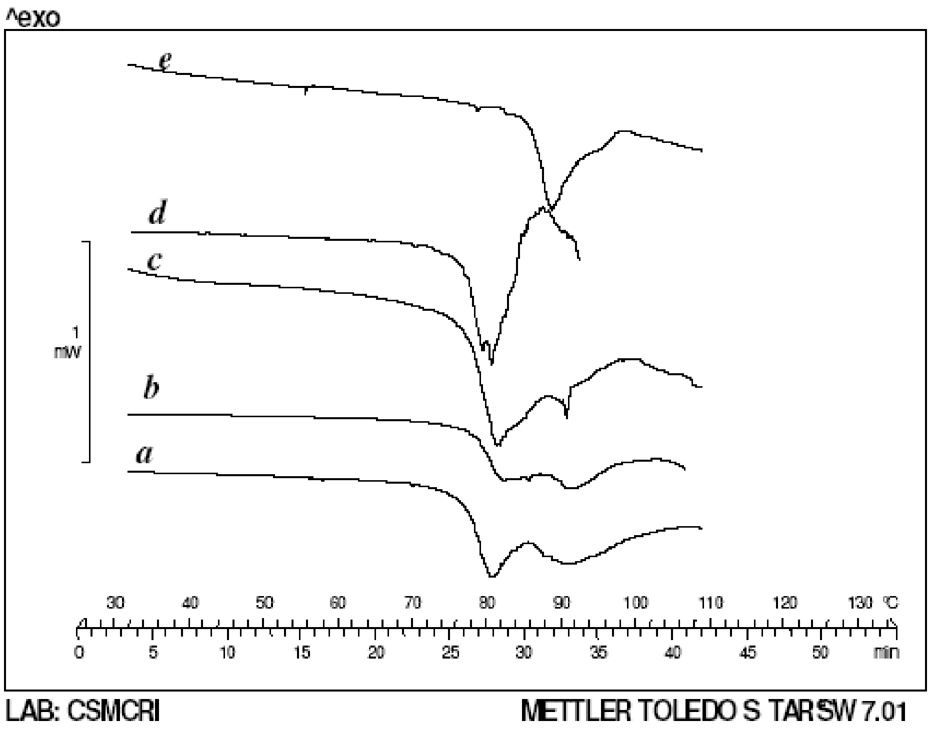

Fig.III.1.4 DSC Heating curves. ***a*** : Control Agar (1.5%) Peak Value -0.58 mW and –0.53Mw at 88.65° and 89.95°C, Sample 10.00 mg; ***b***: Agar gel+Brij35 (0.4%), Peakvalue -0.95 and –0.99 mW at 82.37° and 91.67 °C, Sample 10.60 mg; ***c***: Agar gel+Triton X-100 (0.4%), Peak Value -1.62 and -1.49 mW at 81.75° and 90.72°C, Sample 10.20 mg ***d***:Agar gel+SLS (0.4%), Peak Value -0.30 at 80.66°C, Sample 11.20 mg; ***e***: Agar gel+CTAB(0.4%), Peak Value -0.46 at 88.85°C , Sample 10.40 mg.

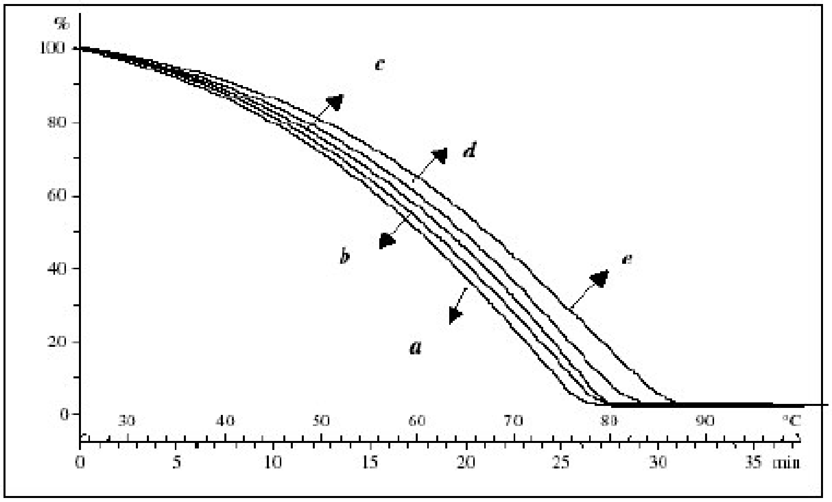

Fig.III.1.5 TGA Profiles. ***a:*** Agar gel + Brij35, Sample 11.0 mg; ***b:*** Agar gel + Triton X-100, Sample 10.6 mg; ***c:*** Agar gel Wt of sample, 10.1mg; ***d:*** Agar gel + CTAB, Sample 10.6 mg ***e*** : Agar gel + SLS, Sample 10.4 mg.

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CHAPTER III.2

SURFACE TENSION STUDIES OF AGAR-CTAB SOLS

III.2.1 INTRODUCTION

The interaction between polymer and surfactants has been a subject of great interest [1,2]. Initially the problem was found to be the studies of interaction of proteins associated with natural lipids, later it dealt with the interaction involving synthetic surfactants. Adding surfactants to polymer solutions with the formation of polymer surfactant complex can substantially change the physical properties of the starting polymer, and this effect can be harnessed to many industrial applications [3-5]. When a polymer carries hydrophobic groups, the effects are dramatically enhanced. Such polymers are often referred to as associating polymers because of their tendency to self-assemble and in extreme cases they form gels by association of hydrophobes. This interaction is utilized in pharmaceutical applications as well as in colloid science [5,6]. It is believed that the principal driving force for the binding between an ionic surfactant and a non-ionic polymer is hydrophobic interactions and the process is quite similar to that of self-association of surfactant into micelle [7-11]. The association of surfactant and polymer chain can be imagined like a necklace, where the surfactants are just like pearls joined to a hydrophobic centre. Actually the interaction between an ionic surfactant and a non-ionic polymer depends upon the nature of the polymer and surfactants. It has also been suggested that electrostatic interactions between the polymer and polar surfactant groups are important [12,13]. In all cases the adsorption of complex polymer/surfactant aggregate structures occur at the air/solution interface. Therefore, changes in the nature of binding of the surfactant to the polymer, even when it occurs in a single-phase region, are generally evaluated by the surface tension studies. The formation and destruction of junctions in presence of low and high amount of surfactants are depicted in Fig.III.2.1.

In this study agar is chosen as the polymer, which is a phycocolloid from a red seaweed *Gelidiella acerosa* (Rhodophyta) and agar is industrially important for its excellent thickening and gelling abilities [14-16]. The backbone of this phycocolloid is made up of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose. The repeating units in agar are shown in Fig.II.1.1 in Chapter II.1.

Surface tension measurements have earlier been used to study the interaction between surfactants and polymers. Jones studied the interaction between sodium dodecyl sulphate (SDS) and polyethylene oxide (PEO) [17] and Svensson et al. studied the interaction between SDS and amylose and amylopectin from potato [18]. From the surface tension measurements the saturation concentration, C_2, was determined and total ammount of surfactant bound to the polymer was calculated. Prasad et al. reported the interaction of various ionic and nonionic surfactants with agar gel [19]. Miguel and coworkers have reported fluorescence study of the interaction between sodium alginate and surfactants [20].

In this work surface tension measurements have been utilized to characterize the interaction/binding between the nonionic agar and the cationic surfactant CTAB (Cetyl trimethyl ammonium bromide). We have already reported in Chapter III.1 the thermal and rheological studies of agar in presence of CTAB in the gel state [19]. Therefore, it was considered appropriate to study the interaction of agar in presence CTAB in sol state by surface tension measurements. The data generated in this study may be useful to explore newer applications of agar e.g in pharmaceutical and oil industries. It was decided that the agar that was extracted in our laboratory from Gelidiella acerosa, which was characterized by IR, NMR studies [21], will be used in the present investigation. This decision was based on the following observations: the surface tension of double distilled water was measured to be 69.2 mN/m, while those of 0.01 % (w/v) Oxoid agar (Oxoid Agar No.1 (Oxoid, UK; Lot/Ch.-B: 810501-2) and Difco agar (Difco Laboratories, 0140-01, Detroit Michigan USA) were 54.8 mN/m and and 51.2 mN/m respectively. The agar that was used in this investigation (AS042901) had surface tension value (in 0.01 %, w/v) 65.1 mN/m, indicating thereby the latter was associated with minimum surface active contamination.

III.2.2 MATERIALS AND METHODS

III.2.2.1 Materials

The agar that was used in this investigation was extracted from a seaweed *Gelidiella acerosa* (Forsskal) J. Feldmann & G.Hamel (Rhodophyta, Gelidiales) by following a method by Roleda et al. [22] with modification, and cetyltrimethylamonium bromide, CTAB (Analytical Grade, S.D fine Chemicals, India) was used as received. The weight average molecular weight of the agar determined by intrinsic viscosity measurements using an Ostwald Viscometer and found to be 2.74 X 10^5D [21].

III.2.2.2 Preparation of agar and CTAB solution

The agar sols were prepared by heating agar powder in water with constant stirring. Then the surfactant solutions were added slowly to the agar sol under agitation. For all the preparations, double distilled water was used. The experiments were performed for selected agar concentrations 0.01 % to 0.16% (w/v) having 0.00392 μM to 15.31 mM of the surfactants. All were obtained at 27.1°C.

III.2.2.3 Equipment

The surface tension in the air-water at 27.1°C (±0.5°C), Kraft point of CTAB, is measured by the static Wilhelmy plate using dynamic contact angle tensiometer (DCAT 21, Germany). To ensure removal of surface-active contaminants, all glasswares in contact with the sample were cleaned in chromic acid and rinsed with double distilled water. The platinum plate was washed in double distilled water, heated on a Bunsen flame and left to cool at room temperature. The solutions were prepared with the double distilled water. Experiments were carried out in triplicate.

III.2.3 RESULTS AND DISCUSSION

Since the surface tension is a measure of the amount of surfactant in solution, with increasing concentration of the surfactant (CTAB) the surface tension will decrease, till the critical micelle concentration (CMC) is reached. The Fig.III.2.2 shows the variation of surface tension with the CTAB concentration. At CMC, the surfactant started to aggregate, generating a break in the curve. Beyond CMC, the surface tension value has not changed with the increase in concentration. The CMC was calculated to be 0.9513mM at 27.1°C, which tallies well with the value reported in the literature [23, 24].

Fig.III.2.3 shows the surface tension as a function of total concentration of CTAB at three different agar concentrations 0.01%, 0.08% and 0.16%. As the total CTAB concentration increased in the polysaccharide sols, the surface tension did not decrease as much as it did in water. The surface tension at the saturation concentration was the same as of the CMC in water. In order to reach the saturation concentration, more amount of CTAB was required in case of higher polysaccharide concentration as reported by Lundqvist et al. in the case of amylose and amylopectin [25]. The saturation concentration was calculated from the plots as the total concentration of CTAB at the breakpoint in the surface tension plots [25]. The data is presented in Table III.2.1.

Table III.2.1 Saturation concentration and binding capacity of agar-CTAB at 27.1°C

Agar concentration (%)	Saturation concentration, mM	Binding capacity, mmol CTAB/ galactose-anhydrogalactose
0.01	1.0232	209
0.08	1.5848	252
0.16	2.0417	218

When CTAB is added to aqueous sol of agar, the interaction is detected up to a specific concentration denoted as saturation concentration of CTAB (foot point). In this study the value of saturation concentration is found to fall in the range 1.0232 to 2.0417 mM of CTAB, which appears to be sensitive to the agar concentration.

The saturation concentration values were plotted against the polysaccharide concentrations which are shown in Fig.III.2.4. It shows that with the polymer concentration, the saturation concentration increases linearly. From the slope it is possible to calculate the amount of CTAB molecules bound to agar. The maximum binding in this case is 218 mmol CTAB per mole of 1,3-β-D-galactose-α-L-3,6 anhydrogalactose units of the agar polymer.

III.2.4 SUMMARY

The principal finding of this work is that CTAB binds with agar and the extent of binding can be determined by surface tension studies. It has been estimated that at CMC level concentration of CTAB in agar sol (at 27.1^{o}C), 218 mmol CTAB was bound to each repeating unit (1,4-linked galactopyranose 3,6-anhydrogalactopyranose moiety) of agar polymer.

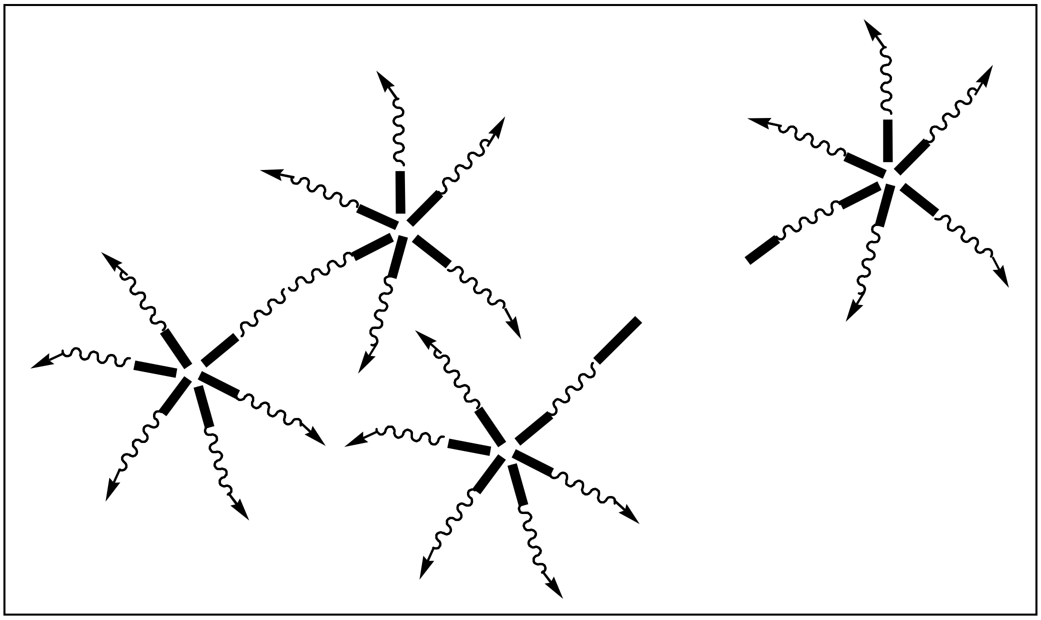

Fig. III.2.1 Schematic representation of (A) formation of junctions with the help of surfactant molecules (B) destruction of junctions by excess surfactant molecules

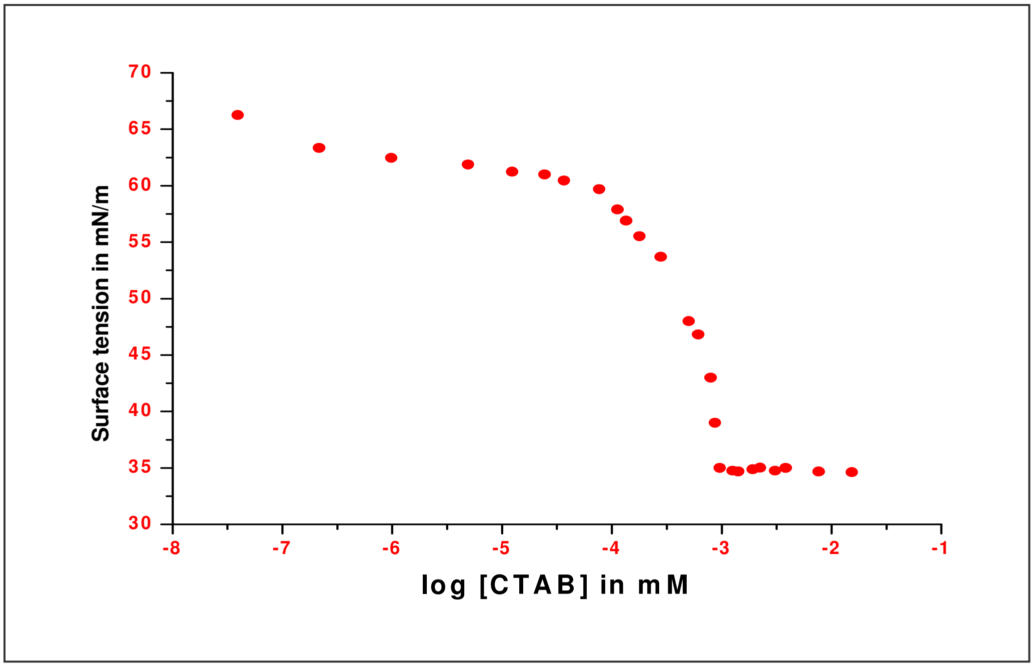

Fig. III.2.2 Variation of surface tension with the logarithmic concentration of CTAB.

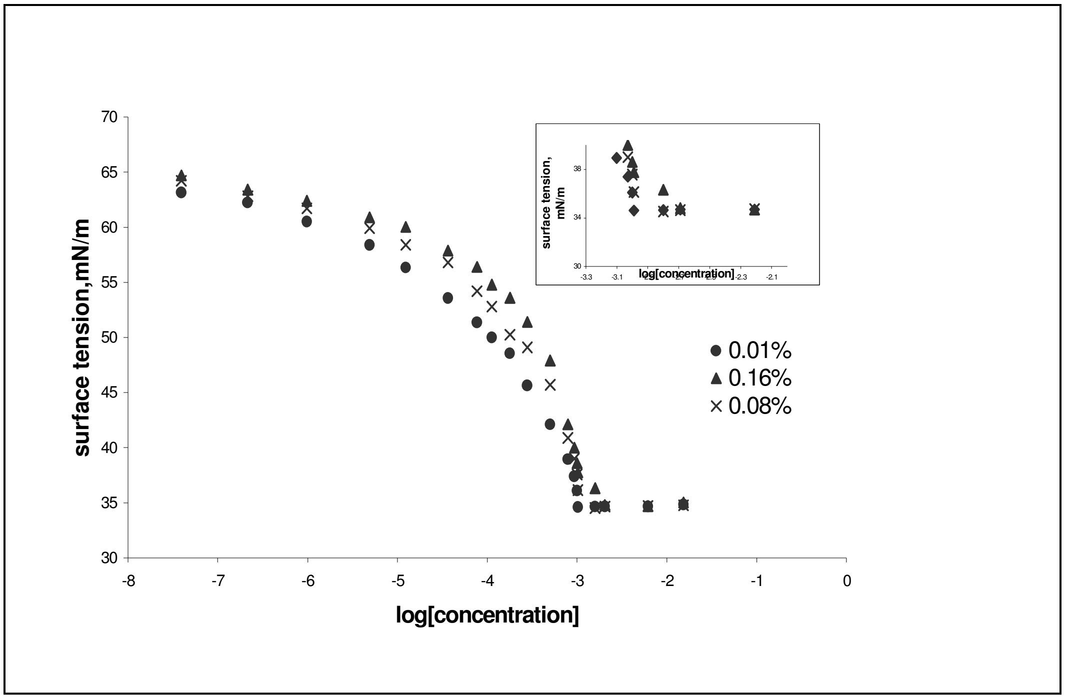

Fig. III.2.3 Variation of surface tension with the CTAB concentration at CMC level for different agar concentration (Inset figure shows the foot point).

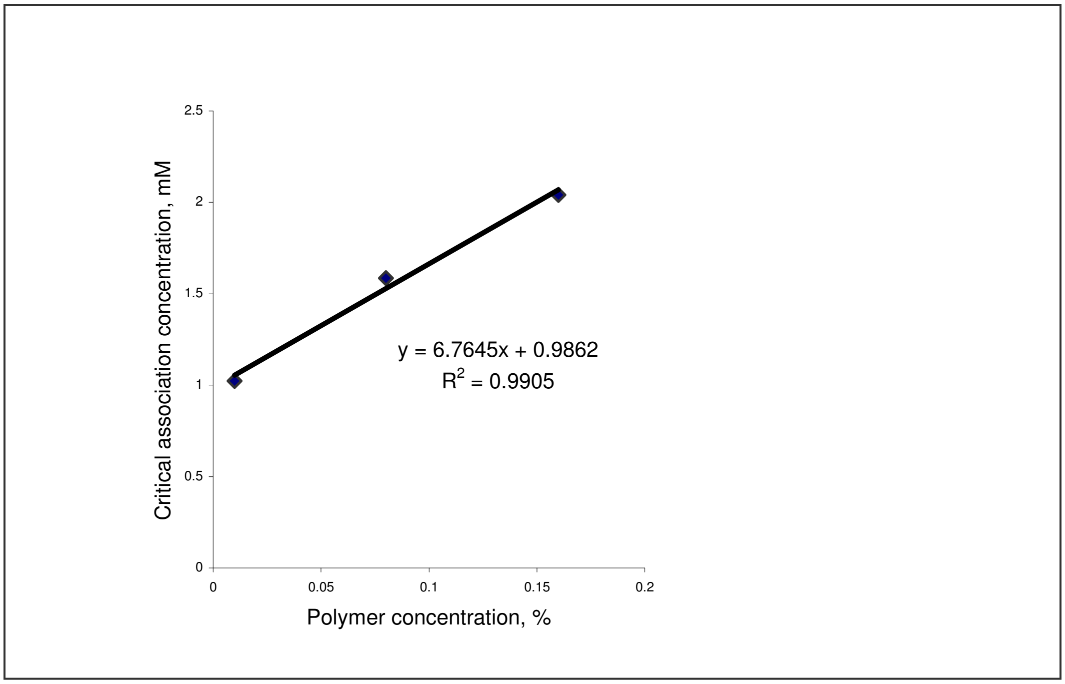

Fig. III.2.4 Variation of critical association concentration with polysaccharide concentration

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CHAPTER III.3

PHYSICAL MODIFICATION OF AGAR: RHEOLOGICAL AND HPLC ANALYSIS

III.3.1 INTRODUCTION

Physical modification of starch to enhance various properties viz., gelatinisation is well known. In many cases the starch is mechanically modified to provide improved properties [1]. Interaction of starch and surfactants are also well studied. Lundqvist et al. has reported binding of surfactant to starch molecules by surface tension measurements [2]. Starch and fatty acid interactions in presence of whey protein is also reported by Zhang and coworkers [3]. The formation of three way complexes between starch and fatty acid has been widely reported and the presence of the fatty acid starch complex can be identified by size exclusion chromatography, large rapid visco analyser (RVA), thermo chemical measurements etc. [4]. Extraction of free fatty acid from starch fatty acid complex by hot aqueous alcohol is reported, the use of less polar solvent like chlorofom was found to be not suitable for such extraction [5]. Starch fatty acid interaction was mainly studied by isolating the complex from the the plants and it was observed that starch form stronger complex with palmitic acid and weaker with linoleic acid [6]. Akuzawa and his coworkers have reported estimation of bound fatty acid by gas chromatography [7].

Agar is a very important naturally occurring biopolymer. The backbone of this phycocolloids is made up of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose (vide Chapter II.1). The increased use of agar in various biological applications including biotechnology and molecular biology makes it more precious. It has been a constant endeavour of many research programmes to modify the physical and chemical properties of commercially important biopolymers. The extensive application of agar as gelling agent in many cosmetics and laxative formulations has made physical modification of agar all the more important. Preparation of low gel strength or creamy agar and use of it as skin moisture is patented by Kojima and coworkers [8].

This prompted us to undertake studies on the physical modification of agar by using fatty acids. Rheological, thermal and viscoelastic properties of the agar fatty acid adduct were studied. The most obvious effect was found in lower gelling and enhanced dynamic flow behaviour of the agar sol. The quantity of fatty acid in the agar fatty acid complex was determined by HPLC and LC-MS analysis of the methanol extract of the adduct. To our knowledge, the studies of interaction of agar-fatty acids have not been reported in the literature. Results of the present study may be useful in exploring newer application areas for agar.

III.3.2 MATERIALS AND METHODS

III.3.2.1 Materials

Bacteriological agar was extracted from *Gelidiella acerosa* collected from Gulf of Mannar at Ervadi (9^{o}15′N, 78^{o}58′E), in March 2000 (Code No. AS0402909) using the method of Roleda et al. [9]. The same agar sample was used for all experiments. The effects of various fatty acids on agar were studied. The following four saturated fatty acids and one unsaturated were used in the study: myristic (C14:0), palmitic (C16:0), stearic (C18:0), lauric (C12:0) and oleic acid (C18:1) from Sigma-Aldrich Inc. Wiscosin, USA. The fatty acids were used as received without further purification.

III.3.2.2 HPLC analysis

The high performance liquid chromatography (HPLC) system used was a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan), including pumps 6AD, a Shimadzu SPD-10A UV-Vis detector attached to a Shimadzu LC-10, chromatography manager. A Discovery C18 stainless steel column of 25cm (length) X 4.6mm (i.d.) packed with C18 reversed-phase material of 5μm particle size, 300 $\overset{\circ}{A}$ pore size (SUPELCO, Sigma-Aldrich, Inc, USA) was used. The column was attached to a 5 cm C18 ODS guard column (Shimadzu, Kyoto, Japan). The samples were injected with a static injector (Rheodyne, Coati, CA, USA) and 20 μl microsyringe (Hamilton, Reno, Nevada, USA). Isocratic elution was performed at 50^{o}C with the solvent system, methanol:5mM phosphoric acid buffer, (9:1, v/v), at a constant flow rate 1.5 ml min^{-1}. The mobile phases were sonicated and filtered over 0.2 μm nylon filter (Cole-Parmer, USA). UV detection was carried out at 210 nm.

III.3.2.3 LC–MS analysis

The liquid chromatography mass selective detector system used was of Waters 2695 separation module with Waters 2487 dual λ absorbance UV-Vis detector at 210 nm wavelength connected to a Waters Q-Tof micro YA-260 mass selective detector (MS/MS). Column used for LC was of Waters Symmetry C18 reverse phase column of 10 cm (length) X 4.6 mm (i.d.) with 3.5 μm particle size and 100 A° pore size. For the LC analysis methanol: water (9:1) was used as the mobile phase with 0.2 ml/min flow rate. The instrument parameters fixed were : 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, using a Waters massLynx version 4.0 Mass analysis programme software.

III.3.2.4 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 solutions taking 1 ml sol on to peltier of the rheometer, for measurements in agar gel done at 25°C. Viscosities at varying shear rate were studied at 45°C. 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 [10].

III.3.2.5 Physical properties

Measurement of pH was carried out using a model No. 535 pH meter from Systronics Scientific Instruments, India. Gelling and melting temperatures of agar gel in presence of various fatty acids were measured in the mixtures as described by Craigie et al. [11].

The gel strength (gcm^{-2}) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd.Tokyo, Japan). The measurements were performed on a 1.5% w/v agar solution (previously cured overnight at 10°C) using a solid cylindrical plunger of 1 cm in diameter [12].

Apparent viscosity was measured at temperature (80°C) using a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No.1 and rpm 60 were used for measuring the apparent viscosity.

III.3.2.6 Thermal measurements

Differential Scanning Calorimetric (DSC) measurements were carried out on a Toledo-Mettler system with STAR^e software, from Switzerland. For this purpose, ca. 10 mg gel was taken in an aluminum crucible weighing 40mg and the measurements were done using a temperature programme of 50^{o}C to 150^{o}C at 5^{o}C min^{-1} heating rate in an air atmosphere.

III.3.2.7 Surface tension measurements

Surface tension was measured by the static Wilhelmy plate using Dynamic Contact Angle Tensiometer (DCAT 21, Data Physics, Germany). To ensure removal of surface-active contaminants, all glass wares in contact with the sample were cleaned in chromic acid and rinsed with the double distilled water. The platinum plate was washed in double distilled water, heated in a Bunsen flame and left to cool at room temperature. The solutions are prepared with the double distilled water. Experiments are carried out in triplicate. For this measurement 0.05% w/v solution of the complex as well as control agar in double distilled water was prepared by heating the powder on a hot plate.

III.3.2.8 Swellability

Swelling properties of the agar and agar fatty acid complexes in water were measured by soaking the samples in water and measuring the weight gain in regular intervals as described by Park et al. [13].

III.3.2.9 Spectral analyses

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

For preparation of sols and gels of the complex for all the experiments, first the complexes were soaked in minimum quantity of hot methanol (ca 50^{o}C) then desired amount of water (final concentration being methanol-water, 1:10) was added. This method of preparation of samples was adapted for ensuring homogeneous dispersion of the adduct in water.

III.3.2.10 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 [15]. Approximately 10 grams of hot 1.5% (w/w) agar and agar fatty acid complexes were poured into test tubes (21 mm dia) and allowed to gel at room temperature (31-33^{o}C), and kept at 5 ^{o}C for 24 h. The initial weights of these gels were measured before placing them on dry Whatman (No. 1) filter papers. Weighing the gels after 2 h monitored the loss of water from the gels. 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. The extent of syneresis was estimated by the amount of water Δw separated from gel phase. The degree of syneresis was estimated by $\Delta w/w_o$, where w_o stands for the initial weight of gel. All measurements were done in triplicates.

III.3.3 RESULTS AND DISCUSSION

III.3.3.1 HPLC Analysis

5 g agar was taken in methanol and 100 mg of fatty acids were added to it. The mixture was stirred at 50-60^{O}C temperatures for 5 hours. The mixture was filtered to separate the product, which was washed with excess hot methanol to remove any free fatty acid. The product (100 mg) i.e. agar-fatty acid adduct was extracted by methanol on a Soxhlet apparatus for 4 h. The extractive was evaporated to dryness on a rotary evaporator. The residue was subjected to HPLC analysis [14]. The amount of bound fatty acid to the agar was determined by calculating the area under the peaks of standard and recovered fatty acids (Table III.3.1). Fig.III.3.1a, Fig.III.3.1b, Fig.III.3.1c, Fig.III.3.1d illustrates the HPLC profiles of the standard and recovered fatty acids from the complexes. The starting material agar was also treated in a similar way and the washings were analysed by HPLC, wherein no peak was observed for the eluents.

The agar that remained after extraction of fatty acids was dispersed in water (0.05%, w/v) by boiling and the surface tension of the dispersion was measured to be 59 mN/m at

27.1oC, which is identical with the control agar dispersion (Table III.3.3). This confirms the fact that the extraction of fatty acid from the complex was complete.

For HPLC analysis of standard fatty acids 100 mg of the acid was dissolved in 2 ml of methanol and 20 µl of this solution was injected. For estimating free fatty acids in the complex, 100 mg of the fatty acid-agar complex was Soxhleted with methanol and the extract was evaporated to dryness. The residue was further dissolved in 2 ml methanol from which 20 µl aliquot was injected for HPLC analysis. It was observed from the HPLC analysis of the extracted free fatty acids from the agar-fatty acid complexes that tiny quantities (142.92 to 305.02 µg per 100 mg of agar-fatty acid complex) of fatty acids were bound to agar forming the complex. Stearic acid (C18) did not show any binding with agar, this may be due to the larger size of stearic acid, which may be preventing this from getting accommodated in the agar structure (pleases see Scheme III.3.1), on the contrary myristic acid (C14) got bound in the maximum quantity (Tab.III.3.1), presumably because of its smaller size. Oleic acid was not detected in HPLC analysis where UV-Vis visible detector was used. But the same was detected in LC-MS analysis, which may be due to higher sensitivity of the MSD system. Fig.III.3.2 shows the analytical data for the bound fatty acids incorporated in the agar fatty acid complexes.

It was observed that the presence of all the fatty acids, except stearic acid, decreases the gel strengths and viscosities (of the sol) of the complex by up to 120 g/cm^2 and 8 cP respectively. No effect of fatty acid on the gelling point of the complex was observed with respect to the control sample, whereas a lowering of melting temperature by up to 5^{o}C was observed (Tab.III.3.2).

Apparent viscosity and gel strength decrease for agar gel in presence of fatty acids, this effect is similar to that reported that in the case of starch [7].

Table III.3.1 HPLC analysis of the recovered fatty acid from the complex

Fatty acid	Retention Time (min)		HPLC Peak area for standard fatty acid	HPLC Peak area for the fatty acid recovered from the complex	Estimated quantity of fatty acid bound in the complex (in µg) per 100 mg agar
	Standard	Recovered			
Lauric acid ($C_{12}H_{24}O_2$)	3.436	3.348	1176236	180855	153.75
Myristic acid ($C_{14}H_{28}O_2$)	4.324	3.426	864958	263828	305.018
Palmitic acid ($C_{16}H_{32}O_2$)	9.211	10.433	2647633	378411	142.92
Stearic acid ($C_{18}H_{36}O_2$)	10.409	ND	1562236	Not detected	---
Oleic acid ($C_{18}H_{35}O_2$)	8.2	ND	ND	------	-----

III.3.3.2 LC-MS Analysis

The extractive obtained after the extraction of fatty acids with methanol from the adducts by Soxhlet extraction were evaporated to dryness on a rotary evaporator. The residues were subjected for LC-MS analysis. Negative electron spray mode (ES $^-$) was used for the analyses. The mass fragmentation patterns obtained are shown in Figures III.3.3a, III.3.3b and III.3.3c. The base peaks (M-1) obtained for each of the extracted fatty acids tallied with the respective molecular weights of the fatty acids. These data reaffirm the phenomenon of binding of fatty acids to agar moiety.

III.3.3.3 Surface Tension measurements

Table III.3.3 includes the value of surface tension for agar (0.05% w/v) and agar and fatty acid complex in water and 1: 10 methanol water mixture at 27.1 ^{o}C. Considerable decrease in the surface tension values for agar in presence of Lauric, myristic, palmitic acid indicates induced surface activity to agar molecule in presence of fatty acids. Stearic acid has no effect on the surface property of agar as evidenced from surface tension measurements.

Table III.3.2 Physical properties of the agar-fatty acid complex

Properties	Apparent Viscosity (Cp at 80 °C) [a]	pH (80 °C)	Gel Strength (g/cm^2)	Gelling T (°c)	Melting T (°c)
Control agar sol/gel (1.5% w/v)	35	6.9	860	40	88
Lauric acid + agar	27	6.5	760	39	85
Myristic acid + agar	29	6.7	740	39	83
Palmitic acid + agar	30	6.7	770	40	83
Stearic acid + agar	35	6.9	850	40	88
Oleic acid + agar	27	6.8	750	38	86

Table III.3.3 Surface tension study of the complex

Sample (0.05% w/v)	Surface tension at 27.1°C in water (mN/m)	Surface tension at 27.1°C in 1:10 methanol/water (mN/m)
Double distilled water	64	27.5*
Control agar	59	26.2
Lauric acid + agar	36	21
Myristic acid + agar	39	19
Palmitic acid + agar	31	16
Stearic acid+ agar	54	27
Oleic acid + agar	37	17
Agar, after extracting the fatty acid from the complex	59	---

* Surface tension value of 1: 10 methanol-water mixture.

III.3.3.4 Synersis

The extent of syneresis as measured by the degree of syneresis values of the agar gel and the fatty acid agar complexes as shown in Table III.3.4. It was observed that agar gel in presence of myristic acid showed maximum syneresis and least in presence of stearic acid. This is evident that agar gel in presence of myristic acid become more water repellent on standing i.e., water holding capacity of agar gel decreases in presence of myristic acid. This attributes to the enhancement of hydrophobicity of the complexes.

Table III.3.4 Degree of Syneresis for agar gel and agar fatty acid complexes

Sample	Initial (w_o) weight (g)	Final weight (g)	Syneresis (Δ w)	Degree of Syneresis (Δ w/w_o)
Control agar	10.58	9.37	1.21	0.117
Agar + lauric acid	10.66	9.18	1.48	0.134
agar + myristic acid	10.64	9.11	1.53	0.143
agar + palmitic acid	10.55	9.20	1.53	0.127
agar + oleic acid	10.50	9.18	1.32	0.125
agar + stearic acid	10.54	9.35	1.19	0.112

III.3.3.5 Swellability

Fig.III.3.4 describes the swelling behaviour of agar and agar fatty acid complexes in water. Dry samples (2 g each) were soaked in water and the weight gained by the samples were monitored and recorded at regular intervals. It has been observed that swelling of agar-lauric acid adduct was maximum and lowest in case of agar-oleic acid adduct. These results confirms that agar-fatty acid complexes are more hydrophobic than agar itself, on the other hand stearic acid has no effect on the swelling ability of agar.

III.3.4 Dynamic viscous behaviour

Flow behaviour: Shear viscosity was measured under applied shear rate from 5-230 1/s. It was observed that agar myristic acid complex has highest gel thinning property. This result indicates myristic acid imparts more fluidity into agar sol. On the other hand palmitic acid impart less fluidity and stearic acid did not induce any fluidity to agar sol. The property of decrement in dynamic viscosity of agar in presence of myristic and palmitic acid can be utilized to prepare low gel strength agar and there by may be used in cosmetic formulations [Fig.III.3.5].

III.3.4.1 Dynamic viscoelasticity

Frequency dependence of G′ and G″ for agar and agar fatty acid complexes in gel state are shown in Fig. III. 3.6. Both moduli decrease for the complexes with myristic, lauric and palmitic acid and become more frequency dependent imparting more liquid like behaviour.

III.3.5 Spectral analyses

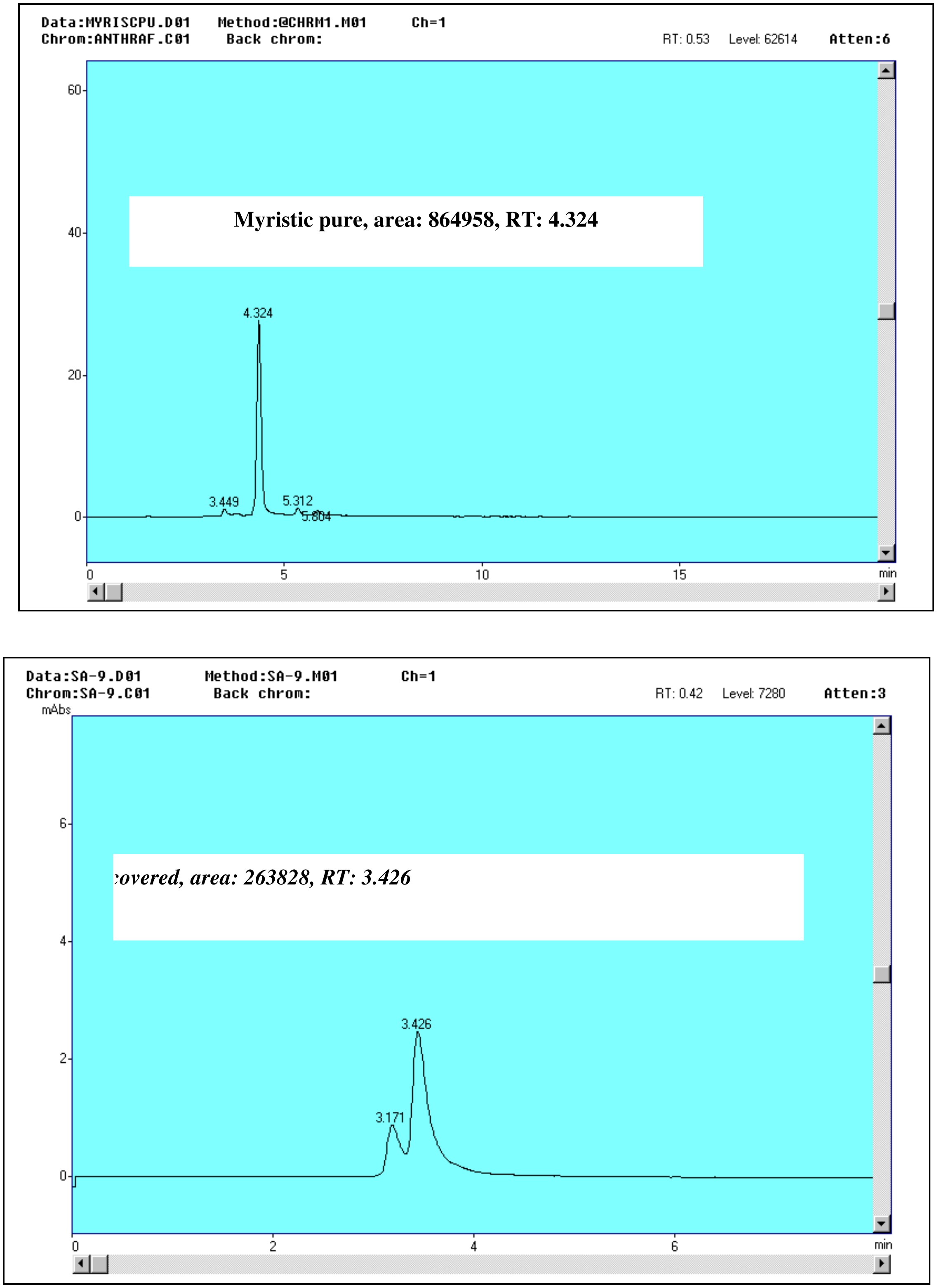
No characteristic IR band for fatty acids was observed in the agar-fatty acid complex as well as in the physical mixture of agar and fatty acid (in the quantity that is present in the adduct). Therefore, the nature of binding of fatty acid and agar is not known. The fatty acid in the adduct can be extracted with refluxing methanol. Threfore, it appears that there exist weak interactive forces like van der Waals force, hydrogen bonding, rather than one of chemical nature. A schematic representation of the fatty acids incorporation in to agar structure is shown in Scheme III.3.1.

III.3.6 Thermal properties

Both TGA and DSC results did not show any changes in the respective thermograms both for agar and for the agar-fatty acid complex. It can, therefore, be concluded that inclusion of tiny amount of fatty acid inside the structure of the agar imparts no effect on its thermal properties in the solid state.

III.3.7 SUMMARY

It can be concluded from above studies that fatty acids bind to agar resulting in an agar-fatty acid adducts. The adduct is more hydrophobic and has more fluidity than control agar in the gel state. This investigation provides a physical method of modification of agar. Such modified agars could be used in various cosmetic and health care product formulations, wherein both the gelling characteristics of agar as well as the merits of fatty acids could be beneficially exploited.



Data:MYRISCPU.D01 Method:@CHRM1.M01 Ch=1
Chrom:ANTHRAF.C01 Back chrom:
RT: 0.53 Level: 62614 Atten:6

Myristic pure, area: 864958, RT: 4.324

Data:SA-9.D01 Method:SA-9.M01 Ch=1
Chrom:SA-9.C01 Back chrom:
RT: 0.42 Level: 7280 Atten:3

covered, area: 263828, RT: 3.426

Fig.III.3.1a HPLC profile for standard and extracted myristic acid from agar-fatty acid complex.

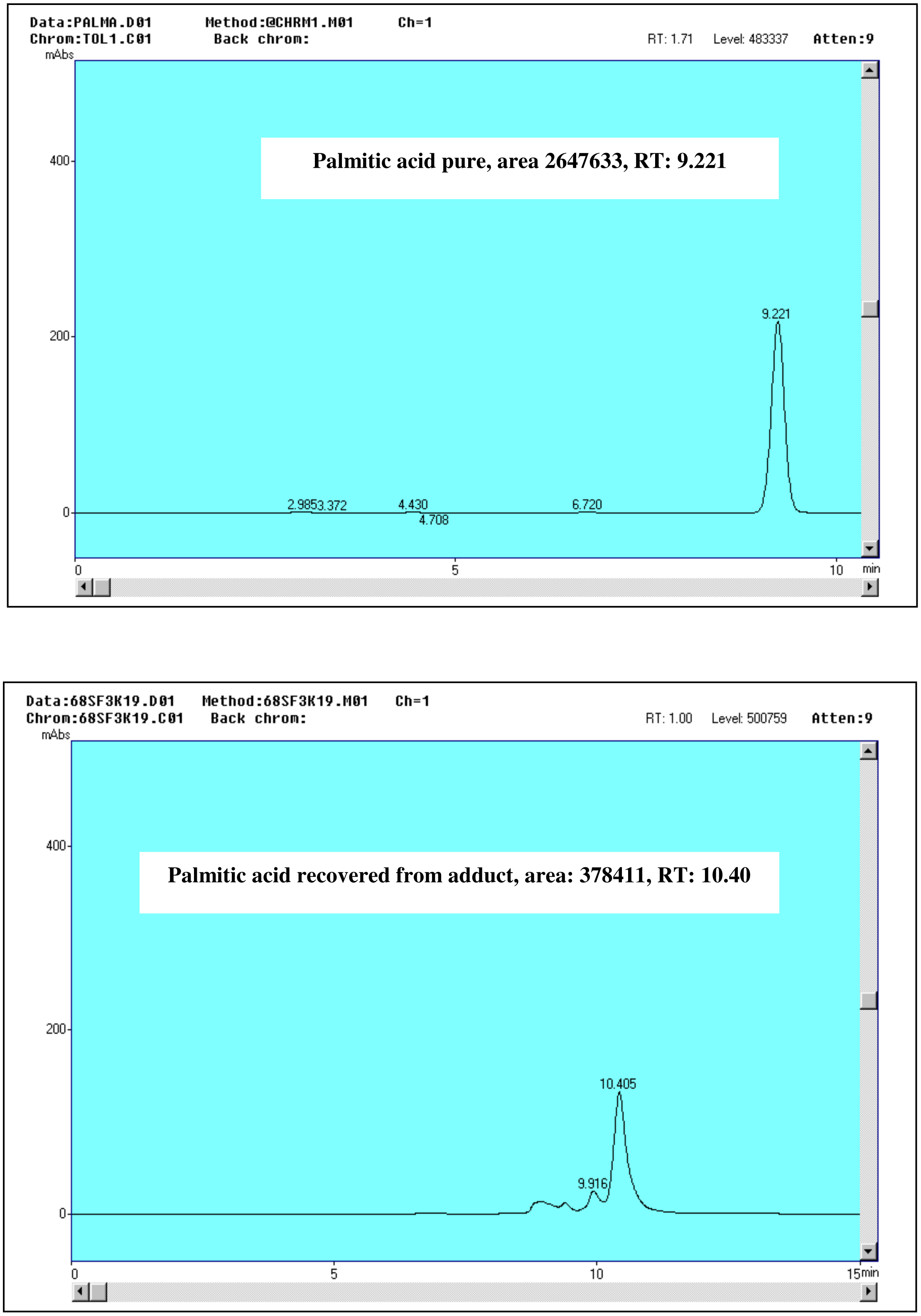


Fig.III.3.1b HPLC profile for standard and extracted palmitic acid from agar-fatty acid complex.

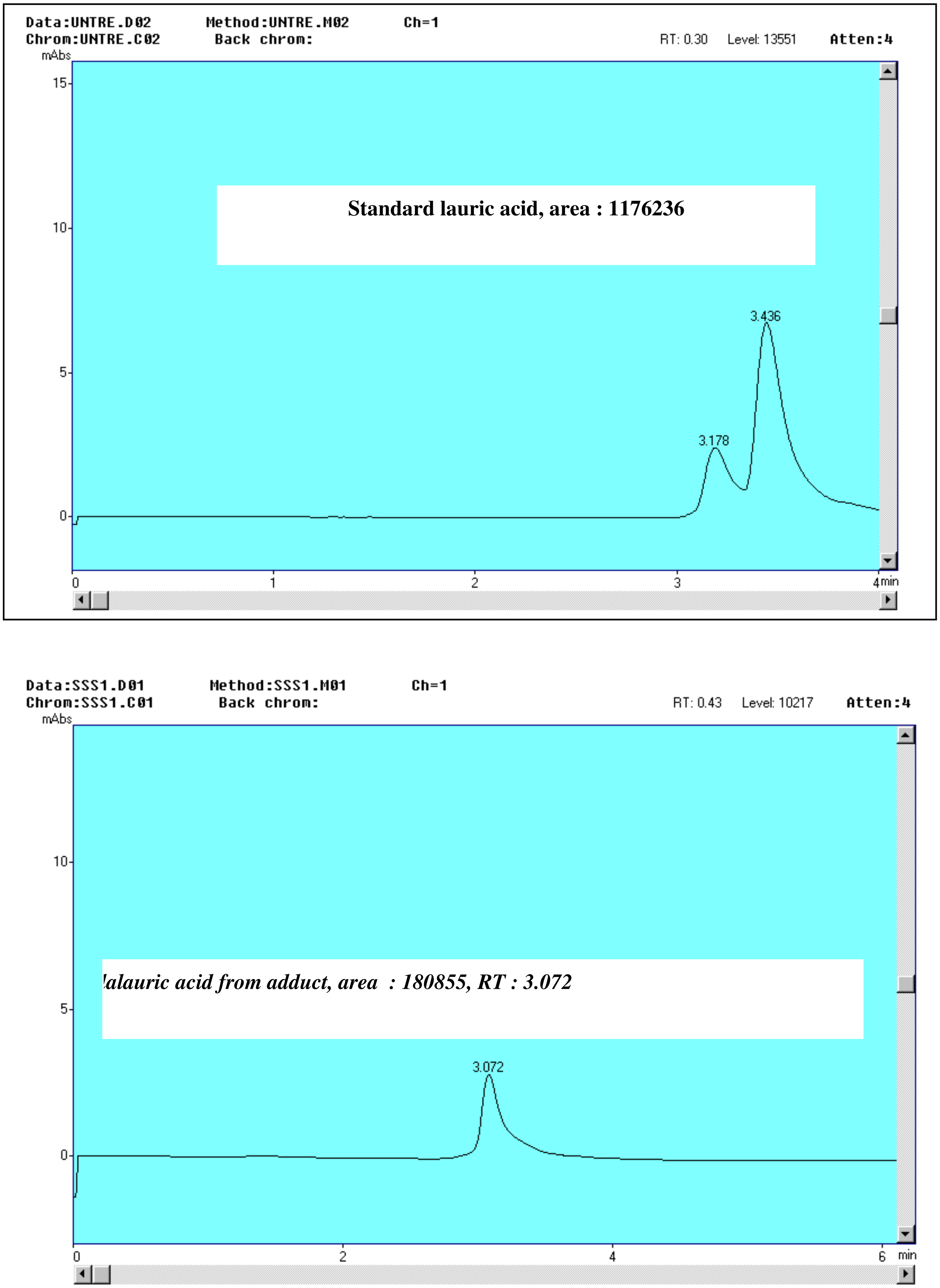


Fig.III.3.1c HPLC profile for standard and extracted lauric acid from agar-fatty acid complex.

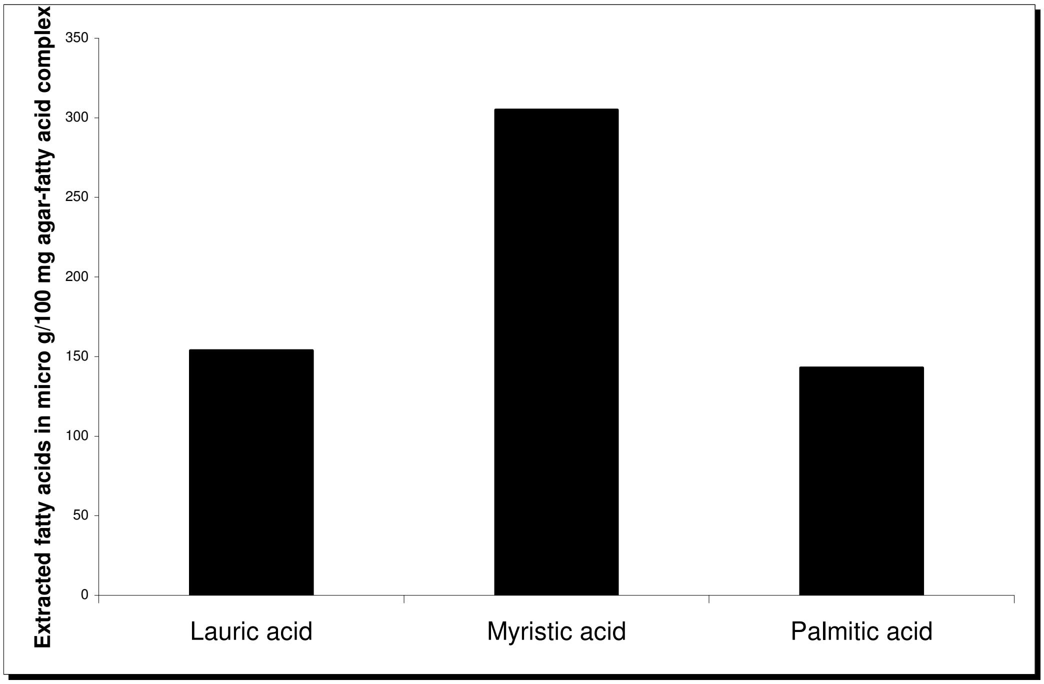


Fig.III.3.2 Analytical data of the bound fatty acids in the incorporated agar.

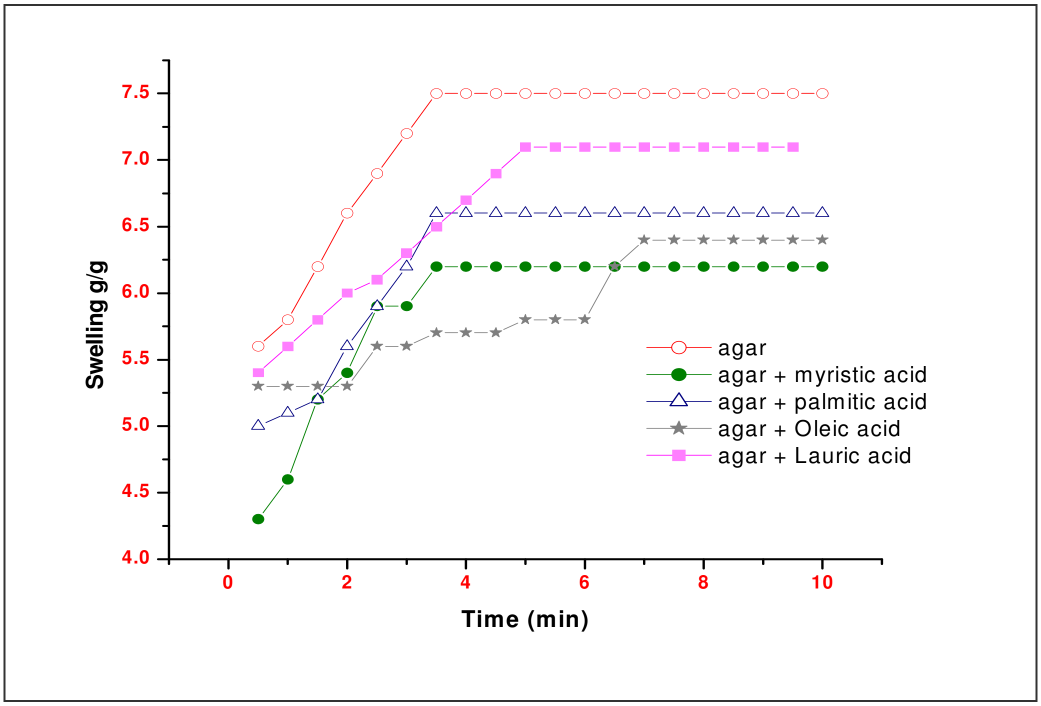


Fig.III.3.4 Swelability of agar and agar fatty acid complexes.

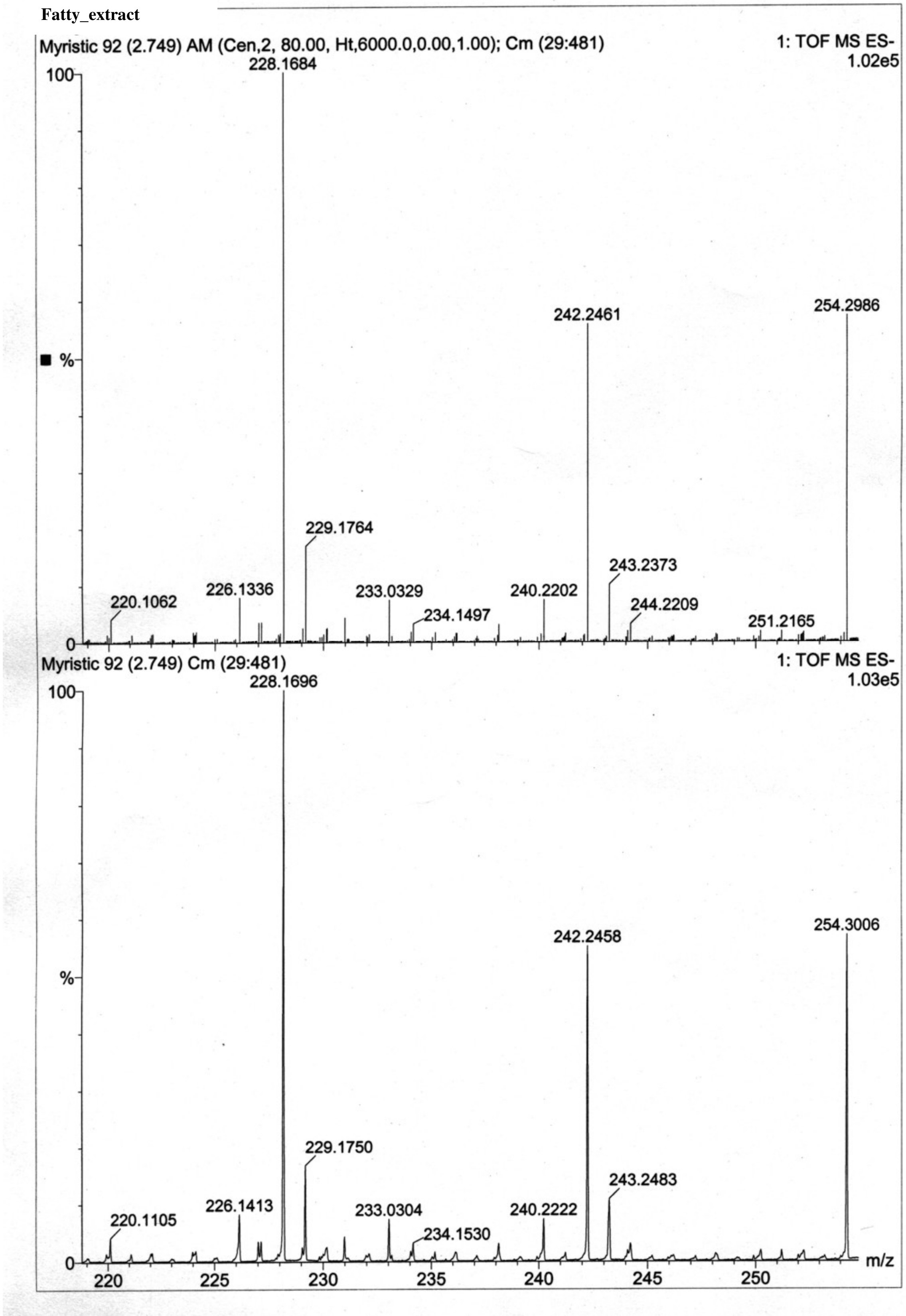


Fig. III.3.3a MS fragmentation for Myristic acid extracted from the agar-myristic acid adduct.

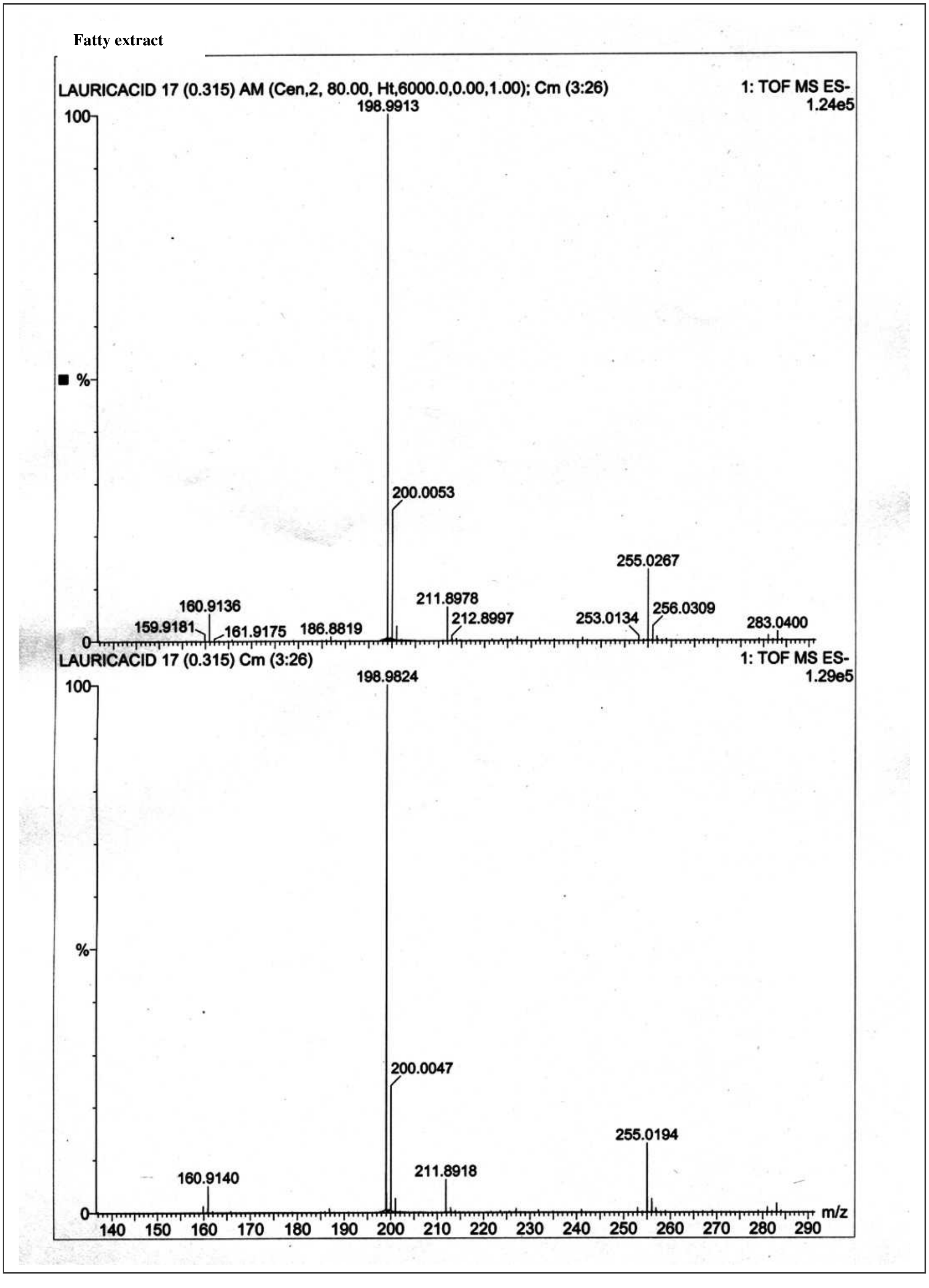


Fig. III.3. 3b MS fragmentation for Lauric acid extracted from the agar-lauric acid adduct.

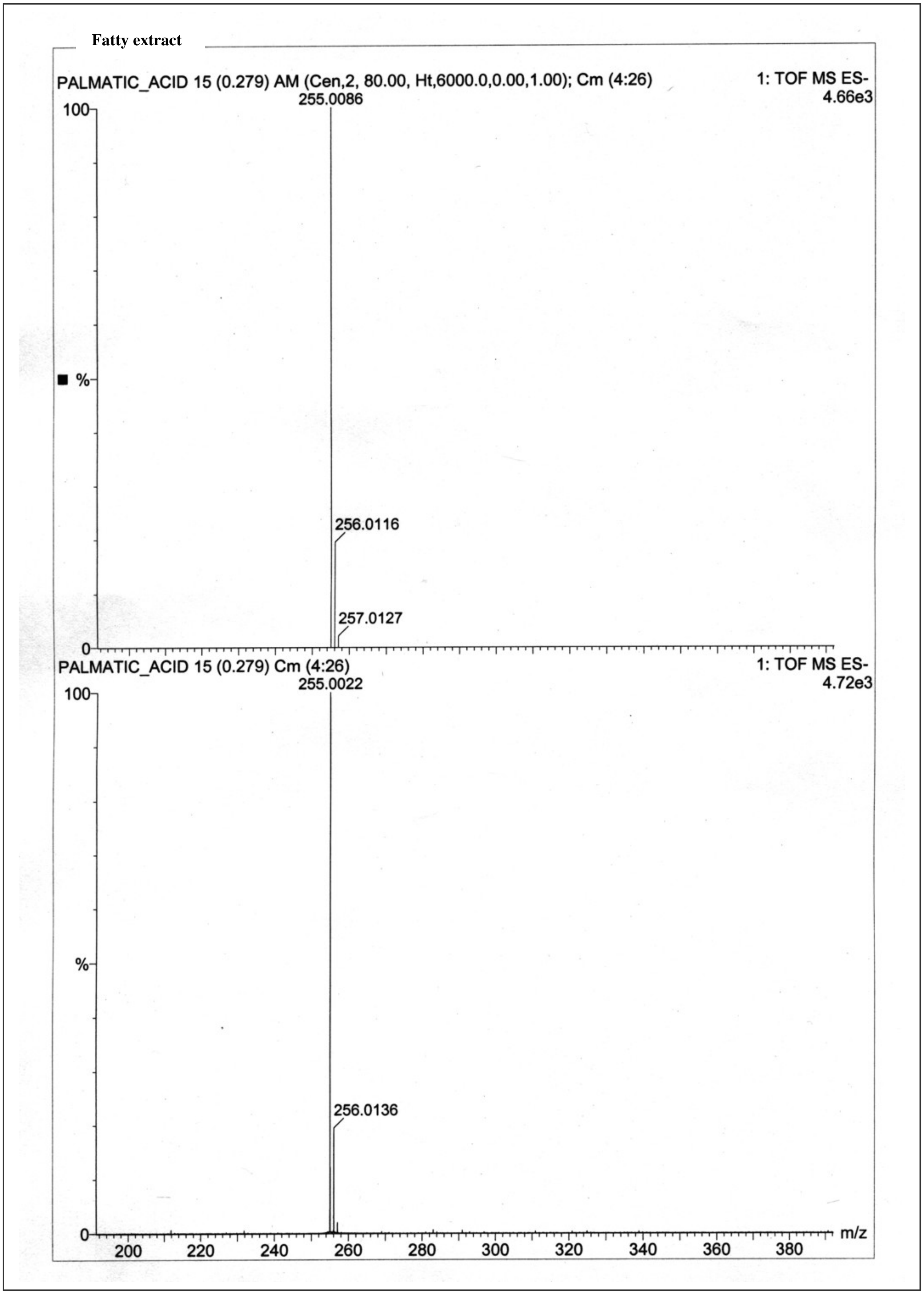


Fig. III.3.3c MS fragmentation for Palmitic acid extracted from the agar-palmitic acid adduct.

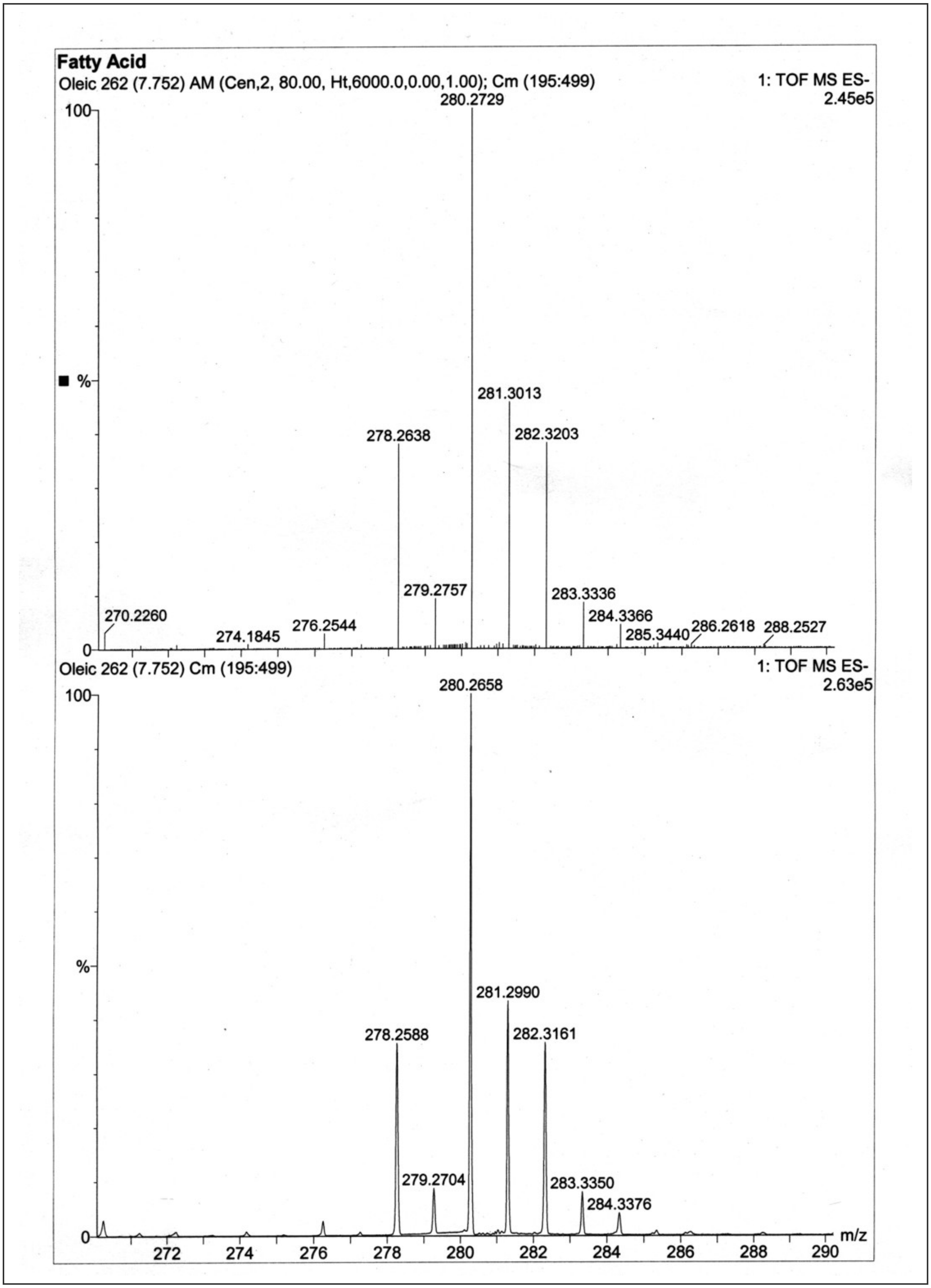


Fig. III.3.3d MS fragmentation for Oleic acid extracted from the agar-oleic acid adduct.

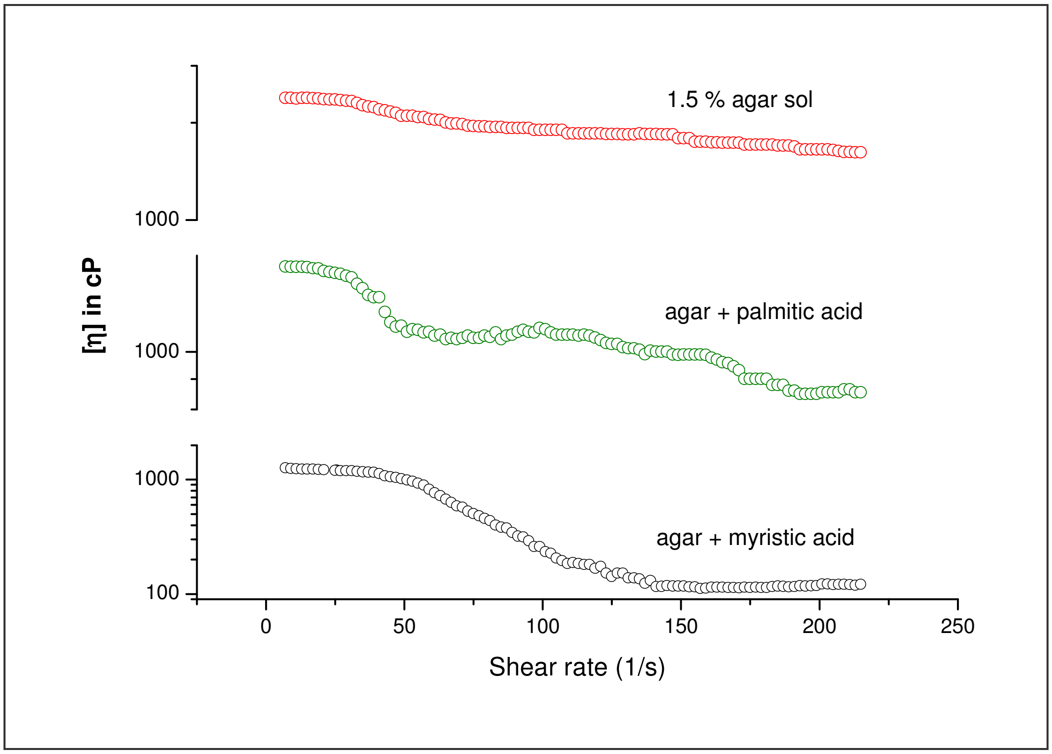


Fig.III.3.5 Dynamic viscosity profile for agar and agar fatty acid complexes in sol

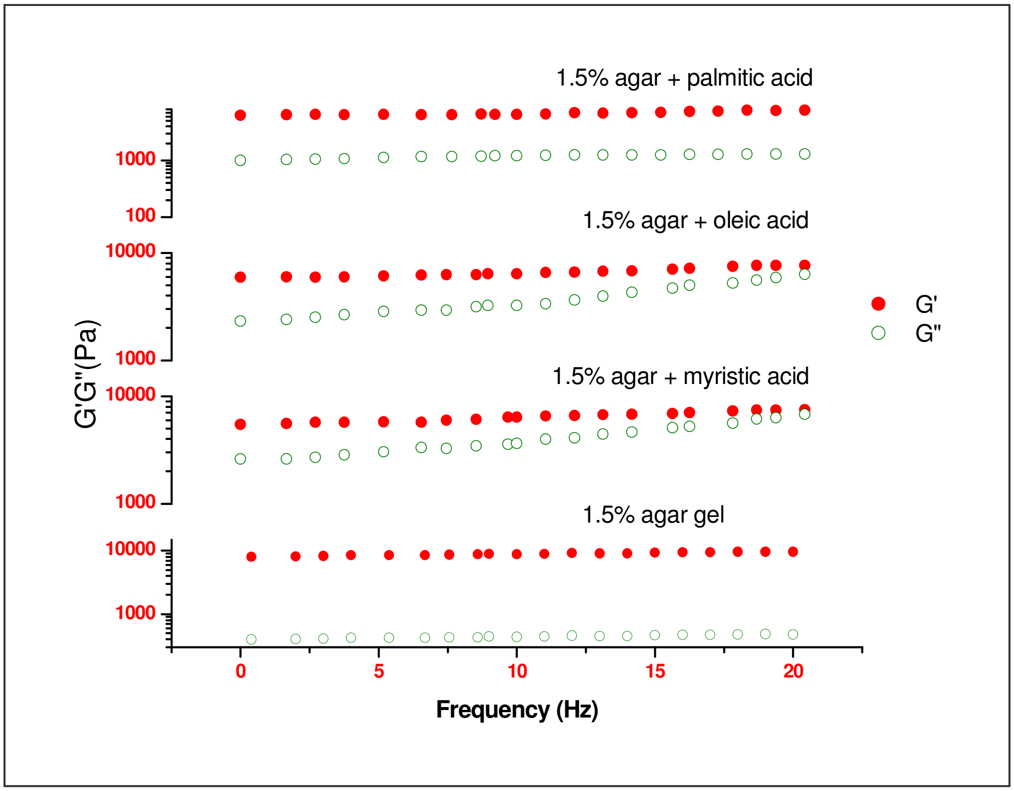
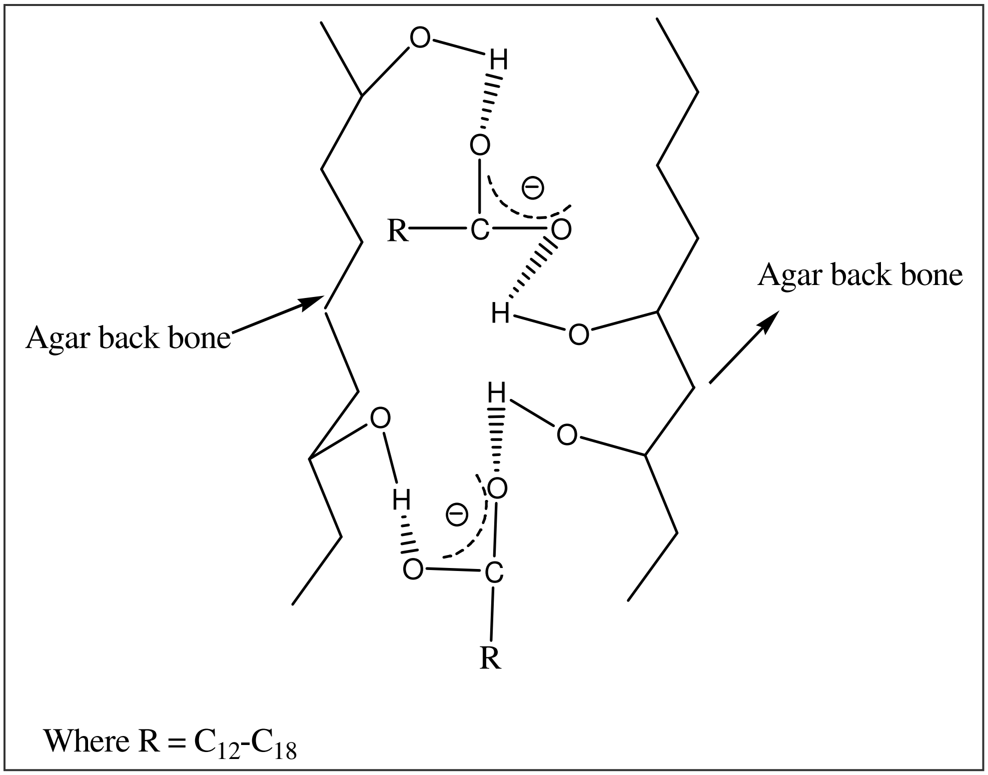


Fig.III.3.6 Frequency dependence of G′ and G″ for agar and agar fatty acid complexes



Scheme III.3.1 Schematic representation of the formation of fatty acid agar complex.

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PART – IV

PREPARATION AND PROPERTIES OF SEAWEED POLYSACCHARIDE BASED THIN FILMS

CHAPTER IV.1

PREPARATION OF SEAWEED POLYSACCHARIDE (AGAR AND REFINED κ-CARRAGEENAN) BASED THIN FILMS.

IV.1.1 INTRODUCTION

Over the past several decades many biopolymers received renewed attention in medical, biomedical and chemical industries. For examples chitin sutures are absorbable in human tissues, therefore chitosan-collagen composite films are prepared and are used as an artificial replacement of human skin, this polymers are not only biodegradable but also edible [1-3]. Butler and coworkers have reported that chitosan based films have very high tensile strength (10-15 MPa) and have excellent oxygen barrier property (4 x10^{-5} cm^3/m.day.atm) [4]. Bai et al. have found that the chitosan-acetic acid composite membranes have excellent selectivity for CO_2 and O_2 permeation the separation factor is lesser than unity indicating their possible application in fruit and vegetable preservations [5]. Among the natural biopolymers popular choice for film formation is chitosan because of the abundance of chitin, from which chitosan is prepared by deacetylation. Chitin is found mostly in marine invertebrates, fungi and yeasts.

Carrageenan is a water soluble phycocolloid extracted from red algae. Used mostly as stabilizer and structure providers in food and ice-cream industries. It is also used in micro encapsulation, to immobilize drugs and enzymes. Carrageenan consists of alternating 1,3- linked α-D galactopyranose and 1-4 linked β-3,6-anhydro D-galactopyranose. Three varieties of carrageenan are known, they are κ, ι, λ, differs in the number and position of sulphate ester groups on the repeating galactose units. κ-carrageenan has one negative charges per disaccharide unit and tends to form strong gel in presence of mono cations, ι-carrageenan has two negative charges and λ-carrageenan has average 2.7 negative charges per disaccharide unit. The gelling properties of κ- and ι- carrageenan impart excellent film forming properties [6].

D. Cade and co workers reported non-animal film compositions suitable for film forming, particularly hard and soft capsules, comprising water soluble cellulose ethers, hydrocolloids and sequestering agents which includes ethylenediamine tetra acetic acid, lactic acid, citric acid, lecithin, beta-cyclodextrin, dihydroxyethylglycine etc. [7]. They have referred to the European patent EP 0714656 describing a film forming composition of cellulose ether, carrageenan and KCl. κ-carrageenan along with dextrin to form a gelled composition (usually a film) enclosing active ingredients, which is water-soluble is described by Genadios in his US patent application [8].

Sadaji Yamashita and Seiki Harada described the production of sustained release capsule and method for preparing the same, using seaweed polysaccharides like alginate and carrageenan besides other gums, produced from terrestrial plants along with polyhydric alcohols [9]. Sun Y. Park and his coworkers have reported production of biopolymer composite films based on κ-carrageenan and chitosan in presence of various organic hydroxy acids (e.g. citric, ascorbic and malic acids) as well as a plasticizer, and studied the properties of the films [10].

Edward Zbygniew Nowak has described production of capsules comprising thermoplastic film of foamed modified cellulose material, preferably hydroxypropylmethyl cellulose. The foamed material dissolves rapidly in the mouth of consumer, releasing the capsule contents into the consumer's mouth [11]. Work of Dong Su Cha et al. also can be quoted in this connection, where they have prepared antimicrobial films based on Na-alginate and κ-carrageenan. They have studied the antimicrobial activity, tensile strength and elongation properties of the films [12]. K. Nakamura et al. have described the thermal properties (DSC) of water insoluble alginate films in presence of di- and trivalent cations e.g. Cu (II), Al (III) and Fe (III)) which were used to replace the sodium (I) ion in sodium alginate. Preparation of polyethylene glycol based alginate oral capsules is also reported by Chandy [14]. Microcapsules derived from polyelectrolyte complexes of sodium alginate with chitosan have been described by K. Y. Lee [15].

During the last three decades, considerable efforts has been devoted to the development of various edible films and coatings [16]. Main purpose of using edible film in food is to provide a barrier to mass transfer (water, gas and lipids), to serve a carrier of food ingredients and additives (pigment flavors and so on) or to provide physical protection [17]. The functional properties of edible films are greatly influenced by parameters such as formulation, film forming technology, solvent characteristics and additives [18]. Cellulose and its methyl derivative are most widely used edible film [19, 20].

There are two distinct mechanisms for film formation i) Dry process mechanisms, based on the thermoplastic properties of polymer when plasticised and heated above their glass transition temperature under low water content. ii) Wet process: In this process polymer is first dispersed in solution then dried. A schematic representation of film formation by wet process is depicted in Fig. IV.1.1. Design of processing equipment for such film formation requires accurate data on rheological properties of film forming solution or dispersion. The presence of defects on the thin films after and before film coating affect their rheological properties and performance [21]. Several types of defects are developed on the film during coating or casting these defects may lead to formation of centers finally responsible for deterioration of the film barrier properties. The coating surface is influenced by the rheology of material in bulk, the method of application of material, and changes in the properties associated with the transition from liquid to gel [17]. For better coating the coating material must possess pseudoelastic, viscoelastic and thixotropic properties. In consequence their rheological state depends on coating process, which involves shears of different magnitude. Agar and carrageenan dispersions are non-Newtonian with little yield stress at low shear rates and exhibit gel thinning behaviour [22].

In the mid-1990s, the outbreak of Bovine Spongiform Encephalopathy (BSE) or “mad cow” disease in Europe drew global attention. Subsequently, it has brought about a major change in the safety perception in the areas of human, cattle and poultry health and welfare. Accordingly, the US Food and Drug Administration (FDA) advisory committee recommended that “gelatin should no longer be considered generally recognized as safe” (GRAS) for either food of pharmaceutical products. Consequent upon these developments, attempts have been under way to find suitable alternatives to gelatin for making soft capsules. This has prompted this investigator to undertake studies for developing seaweed polysaccharide based thin films which would have applications in pharma and food packaging industries. This chapter summarizes the work done in this aspect.

IV.1.2 MATERIALS AND METHODS

Extraction of agar and refined κ- carrageenan: Agar and refined κ- carrageenan used in this film formation was extracted from *Gelidiella acerosa* and *Eucheuma cottonii* following methods described in literature [23, 24]. All the chemicals were used as received without any further purification.

IV.1.2.1 Preparation of film forming dispersions and films therewith

Preparation of films

Films with agar (with various plasticizers)

1. Agar, water, glycerol, glycine (6:80:20:0.2: 0.1; w/v/v/w/w): Mixture of agar and water were autoclaved at 100 °C (9 psi) for 20 min and mixed well. Then glycerol was added, stir mixed and then put in a microwave oven (100%, 60 s). The film was cast using a TLC spreader on a Teflon sheet. The film was left overnight at 20° C (A).

2. Agar, water, mannitol and glycine (6:80:20:0.1; w/v/v/w): Mixture of agar and water were autoclaved at 100 °C (9 psi) for 20 min and mixed well. Then ethylene glycol and glycine were added, stir mixed and then put in a microwave oven (100% exposure, 60 s). The film was cast using a TLC spreader on a Teflon sheet. The film was left overnight at 20°C (B).

3. Agar, water, sorbitol and glycine (6:80:20:0.2; w/v/v/w): Mixture of agar and water were autoclaved at 100°C (9 psi) for 20 min and mixed well. Then sorbitol and glycine were added, stir mixed and then the mixture was put in a microwave oven (100% exposure, 60 s). The film was cast using a TLC spreader on a Teflon sheet. The film was left overnight at 20°C (C).

4. Agar, water, polyethylene glycol and glycine (6:80:20:0.2; w/v/v/w): Mixture of agar and water were autoclaved at 100°C (9 psi) for 20 min and mixed well. Then PEG and glycine were added, stir mixed and then put in a microwave oven (100% exposure, 60 s). The film was cast using a TLC spreader on a Teflon sheet. The film was left overnight at 20°C (D).

Composition of the above films (A), (B), (C) and (D) are summarised in Table.IV.1.1.

Films with refined κ- carrageenan

1. Refined κ-carrageenan (0.96g) in 1% KCl (80 ml solution), glycerol (Refined κ-carrageenan, water, KCl, glycerol, glycine 0.96:80:0.8:20:0.1), w/v/w/v/w) was boiled on a hot plate and mixed well. Film was prepared with a TLC spreader on Teflon sheet. The film was left overnight at 20°C (E).

2. Refined κ-carrageenan (0.96g) in 1% KCl (80 ml solution), mannitol (Refined κ-carrageenan, water, KCl, mannitol, Glycine 0.96:80:0.8:20 ;0.1, w/v/w/v/w) was boiled on a hot plate and mixed well. Film was prepared with a TLC spreader on Teflon sheet. The film was left overnight at 20°C (F).

3. Refined κ-carrageenan (0.96g) in 1% KCl (80 ml solution), sorbitol (Refined κ-carrageenan, water, KCl, sorbitol, glycine 0.96:80:0.8:20:0.1, w/v/w/v/w) was boiled on a hot plate and mixed well. Film was prepared with a TLC spreader on Teflon sheet. The film was left overnight at 20°C (G).

4. Refined κ-Carrageenan (0.96g) in 1% KCl (80 ml solution), ethylene glycol (Refined κ-carrageenan, water, KCl, ethylene glycol, glycine 0.96:80:0.8:20:0.1, w/v/w/v/w) was boiled on a hot plate and mixed well. Film was prepared with a TLC spreader on Teflon sheet. The film was left overnight at 20°C (H).

Composition of the above films (E), (F), (G) and (H) are summarised in Table.IV.1.1.

Plasticizers used were glycerol, ethylene glycol, mannitol, sorbitol, polyethylene glycol, all of them were of LR grade from S.D. fine chemicals, Mumbai and were used without further purification.

IV.1.2.2 Rheological properties

Rheological properties of film forming dispersions were carried out by using Haake Rheometer, geometry selected were cone/plate with 60 mm dia of cone (1°angle) and plate / plate with 35 mm dia. Strain kept for the oscillatory measurements were 0.05%.

The frequency kept from 1-20 Hz. cooling curve was done by varying temperature from 90 to 10 °C. Shear viscosity with shear rate were determined keeping shear from 5-200 1/s. Each experiment was carried out in triplicates and slippage of material during rotation was controlled by selecting appropriate measuring conditions. For high temperature measurements outer surface of the sols were covered with silicon oil to avoid losses due to evaporation.

Mechanical properties e.g. tensile strength and % elongation of the films were measured on an Instron Tensile Strength Tester, Model 1072, Instron Inc, MA, taking 1 cm^2 of sample.

IV.1.2.3 Wettability

Wettability was measured using a static Wilhelmy plate on a Dynamic contact angle Goniometer (DCAT 21, Germany). To ensure removal of surface-active contaminants, all glassware in contact with the sample were cleaned in chromic acid and rinsed with the double distilled water. The platinum plate was washed in double distilled water, heated in a Bunsen flame and left to cool at room temperature. The solutions are prepared with the double distilled water. Experiments are carried out in triplicate.

IV.1.2.4 Thermal analyses

DSC and TGA were measured on a Toledo Mettler DSC system and on a Toledo Mettler TGA system using $STAR^e$ software, Switzerland. For DSC measurements 10-12 mg material was taken in aluminum crucible under argon atmosphere. For TGA measurements 4-5 mg material was taken in ceramic crucibles. Measurement was done under air atmosphere. Temperature programme used was 30-160°C with 5 °C/min for both DSC and TGA.

Moisture content was measured on a Sartorius MA30 moisture analyzer.

IV.1.2.3 Water vapour permeability (WVP)

Water vapour permeability was measured adapting the cup method described by Park et al. [22]. In this method 100 ml graduated glass beaker of known weight with ID 4.9 cm. OD 5.2 cm and depth 7.2 cm were taken. Beakers were filled to a depth 3.0 cm with distilled water and covered with films to be tested and films were fixed tightly with paraffin films. Weight changes of the beakers vs. time were measured and plotted. Linear regression was used to calculate the slope of the best fitted straight line.

IV.1.2.4 Physicochemical properties

Transparency (40-70%) of the films was measured at 640 nm on a Shimadzu UV-160A Spectrophotometer using a clear OHP transparency Sheet (100 micron) as reference [25].

Swelling properties of the film in water was measured by soaking the film in water and measuring the weight gain in regular intervals as described by Park et al. [22]. The swelling weight vs. time were plotted and 'Voigt-based model' equation was applied to determine rate parameters of each film [26].

Scanning electron micrographs were taken on a Philips, Leo model LEO 1430 VP with 15 -20KV accelerating voltage and 2000 x and 1000 x magnification.

Thickness of the film were measured by Digital Vernier calipers, Mitatoyo, Japan and taken as an average of six measurements.

Elemental Analyses were performed on a Perkin-Elmer CHNS/O Analyser, Series II, A2400, Perkin-Elmer USA, using around 5 mg of the sample.

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

Table. IV.1.1 Composition of thin films

Films with agar		
Film code	Additives	Observation
A	Glycerol and Glycine	Good film could withstand mild manual stretching
B	Mannitol and Glycine	Film was weaker than ‘A’ above.
C	Sorbitol and Glycine	Film was brittle after drying.
D	Polyethylene glycol and Glycine	Film was weaker than ‘A’ above.
Film with Refined κ- carrageenan		
E	Glycerol, KCl, Glycine	Good film could withstand manual stretching.
F	Mannitol, KCl, Glycine	Weaker than ‘E’ above.
G	Sorbitol, KCl, Glycine	Same as ‘F’ above.
H	Ethylene glycol, KCl, Glycine.	Weaker than ‘E’ above.

IV.1.3 RESULTS AND DISCUSSION

IV.1.3.1 Mechanical properties

Tensile strength, % elongation, thickness are furnished in Table.IV.1.2 below.

From Table.IV.1.1 and Table.IV.1.2 it is evident that refined κ-carrageenan gave film with good tensile strength and % elongation with glycerol. Agar too gave good film with glycerol but the strength and % elongation is poor. Refined κ-carrageenan gave formation of film with mannitol, sorbitol, PEG, Ethylene glycol, but agar did form film with glycerol, mannitol, sorbitol but with PEG and ethylene glycol it did not form any film. It is also evident that all other plasticisers except poly ethylene glycol formed good films in presence of glycerol.

Table.IV.1.2 Mechanical properties of agar and refined κ-carrageenan based film.

Film code	Description	Thickness[a] (mm)	Tensile strength (g/cm^2)	Elongation (%)
A	Agar + Glycerol + Glycine	0.17	56.0	140.0
B	Agar + Mannitol + Glycine	0.25	58.0	100.0
C	Agar + Sorbitol + Glycine.	0.17	50.0	85.0
D	Agar + PEG + Glycine.	0.15	-----	----
E	κ-carrageenan + KCl + Glycine.	0.17	120	185.00
F	κ-carrageenan + KCl + Mannitol + Glycine.	0.20	90	100.0
G	κ-carrageenan + KCl + Sorbitol + Glycine.	0.19	75	96.0
H	κ-carrageenan + KCl + Ethylene glycol + glycine.	0.20	85	110.0

[a] An average of six measurements has been taken; measured on a Digital Vernier calipers, Mitatoyo, Japan

IV.1.3.2 Physicochemical properties

Elemental Analysis: Elemental analysis of agar and refined κ-carrageenan based films shows following compositions for both the films.

Agar film with glycerol : C 33%, H 8.64%, O 58.2% and refined κ- carrageenan film with glycerol : C 35.12%, H 9.69%, N 0.21% and O 53.2%. The transparency of the thin films was found as follows- agar film with glycerol (thickness 0.11 mm) = 61%.

Refined κ-carrageenan film with glycerol and thickness (0.11 mm) = 55% ; Refined κ-carrageenan film with ethylene glycol (thickness 0.14 mm) = 67% . Therefore, film made with refined κ-carrageenan and ethylene glycol is the most transparent one.

IV.1.3.3 Water vapour permeability

The permeation of gases and vapors in membranes and plastics can be described as a two stage process. First, the permeating substance dissolves in the surface of the membrane. Second, the molecules diffuse through the material, forming a concentration gradient, and then evaporate again. The steady state diffusion of a gas or vapor is described by Fick's first law (Eq. IV.1.1).

$$dQ/dt.1/A = -D.dc/dx \text{ ------------------ Eq.IV.1.1}$$

where dQ/dt is the flow velocity of the medium, A is the flow area, D is diffusion constant, and dc/dx is the concentration gradient. Unit of diffusion that is used here is $g/cm^2.h^{-1}.bar$ [cf. 27].

WVP was determined by cup method. Weight changes of cup vs. time were plotted. Linear regression was used to calculate the slope of the best fitted straight line.

Following table presents the WVP values of various films. WVP vs time plots and regression graphs derived thereof are depicted in Fig.IV.1.2a, Fig.IV.1.2b, Fig.IV.1.2c respectively.

Table.IV.1.3 WVP of thin films

Film constituent	WVP ($g/cm^2.h^{-1}.bar$)
Refined κ-carrageenan, glycerol, glycine and KCl	0.0053
Refined κ-carrageenan, glycine, KCl and polyethyle glycol	0.0085
Agar, glycerol and glycine	0.017

Scrutiny of the data presented in the table above reveal that refined κ-carrageenan films with glycerol and glycine have the lowest vapor permeation on the other hand agar film with glycerol has the highest vapor permeation.

IV.1.3.4 Water swellability

Swelling or water absorption of this films were studied by following a method described by Park et al. [22]. A weighed piece of dry films of dimension 4 x 2 cm were dipped in 50 ml distilled water in a beaker. The weight of the swollen film was taken after time to time. The water content of the swollen films were calculated using following equation.
$W_c = (W_s - W_d) / W_d$ ------------------------ Eq.IV.1.2

Where W_c is the water uptake in grams per gram of dried film, and W_s and W_d are the weights of the swelled films and after press drying the swelled film on filter papers respectively.

Fig.IV.1.3 represents the swelling capacity of the agar, refined κ-carrageenan films with glycerol and ethylene glycol at consecutive time intervals. According to this figure, the swelling rate sharply increases and then begins to level off. The data may fit well with a 'Voigt-based model' equation as follows [24].

$S_t = S_e (1 - e^{-t/T})$ ------------------------- Eq.IV.1.3

Where S_e and S_t are equilibrium swelling and swelling at time t, respectively. T (in s) stands for the 'rate parameter'. Using the equation, the rate parameter for agar film in water was deduced to be 6.11 min, for refined κ- carrageenan film with glycerol it was 5.3 min and for refined κ- carrageenan film with ethylene glycol 3.5 min. This indicates that agar film with glycerol has the maximum water swelling capacity. All the films get completely dispersed in water beyond 7.0 min.

IV.1.3.5 Rheological behaviour for agar and refined κ-carrageenan based film forming dispersions

The typical gel behaviour of agar film forming dispersion is shown in Fig.IV.1.4. The storage modulus (G′), loss modulus (G″) are plotted against time on cooling from 90 to 10 °C. It was observed that with decrease in temperature the values of G′ and G″ change with decrement of temperature. At the temperature near gel point of the dispersion (32-35°C) there appears a sudden increase of these values, indicating formation of elastic network (G′>G″). The moduli continue to increase even after gelling; eventually they reach the equilibrium after long time indicating thereby the completion of the process of networking in the gel state . Similar rheogram was observed for refined κ- carrageenan film forming dispersion and depicted in Fig.IV.1.5. Here the sudden increase in the G′ and G″ values were observed at (40 – 43°C). This indicates that the formation of elastic network in case of refined κ-carrageenan based thin film dispersion took place at higher temperature in comparison to that with agar. It also took long time to achieve the equilibrium in the gelling process.

IV.1.4 RHEOLOGICAL MODELS

IV.1.4.1 Rheological model based on flow behaviour

The steady-state flow curves of the film forming dispersions showed shear-thinning behaviour under steady state shear flow. Viscosity-shear rate plots show different profiles for following film forming dispersions: 2% agar in presence of glycerol and 2% refined κ- carrageenan in presence of glycerol.

Flow diagrams for agar and refined κ-carrageenan film forming dispersions are shown in Fig.IV.1.6 and Fig.IV.1.7 respectively. Both shows gel thinning or pseudoplastic behaviour. The experiment was conducted at 45°C. Agar thin film forming material was found to have more gel thinning property than its refined κ-carrageenan counterpart. This indicates that agar film can be cast at lower temperature in comparison to refined κ-carrageenan due to the former's relatively higher fluidity at lower temperature under the applied shear.

When the shear stress-shear rate function is nonlinear, the data are often fit by a Herschel-Buckley model. This is often true for many non-Newtonian fluids. Flow property modeling for film forming dispersions is useful technologically for identifying the most appropriate coating system design and to optimize the operating conditions. Constitutive equations are also important for providing the material parameters required by process control [12,28]. Herschel-Buckley model can be used for highly concentrated thick film forming dispersions.

$$\tau = \tau_o + K\dot{\gamma}^n \text{ ------------------------- Eq.IV.1.4}$$

Where τ (Pa) is the shear stress, $\dot{\gamma}$ (s^{-1}) is the shear rate, τ_o is the apparent yield stress, K (Pa S^n) and n (dimensionless) are the consistency and flow indices, respectively. But for relatively dilute film forming dispersions like those containing agar and refined κ-carrageenan, Oswald-de Waele model will fit better.

For pseudoplastic or non-Newtonian fluids the ratio τ/γ i.e., η is a decreasing function of γ. The decrease in viscosity with increasing rate of deformation can usually be attributed to the breakdown of a structure at the colloidal or molecular level. Macromolecules will become more aligned and hence less entangled and less resistant to deformation as the deformation rate is increased. It is very difficult experimentally to acquire viscosity data at zero shear and infinite shear values. Between these two limits the shear stress (or viscosity) is often linear with shear rate over several decades on logarithmic coordinates. In this restricted region

$\log \tau = \log k + n \log \dot{\gamma}$ ------------------------- Eq.IV.1.5

$\log \eta = \log k + (n - 1) \log \dot{\gamma}$ ------------------------- Eq.IV.1.6

For agar film forming dispersion, log η plotted vs. log $\dot{\gamma}$' from the slope of linear regression values of 'n' and 'k' can be calculated, and thus

$\tau = k\,|\dot{\gamma}|^{n-1}.\dot{\gamma}$ ------------------------- Eq.IV.1.7

$\eta = k\,|\dot{\gamma}|^{n-1}$ ------------------------- Eq.IV.1.8

Equation.IV.1.8 is called Power law or Oswald-de Waele model. This law is empirical and must fail for very low and high shear rates. The factor k is temperature sensitive (and concentration sensitive for suspensions), but 'n' is typically insensitive to temperature (and concentration) changes.

Attempts were made to fit both the above mentioned models for agar and refined κ-carrageenan gels as well as for the respective film forming dispersions but they did not fit well unlike other film forming dispersions of methylcellulose, and macromolecular dispersion like polyacrylamide gels. It may be due to completely different behavior of agar and refined κ-carrageenan gels in solutions at higher and lower temperatures. At higher temperature agar and refined κ-carrageenan gels behaves as sols with random single strands, as temperature decreases the single strands come closer and form double helices, which finally forms gel network by means of developing junction zones. Since film casting with such polysaccharides are made only at higher temperature hence models were examined at the sol states for these polysaccharide dispersions. As Marcotte and coworkers have observed that shear rate and shear stress corrections have not much effects on the validity of models, hence the shear rate corrections were not done [28]. Fig.IV.1.6 and Fig.IV.1.7 depicts the validity of Ostwald de Waele model for 2% agar and 2% refined κ-carrageenan film forming dispersions at 45°C i.e. at sol state. On the other hand Fig.IV.1.8 and Fig.IV.1.9 depicts the validity of Herschel –Buckley model for 2% agar and 2% refined κ-carrageenan film forming dispersions at 45°C i.e. at sol state. The values of flow and consistency indices both for Ostwald de Waele and Herschel-Buckley models' are furnished in Table.IV.1.4 and Table.IV.1.5. It has been observed that for 2% agar film forming dispersions consistency and flow indices for stress and strain were found to be 43.74 and 0.1806 respectively considering the Oswald de Waele model. On the other hand the corresponding values are for viscosity of the same dispersion were 18.40 and 0.4478 respectively. But considering the Herschel-Buckley model the respective values reached 2330, 0.0066 and 124.3, 0.116 with very high level uncertainty (Table IV.1.5). These results indicate that the flow behaviour of 2 % agar film forming dispersion fits well on the Oswald de Waele model, the determination constant (R^2) is also near unity.

Similarly for 2% refined κ-carrageenan film forming dispersions in Ostwald de Waele model for stress and strain consistency and flow indices are 20.43 and 0.4177. Corresponding values in Herschel-Buckley model were 1.47 and 0.8910 respectively. These values indicate that refined κ- carrageenan film forming dispersion fits well on the Herschel-Buckley model.

On the other hand the trend of dynamic viscosity values indicate that in refined κ-carrageenan dispersion Ostwald de Waele model suits well, showing no yield stress. The consistency and flow indices values were 18.53 and 0.5240 respectively.

Table.IV.1.4 Validity for Ostwald de Waele model for 2% agar and refined κ-carrageenan film forming dispersion.

m	Uncertainty	n	Uncertainty (p)	R^2
2 % agar film forming dispersion (Stress and Strain)				
43.74	6.16	0.1806	7.4	0.9227
Viscosity				
18.40	5.513	0.4478	5.048	0.9362
2 % refined κ-carrageenan film forming dispersion (Stress and Strain)				
20.43	15.39	0.4177	8.158	0.9225
Viscosity				
18.53	4.532	0.5240	3.818	0.9596

Note: Measurements were done at 45°C; R^2 near unity means higher validity.

Table.IV.1.5 Validity for Herschel-Buckley model for 2% agar and refined κ-carrageenan film forming dispersion.

m'	Uncertainty	n'	Uncertainty (p)	τ_o	R^2
2% agar film forming dispersion (Stress and Strain)					
2320	736	0.0066	719	-2292	0.8912
Viscosity					
124.3	25.42	0.116	22.94	-12.0	0.9362
2% refined κ-carrageenan film forming dispersion (Stress and Strain)					
1.47	75.25	0.8910	16.38	50.32	0.96
Viscosity					
32.5	17.39	0.3895	10.68	-12.5	0.91

Note: Measurements were done at 45°C; R^2 near unity means higher validity.

IV.1.5 Thermochemical Analysis

IV.1.5.1 TGA

The TGA analysis of the refined κ-carrageenan and agar gel forming materials were carried out and the respective thermo grams are depicted in Fig.IV.1.10a and Fig.IV.1.10b. It has been observed that 100% mass loss took place at 108°C, on the other hand for refined κ-carrageennan based thin film the corresponding temperature was 122°C. This indicates that the latter is more thermally stable than the former.

IV.1.5.2 DSC

The DSC thermograms for refined κ-carrageennan and agar film forming materials are depicted in Fig.IV.1.11a and Fig.IV.1.11b respectively. It was observed that with both the film-forming materials there appeared two endotherms at temperatures 80°C and 90°C for agar film forming material and 95°C and 100°C for the respective refined κ-carrageenan containing material. The total enthalpy for agar film forming material was –3.36 J/g (9 mg gel) and that for refined κ-carrageenan film forming material the value was –163.07 J/g (13 mg gel). This indicates that heat content for refined κ-carrageenan film forming material is more in comparison to agar film forming material, indicating enhanced molecular association in refined κ-carrageenan film forming material at glass transition temperature (T_g).

IV.1.6 Wettability

IV.1.6.1 Contact angle mesurements

Contact angles of various films to measure hydrophobicity on the surface of the films were done. For these measurements films of 4 x 2 cm dimensions were dipped it water and the corresponding advancing and receding angles were measured, the values obtained are furnished in Table.IV.1.6.

Table IV.1.6 Values of advancing and receding angles for various thin films

Sample	Solvent	Advancing angle (degrees)	Receding angle (degrees)	Mean (degrees)	Remarks
Agar + glycerol + glycine film (A)	Water	47.25 48.42 47.21	39.10 40.20 39.75	43.10 44.31 43.48	Agar film with glycerol is most hydrophilic film.
Agar + mannitol + glycine film (B)	Water	44.15 50.56 56.42	36.13 45.19 46.29	40.13 47.87 51.35	
Refined κ-carrageenan + Glycerol + KCl + glycine (E)	Water	59.50 58.17 59.76	44.77 46.23 49.23	52.13 52.2 54.49	
Refined κ-carrageenan + KCl + mannitol + glycine (H)	Water	60.03 63.98 67.66	50.31 51.96 57.90	55.17 57.97 62.78	

From contact angle measurements on the film surface it was observed that agar film with glycerol was the most hydrophilic while the refined κ-carrageenan film with ethylene glycol had the least hydrophilicity. The more hydrophilicity of agar film can also be inferred from its higher water swellability.

IV.1.7 Electronmicrographic studies of films

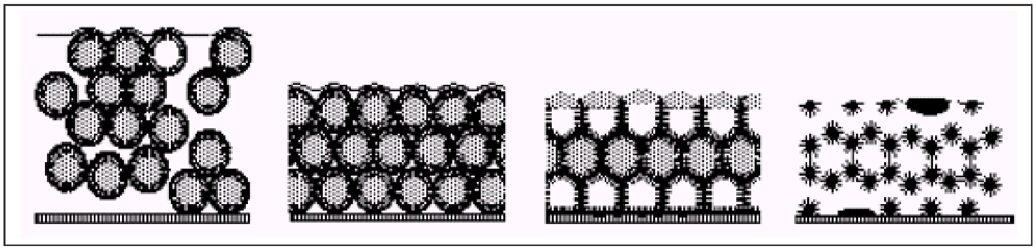
IV.1.7.1 Scanning Electron Micrographs (SEM)

The scanning electron micrographs of agar and refined κ-carrageenan film on the surface and cross sectional area were taken by applying 20 KV accelerating voltage and 2000 x magnification. The micrographs of the films of agar and refined κ-carrageenan are depicted in Fig.IV.1.12 and Fig.IV.1.13 respectively. From the micrographs of the surface and cross section area for both the films it appears that refined κ-carrageenan molecules are more ordered in the film made with refined κ-carrageenan than that of agar molecules in the film made with agar. Potassium chloride crystals are also visible on the surfaces of refined κ-carrageenan based film image.

IV.1.8 SUMMARY

It has been demonstrated in the present investigation that it is possible to prepare thin films with agar and refined κ-carrageenan in presence of plasticizers (e.g. glycerol, ethylene glycol polyethylene glycol, mannitol and sorbitol). In the case of refined κ-carrageenan additives e.g. KCl and glycine were added for enhanced film strength. Thermal measurements revealed that refined κ-carrageenan films were more thermally stable than agar films. Scanning electron micrographs showed that refined κ-carrageenan films constituted of more ordered molecular association than agar films. It has been observed that κ-carrageenan produces superior films in presence of plasticizer and additives. Rheological measurements revealed that the film forming material containing refined κ-carrageenan possesses enhanced thixotropic properties compared to the film forming material containing agar. The carrageenan containing material required higher temperature (ca. 50^{o}C) while casting the films and in case of agar the casting of films could be done ca. 45^{o}C. Wettability and swelling measurements showed that agar films were more hydrophilic and have higher water absorbing capacity than its refined κ-carrageenan counterpart.

Various rheological models were considered to find out the best one that suits the flow behaviour of the film forming materials containing agar and refined κ-carrageenan. It has been found that in the sol state the film forming material containing agar is best fitted to Ostwald de Waele model for stress, strain and viscosity parameters. On the other hand, Herschel-Buckley model fits well for the film forming material containing refined κ-carrageenan in sol state for stress and strain parameters, and its viscosity parameter suits well with the Ostwald de Waele model. These measurements would be useful for drawing out the operational conditions for preparing films from these polysaccharides. Technologically speaking, these results would be of help in predicting the operational parameters for unknown or little known systems. The seaweed polysaccharide systems studied herein would be useful for developing non-animal gelatin based formulations suitable for pharma and food packaging industries.



Random polysaccharide (PS) molecules in solution

Ordered PS molecules in a cast film

PS molecules in a stable semi dry film

PS molecules in a dry film

Fig. IV.1.1 Schematic diagram of formation of polysaccharide based thin films

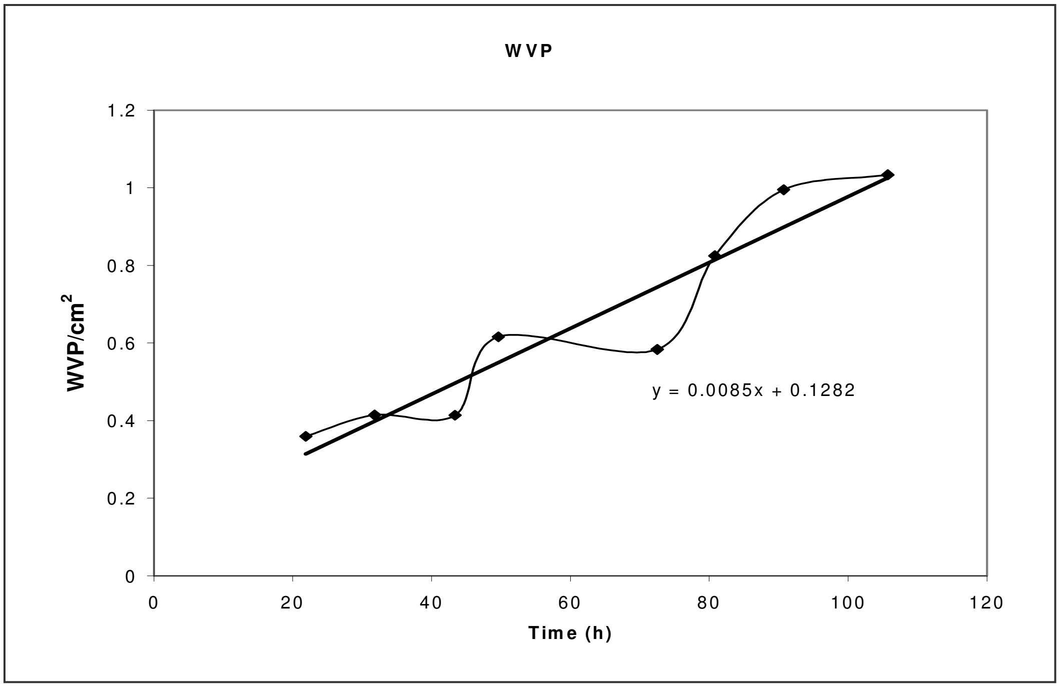


Fig.IV.1.2a WVP determination of Film with refined kappa carrageenan, glycerol and KCl.

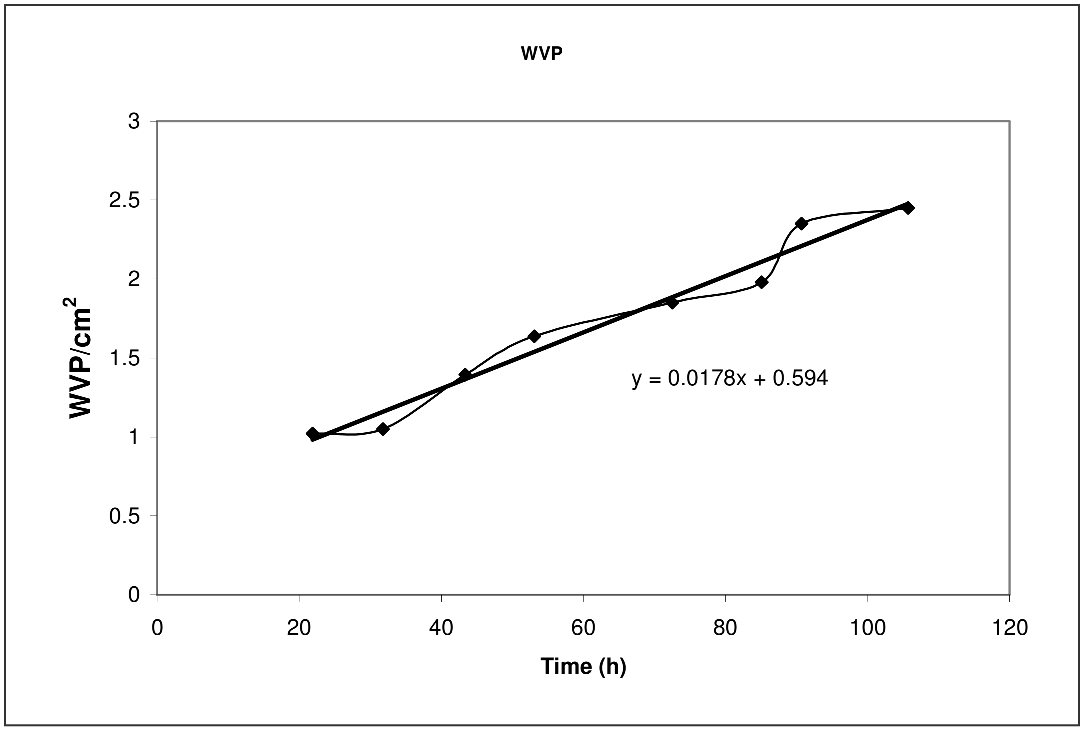


Fig.IV.1.2b WVP determination of film with refined carrageenan and polyethylene glycol

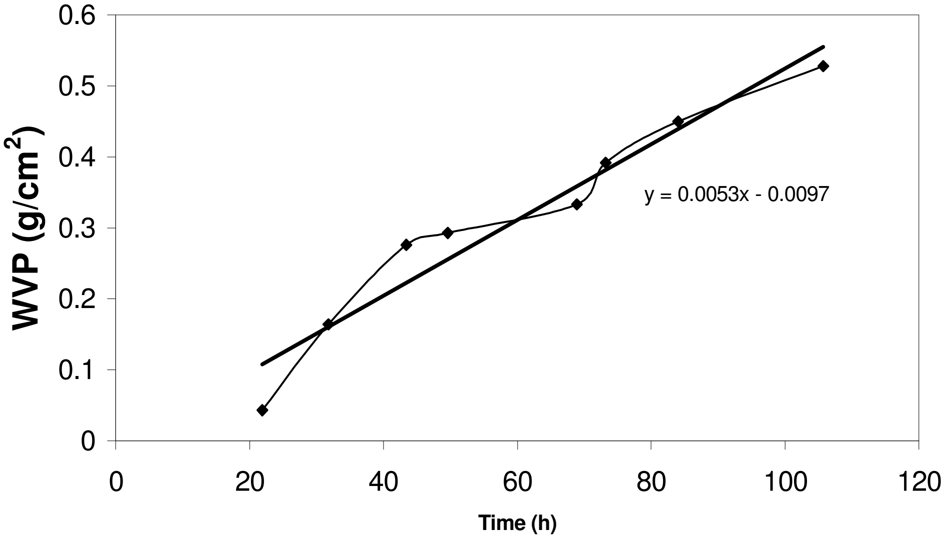


Fig.IV.1.2c WVP determination of film with agar and glycerol

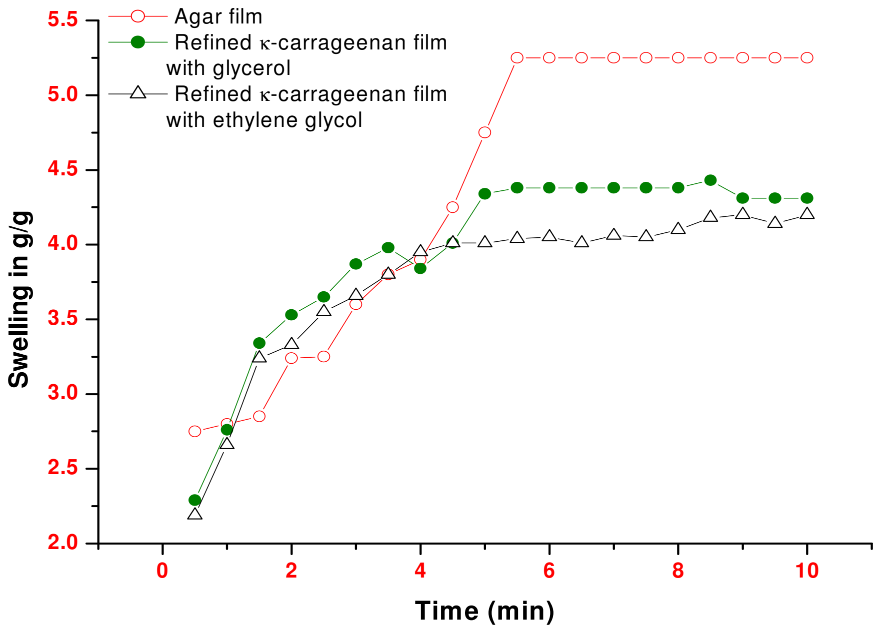


Fig. IV.1.3 Swellability of polysaccharide based thin films

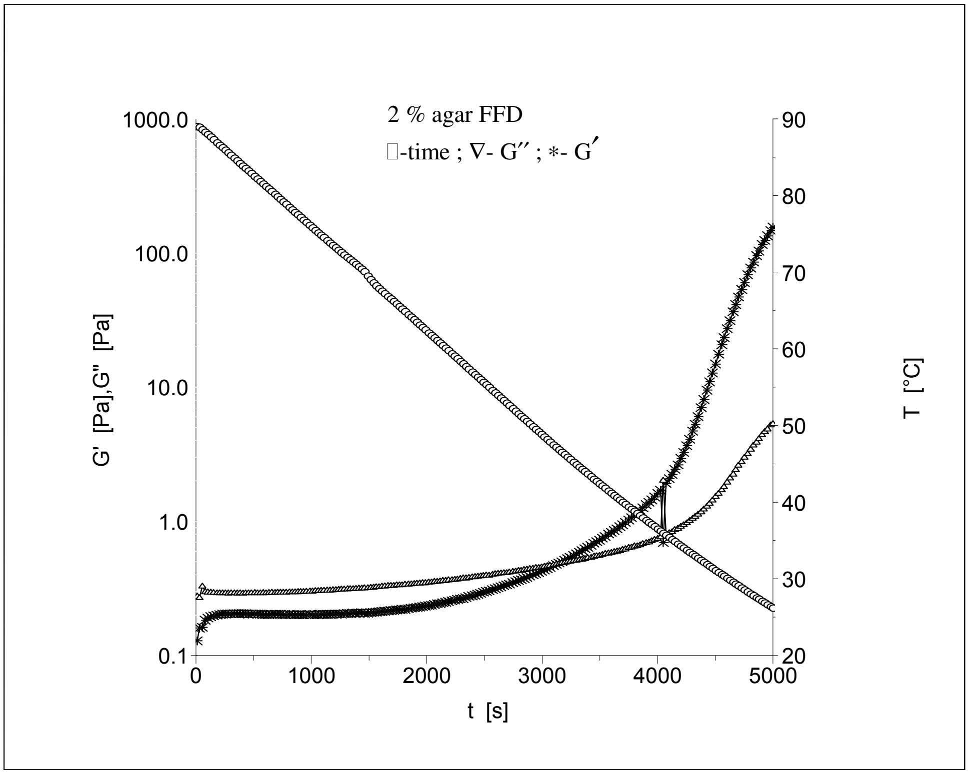


Fig. IV.1.4 Rheograms for G′, G″ dependency on temperature and time for gel forming dispersion of 2% (w/v) agar.

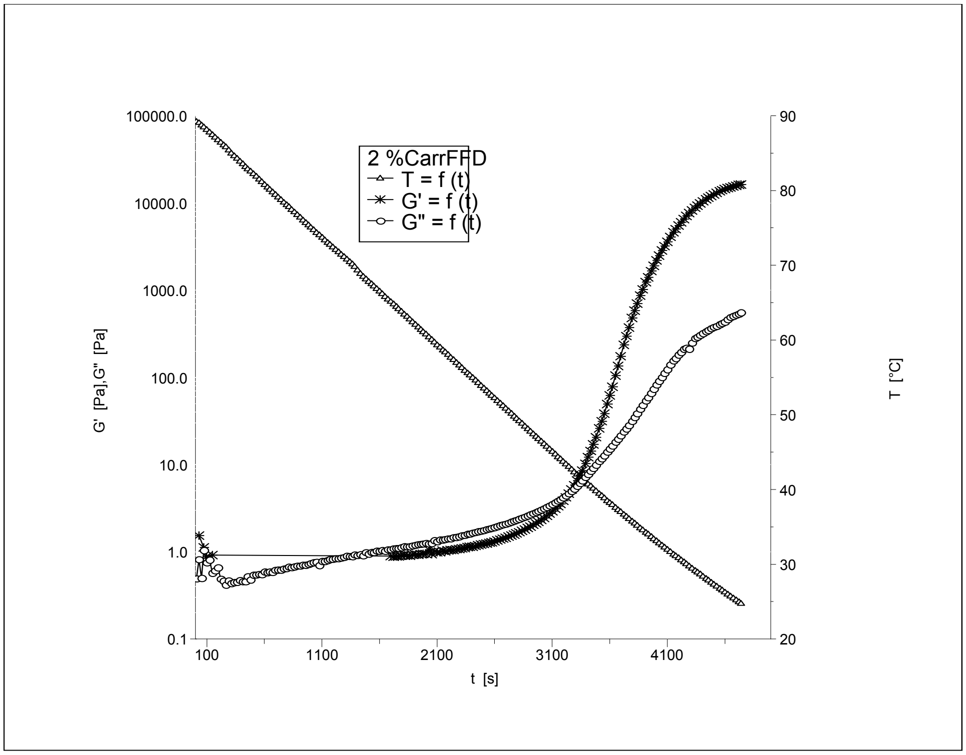


Fig. IV.1.5 Rheograms for G′, G″ dependency on temperature and time for gel forming suspension of 2% (w/v) refined κ-carrageenan.

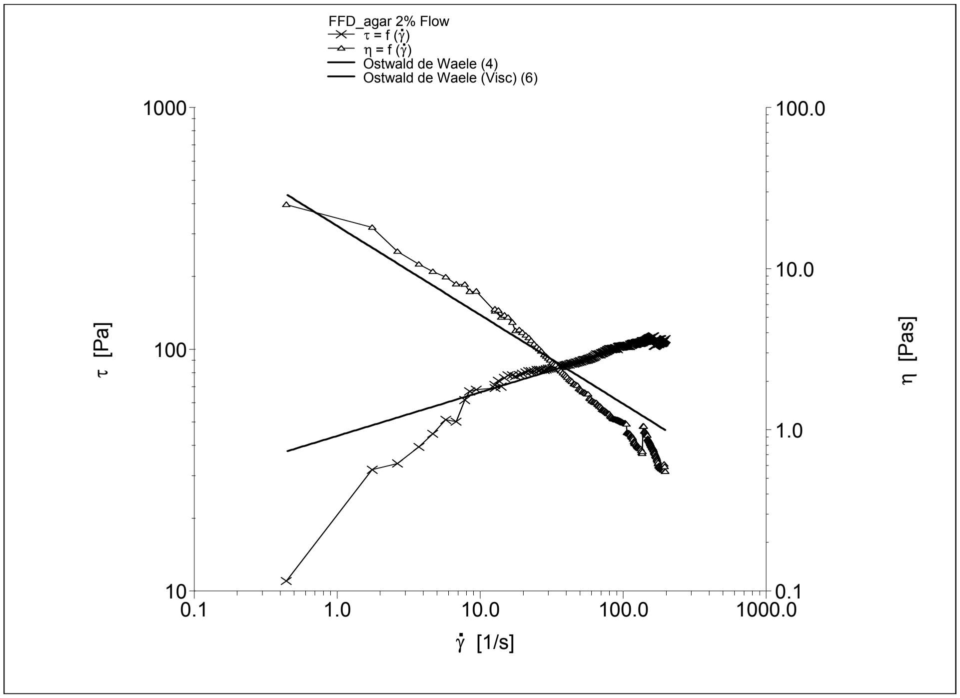


Fig. IV.1.6 Logarithmic plots of shear rate vs shear stress and viscosity, regression analysis according Ostwald de Waele model for 2 % agar film forming dispersion at 45 °C.

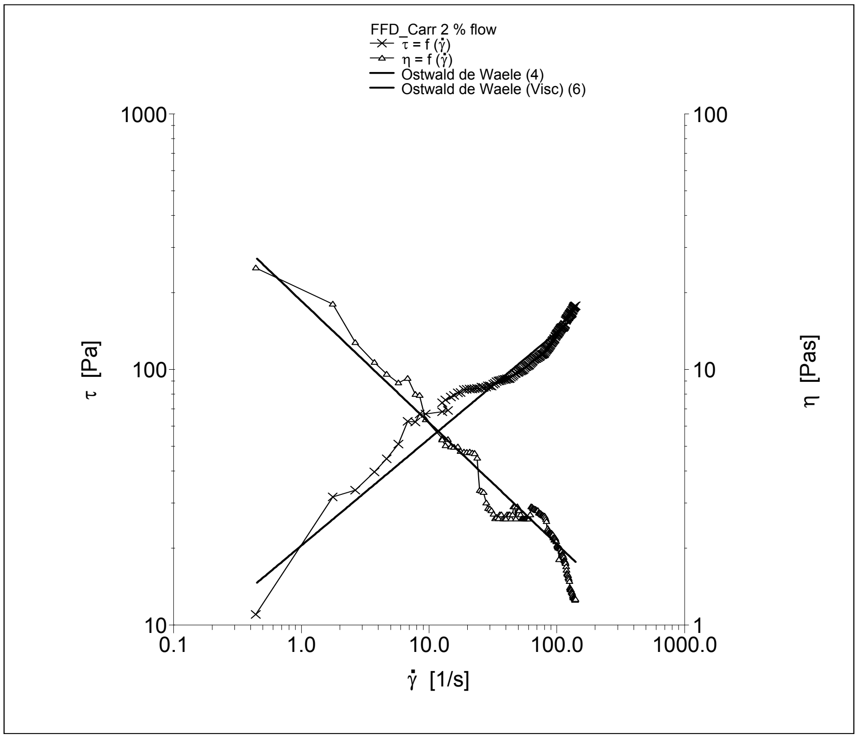


Fig. IV.1.7 Logarithmic plots of shear rate vs shear stress and viscosity, regression analysis according Ostwald de Waele model for 2 % κ-carrageenan film forming dispersion at 45 °C.

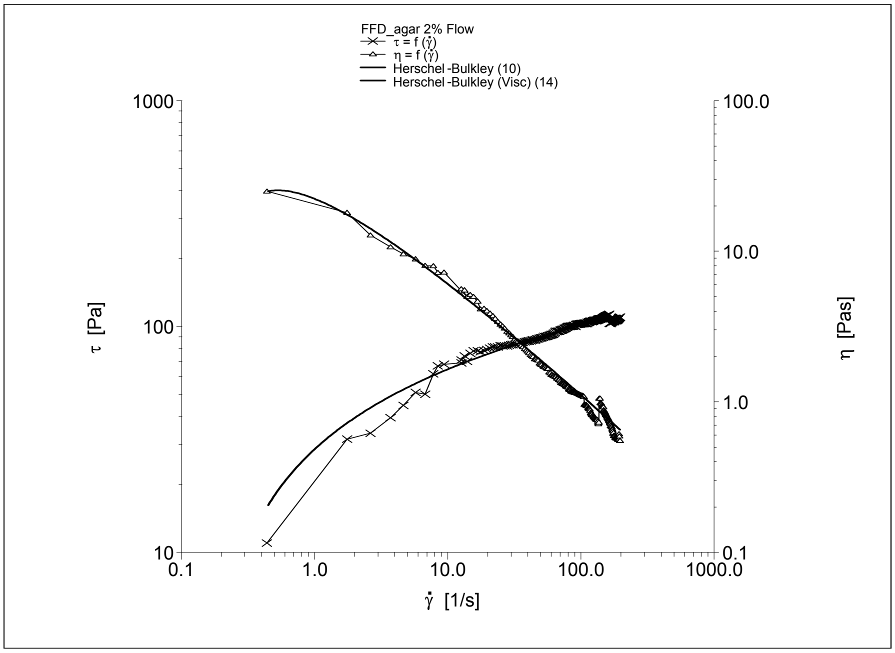


Fig. IV.1.8 Logarithmic plots of shear rate vs shear stress and viscosity, regression analysis according Herschel-Buckley model for 2 % agar film forming dispersion at 45 °C

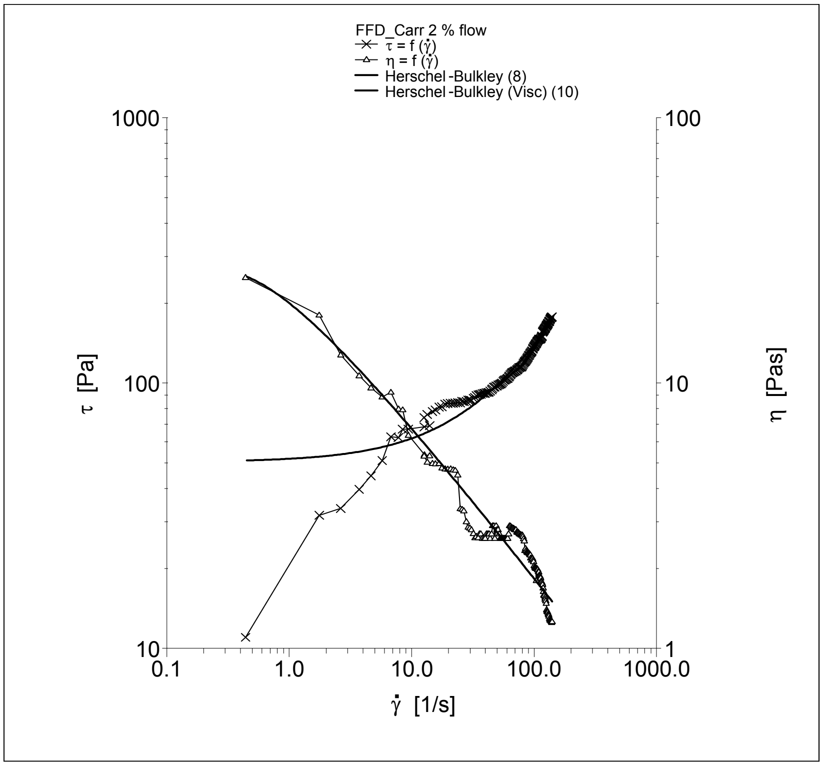


Fig. IV.1.9 Logarithmic plots of shear rate vs shear stress and viscosity, regression analysis according Herschel-Buckley model for 2 % κ-carrageenan film forming dispersion at 45 °C

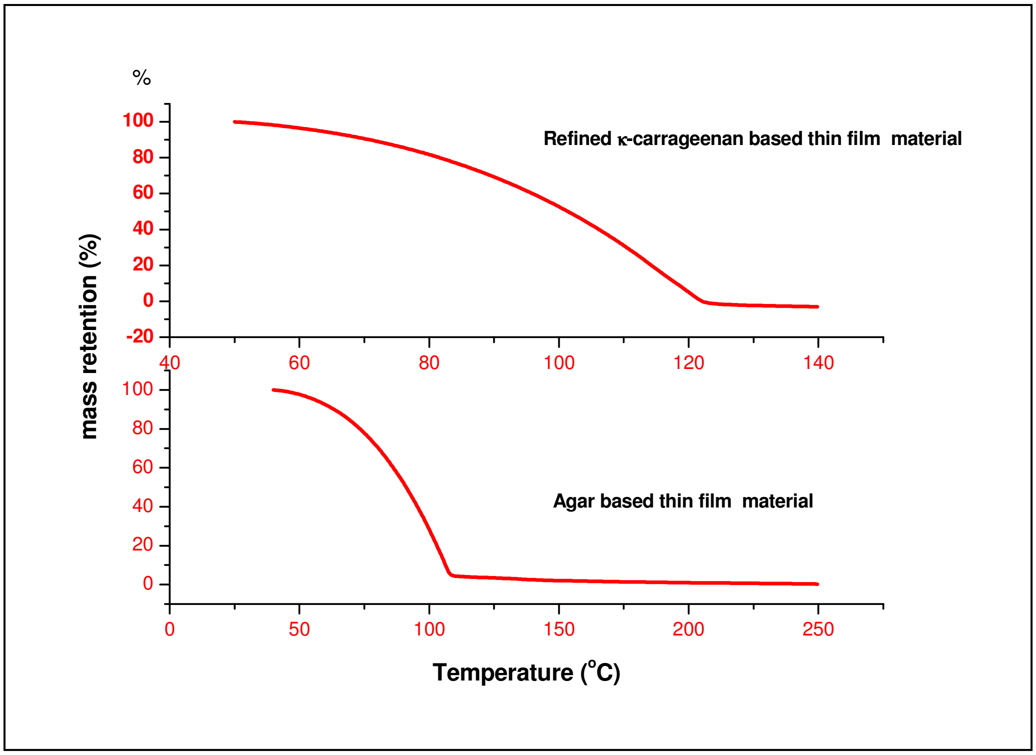


Fig.IV.1.10 (a) TGA thermo grams for 2 % Refined k-carrageenan and (b) 2 % agar film forming material.

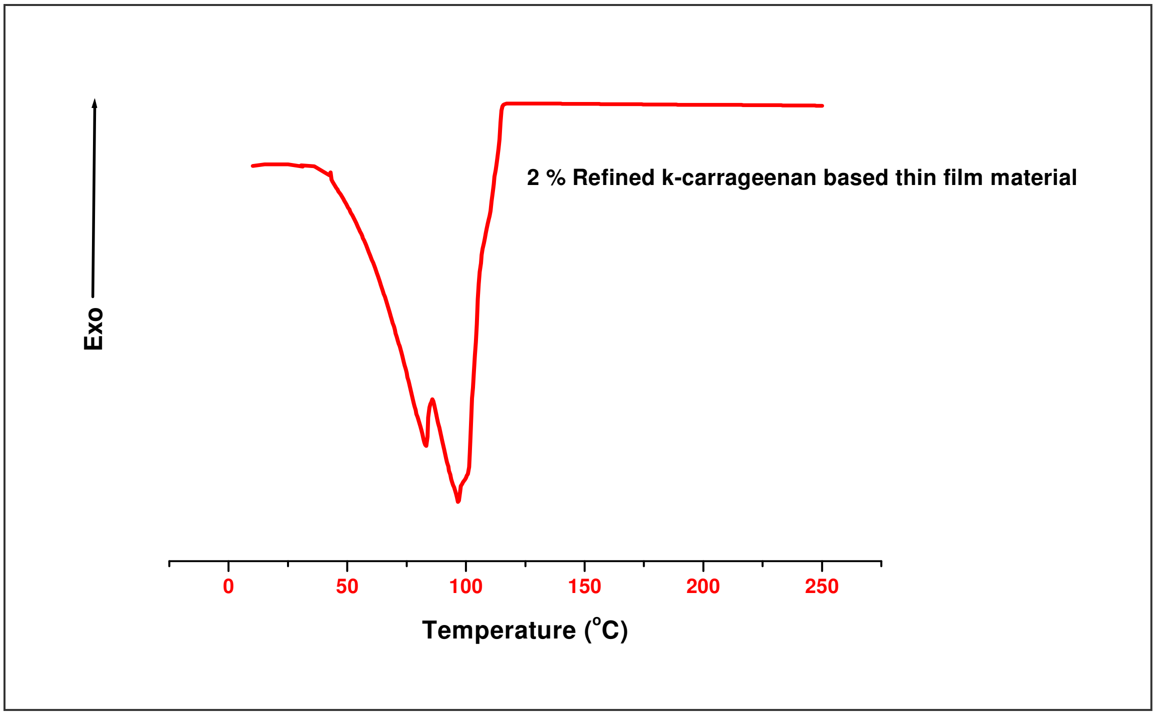


Fig.IV.1.11a DSC thermograms for 2% refined k-carrageenan film forming material

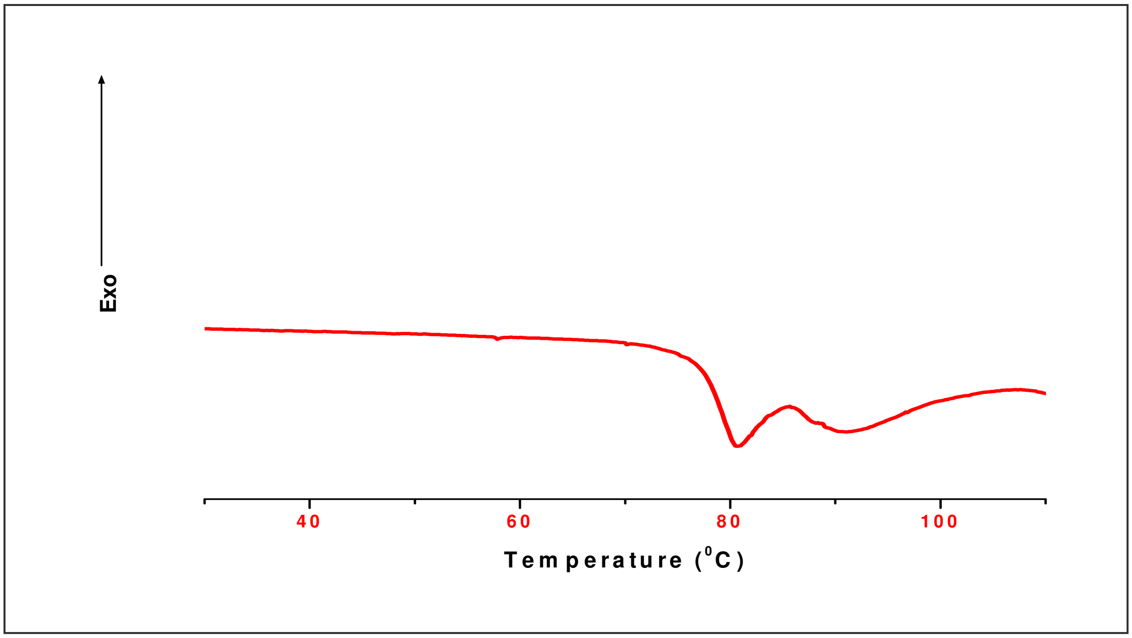


Fig.IV.1.11b DSC thermograms for 2% agar film forming material

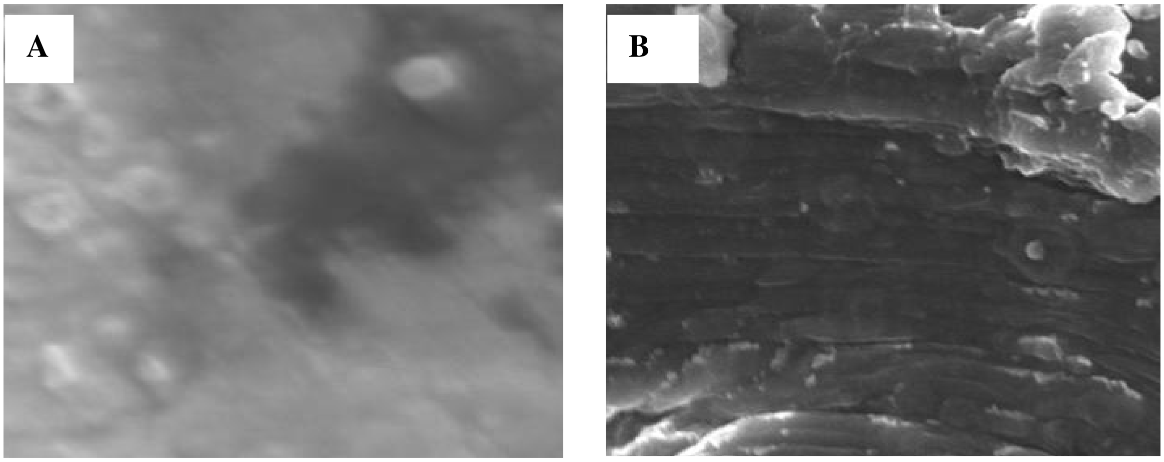


Fig. IV.1.12 Scanning electron micrographs for agar based thin films A) Surface of the film B) Cross sectional view of the film.

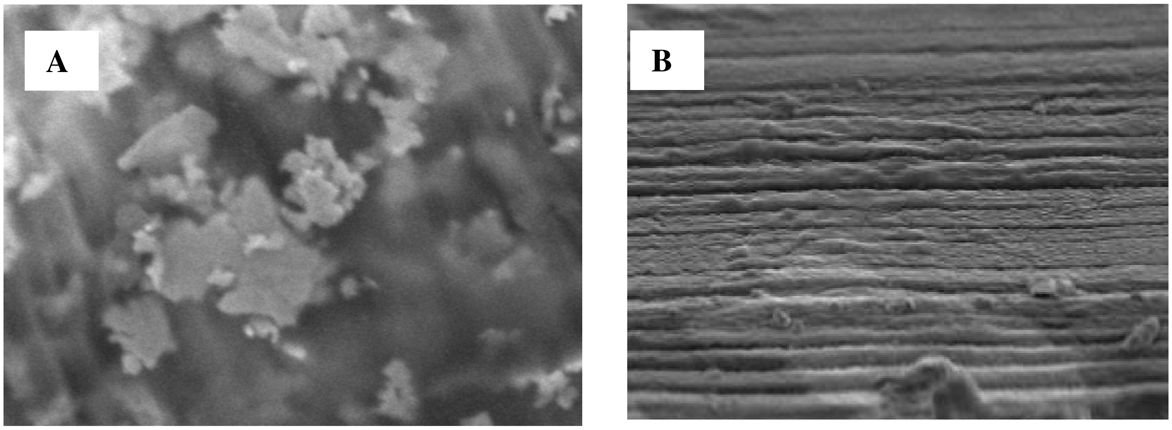


Fig.IV.1.13 Scanning electron micrographs for refined κ-carrageenan film. (A) Surface (Crystals of KCl are visible, confirmed by EDAX measurements) (B) Cross sectional view.

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CHAPTER IV.2

CHEMICAL MODIFICATION OF SEAWEED POLYSACCHARIDES AND THIN FILMS DERIVED THEREOF

IV.2.1 INTRODUCTION

Materials from renewable resources, with polysaccharides as a very important group, have gained increasing scientific and technical interests. There are various types of potential applications of these biopolymers. Biomimetic and ultra thin soft polymer films based on polysaccharides have been proposed as ideal “interface” between biological systems, for instance receptor proteins, sensor electrodes or optical devices [1]. Several polysaccharide films have been shown to have low-adsorbing and biocompatible properties [2].

Attempts have been made via chemical derivatization (before and after film formation), blending (before film formation) as well as cross linking (after film formation) to modify the film properties. In all cases, the structural modification is more or less evenly distributed through the film thickness, which can deteriorate the mechanical properties. Also, there are tradeoffs for these type of bulk modifications, i.e. only large changes will be effected but will at the same time have unwanted side-effects [3].

Therefore, surface modifications or thin-layer coatings should provide better solutions because only the interface or inter phase and barrier properties are changed while the bulk properties should remain unchanged. Plasma-supported technologies have been proposed for such modifications. However, with plasma it is rather complicated to control the balance between desired effects (which are also often rather empirical) and undesired degradation. Also, special and expensive facilities and conditions are required.

Modification of polysaccharides can be achieved by various ways, grafting or crosslinking is one among the most popular routes [4]. Grafting a synthetic and natural polysaccharide is a way of creating large molecules, which have some of the properties of each polymer. This was mainly done to obtain good water absorbents and biodegradable polymers that can be cast in sheets [4]. 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 [5]. Preparation of cellulose graft copolymers were reported for the first time by Ushakov in 1943 [6].

Agar and carrageenan are seaweed polysaccharides and chemically consists of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose for agar and 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-D-galactopyranose for carrageenan. There are many reports of chemical modification of agar, agarose by inducing hydrophobic groups like alkyl, acetyl, hydroxy alkyl, to decrease gelling and melting temperature which is very crucial for bacteriological applications [7]. Low gel strength or cream like agar gel is also reported, such low gel strength agars are prepared by reacting high gel strength agar with salts of weak acids viz., citric and ascorbic acids. They are mainly used as massage gels, skin moisturizers, or as active carrier for pharmaceuticals which have to be applied through skins [8]. A novel biopolymer based super absorbent hydrogel was synthesized through chemical cross linking graft polymerization of acrylic acid on to κ-carrageenan in presence of cross linking agent (N, N'-methylene bis acrylamide) and an initiator (ammonium persulphate) is reported by Pourjavadi and co workers [9]. Shchipunov from Russia has reported a sol-gel biomaterial based on carrageenan and silica [10]. Prasad and co workers have reported physical modification of agar gels by interacting agar with ionic and nonionic surfactants [11].

There are number of reports where natural polysaccharides or gums are modified to impart enhanced functional properties [12]. Polysaccharides like chitosan are also been tried for modification in gel state by adding mono- or dibasic phosphate salts [13]. Pourjavadi et al. have reported synthesis of κ-carrageenan based super absorbent hydrogel [14].

All the grafting reactions carried out on carrageenan or agar used hectic and vigorous reaction conditions as well as long duration and often reaction product leads to degradation of polysaccharides [15]. There are numerous reports of chemical modification of industrially important polysaccharides like cellulose and starch [16-21].

From the above mentioned prior art it is seen that, inducing hydrophobicity on hydrophilic polysaccharides has tremendous applications specially in microbiology, pharma, agriculture, membrane technology. This promoted us to undertake modification of seaweed based polysaccharides and films derived thereof.

This chapter consists of Section A and Section B dealing with "Chemical modification of seaweed polysaccharides" and "Surface modification of κ-carrageenan based thin films" respectively.

SECTION A: CHEMICAL MODIFICATION OF SEAWEED POLYSACCHARIDES

In this section modification of agar and κ-carrageenan are described. Carbanilation reaction was carried out on κ-carrageenan to induce hydrophobicity. Carbanilates of polysaccharides are useful in various fields as they are soluble in organic solvents. Cellulose carbanilates are used as surface sorbents in the separation of enantiomers by chromatography [27]. To our knowledge this is first report of preparation of κ-carrageennan carbanilate derivative.

Modification of polysaccharide by grafting is popular for the preparation of new polysaccharide derivatives. Many grafting reactions reported so far in the literature are time consuming and the conditions are harsh often leading to degradation of parent polysaccharides. In this chapter microwave irradiation technique for conducting grafting reactions on acid sensitive seaweed polysaccharides is described. This was found to be a rapid and simple method. The microwave conditions were optimized to achieve polysaccharide copolymers with minimum degradation.

IV.2.2 MATERIALS AND METHODS

IV.2.2.1 Materials

κ-Carrageenan and agar used in this reaction was extracted from a red seaweed *Eucheuma cottonni* and *Gelidiella acerosa* respectively by following methods available in the literature [22, 23]. Agar and κ-carrageenan were vacuum dried prior to reaction. Analytical grade pyridine, phenyl isocyanate (all from Sigma-Aldrich), and potassium persulphate, DMSO methyl methacrylate, isopropanol, methylene chloride and toluene (all from Ranbaxy Chemicals, Mumbai) were used. Kitchen microwave oven (LG) having temperature range 40-100^{o}C (magnetrons are set at a frequency of 2450 MHz). Infrared spectroscopy was recorded on a Perkin-Elmer Spectrum GX, FT-IR System, USA by taking 2.0 mg of sample in 600 mg of KBr and for films IR-ATR technique was adopted. All spectra were the average of two counts with 10 scans each and a resolution of 5 cm^{-1}. Elemental Analyses were performed on a Perkin-Elmer CHNS/O analyser, Series II, A 2400, Perkin-Elmer USA, using around 5 mg of the sample. The TGA of agar (3.18 mg), κ-carrageenan (3.32 mg) and their copolymers (3.28 mg and 3.44 mg respectively) were carried out on a Toledo Mettler TGA system, Switzerland, machine using a temperature programme (30^{o}C to 600^{o}C at a heating rate 10^{o}C min^{-1}) in an air atmosphere. Powder X-ray diffractions were recorded on a Philips X'pert MPD X-ray powder Diffractometer using $2\theta = 10$ to 60^{O}. Optical microscope used is of Olympus model SZH 10, Japan with 70x magnification. Demineralised water was used in all the above experiments wherever required.

^{13}C NMR spectra (noise-decoupled) was recorded on a Bruker Advance DPX 200 Spectrometer at 50 MHz. Carrageenan (50 mg/ml) was dissolved in D_2O and spectrum was recorded at 70°C with 7565 accumulations, pulse 5.9 μs, acquisition time 1.2059 s and relaxation delay 6 μs using DMSO (δ39.5) as internal standard. In the case of carbanilate derivative the spectrum was recorded in $CDCl_3$ with TMS as internal standard.

IV.2.2.2 Carbanilation of κ-carrageenan

Carbanilation reaction of κ-carrageenan (AS-V-5-2) activated by ammonia at -12°C, was carried out by reacting with phenyl isocyanate adapting the method described by R.L Whistler [26]. The activated κ-carrageenan (5 g) was taken in 75ml dry pyridine (dried overnight on KOH pellets) to make a suspension. Phenyl isocyanate (25 ml) was added, refluxed at 100°C for 5 h. Then 20 ml DMSO was added to aid solubilization, then refluxed for additional 9 h.

IV.2.2.3 Microwave induced rapid one pot synthesis of agar-graft-PMMA and κ-carrageenan-graft-PMMA copolymers by potassium persulphate Initiating system

IV.2.2.3.1 Graft copolymerisation

Graft copolymerisations were carried out in 100 ml narrow mouth conical flasks. 1 g of agar and κ-carrageenan was first dissolved separately in 100 ml of distilled water followed by addition of 0.06 g (0.0022 mol/L) of potassium persulphate. Methyl methacrylate (2.0g) (w/w) [0.1997 mol/L] was added to each of the reaction mixture. Then the mixtures were irradiated under microwave irradiation for 120 seconds with constant stirring. A milky white solution appeared which was further cooled and precipitated in isopropyl alcohol, followed by centrifugation at 8000 rpm for 3 min. The off white precipitate thus obtained was vacuum dried. Un reacted MMA homopolymer was extracted from the product by Soxhlet extraction with toluene. Unreacted MMA was also recovered from the supernatant remained after precipitation by solvent extraction with toluene. The toluene thus obtained was evaporated and weight of unreacted MMA was determined. Product was washed with isopropyl alcohol and vacuum dried.

IV.2.3 RESULTS AND DISCUSSION

IV.2.3.1 Carbanilation of κ-carrageenan

Refined κ-carrageenan (AS-V-5-2) (5 g) was taken in 75ml dry pyridine (dried overnight on KOH pellets) to make a suspension. Phenyl isocyanate solution (25 ml) was added to the latter dropwise with constant stirring, which was then refluxed at 100°C for 5 h. Then DMSO (20 ml) was added to the reaction mixture at 50°C to aid solubilization. Then the mixture was refluxed for an additional 9 h. The reaction mixture assumed dark chocolate colour. TLC showed the appearance of a spot at R_f 0.4 (Si gel/1:1 methylene chloride-hexane) visualized with iodine vapour, the spot of starting material remained at the point of application. After cooling, the reaction mixture was extracted with methylene chloride (4 x 50 ml). The methylene chloride layer was washed with Cu (II) sulphate solution (x 3) to remove pyridine, followed by washing with water (x3) to remove copper sulphate solution. The methylene chloride extract, after dehydrating with solid anhydrous sodium sulphate, was evaporated to obtain a solid powdery product (designated KP-II-61-3). Yield of product was 26.3% (with respect to κ-carrageenan). The product obtained was insoluble in water and soluble in organic solvents like chloroform and acetone and insoluble in DMSO, DMF and water. The melting was very high and therefore TGA was carried out which has been described below.

The DS (degree of substitution of the product was determined by following the equation as described by Whistler et al. [26].

Degree of Substitution [D.S.] = (MW of repeating disaccharide unit of carrageenan) x % of N or S or X / Substituent elemental weight x 100 – Substituting group x % N or S or X. For κ-carrageenan carbanilate derivative D.S = (403x 3.9) / 1400 - (120 x 3.9) = 1.68. The D.S. more than 1 indicates that the κ-carrageenan was modified substantially [cf. 27].

Elemental analysis of the product was done and the results are given in Table IV.2.1. Almost 15 fold increase of the N content in the product indicated that this is predominantly consisted of dicarbanilate derivative, for the calculated nitrogen content in mono-derivate would be 2.17 %. The observed percentage of C also corroborates this conclusion. In Table IV.2.1, in both the cases the observed percentages of H were higher than the calculated values.

Table IV.2.1 Elemental analysis of carrageenan carbanilate derivative

Sample	C %		H %		N %	
	(Observed)	(Calcd.)	(Observed)	(Calcd.)	(Observed)	(Calcd.)*
κ-Carrageenan	35.80	35.73	6.4	4.21	0.21	---
κ-Carrageenan carbanilate	43.10	52.50	8.2	4.88	3.90	4.71

*Assuming exclusively dicarbanilate derivative.

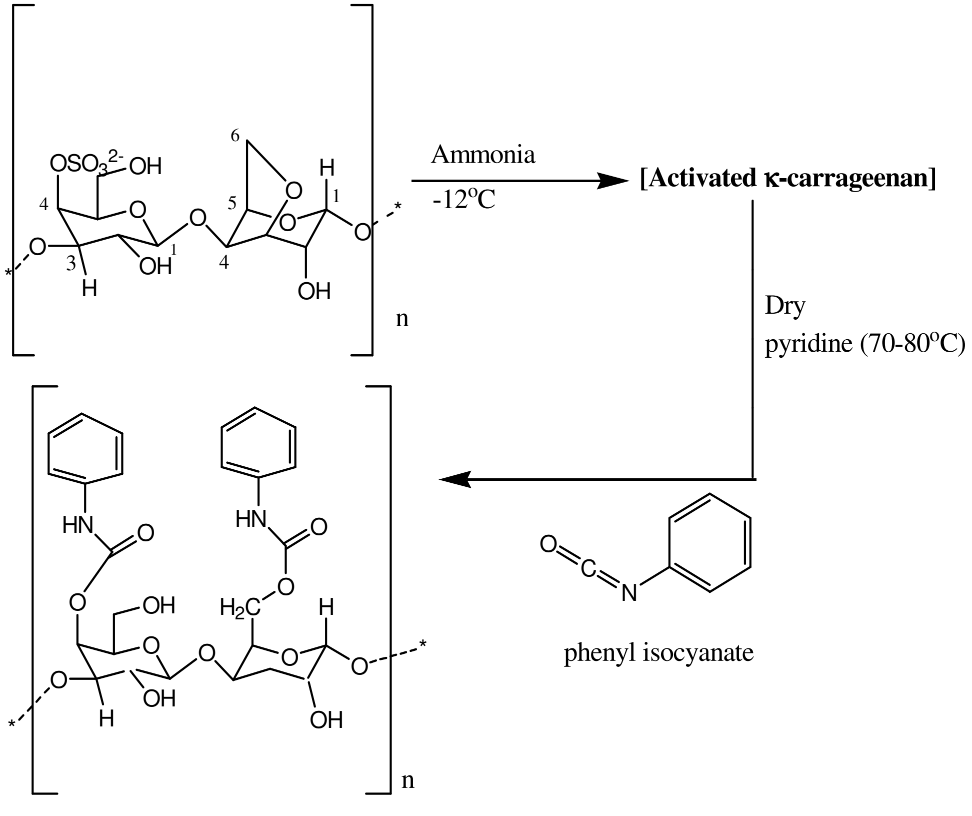
IR (ν_{max} , cm^{-1}): 3358 (OH/N-H), 1705 (ester carbonyl), 1443, 1598, 1499 (aromatics) as shown in Fig. IV.2.1. Absence of bands at 847 cm^{-1} and 928 cm^{-1}, which are due to C-4 sulphate and 3,6 anhydrogalatose ring stretching vibrations (of κ-carrageenan) respectively indicating that most probably the reaction took place at C-4 sulphate and 3,6-anhydro ring of κ-carrageenan.

^{13}C NMR ($CDCl_3$, δ): 153.9 (N-*C*=O), 121.1, 123.9, 124.9, 128.8, 129.8, 130.2, 130.9, 137.0, 137.6 (aromatic carbons) δ 61.4, 64.2, 82.2 (C-1, C-2, C-6 of κ-carrageenan) [cf. 27], other peaks due to other carbon atoms in κ-carrageenan are not visible because of low resolution. A probable route of the reaction is depicted in Scheme IV.2.1. Fig. IV.2.2a and Fig.IV.2.2b illustrate the ^{13}C spectra of control κ-carrageenan and κ-carrageenan carbanilate derivative respectively.

^{13}C NMR resonances (δ) of κ-carrageenan are: 69.8 (anomeric carbon); 61.4, (C-6 of galactose unit); 68.7 (C-4 of galactose unit); 69.4 (C-6 of 3,6-anhydrogalactose unit); 76.1 (C-5 of galactose unit); 77.1 (C-5 of 3,6-anhydrogalactose unit); 80.1 (C-3 of 3,6-anhydrogalactose unit); 98.2 (C-1 of 3,6-anhydro-α-L galactose residue); 102.4 (C-1 of D-galactose) [unpublished work; cf. Miller, I. J. *Botanica Marina* **44**:583-587, 2001]. ^{13}C NMR resonances (δ) of phenyl isocyanate are: 133.6, 129.6, 125.7, 124.8 (all aromatic carbons); 122.2 (N-*C*=O) [after ChemDraw Ultra 7.0, CambridgeSoft, UK]. A comparison of the 13C NMR data reveals that the product is probably a dicarbanilate derivative of κ-carrageenan.

On the basis of the above mentioned evidence it may be concluded that the product (KP-II-61-3) is predominantly consisted of dicarbanilate derivative of κ-carrageenan. The reaction is depicted in Scheme IV.2.1.

TGA thermograms of κ-carrageenan and its carbanilate derivative is shown in Fig.IV.2.3. It is observed that in both the cases mass loss took place at two stages. For κ-carrageenan 10-25 % mass loss took place at temperature range 163-275°C, on the other hand for the carbanilate derivative 5-20% mass loss took place at temperature range 347-455°C. It is obvious, as expected, that carbanilate derivative has enhanced thermal stability in comparison to control κ-carrageenan.



κ-carrageenan dicarbanilate
(KP-II-61-3)

Scheme IV.2.1. Formation of dicarbanilate of carrageenan

IV.2.3.2 Microwave induced rapid one pot synthesis of agar-g-PMMA and κ-carrageenan-g-PMMA copolymers by potassium persulphate initiating system

IV.2.3.2.1 Yield and grafting parameters

Yield obtained for agar-g-MMA: 2.76g; κ-carrageenan-g-MMA: 2.86g. Weight of unreacted MMA homopolymer: 0.21 g for agar copolymerisation and 0.12 g for κ-carrageenan copolymerization reactions respectively. Total conversion Percentage C %: (Total weight of polymerized MMA/Weight of MMA charged) x 100 ; C % for agar-g-MMA is 88% and C % for κ-carrageenan-g-MMA is 93%.Grafting efficiency (E%): (Weight of Poly MMA grafted /total weight of Poly MMA) x 100 ; E % for agar-g-MMA is (1.76/2-0.21) = 98.32 % and E % for κ-carrageenan-g-MMA is (1.86/2-0.12) = 99 %.

Grafting Percentage: G% = (Weight of Poly MMA grafted/Weight of Polysaccharide) x100 ; G% for agar-g-MMA is (2.76-1/1)= 176 % and G % for κ-carrageenan-g-MMA is (2.86-1/1) = 186 %.

IV.2.3.2.2 Effect of the ratio of MMA to Polysaccharides (Agar and κ-carrageenan)

For simplification agar and κ-carrageenan are collectively mentioned as polysaccharides in the following sections. The influence of the ratio of MMA to agar and κ-carrageenan on the graft parameters were studied and shown in Fig IV.2.4a and Fig. IV.2.4b. It was found that G%, C% and E% reached a maximum value when the ratio of MMA to polysaccharides were 2:1 and then decreased gradually (degradation of polysaccharides!). Because of the limited solubility of MMA in reaction medium, the ratio of MMA to polysaccharide was low, resulting in lower collision probability between the polysaccharides and MMA, leading to the low values of C%, G% and E%. It was observed that in the absence of the water soluble initiator the reaction did not take place at all. In presence of the initiator in the aqueous solution of polysaccharide, when the MMA to polysaccharide ratio was increased then polysaccharide-g-MMA could play the role of self emulsifier so as to absorb more monomers (MMA) on polysaccharide surface, which subsequently enhanced the rate of the graft reaction, thereby sharply increasing C%, G% and E% values. If the ratio of MMA to polysaccharide exceeds 2, the adsorption of monomers onto polysaccharide surface is so much that it generates sulphuric acid degrading the copolymer resulting in decline in the C%, G% and E% values. When the ratio of MMA to polysaccharide was higher (than 2), which accelerate the chain transfer reactions decreasing E%. The copolymers were soluble in hot DMSO, DMF and were insoluble in water even upon boiling.

IV.2.3.2.3 Effect of Reaction Time (Duration of microwave irradiation)

Fig. IV.2.5a and Fig. IV.2.5b illustrates the influence of the reaction time or time of microwave irradiation on grafting parameters. As expected, up to 120 seconds of microwave irradiation the C%, E% and G% increased. After 120 seconds or beyond, these values declined gradually. This phenomenon may be explained as follows. Microwave irradiation produces hydrogen and hydroxyl free radicals. The sulphate free radicals generated from potassium persulphate absorbed the hydrogen radical and formed sulphuric acid, The hydroxyl radical produced KOH by reacting with potassium radical. At lesser duration of microwave exposure there is a balance in formation of sulphuric acid and KOH leading to neutral pH of the reaction medium, which was actually observed. But as the duration of microwave exposure increased the balance of suphuric acid and KOH tripped towards acidic pH, which was actually observed, leading to the degradation of the product.

IV.2.3.2.4 Effect of temperature

When the other reaction conditions were constant (MW time 120 sec, MMA: PS = 2:1), the grafting parameters at various temperatures are shown in Fig.IV.2.6a and Fig.IV.2.6b. In accordance with the general rule of radical polymerization, E% increases and then levels off. In this type of microwave induced reactions with increasing temperature the vibration of reactant molecules as well as the reaction medium increases, as a result the activation energy required to form the product decreases increasing the reaction rate. But after a certain temperature the vibration of the molecules reach the optimum condition and reaction rate levels off. Again when temperature increases the excessive vibration leads to destruction of polymer chains and as a result products with lower C%, E% and G% values are obtained. In this type of reaction there is no product formation in the reactions carried out at temperatures less than 70^{o}C.

IV.2.3.2.5 Effect of initiator concentration of grafting parameters

Potassium persulphate was added in the range 0.009 to 0.14 g [0.0003 to 0.005 mol/L] in the reaction mixture. It was observed that initially when initiator concentration was low the C %, E % and G % values were also low, this may be because of small amount of initiator is not sufficient to fully cleave the bond between O and H in the polysaccharide (at C-6) under microwave irradiation conditions. The values gradually increased up to 0.06 g [0.0022 mol/L] of initiator concentration and then leveled off to 0.09 g [0.0033 mol/L], but beyond this due to excess initiator concentration cleavage of more bonds (at C-2, C-4) and as a result the values declined. Fig.IV.2.7a and Fig.IV.2.7b illustrates the same for agar-g-MMA and κ-carrageenan-g-MMA respectively.

IV.2.3.2.6 IR spectral studies

The grafting was confirmed by comparing the IR spectrum of agar and κ-carrageenan with that of the grafted product and the results obtained are shown in Fig.IV.2.8a and Fig.IV.2.8b. The main difference observed is the appearance of a carbonyl absorption band at 1733 cm^{-1} for carrageenan-g-MMA and 1739 $cm^{-1}$for agar-g-MMA copolymers. Appearance of a carbonyl absorption band at 1733 cm^{-1} for carrageenan-g-MMA and at 1739 cm^{-1} for agar copolymer indicated insertion of the MMA unit in the polysaccharide. Characteristic IR bands at 740, 773, 930 cm^{-1} due to 3,6-anydro-β-galactose skeletal bending of κ-carrageenan and agar [29,30], in the copolymer indicated that during grafting κ-carrageenan and agar was not decomposed. Presence of band at 846 cm^{-1}, in the κ-carrageenan-g-MMA copolymer, which is due to C-4 sulphate indicates that the grafting has taken place on the hydroxyl groups of κ-carrageenan rather than on sulphate group at C-4 position.

IV.2.3.2.7 Thermal analysis

Thermo gravimetric analysis (TGA) of pure agar and κ-carrageenan and the grafted copolymer is shown in Fig. IV.2.9a and Fig. IV.2.9b. In case of κ-carrageenan there were two stages of mass loss. First stage was from 30-280°C. The initial weight loss (ca 5%) may be due to bound water in κ-carrageenan. Again there was a 10-20 % mass loss between 240-280 °C, this may be due to degradation of the κ-carrageenan product. In the next step at ca 40-50 % mass loss observed in temperature range 320-410 °C. But for the graft copolymer the mass loss takes place only in one stage. ca 60 % mass loss took place at temperature ranges between 250-500 °C. In case of control agar 10-60 % mass loss took place in the temperature range 150-350 °C, on the other hand for agar-copolymer 10-70 % mass loss took place in the temperature range 200-480 °C. It is evident that grafting of MMA on κ-carrageenan and agar can improve the thermal stability of κ-carrageenan and agar.

IV.2.3.2.8 X-ray diffraction analysis

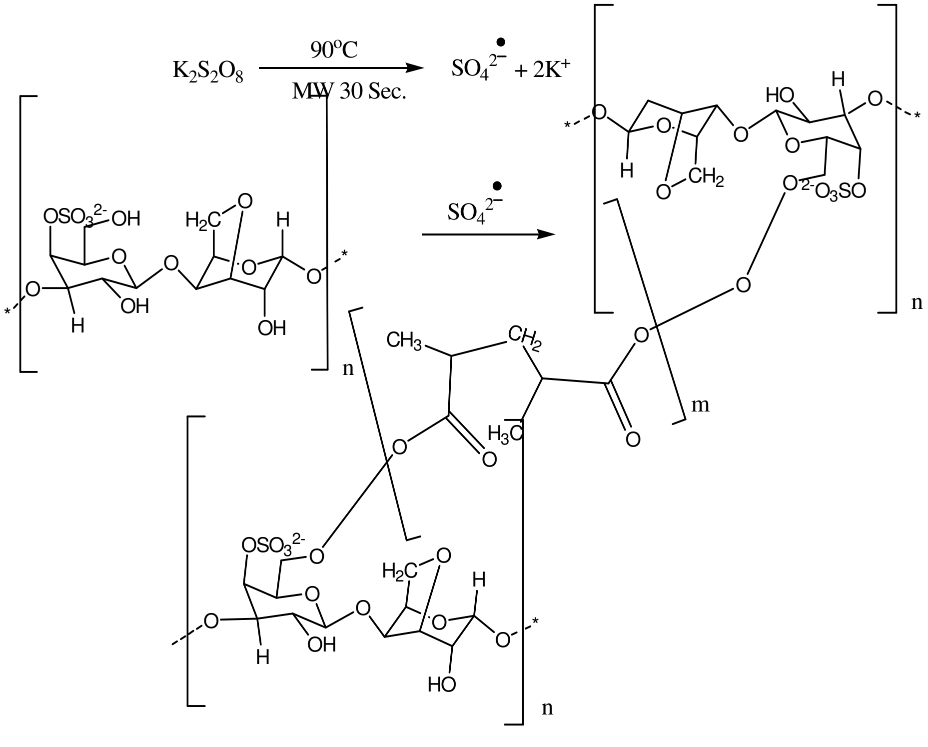
The X-ray diffraction spectra of pure agar and κ-carrageenan and their copolymers are shown in Fig.IV.2.10a and Fig.IV.2.10b respectively. It was observed that the X-ray diffraction spectrum of native κ-carrageenan that it has one sharp peak, after grafting with MMA the copolymer shows three sharp peaks. This result manifested that along with amorphous part the crystalline part of κ-carrageenan also took part in grafting. The X ray diffraction pattern of native agar and agar-g-MMA copolymer is same (broad blunk peaks) indicates that in case of agar the only amorphous part took part in grafting [Fig.IV.2.10a]. The crystallinity indices were determined using the methods described by Hermans and Weidinger [31]. The crystallinity index for native κ-carrageenan is 0.0847 and for graft copolymer is 0.2536 i.e., crystallinity index is increased by 3 times in the graft copolymer of κ-carrageenan, this indicates that in the grafted product the molecular association pattern has changed in the crystalline region of κ-carrageenan polymer.

IV.2.3.2.9 Morphology

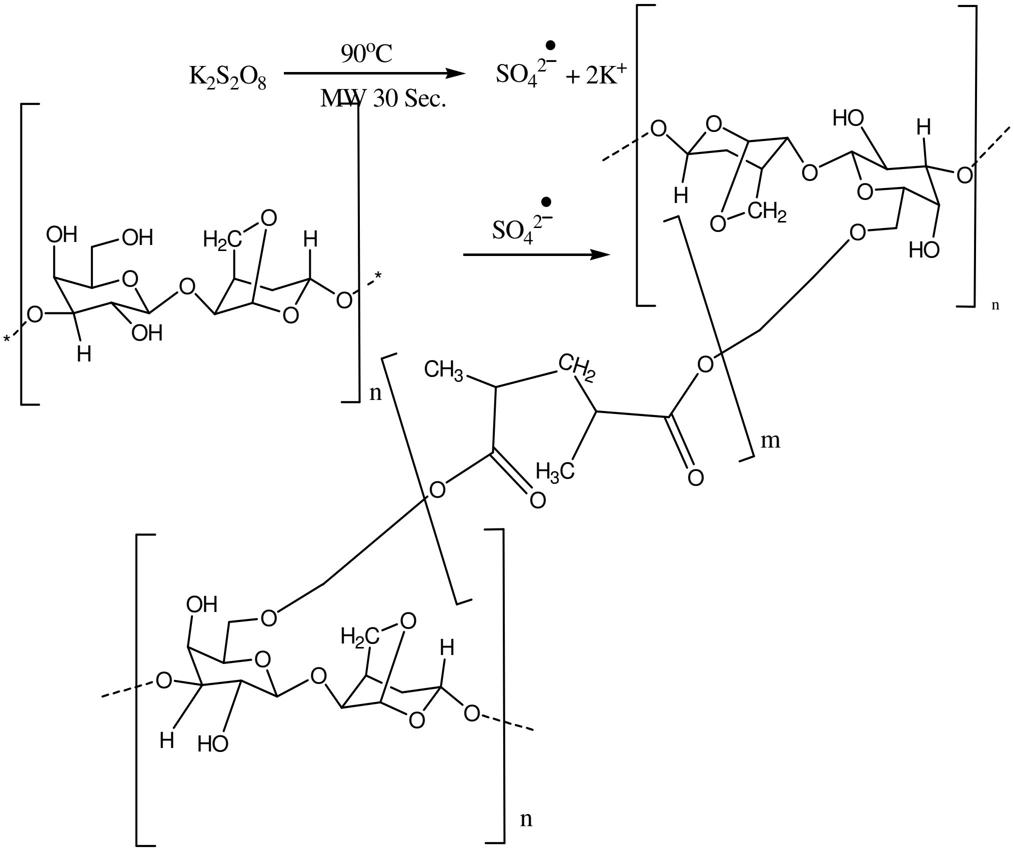
Optical micrographs of the copolymers of agar-g-MMA and κ-carrageenan-g-MMA were taken with 70 X magnification and compared with agar and κ-carrageenan (Fig. IV.2.11a and 11b) Scrutiny of these figures reveals that the morphology of κ-carrageenan and agar were modified considerably.

IV.2.3.2.10 Proposed mechanism of copolymerization

The above results illustrated that MMA was grafted on agar and κ-carrageenan. So, it can be concluded that, single electron produced in the process of microwave irradiation of potassium persulphate. Agar and κ-carrageenan were also initiated by the initiator under microwave irradiation and a free radical generates over the polysaccharide moiety. During propagation step the free radical thus formed combine with the free radical of MMA which it self polymerized. The proposed mechanism for carrageenan copolymer is depicted in scheme IV.2.2 and for agar copolymer in Scheme IV.2.3.



Scheme IV.2.2 Proposed mechanism for the formation of κ-carrageenan-g-PMMA copolymer



Scheme IV.2. 3 Proposed mechanism for the formation of agar-g-PMMA copolymer

SECTION B: SURFACE MODIFICATION OF AGAR AND κ-CARRAGEENAN BASED THIN FILM

Several reactions viz., methylation, silylation, carbanilation, hydroxypropylation, were carried out on κ-carrageenan derived thin films, but in all the cases degraded brittle films were obtained. But grafting parameters under microwave induced reaction were optimized in such a way that it afforded undegraded film formation after casting. The films had enhanced hydrophobicity and would be useful for newer applications. To our knowledge, this is the first report of preparation of polysaccharide films using modified polysaccharides.

IV.2.4 MATERIALS AND METHODS

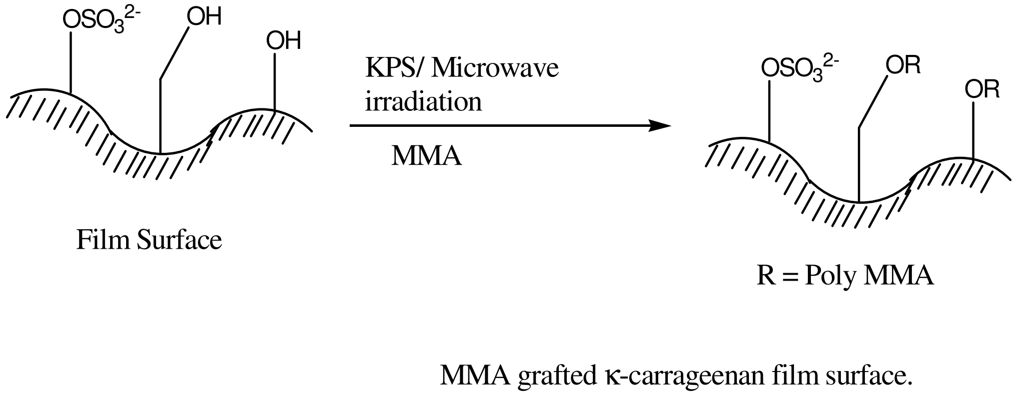
IV.2.4.1 Preparation of grafted thin films

Agar or refined κ-carrageenan (5 g) was dissolved in 100 ml demineralised water. Then 0.3 g [0.01mol/L] potassium persulphate (Ranbaxy Chemicals) and 10 g [1.0 mol/L] MMA (Ranbaxy Chemicals) was added and irradiated under microwave for 2 min. To the white milky viscous solution thus obtained was added 5 % (w/w) glycerol, and mixed well which was directly used for casting the film at 80-90°C on a Teflon sheet employing a TLC plate spreader. The film was dried overnight under ambient conditions. Infrared spectroscopy (ATR) was recorded on a Perkin-Elmer Spectrum GX, FT-IR System, USA by directly using the films. All spectra were the average of two counts with 10 scans each and a resolution of 5 cm^{-1}. Demineralised water was used in all the above experiments wherever required. The methods for measuring swellability and wettability have been given in chapter IV.1.

IV.2.5 RESULTS AND DISCUSSION

IV.2.5.1 Formation of grafted thin film

A schematic representation of grafting on refined κ-carrageenan film surface is depicted in Scheme 4 below.



Scheme 4: A schematic representation of grafting on film surface.

IV.2.5.2 Spectral analysis: FT-IR

The dried films were opaque and white color, on which IR-ATR analysis was carried out. Appearance of strong bands at 1731 cm^{-1} and 1735 cm^{-1} (ester carbonyl stretching) in case of agar and κ-carrageenan based films respectively, indicated the formation of modified film containing –O-MMA moiety. FT-IR/ATR spectra of the grafted and control films are shown in Fig. IV.2.12a and Fig. IV.2.12b.

IV.2.5.3 Swellability

Method: Swelling or water absorption of this films were studied by following a method described by Park et al. [32], detail of the procedure is described in Chapter IV.1. A weighed piece of dry films of dimension 4 x 2 cm was dipped in 50 ml distilled water in a beaker. The weight of the swollen film was taken after definite interval of time. The water content of the swollen films were calculated using following equation.

$W_c = (W_s - W_d) / W_d$ ------------------ Eq.IV.2.1

Where W_c is the water uptake in grams per gram of dried gel film, and W_s and W_d are the weights of the gel films after swelling and after subsequent drying respectively. The results are shown in table IV.2.4 followed by graphical representation.

Fig. IV.2.18 represents the swelling capacity of the control agar, carrageenan films and their respective copolymers with MMA at consecutive time intervals. According to this figure, the swelling rate sharply increases and then begins to level off. The data may fit well with a 'Voigt-based model' equation as follows [33].

$S_t = S_e (1-e^{-t/T})$ ------------------ Eq.IV.2.2

Where S_e and S_t are equilibrium swelling and swelling at time t, respectively. T (in s) stands for the 'rate parameter'. Using the equation, the rate parameter for control agar film in water is 6.11 min, for control carrageenan film is 5.3 min , for agar-g-MMA film is 3.09 min and for carrageenan-g-film is 2.394 min. From the values of rate parameters it is evident that films prepared from the grafted copolymers have less water absorption capacity to their respective control polysaccharides. Figure IV.2.13 includes the swelling behaviour of the control and grafted films. Table IV.2.2 consists the corresponding values.

Table IV.2.2 Swelling behaviour of control and grafted films

Time (t) (min)	Ws (g)				W_d (g)				Wc (g) /(g) dry film			
	A	B	C	D	A	B	C	D	A	B	C	D
0.5	5.81	5.42	4.06	3.75	1.55	1.65	1.5	1.4	2.75	2.29	1.71	1.68
1.0	5.84	6.20	4.38	3.76					2.8	2.76	1.92	1.694
1.5	5.96	7.16	4.51	3.85					2.85	3.34	2.01	1.75
2.0	6.57	7.47	4.71	4.06					3.24	3.53	2.145	1.9
2.5	6.58	7.67	5.68	4.13					3.25	3.65	2.79	1.946
3.0	7.13	8.03	6.13	4.24					3.6	3.87	3.09	2.03
3.5	7.44	8.21	6.13	4.45					3.8	3.98	3.09	2.18
4.0	7.59	7.98	6.13	4.55					3.9	3.84	3.22	2.254
4.5	8.13	8.256	6.33	4.55					4.25	4.01	3.22	2.394
5.0	8.91	8.81	6.33	4.55					4.75	4.34	3.22	2.94
5.5	9.68	8.81	6.33	5.51					5.25	4.38	3.22	2.94
6.0	9.68	8.81	6.33	5.51					5.25	4.38	3.22	2.94
6.5	9.68	8.81	6.33	5.35					5.25	4.38	3.22	2.97
7.0	9.68	8.81	6.33	5.57					5.25	4.38	3.22	2.98
7.5	9.68	8.81	6.33	5.57					5.25	4.38	3.22	2.98
8.0	9.68	8.81	6.33	5.57					5.25	4.38	3.22	2.98
8.5	9.68	8.95	6.33	5.57					5.25	4.43	3.22	2.98
9.0	9.68	8.95	6.33	5.57					5.25	4.31	3.22	2.98
9.5	9.68	8.95	6.33	5.57					5.25	4.31	3.22	2.98
10.0	9.68	8.95	6.33	5.57					5.25	4.31	3.22	2.98

A = Control agar film, thickness 0.43 mm
B = Control refined carrageenan film, thickness 0.47 mm
C = Agar-g-MMA based film, thickness 0.53 mm
D = κ-carrageenan-g-MMA based film, thickness 0.50 mm

IV.2.5.4 Contact angle measurements

Contact angle measurements were carried out on the grafted thin films for evaluating the hydrophobicity of the films. For these measurements films of 4 x 2 cm dimensions were dipped in water and the corresponding advancing and receding angles were measured, the values obtained are given in Table IV.2.3. From the values of contact angles it is evident that grafted films become more hydrophobic than the control.

Table IV.2.3 **Measurement of contact angles for control and grafted thin films**

Sample	Solvent	Advancing angle (deg)	Receding angle (deg)	Mean (deg)	Remarks
Agar + glycerol film	Water	44.15	36.13	40.13	Grafted films are more hydrophobic.
		50.56	45.19	47.87	
		56.42	46.29	51.35	
Agar-g-MMA film	Water	78.12	68.25	73.18	
		78.90	68.14	73.54	
		76.23	65.14	70.68	
Refined κ-carrageenan + Glycerol	Water	59.50	44.77	52.13	
		58.17	46.23	52.2	
		59.76	49.23	54.49	
Refined κ-carrageenan-g-MMA	Water	70.03	60.31	65.03	
		73.98	63.96	68.97	
		77.66	67.90	72.78	

IV.2.6 SUMMARY

Water insoluble derivative of κ-carrageenan were prepared and characterized by modifying the polysaccharide with phenyl isocyanate. Grafting of natural seaweed polysaccharides agar and κ-carrageenan by microwave irradiation has been demonstrated. Most important advantage of this method is that the reaction conditions are very simple and duration of reaction is also very brief. Many chemical reactions leads to degradation of these types of polysaccharides due to their very high acid sensitivity but in this study it has been shown that microwave irradiation under controlled conditions can result in the formation of undegraded copolymer of polysaccharides. This work would promote microwave irradiation technique for graft copolymerisation reactions. Hydrophobized biopolymers have been reported to be of great potential in bone tissue engineering applications [34].

Thin films were prepared with grafted agar and κ-carrageenan. The hydrophobicity, swellability, FT-IR analysis of the films showed that the films thus prepared became hydrophobic compared to the control polysaccharides. This study thus provides a method of preparing hydrophobic thin films which would have wider applicability in comparision ot the hydrophilic films prepared by the control polysaccharides.

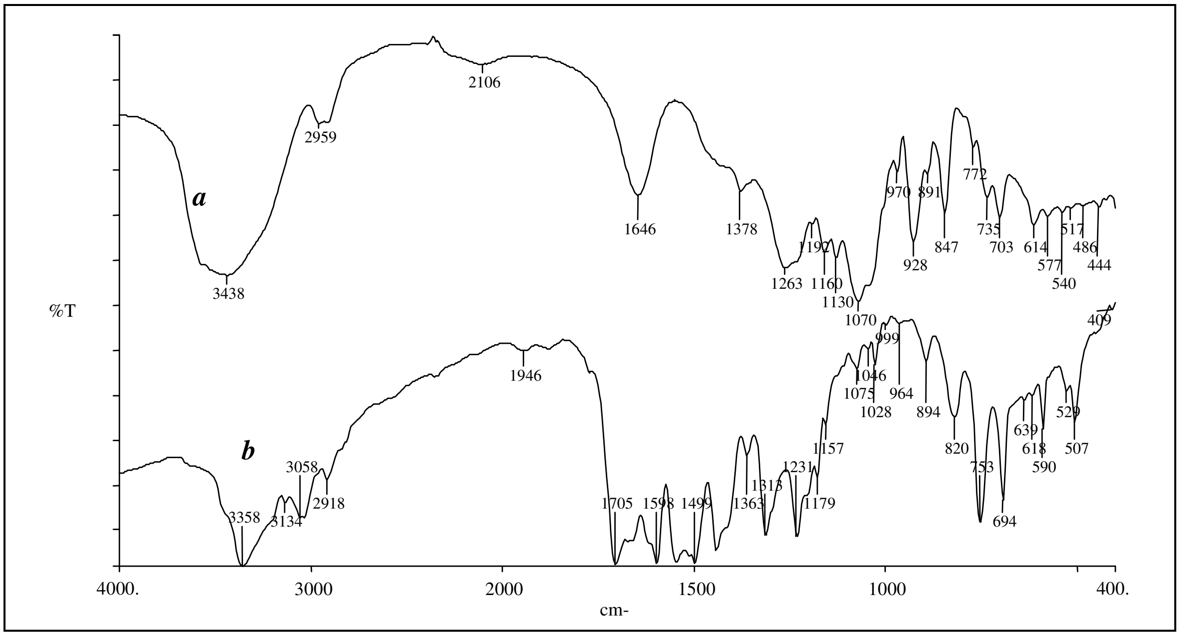
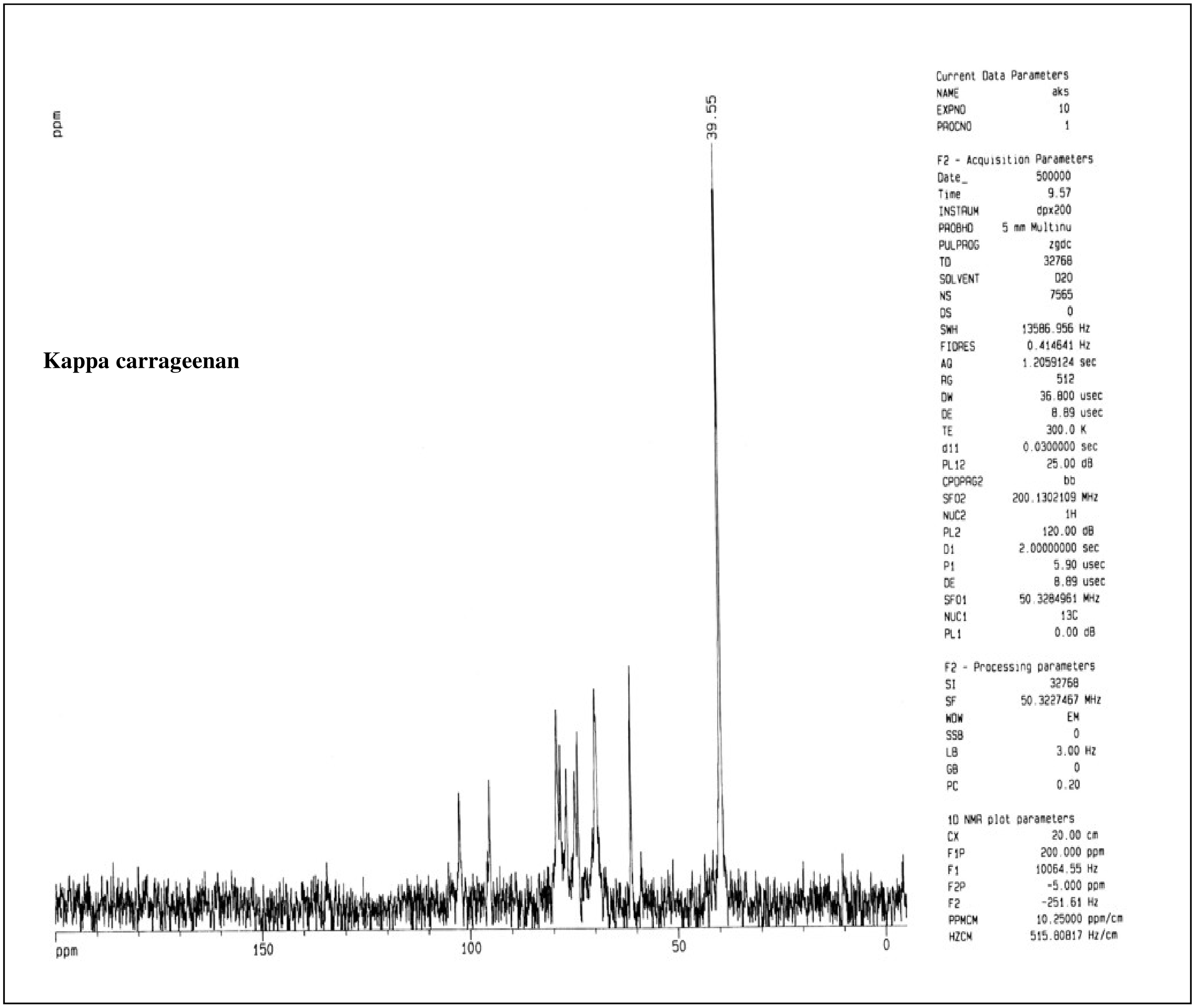


Fig. IV.2.1 FT-IR (a) Control κ-carrageenan (b) κ-carrageenan dicarbanilate



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FIDRES         0.414641 Hz
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DE                 8.89 usec
TE                300.0 K
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SF02        200.1302109 MHz
NUC2                 1H
PL2              120.00 dB
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1D NMR plot parameters
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F1             10064.55 Hz
F2P              -5.000 ppm
F2              -251.61 Hz
PPMCM          10.25000 ppm/cm
HZCM          515.80817 Hz/cm
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Fig. IV.2.2a ^{13}C NMR Control κ-carrageenan : DMSO-d_6 is used as internal standard.

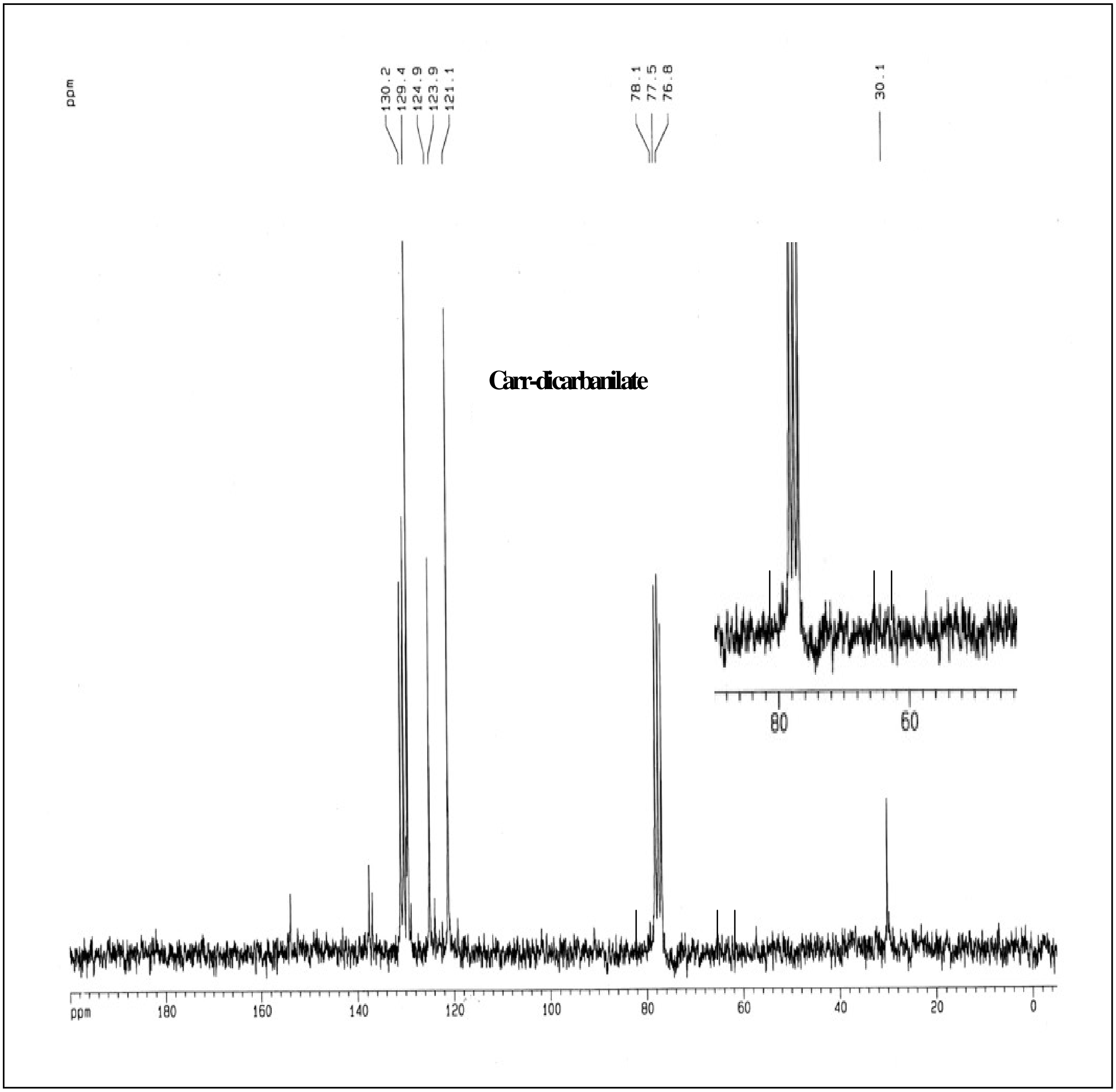

Fig. IV.2.2b ^{13}C NMR κ-carrageenan dicarbanilate in $CDCl_3$

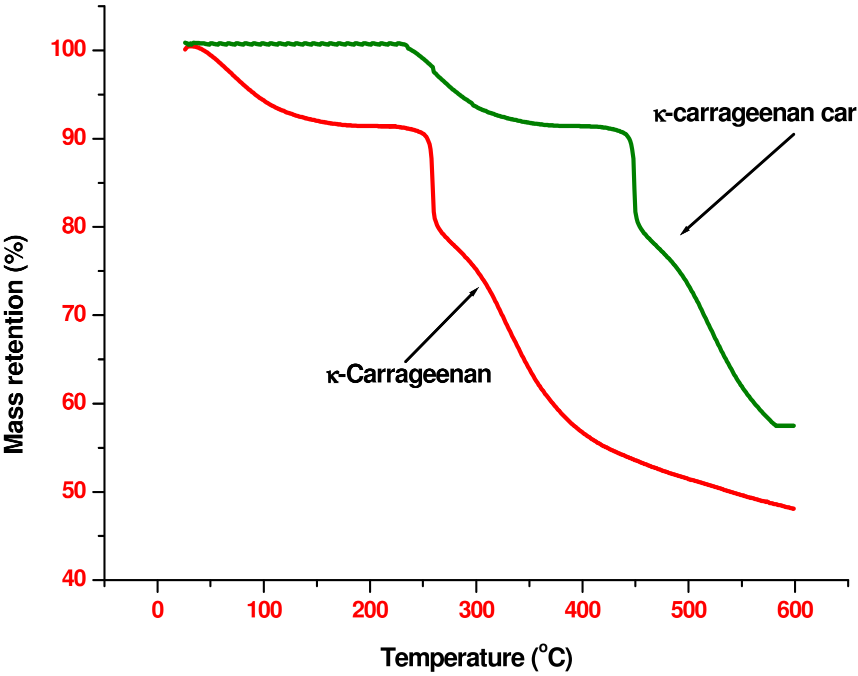

Fig.IV.2.3 TGA thermograms of κ-carrageenan and κ-carrageenan-dicarbanilate

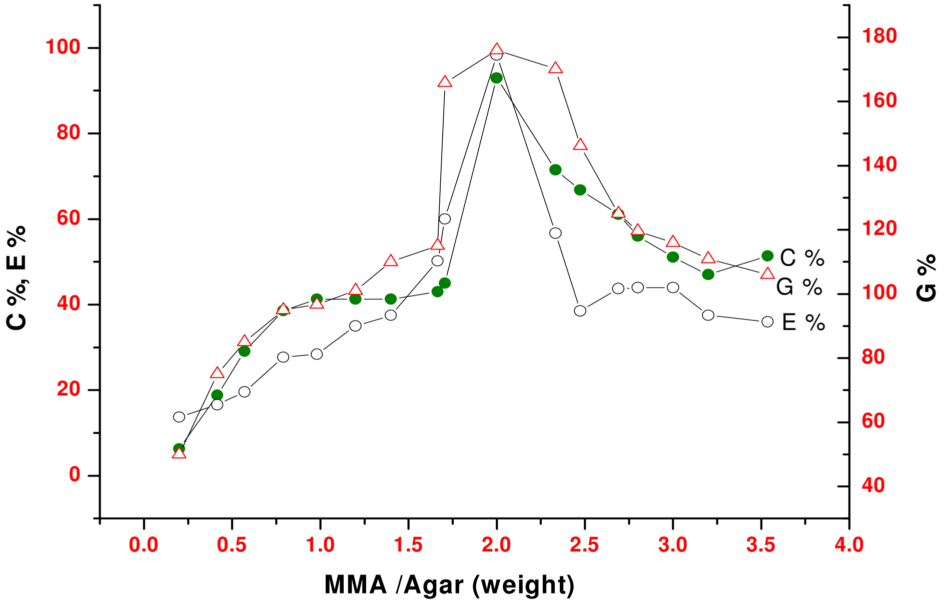

Fig. IV.2.4a Effect of MMA/agar ratio on Grafting parameters in the formation of agar-g-MMA.

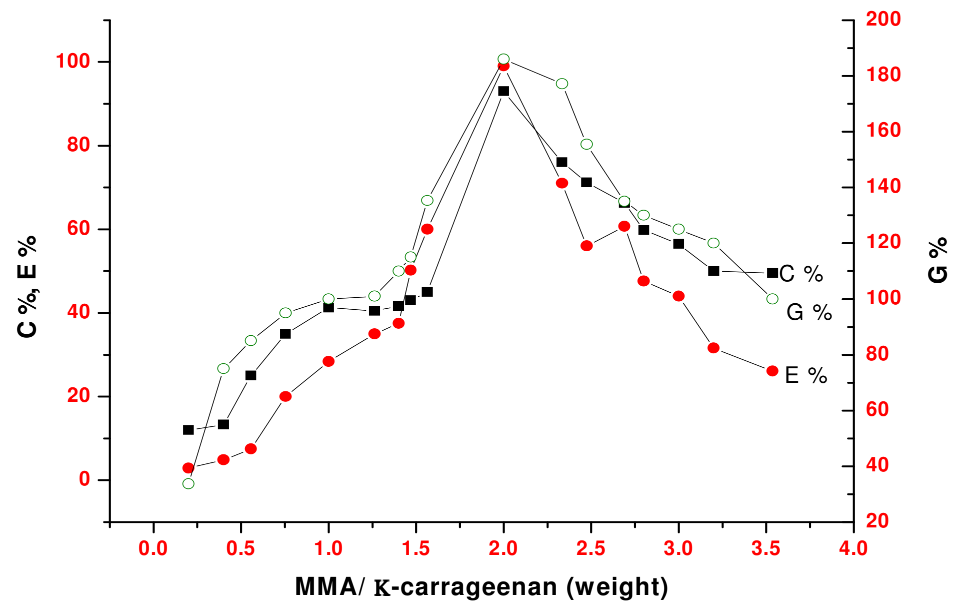

Fig. IV.2.4b Effect of MMA/κ-carrageenan ratio on grafting parameters in the formation of κ-carrageenan-g-PMMA.

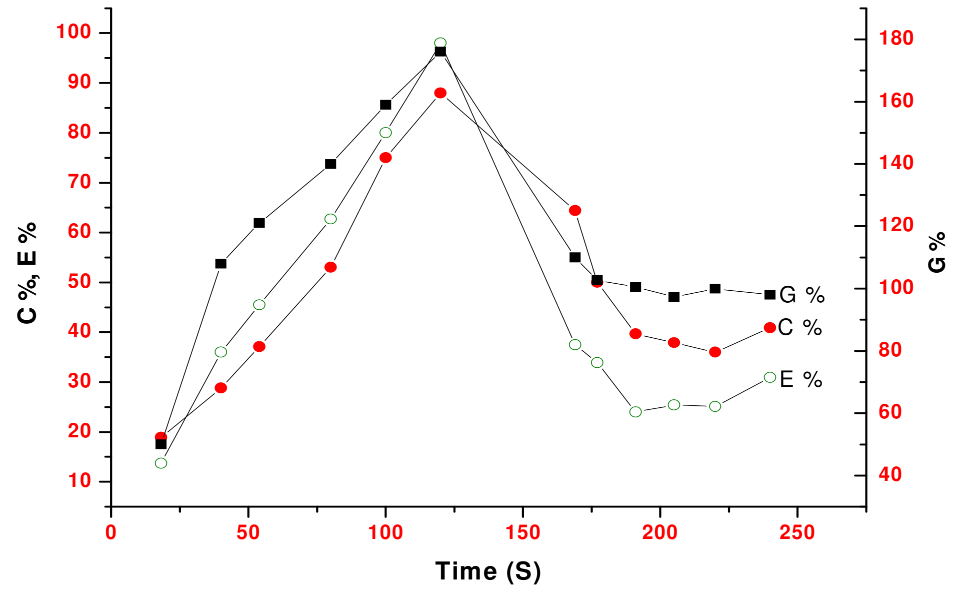

Fig. IV.2.5a Effect of reaction time on grafting parameters in the formation of agar-g-PMMA.

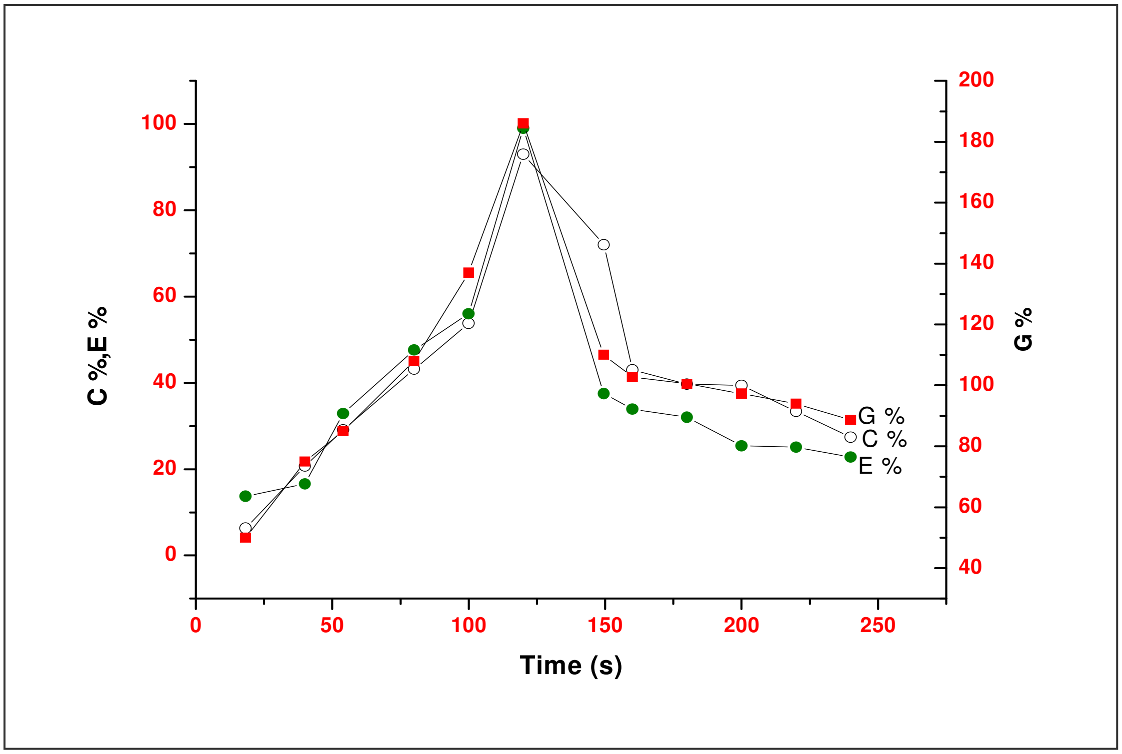

Fig. IV.2.5b Effect of reaction time on grafting parameters for the formation of κ-carrageenan-g-PMMA

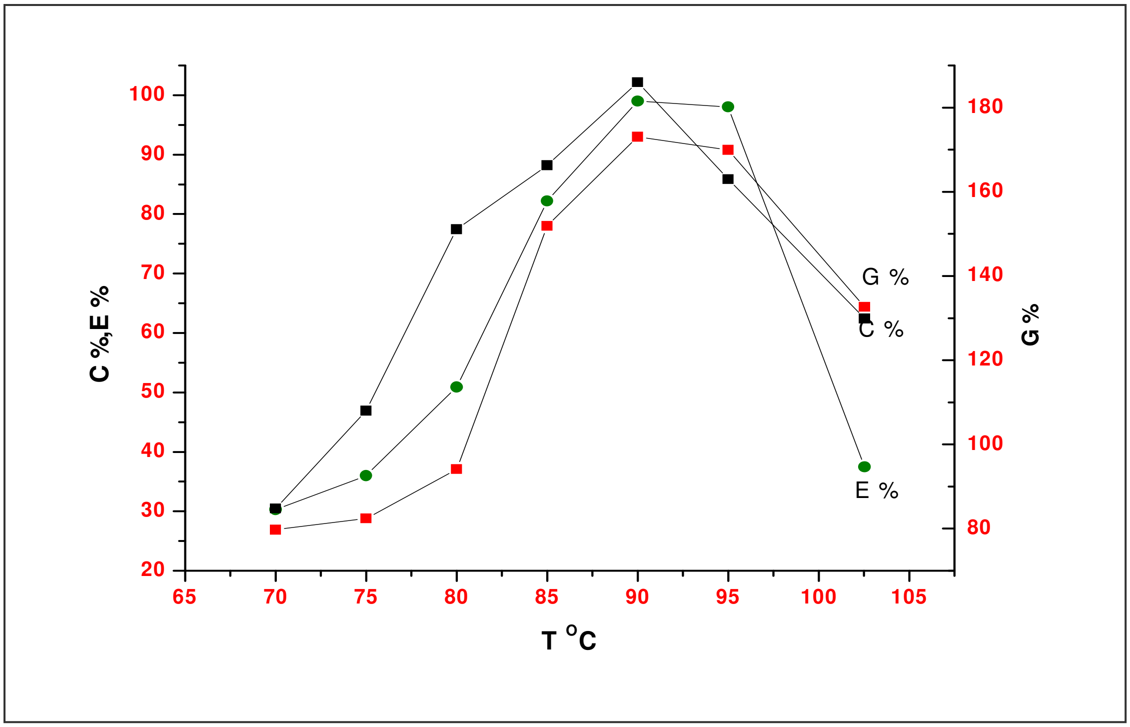

Fig. IV.2.6a Effect of reaction temperature on grafting parameters for the formation of agar-graft-PMMA.

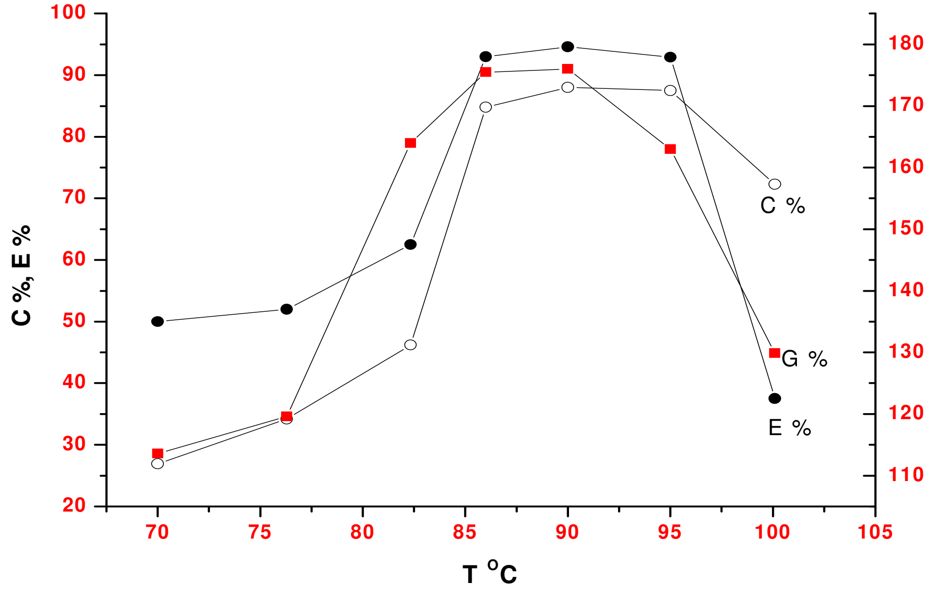

Fig. IV.2.6b Effect of reaction temperature on grafting parameters for the formation of κ-carrageenan-graft-PMMA

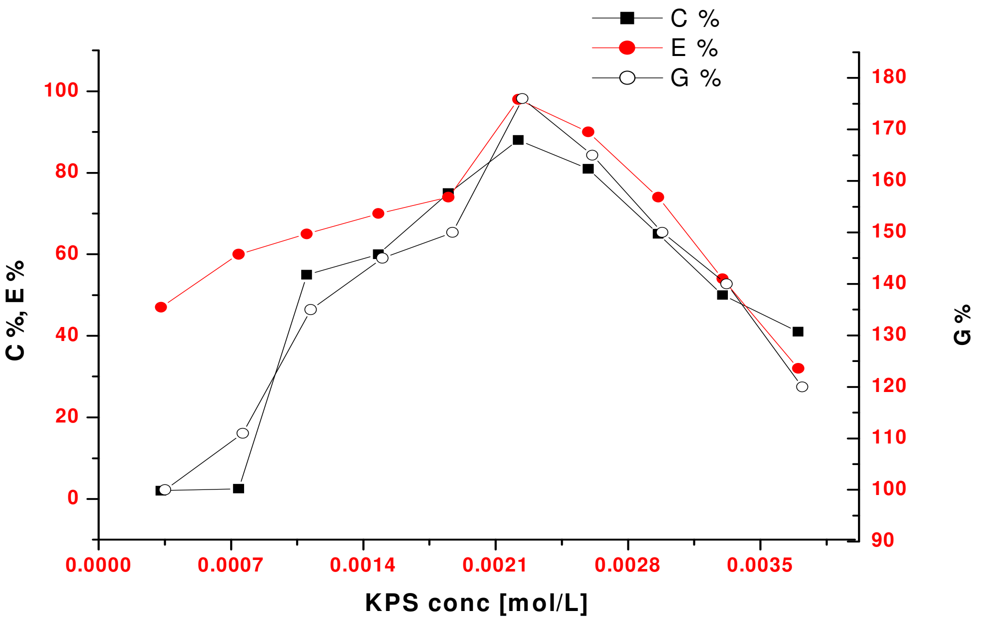

Fig.IV.2.7a Optimisation of quantity of initiator in the formation of agar-graft-PMMA.

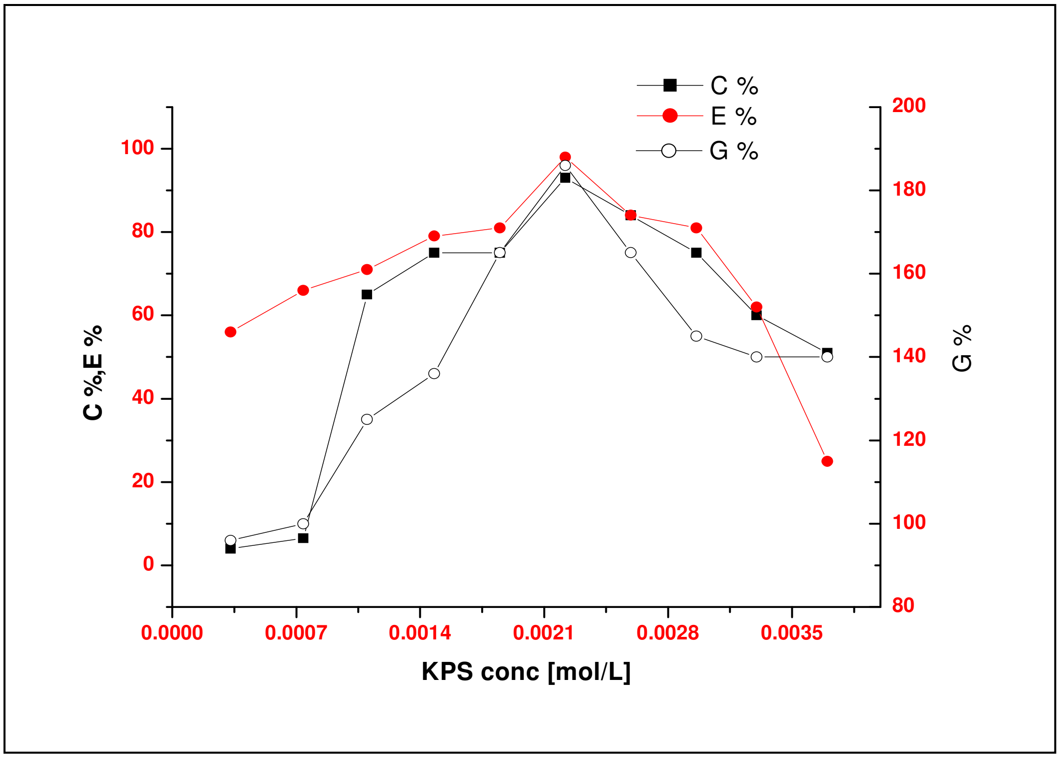

Fig.IV.2.7b Optimisation of quantity of initiator in the formation of κ-Carrageenan-g-MMA.

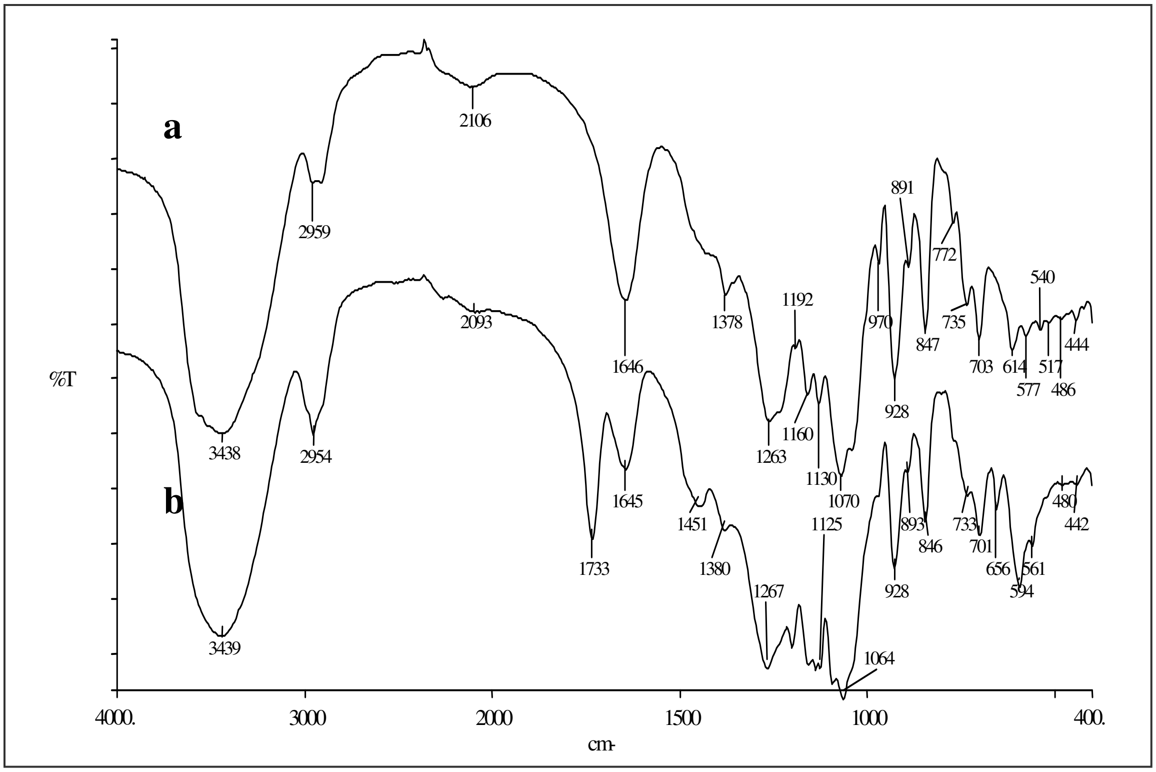

Fig.IV.2.8a FT-IR for (a) Control refined κ-carrageenan film (b) κ-carrageenan-graft-PMMA

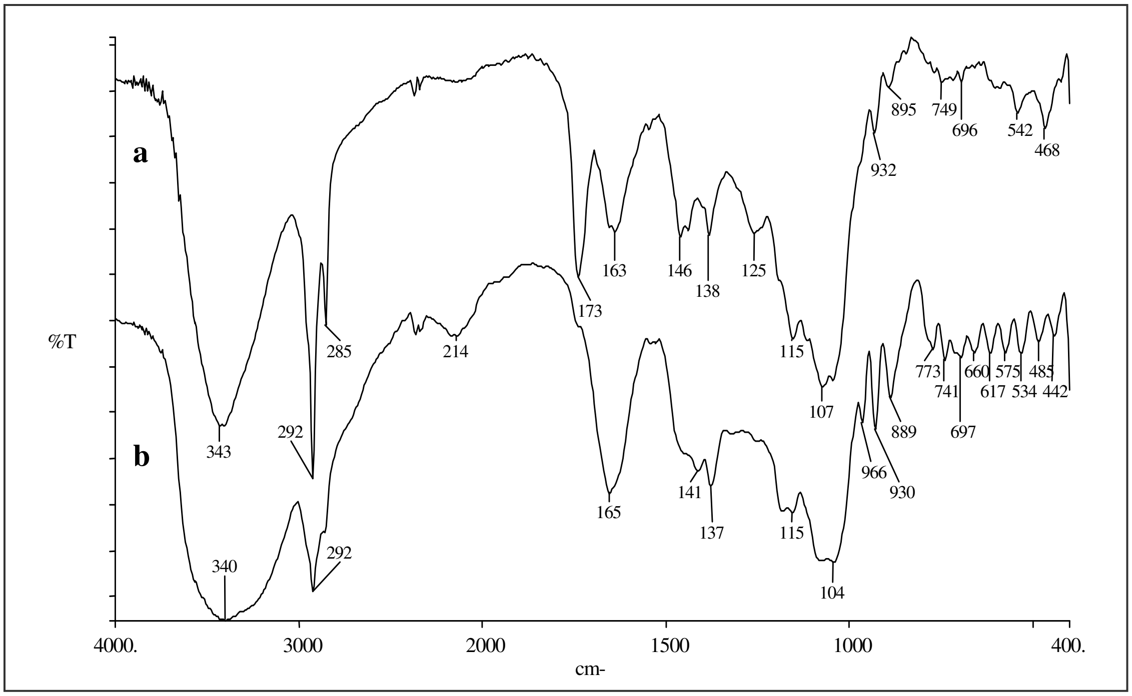

Fig.IV.2.8b FT-IR for a) Control agar film b) agar-graft-PMMA

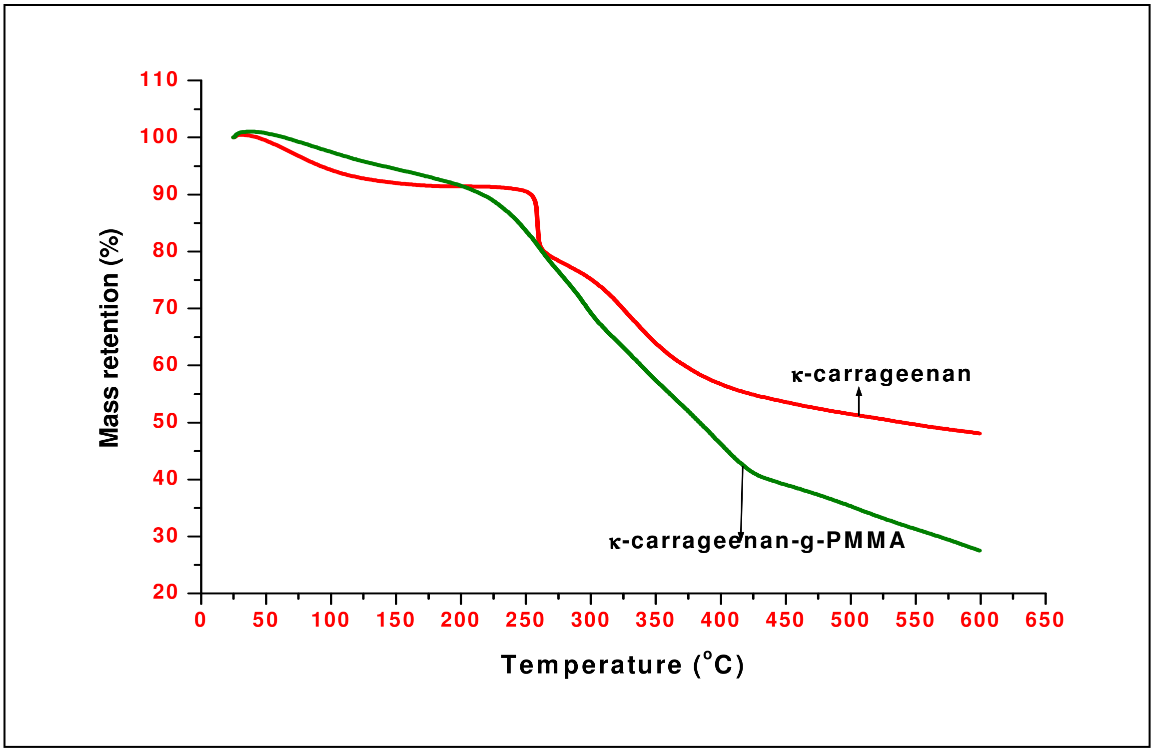

Fig.IV.2.9a Thermogravemetric (TGA) profile for pure κ-carrageenan and κ- carrageenan -graft-MMA copolymer.

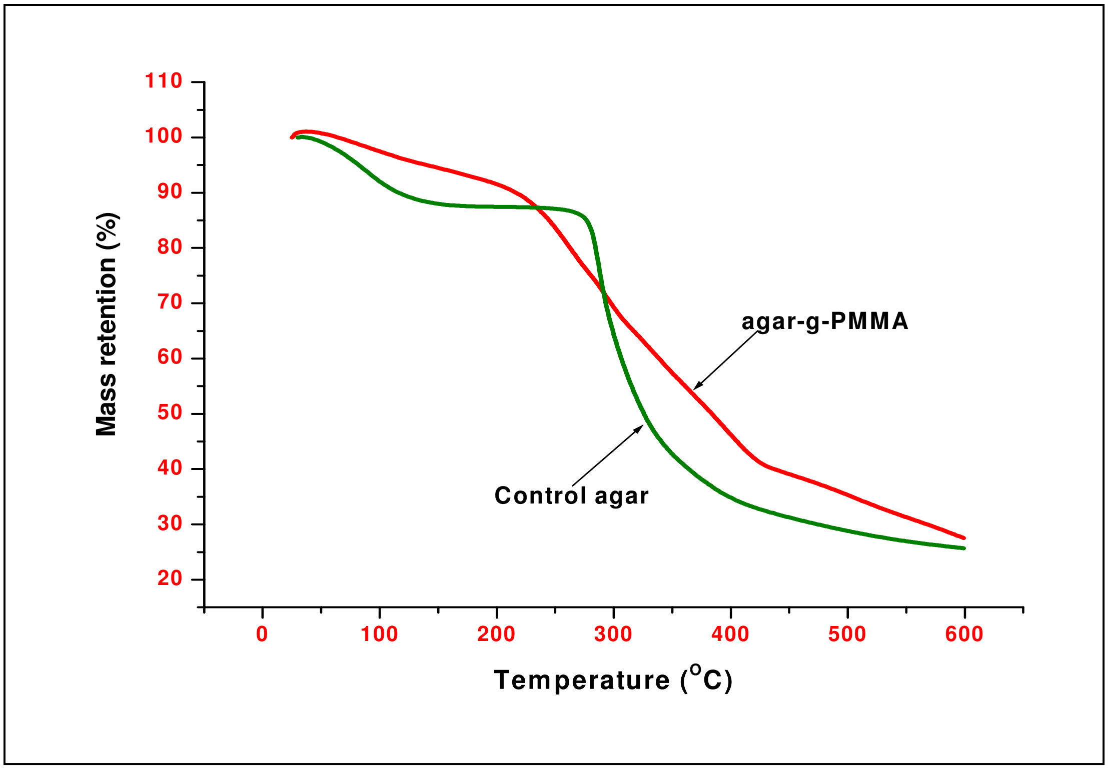

Fig.IV.2.9b Thermogravemetric (TGA) profile for pure agar and agar-graft-PMMA copolymer.

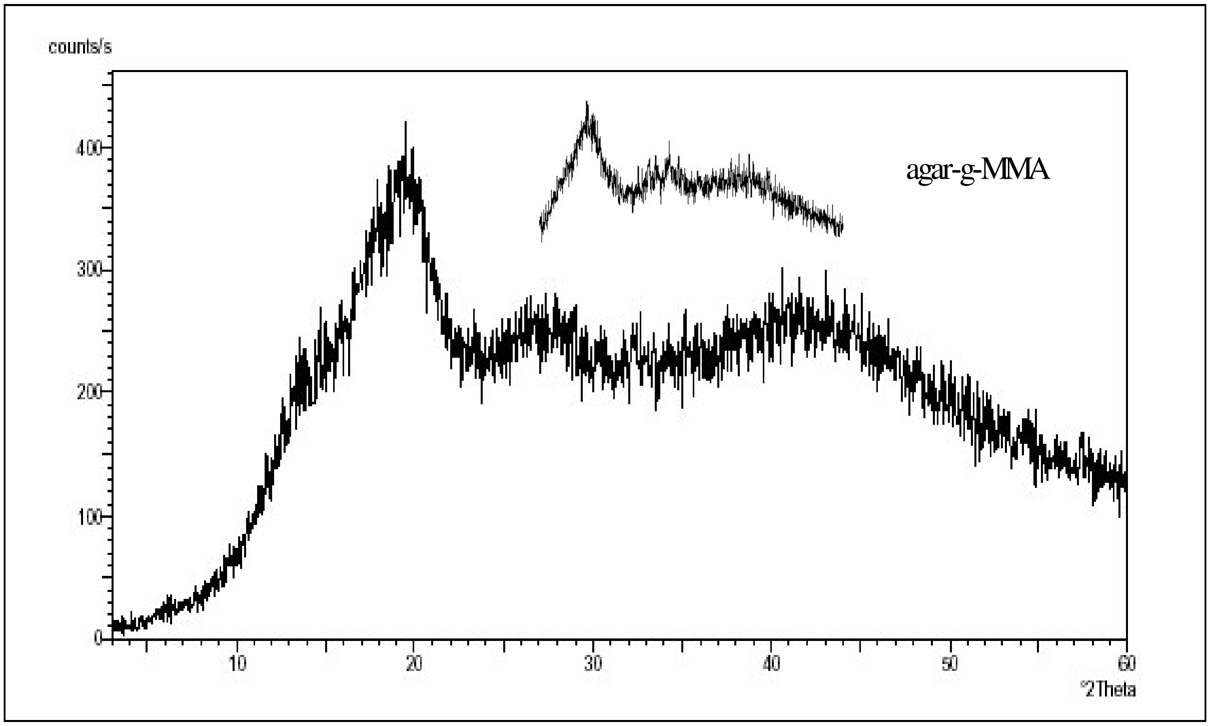

Fig.IV.2.10a X-ray diffraction of agar and agar-graft-PMMA.

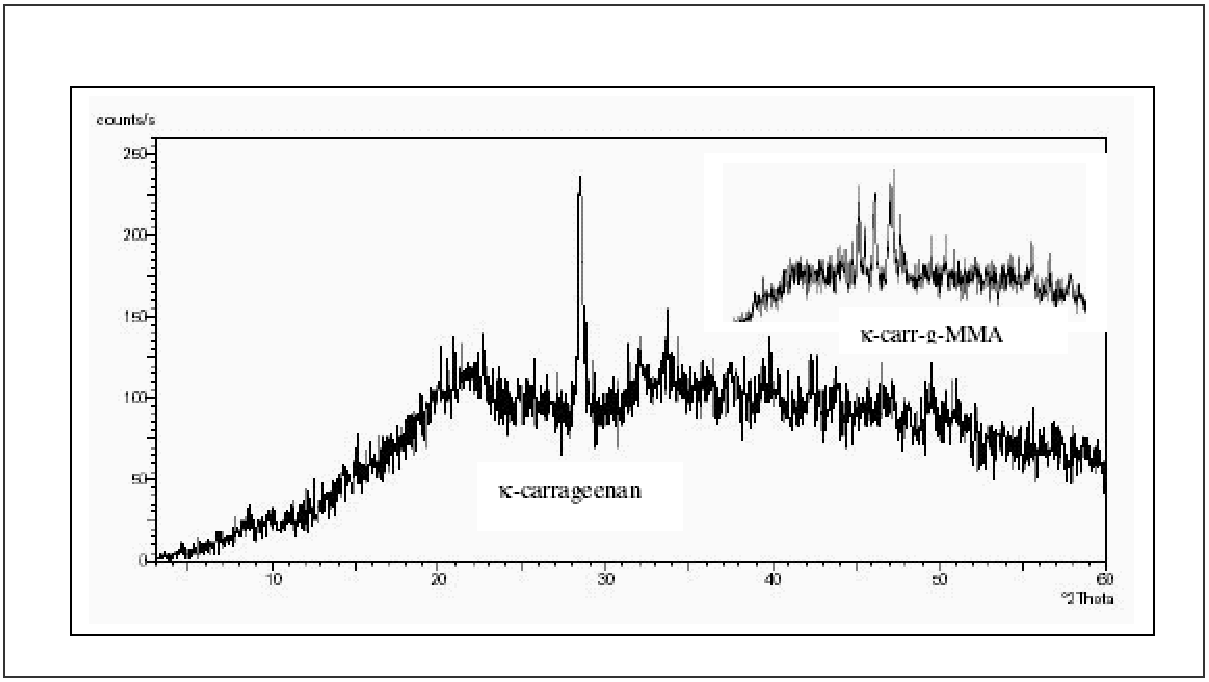

Fig.IV.2.10b X-ray diffraction of κ-Carrageenan and κ-Carrageenan-g-MMA.

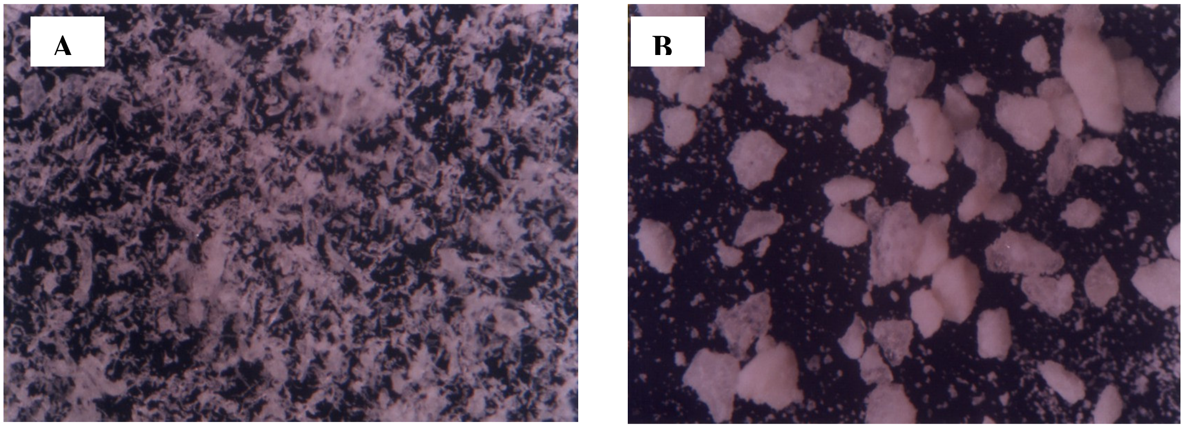

Fig. IV.2.11a Optical micrographs for (a) Control κ-Carrageenan (b) κ-Carrageenan-graft-PMMA

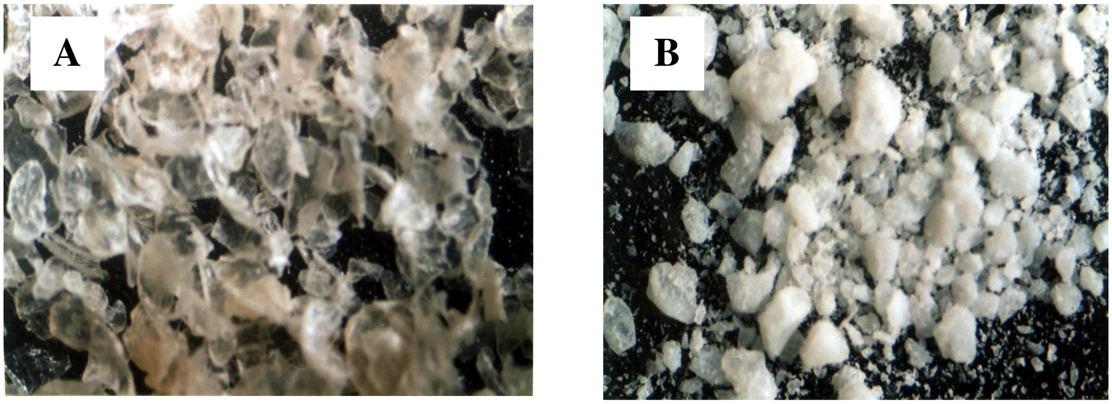

Fig. IV.2.11b Optical micrographs for a) Control agar b) agar-g-PMMA

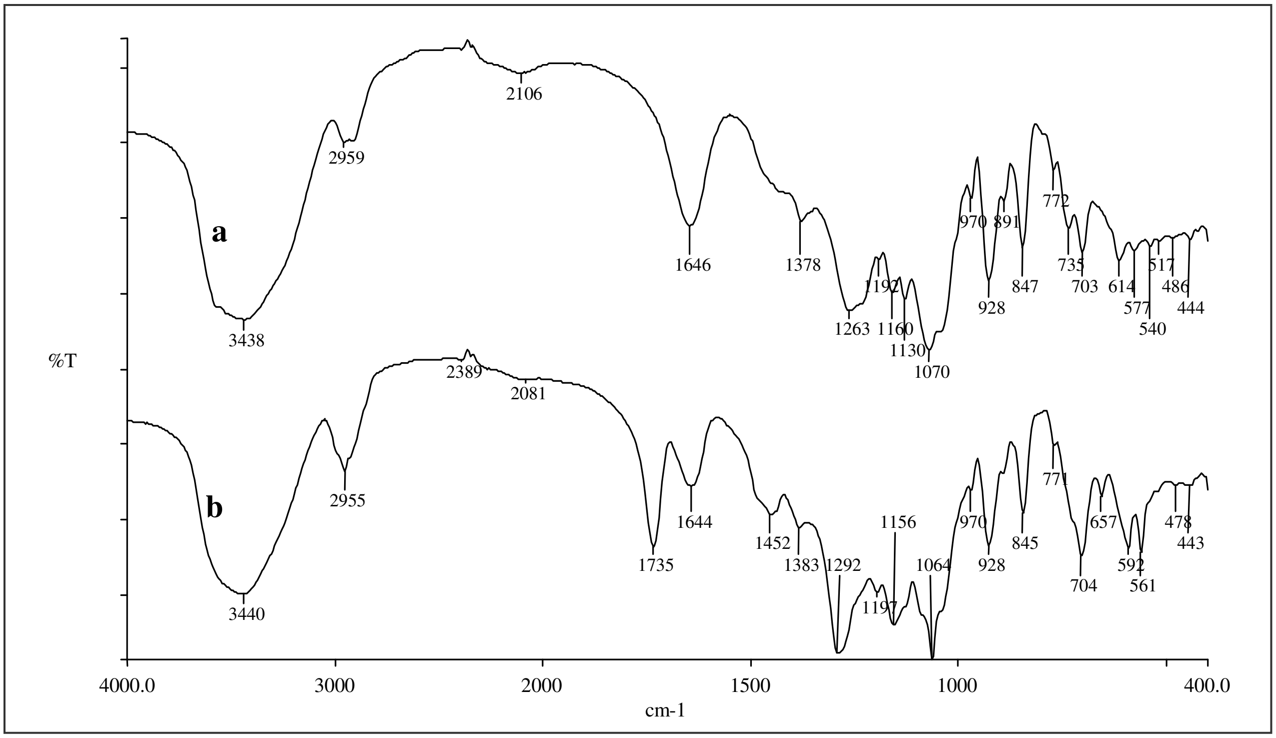

Fig.IV.2.12a FT-IR spectra (a) Control κ-carrageenan film; (b) κ-carrageenan-g-MMA film

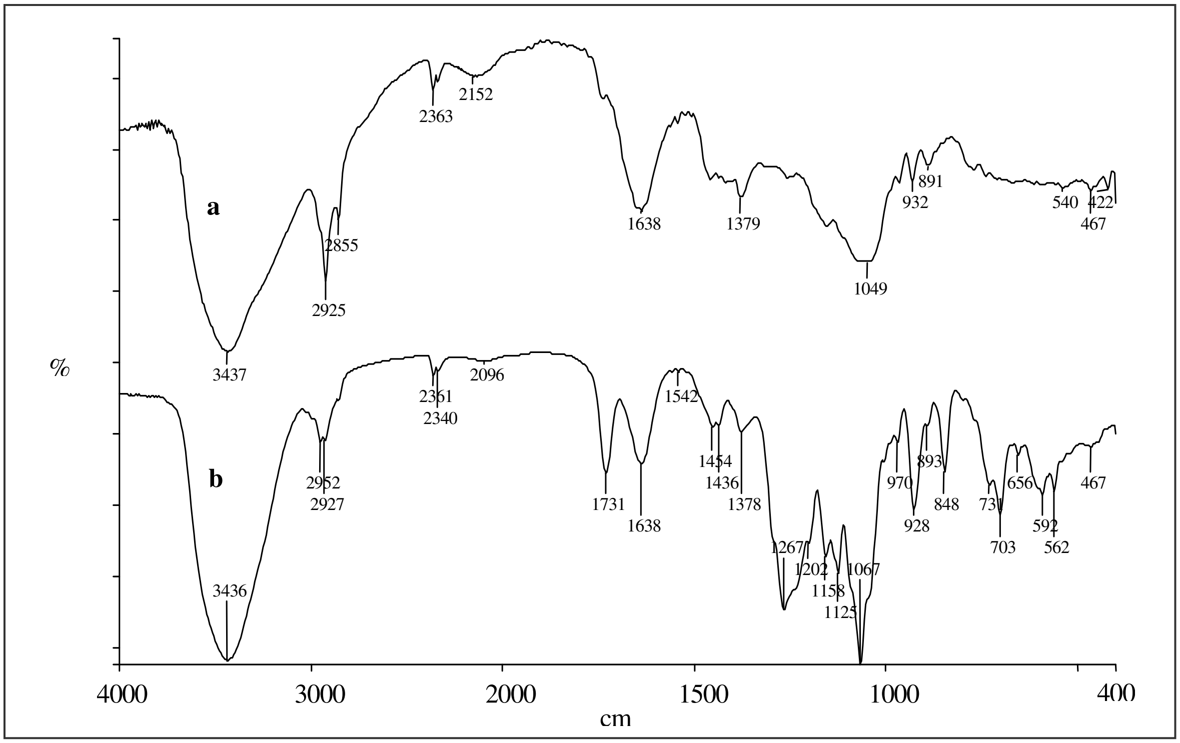

Fig.IV.2.12b FT-IR spectra a) agar-g-PMMA film b) Control agar film.

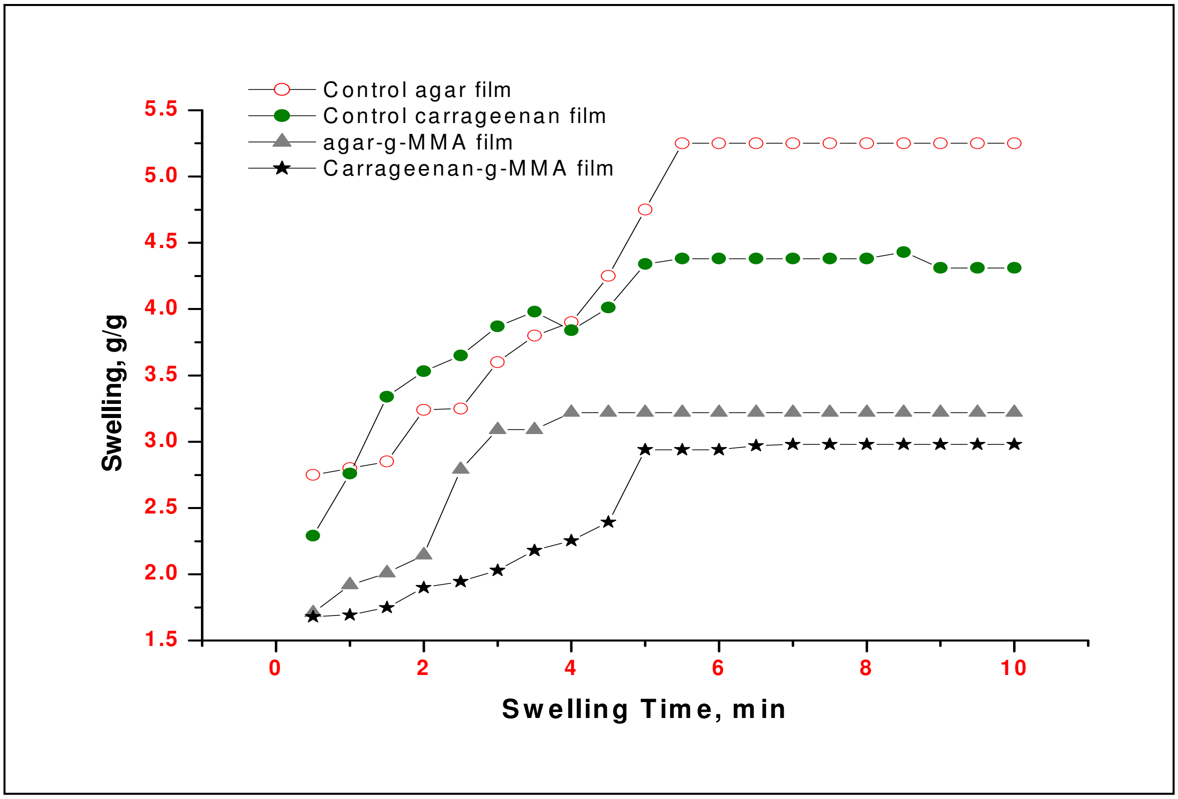

Fig.IV.2.13 Swelling kinetics of agar-g-PMMA and carrageenan-g-PMMA films

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