PRODUCTION, CHARACTERIZATION AND UTILIZATION OF GELLING POLYSACCHARIDE 0 F BACTERIA

By

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CERTIFICATE

It is certified that this thesis entitled "Production, characterization and utilization of gelling polysaccharide of bacteria" which is submitted to the University of Mysore, Mysore for the award of the Degree of Doctor of Philosophy in Microbiology is the result of research work carried out by Miss R. Triveni, under my guidance and supervision during the period from June 1995 to June 2000 in the Department of Food Microbiology, CFTRI,
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Guide

DECLARATION

I, Miss R. Triveni, hereby declare that the data presented in this thesis entitled "Production, characterization and utilization of gelling polysaccharide of bacteria" which is submitted to the University of Mysore, Mysore for the award of the Degree of Doctor of Philosophy in Microbiology is the result of research work carried out by me under the guidance and supervision of Dr. T.R.Shamala during the period from June 1995 to June 2000 in the Department of Food Microbiology, CFTRI, Mysore.

I further declare that the work presented in this thesis has not been submitted previously for the award of any other degree or diploma or any other similar titles.

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1 **INTRODUCTION**

Historical background

Polysaccharides also referred to as gums or hydrocolloids which consist mostly of water soluble or water swellable macromolecules, have broad applications in both food and non-food industries. They are used commercially to thicken, suspend or stabilize aqueous systems, to produce gels and to act as flocculent, binders, film formers, lubricants and friction reducers.

Polysaccharides are documented in the Bible as famous "manna from heaven" which sustained the Israelites in their escape from Egypt. In Australia, the natives eat wattle gum in combination with fish. According to

Maiden (1890) in the city of Sydney, youngsters, enjoy a home made confection known as "gum jelly" which is prepared by soaking wattle gum in water and adding sugar. Thus the "gum-sucker" evolved in about 1820 from the habit among small boys eating/chewing a gum obtained from eucalyptus/acacia trees.

In India, similar gum exudate is used in foods like *laddu* and other sweetmeats, as well as in *sherbet* and syrups. Similarly seaweeds also have an ancient history of use as food and medicine. A poem in the "Chinese book of poetry" written in the time of Confucius (800-600BC) mentions that a housewife cooked seaweeds [1,2].

Naturally occurring polysaccharide from higher plants and seaweeds have been in use for many decades in large quantities. Due to increase in demand over the availability of seaweed/plant gum lead to discovery of microbial polysaccharides. In 1940 dextran was tile first microbial polysaccharide to be commercialized, followed by xanthan in 1964.

Classification

Polysaccharides can be classified based on

1. Origin

2. Morphological localization and
3. Charge properties.

Based on origin, polysaccharides are classified as those obtained from exudates of trees/ bushes, plant extract, seaweeds, flours from seeds or grains, gummy slimes from fermentation process and modified gums (Table-I).

Plant exudate

Gums of the ancient world were large plant exudates of highly viscous gummy liquids which when exposed to air and allowed to dry, formed hard glassy masses. For example, gum arabica is the oldest and natural exudate produced by various species of thorny Acacia trees from the stem and branches of *Acacia senegal* (L) and related species3.

Table 1: Classification of polysaccharides

Exudates Extracts Flours Biosynthetic/ Chemical fermentation modification

Gum Arabic, *From Seeds* Dextran, Cellulose Tragacanth, *Seaweeds* Guar gum, Xanthan, derivatives Karaya, Agar, Locust bean Curdlan such as
Ghatti Alginate, Gellan, etc. carboxymethyl carrageenan, cellulose Furcellaran

Form Cereals Other
" Pectin Starch, *Derivatives*
Cellulose Modified starches, Low Methoxyl
ectin etc,.

Plant seed polysaccharides

Plant seeds are traditional and ancient sources of polysaccharides. Most seeds contain starch as the principle reserve food stored for use by the embryonic plant, but many seeds contain polysaccharide polymers with functional gum-like properties which have served as a useful source of commercial polysaccharides [4,5]. This includes quince, psyllum, flaxseed, locust bean, guar gum, etc. For example, in locust or carob, after efficient removal of the outer tough, dark brown husk and germ from the bean kernel/seed, the endosperm is powdered by selective milling to yield a range of finished products of various particle size6.

Plant extract

Pectin is a structural carbohydrate product present in all plants [7]. Pectic substances are abundant in fruits and vegetables and to a large extent responsible for firmness and form retention of their tissue, e.g. Apple pomace and citrus peel. Pectin is also an integral component of the cell structure and plays an important role as cementing material in the middle lamellae of primary cell wall.

Cellulose derivative

Cellulose is a readily available polysaccharide produced in great volume and is an excellent starting material for the production of gums. Developed chemical procedures may properly modify a polysaccharide to the desired product such as CMC, HEC. Low-cost modification practices offer a stimulating challenge to chemists and consequently industrial chemists will ever seek to devise simpler and more ingenious modification procedures. Hence products resulting from the modification of cellulose will continually vie with natural gums for important industrial markets.

Seaweed polysaccharides Gum extraction from seaweeds originally practiced in oriental countries, has spread to different parts of the world where shallow waters and seaweeds are abundant. Agar and carrageenan are examples of seaweed polysaccharides. Agar is a cell wall constituent and the method involved in production and purification consists of the following steps. Cleaning of raw material, chemical pretreatment, pressure extraction, chemical post- treatment, filtration,

gelation, freezing, post treatment, washing, drying, sterilization, bleaching, washing and drying9.

Microbial polysaccharides Polysaccharides obtained from plant and seaweeds have been in use tl1foughout the world since ancient days. Although nearly all living organisms produce polysaccharides, bacteria and fungi are the most easily adapted to large scale commercial production. Bacteria and fungi can be readily grown as submerged pure cultures in large fermentors. In order to avoid costly purification procedures, microbial polysaccharides that are produced unattached to cells have been sought first for commercialization. Naturally, exopolysaccharides from mammalian pathogens have been avoided. It has been only with in the last decade that the practical potential of microorganisms as sources of industrial gums has been given serious consideration. Since microbial gums are produced under controlled conditions, problems of plant and seaweed gums associated with climatic conditions, collection, supply and quality can be avoided. Microbial gums are usually produced by aerobic fermentation, and are further processed as shown in flow diagram. (Fig.1)

Dextran was the first microbial polysaccharide to be commercialized in 1940[10] which was followed by xanthan. Even though a few microbial polysaccharides have been commercialized, many microbial polysaccharide exist which can also be commercialised (Table-2).
Microbial polysaccharides can be divided into 3 main groups according to their morphological localization:
1. Intracellular polysaccharide located inside/as part of the cytoplasmic membrane.
2. Cell wall polysaccharides forming a structural part of the cell wall.
3. Extracellular polysaccharides located outside the cell wall.
Extracellular polysaccharides occur in two forms:
a. Loose slime which is non-adherent to the cell. It imparts a sticky consistency to bacterial growth in a solid medium and an increased viscosity in a liquid medium

Fig 1: Production of microbial polysaccharides

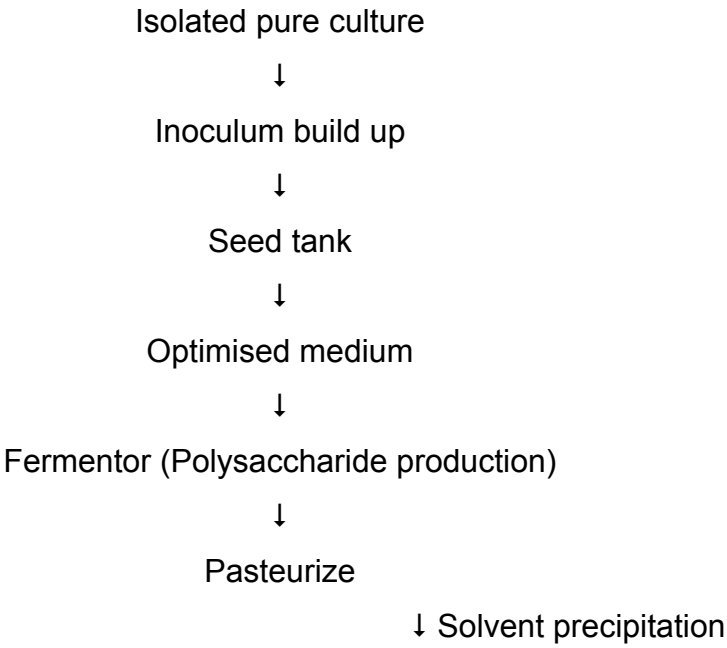


Table 2: Polysaccharides of microbial origin

Polysaccharide	Microorganisms (source)
Xanthan	Xanthomonas campestris
Dextran	Leuconostoc mesenteroides
Pullulan	Aureobasidium pullulan
Baker's yeast	Saccharomyces cerevisiae
Scleroglucan	Sclerotium rolfi'ii
Elsinan	Elsinoe leucospila
Gellan	Pseudomonas elodea
Curdlan	Alcaligenes faecalis var myxogenes
Welan	Alcaligenes sps
Rhamsan	Alcaligene sps
Alginate	Azotobacter vinelandii
Alginate	Pseudomonas aeruginosa
PS.7	Beijierinckia indicia var myxogenes
S-130	Alcaligenes ATCC 31555
S-194	Alcaligenes ATCC 3196
S-198	Alcaligenes ATCC 31853

b. Microcapsules which adhere to the cell wall. They have a definite form and a boundary being only slowly removed by shaking in water/salt solutions.

Based on their charge properties exopolysaccharide may be classified s anionic, neutral or cationic.

Disadvantages of plant gums

In ancient days polysaccharide obtained from plants were used by 1an. The disadvantages of plant gums over microbial gums are as follows.

The quality and quantity of plant gums depends on environmental condition for e.g., gum tragacanth obtained from Astragalus gummifer requires abundance of water during the growing season but needs a relatively dry climate during the collection

season 11. Excessive rain and wind storms during the collection season will cause discoloration of the gum and reduce solution viscosity typical of poor quality gum.

The availability of plant gum is fixed to particular area. For e.g., gum karaya is found in dry rocky hills and plateaus of Central and Northern India.

3. Starting from harvesting to packaging of plant gums, all the operations are managed by labourers, therefore it is a time consuming and more laborious process. Gum karaya, the dried gummy exudate which hardens into lumps is collected by hand and brought to village collection stations for crude sorting and classification [12] .

4. Plant gums are distributed in the raw state. So it requires further processing including crushing and cleaning by the end user.

Advantages of microbial gums over plant gums

Due to rise in demand, limited availability of plant, seaweed gums and consequent very high price provoked to obtain polysaccharide by alternative sources such as microorganisms. The advantages are:

1. In microbial gums the quality and quantity can be maintained by fermentation technique.

2. Microbial gums of known quality can be obtained as and when required.

3. It is a highly time saving and non laborious process.

4. These gums have diversified rheology, chemical structures and functions.

REVIEW OF LITERATURE

GELLING POLYSACCHARIDES FROM SEAWEEDS

Various seaweed gums have been used in food and other industries due to their excellent rheological properties. Agar was the first seaweed polysaccharide which came into the market. It was discovered in 1660 by Minoya Tarozaemenon, a Japanese inn keeper who found a porous jelly like mass from the seaweed discarded in the soil. When reboiled in water

and cooled, it yielded a gel, and thereby he had discovered agar. Later in 19th century, Irish moss carrageenan extracted from Chondrus crispus was in market for beer clarification and textile sizing. Similarly in 1880 Algin was discovered by E.C. Standford.

AGAR

Agar is produced from seaweeds such as Acanthopeltis japonica, Gelidium sps, and Pterocladia sps, etc,. It occurs as a structural carbohydrate in the cell wall and performs the functions of ion-exchange and dialysis process.

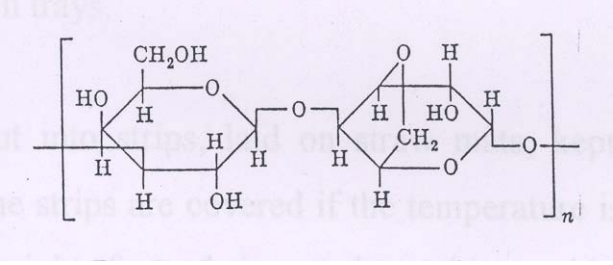


Fig.2: Structure of agar

Agar consists of predominantly repeating units of alternating ß-D-galactopyranosyl and 3,6-anhydro-a-L-galactopyranosyl units coupled by 1,3- linkages as shown in Fig.2. Uronic acids may be absent or present in amounts less than 1 %.

Processing of agar

In traditional process[13] , the weeds are washed in batches of 8kg and pounded for 20 min to remove sand and epiphytes. For each 200 kg of seaweed, about 2200 liters of water are boiled in an open iron caldron over a pine log fire. The tougher types of algae are introduced into the caldron first and the softest last. When the intermediate weeds have been added, the mixture is treated with 19 of sulfuric acid or 0.3g of poly phosphate per kg of seaweed to adjust the pH to 5-6 and extraction continues at 80°C for 8-9 hours during which time, weak liquor from the previous day is added.

After approximately twelve hours, calcium hypochlorite or sodium bisulfate is introduced for bleaching at a rate of about 2g per kg of weed, and the cooking is continued for fifteen hours.

The entire material is strained through cloth of 3mm mesh and the cake is pressed and retained for recooking. The liquor containing 1 % of agar is cleared by sedimentation, after which it is allowed to solidify in 170x30x1cm wooden trays.

The gel is cut into strips, laid on straw mats, kept out doors and allowed to freeze, the strips are covered if the temperature is too low. Each ay some of the night formed ice melts, taking with it some salts, nitrogenous material, and residual color. Sprinkling is used when needed to prevent excessively rapid drying. After 5-6 days, the racks carrying the mats placed in the sun in such a manner that final drying is complete in mother 15-30 days.

Properties

Agar is unique among the gelling agents in that gelation occurs at a temperature relatively far below the gel melting temperature14. A 1.5% solution of a good quality agar, for example, forms a gel upon cooling to about 30°C and the gel does not melt below 85°C. Agar is probably the strongest gelling agent known and will form rigid, tough gels at concentrations of as low as 0.5%.

Threshold gels of agar are useful for their protective action, diffusion prevention and texture enhancement. Stronger gels are valuable because of their strength, resilience, transparency permanence, and reversibility.

Viscosity

The viscosity of agar is markedly dependent upon the raw material source and varies widely. The viscosity of an agar solution at temperature - above its gelation point is relatively constant from pH 4.5-9 and IS not greatly affected by age or ionic strength within the limits of pH 6-8. Once gelation begins, however, viscosity increases with time at constant temperature.

CARRAGEENANS

Carrageenan is not a single polysaccharide but a group of sulfated. galactans extracted from a number of seaweeds belonging to class Rhodophyceae (red algae). Seaweeds used for extraction of carrageenan are species of Chondrus, Eucheuma, Iridaea, Gigartina and Gloiopeltis etc, Carrageenan is located in the cell wall and intracellular matrix of the plant tissue. It was first produced commercially from the red

algae, Chondrus crispus found along the northern shores of V.S and Canada in 1937 and referred to as Irish moss extract.

The carrageenans (Fig.3) differ from one another in their content of 3,6 anhydro-D-galactose, number and position of ester sulfate groups. Kappa carrageenan is composed of alternating 1,3-linked galactose-4-sulfate and 1,4-linked 3,6-anhydro-D-galactose units. Iota-carrageenan polymer is composed of alternating 1,3,linked D-galactose-4-sulfate and 1,4-linked 3,6anhydro-D-galactose-2-sulfate. Lambda carrageenan, is a non-gelling carrageenan and is composed of alternating units of.} ,3-linked galactose and 1,4-linked galactose 2,6-disulfate.

Production of carrageenan

The dried seaweed is given a cold-water wash to remove extraneous undesirable material. This is followed by a hot alkaline extraction under vigorous agitation sufficient to disintegrate the plants. The hot extract is then filtered through plate-and-frame or rotary filter presses. It may or may not have been decolorized with charcoal prior to filtration, depending on the grade and end-use requirements. The filtrate is then concentrated by vacuum evaporation to about half its volume and then recovered by either drum drying or by alcohol precipitation, followed by vacuum drying. The final product is then ground and packed

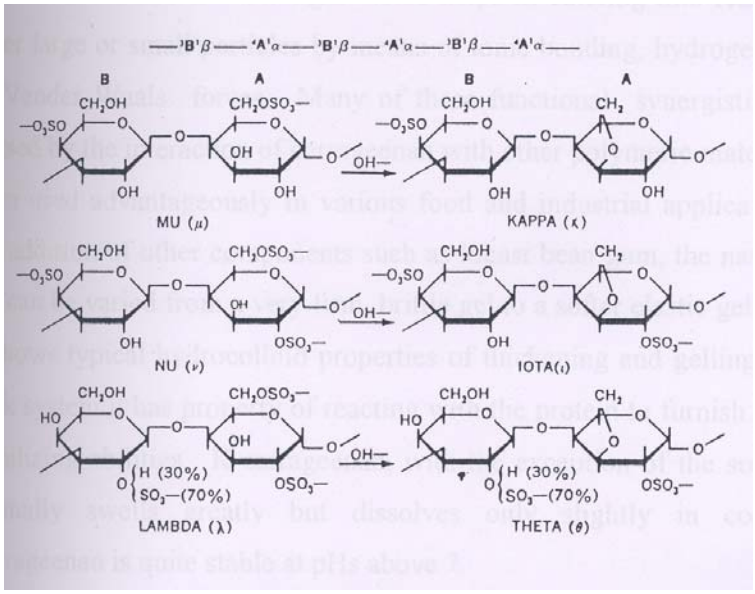


Fig. 3: Structure of carrageenans

(Ref: Glicksman, M., 1982)[178] Properties

According to Frieden and Werbinl6, Smith[l7], the gelling and thickening properties of carrageenan can be enhanced by the addition of potassium salts. Carrageenan is a

strongly charged anionic polyelectrolyte of large size and molecular weight. This helps in entering into reaction with other large or small particles by means of ionic bonding, hydrogen bonding or Vander Waals forces. Many of these functional synergistic changes caused by the interaction of carrageenan with other polymeric materials have been used advantageously in various food and industrial applications. By the addition of other components such as locust bean gum, the nature of the gel can be varied from a very firm, brittle gel to a softer elastic gel. In water it shows typical hydrocolloid properties of thickening and gelling, while in milk system it has property of reacting with the protein to furnish additional stabilizing abilities. K-carrageenan, with the exception of the sodium salt, normally swells greatly but dissolves only slightly in cold water. Carrageenan is quite stable at pHs above 7.

ALGIN

Algin is found in all species of brown algae, in which it is the main structural constituent of the cell walls, but only a few species are of commercial importance. The principal source, Macrocystis pyrifera is a giant kelp which occurs in large beds of nearly pure stands of this single species. Other seaweeds which contain algin are: Laminaria sps, Ascophyllum nodosum, Ecklonia maxima and Lessonia nigrescens.

Current methods of production of algin in the United States are based on modification of algin in the processes of Green (1936)18 and Legloachec and Herter (1938)19. In this process freshly harvested Macrocystis pyrifera is leached for several hours in dilute HCl to reduce the salt content, then drained and shredded. The macerated weed is then digested with 2-25% soda ash at pH 10. A gelatinous mass is produced, which is redigested with more soda ash and then disintegrated in a hammer mill. Six volumes of hot water are added and the pH is maintained between 9.6 and 11. After clarifying and filtering, the sodium alginate solution is treated with 10% $CaCl_2$ to precipitate calcium alginate. The recovered calcium alginate is converted to alginic acid by treating with 5% HCl. After washing and purifying it can be converted to the desired salt by treatment with the proper carbonate, oxide, or hydroxide, and then dried.

Properties

1. Alginic acid is insoluble in cold water and only slightly soluble in hot water. The sodium, potassium and ammonium salts of alginic acid and propylene glycol ester, are readily soluble in hot/cold water.

2. It forms lump upon addition of water. Blending with other ingredients, such as sugars and dextrin, facilitates dispersion, as does tagglomeration, a technique that is currently gaining wide use in the food industry

3. The viscosity of alginate solution are primarily dependent on concentration, temperature, molecular weight, pH and the presence of, polyvalent metal cations.

4. Alginate solutions decrease in viscosity on heating, a 2.5% decrease in viscosity for each centigrade degree rise for extended periods, a degradation of the molecule and resultant decrease in viscosity may Occur. However, if degradation is avoided, algin solutions will regain their original viscosities on cooling.

5. Solutions of the water-soluble alginate salts exhibit little changes in viscosity over the pH range of 4-10. Maximum viscosity is obtained 'near neutrality. Below pH 4 the viscosity tends to increase because of the lower solubility of the free acid. Eventually alginic acid is precipitated. Solutions are also unstable above pH 10.

6. Polyvalent metal ions increase the viscosity of soluble alginate and can cause gelation if present in sufficient concentration. Gels are usually formed by the gradual release of calcium or hydrogen ions, or by a combination of the two. Gel formation can be controlled by the presence of sequestrant, such as phosphate or polyphosphate, that is capable of combining with calcium or other polyvalent metal ions, clear transparent gels which do not melt at room temperature are obtained in this manner

7. Alginate films can be prepared by drying a thin layer of a soluble alginate solution, by treating a soluble film with a di- or tri-valent metal or with an acidic solution, or by extruding a solution of soluble alginate into a precipitating bath which produces an insoluble alginate. These films are tough, clear, flexible and resistant to grease, fats, oils and organic solvents.

MICROBIAL GELLING POLYSACCHARIDES

Exopolysaccharides are produced by many species of Gram positive and Gram negative bacteria, some algae and a few fungi. These organisms are found in effluents from paper, sugar mills, soil, fresh water, sea water, infected plant material spoiled food etc.21,22. A few microorganisms which produce viscous/film/gelling polysaccharide have been isolated from the environment. They differ from one another in physical and chemical characteristics. These polysaccharides are mentioned below:

S-119 is an extracellular polysaccharide produced by Agrobacterium radiobacter A TCC 31643. It is composed of glucose and galactose in the ratio of 7: 1 with p-linkage. 8-119 gives viscous solution of 350-600 cpo at 1% concentration in deionized water. A pourable solution can be obtained at 20% gum concentration. Therefore it can be used as excellent film with good tensile strength, sole sizing agent in textile industry to replace gum arabic in lithography and paper making industry23. S-130 produced by Alcaligene ATCC 31555 is composed of glucuronic acid, glucose, mannose and rhamnose in the molar ratio of 1:2: 1 :2. It exhibits pseudoplasticity, thermostability, excellent pH stability.

It has good suspending ability similar to xanthan gum. Therefore it is used as drilling fluid additive and completion system for wells with high temperature24. Another species of Alcaligenes (A TCC 3196) produces polysaccharide which is composed of glucuronic acid, glucose and rhamnose in a molar ratio of 1:4:1 and 9-10% of acetate as glycosidically linked 0-acetyl but it does not contain pyruvate. It exhibits high viscosity at low concentration (0.1%), good thermostability upto 100°C, pseudoplastic, good shear stability, and excellent suspending ability. Therefore it is used as suspending agent in suspension fertilizer.

S-198 is produced by Alcaligenes sps (ATCC 31853). It contains glucuronic acid, glucose, rhamnose and mannose in a molar ratio of 1 :2:2:1 with a-acetyl and O-succinyl groups. It has an excellent shear stability compared to xanthan gum. It exhibits great potential for high water fluids, high viscosity at low product concentration. Therefore it is used as a thickener in high water based fluids24.

PS- 7 is an extracellular polysaccharide produced by Beijerinckia var myxogenes. It is composed of 73% glucose, 6% rhamnose and glucuronic acid. It dissolves both in hot and cold water to produce high viscosity solutions even at low concentration. The polysaccharide is highly pseudoplastic, has excellent pH stability between 3-12 and temperature stability of 25oF-200oF. It is highly compatible with wide variety of salts.

For e.g. it is compatible with 2% solutions of NaCl and 32% CaCl2. It is used in drilling mud, dripless water based latex paint, wall joint cement adhesives and textile printing. 25

Curdlan is a linear polymer of (3-1,3-D-glucose with a degree of polymerization of 400. It is produced by A.faecalis. Curdlan is a tasteless, odorless, white powder which swells upon the addition of water. Below 2%,curdlan gum suspension on heating at 80°C gives a cloudy, opaque irreversible gel which is not affected by

heating, cooling/freezing. It has a good pH stability (2.5 to 10.0). The gel texture lies between the hard, brittle texture, of agar and the soft, elastic texture of gelatin. It is a good gelling agent in jelly and dry mixes, slimming additives, forms films which are edible, insoluble in water, biodegradable and impermeable to oxygen. It is used for improvement of visco-elasticity, binding agent, water-holding agent, retention of shape, to mask malodors/aromas, etc.

Gellan gum is an extracellular polysaccharide produced by Pseudomonas elodea. It is composed of glucose, galactose and rhamnose. Gellan gels both in the presence of monovalent/divalent cations. Divalent cations such as magnesium/ calcium have a much more profound effect on gel strength than monovalent ions such as sodium/potassium. By using 0.1% CaCl2 gellan is reported to be stable at 80°C for at least 10 days. Gellan gum is used as microbiological agar substitute, in plant tissue culture in food industry and as gel for antibody diffraction techniques[15,87,122].

NUTRITIONAL REQUIREMENTS FOR FERMENTATION

Carbon source

Carbon source helps in biosynthesis of polysaccharide and cell polymer maintenance. Most of the microorganisms require wide range of carbon substrates such as glucose, maltose, sucrose, fructose, xylose, arabinose, com steep liquor, hydrolysed cereal grains, molasses, starch and hydrolysed acid whey for polymer production but some species require specific carbon substrates, for po]ymer synthesis28. According to Ozawa et al (1972)29 starch and dextrose are essential carbon substrates for the production of polysaccharide by Achromobacter mucosum. According to Bushel124 sucrose is the specific substrate for the production of dextran by Leuconostoc spp.

According to Lilly et al.30, 1.5% of glucose was found to produce the best yields of xanthan. The conversion efficiency of glucose to polymer by X.campestris decreases when higher glucose concentrations are used32. This problem can be overcome by gradual addition of the carbohydrate substrate to the fermentation broth33.

For dextran production many sugars like glucose, invert sugar and maltose serve as energy source for the organism but only sucrose serves to induce dextran sucrase synthesis34 followed by polymer production.

Alcaligenes can produce curdlan from low concentration of sugars and inorganic salts at pH 6-7, It has been shown that l00mg of cells in l00ml of growth medium

containing 4g of glucose and 500mg of $CaCO_3$ can produce 1.2g of curdlan during incubation for 3 days, It is also reported that the salt medium containing 8% glucose (w/v) is converted to 4g of curdlan over 4 days of fermentation36.

In Azotobacter vinelandii 50gl-1 sucrose gave good growth of the organism compared to that obtained with other concentration of sucrose 37.

Similarly Sag I-I of glucose/lactose in media gave maximum growth and exopolysaccharide production but high concentration of glucose and lactose were inhibitory for growth and exopolysaccharide production.

Rhizobium hedysari is reported to produce acidic polysaccharide on mannitol followed by glucose and sucrose 38.

Nitrogen source

Nitrogen source used in culture media is directly related to cell growth. The cell quantity produced is proportional to the initial concentration of nitrogen source as seen in yeast and bacteria39,40. Suitable nitrogen sources for the production of polysaccharide are yeast extract, corn steep liquor, acid hydrolyzed casein, malt extract, peptone, tryptone, nitrate, corn gluten, soy protein, ammonium nitrate, ammonium chloride, ammonium hypophosphate, potassium nitrate and urea.

It has been reported in Azotobacter vinelandii that addition of sodium nitrate to medium decreases the lag phase and generation time of cells and the fermentation period is also reduced. It was observed that as sodium nitrate concentration was increased to 0.5g 1-1 there was an increase in growth as well as exopolysaccharide recovery. But further increase in concentration did not alter the growth pattern and exopolysaccharide production. Potassium nitrate gave similar result. Ammonium chloride gave increased growth but recovery of exopolysaccharide was less. In case of urea, growth and exopolysaccharide production were similar at all concentrations. Alcaligenes also grew well on potassium nitrate or urea. In contrast to these, Rhizobium hedysari required glutamate and lysine for cell growth.

Mineral salt

Polysaccharide producing bacteria are known to require mineral salts such as calcium, magnesium, manganese, ferrous sulphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, magnesium sulphate, sodium chloride, calcium carbonate for growth, yield and viscosity of polysaccharide. Potassium, magnesium or calcium ions are essential for the maximum polysaccharide synthesis. Sulphate can either stimulate or inhibit depending on the strain and polymer formed. The

omission of Fe2+ ions from the growth medium of Chromobacterium violaceum led to increased polysaccharide synthesis.45,46 Phosphate and iron were essential for polysaccharide production by strains of P.aeruginosa.47,48

The most critical factor for production of polysaccharide of Arthrobactor species (NRRLB-1973) was the presence of magnesium sulphate in tlle growth medium. It was found that a minimum of 0.04% was required to obtain good yields of gum, with 0.01% magnesium sulphate yields were reduced by 40%. No product was produced when the salt was omitted though the Arthrobacter strain showed satisfactory cell growth.49

In Azotobacter vinelandii the low concentration of Mg2+ inhibited the growth in culture maintained at high dilution rate in continuous culture. Addition of 1g 1^{-1} of potassium dihydrogen phosphate showed an increase in polysaccharide production whereas at 2 g 1-1 there was a drastic fall in the exopolysaccharide formation.

It has been reported that low calcium concentration inhibited growth of Azotobacter vinelandii maintained at high dilution rates in continuous culture.52. It is suggested that the Ca ++ uptake stimulated sugar uptake by decreasing the apparent Km of sugar transport uptake.53 According to Martins et al.54 the alginate biosynthesis in Pseudomonas aeruginosa is stimulated in the presence of Ca ++ and Mn ++ ions due to enhancement of growth and subsequently EPS synthesis. Mg++ ions are also required for activating certain enzymes leading to exopolysaccharide synthesis.55

In addition to these, calcium carbonate has been found essential for exopolysaccharide production and growth of the organism is also affected by its absence. Addition of 0.05g/l ferrous sulphate shows only a marginal increase and at higher concentration a gradual reduction in exopolysaccharide production. Manganese and iron are essential for a high yield of curdlan. Na2HPO4/CaCO3 maintain pH of the broth and simultaneously there is an increase in the yield of EPS.

CULTURAL PARAMETERS AFFECTING FERMENTATION OF EXOPOLYSACCHARIDE

Aeration

It is required for the growth and polysaccharide production from lightly aerobic to less aerobic organisms. In Xanthomonas campestris fermentation, the uptake of sugar ceased after 3 days of incubation in shake flask because of increase in broth viscosity.56 Pyruvate content of xanthan decreases with low level of aeration57. Enterobacter aerogenes also required high aeration for optimal polysaccharide

production, but in case of Rhizobium meliloti the growth and polysaccharide production were best in fermentors supplied with low aeration.

pH

pH of the fermentation medium is one of the important parameters that can affect polysaccharide production. Absence of medium buffering and lack of pH control result in rapid fall in the pH of fermentation with cessation of growth and production of the polysaccharide. Optimum pH reported for the production of acidic polysaccharides by Pseudomonas NC1BI 1264 is around 7.0. Here the cell growth is less sensitive to pH than product synthesis.

KINETICS OF EXOPOLYSACCHARIDE FORMATION

The rate of polysaccharide production varies with different stages of growth. In P.aeruginosa, polysaccharide production occurred throughout the growth phase.58 Where as in Azotobacter vinelandii the specific rate of exopolysaccharide synthesis has been shown to be largely independent of growth rate. The exoplysaccharide formation by Sphingomonas paucimobilis59 and secretion of numerous other polysaccharide take place only either in the late exponential or in the stationary phase of growth.60 During batch cultivation of organism, two phases are encountered, namely. (i) Propagation of cells and (ii) Production of gum. Various parameters like temperature, pH and C/N ratio required for growth and production vary from one organism to the other.

A two-stage fermentation process with temperature shift from 27-32°C is suggested to optimize cell growth and polysaccharide production, respectively at each stage and to improve overall polysaccharide formation.61 It has been shown that by using two separate fermentors for the production of the cells and product, the total fermentation time could be reduced by 50%.[62]

FERMENTATION METHODS

In fermentation processes, microorganisms producing polysaccharide is considered as a factory. This factory depends upon three factors, viz., (I) selection of good strain, (2) environmental conditions which includes best medium and fermentation conditions that suit for large scale production of the metabolite, (3) high level sophisticated technology which optimize fermentation conditions and reduce fermentor occupancy period.63

Batch culture

In batch culture one can determine optimal medium, composition and pH. Large number of flasks can be kept under almost identical conditions. In addition to its use in initial assessment of the culture requirements of selected microorganism, batch cultures enable the rapid production of sufficient polysaccharide for examination of physical characteristics, chemical composition and structure. At present, polysaccharides are produced via conventional batch-scale operation. Such fermentations take about 80h with a conversion efficiency of75-80% of carbon substrate.

Continuous culture

In continuous fermentation, continuous addition of fresh medium to the culture is carried out, with simultaneous harvesting of the desired products. The advantage represent more economic manufacturing owing to increased overall productivity of selective operations of the fermentation at the most effective physiological state of the microorganism with regard toproduct formation, in the steady state mode. However, despite this technical information, polysaccharide production by continuous fermentation is not used because of the difficulty of maintaining sterility and culture vigour.[65]

Industrial-scale production

For industrial production of exopolysaccharide each producer uses particular strain, growth conditions and fermentor design. Substrate of less [purity which is readily obtainable and utilized by microorganism without affecting growth, yield and properties of the polysaccharide are usually used. For e.g., X. campestris can use wide range of carbon substrates like glucose, sucrose and acid whey.66 It is feasible to use industrial waste as carbon substrate since it is cheaper and yields obtained are comparable.

BIOSYNTHESIS OF MICROBIAL EXOPOL YSACCHARIDE

The biosynthesis of microbial exopolysaccharide is divided into two distinct mechanisms. The extra cellular process e.g., formation of dextran and levan (2) the intracellular synthesis. e.g., xanthan.

Extracellular synthesis

The production of dextran depends upon an enzyme dextran sucrase secreted by dextran producing organism which is active outside the bacterial cells.67 It enters

into the culture medium either through secretion or from autolysis of the cells. This trans glycosidase enzyme transfers the D-glycosyl group from a sucrose molecule to an enlarging dextran chain and liberates the D-fructose portion

Intracellular synthesis

Substrate uptake is one of the first step in exopolysaccharide synthesis. Substrates may enter by one of the three mechanisms, i.e., facilitated diffusion/active transport/group translocation.68

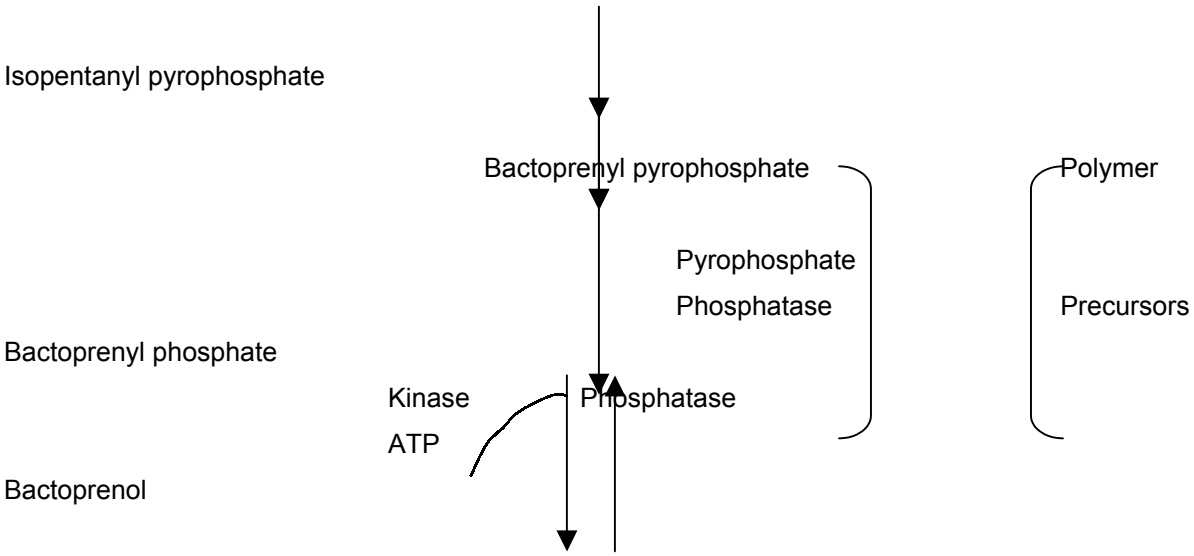
Following the entry of the substrate into the cell, the substrate is phosphorylated by group translocation by hexokinase enzyme utilising ATP. Phosphorylated substrate can enter either catabolic process for cell energy or anabolic process leading to the fonnation of polysaccharide. Utilisation of substrate for polysaccharide synthesis is controlled by regulation of the synthesis 70 and hydrolysis of nucleotide 5-glycosyl diphosphate.71

Formation of exopolysaccharide .

Phosphorylated substrate is converted into monosaccharides, which are the immediate precursors of the repeating units of sugar residues. The construction of the repeating units of the polymer is dependent on transfer of the approximate monosaccharide from sugar nucleotides to a carrier lipid isoprenoid alcohol phosphate to form the basic repeat unit72,73 as shown in flow diagram (Fig 4).

The exact mechanism involved in further elongation of the chain and tile release of the exopolysaccharide are still unknown, but it is suspected that the site of exopolysaccharide biosynthesis lies in the cytoplasmic membrane.74

Fig.4: Formation of microbial exopolysaccharide



Polymer = Exopolysaccharide, lipopolysaccharide, or peptidoglycan

As there is competition for isoprenoid lipid between lipopolysaccharides, peptidoglycan and teichoic acids, the availability of isoprenoid lipid phosphate is one of the most critical factors affecting the synthesis of exopolysaccharide. 75

Many polysaccharides contain acyl, acetyl and pyruvyl substituents. The proportion of these groups in exopolysaccharide is variable and as the properties of these polymers can also vary with their substitute groups 76, more attention has been given to factors governing the attachment of these groups. Pyruvic acid acetal residues in xanthan are formed by transfer from phosphoenol pyruvate to the pentasaccharide-p-p-lipid.

The regulatory mechanisms controlling the release of polysaccharide from the cell and size of the polymer are unknown, presumably, some specific enzymes are present which leaves the terminal monosaccharide residue from the isoprenoid alcohol phosphate, to allow return of the carrier lipid for further polymer synthesis. It has been suggested that the chain length (molecular weight) of microbial polysaccharides may depend upon the growth rate of the microorganisms. In particular, a higher growth rate may lead to more rapid turn over of the carrier lipid and therefore earlier release of the polymer with a shorter chain length.

TREATMENT OF POLYSACCHARIDES

Polysaccharides obtained by fermentations are treated by different methods to get the desired end products.

Microbial cell removal

Cell removal from the polysaccharide is essential as many food, cosmetic, pharmaceutical industry require optically clear gum. Cells from broth can be readily removed by filtration where fungi are employed for polysaccharide production because of their large size. It is not easy to remove bacterial cells by filtration 77.

Physical method of purification

The separation of polysaccharide from fermentation broth can be achieved by dilution and centrifugation or ultracentrifugation or ultrafiltration. Further separation of polysaccharide from low molecular weight carbohydrates and inorganic salts can be achieved by dialysis. For maximum purity, ion exchange or affinity chromatography could be used. The process is highly expensive and time consuming.

Chemical methods of purification

Solvent precipitation: Polysaccharide can be precipitated by lowering the solubility of the polymer either by addition of a water miscible solvent, i.e., methanol, ethanol, isopropanol, acetone or by addition of salt (KCI) and acid (poly hexamethylene biguanide HCI) 78.

Salt precipitation: Multivalent cations such as calcium can also precipitate polysaccharide from culture broths. Xanthan can be precipitated by the addition of calcium salt at pH 10.0. But further removal of salt from polysaccharide is difficult79.

Recovery with quaternary ammonium compounds: Long chain quaternary ammonium salts have been proposed as polysaccharide precipitants. This process requires essentially complete recovery of the relatively expensive quaternary ammonium salt.

Spray drying: Spray dried preparations can be made directly from culture filtrates wherein large amounts of salts and some unused medium components are also present. Spray dried material can also be obtained by drying ion free broths[80].

Enzymatic method

Removal of bacteria can be achieved through the use of proteolytic and cell lytic enzymes obtained from microbial source such as Bacillu.5 subtilis, Aspergillus niger, Pellicularia filamentoso and Trichoderma viride which will lead to lysis of bacteria. Alkaline protease obtained from Bacillus subtilis can hydrolyse the cells of X.campestris81. An enzyme mixture containing protease and p (1->3)glucanase obtained from Pellicularia filamentosa and Pellicularia sasaki have also been employed in the clarification of xanthan solution82.

After the initial process of xanthan fermentation, a second fermentation is carried out under acidic conditions with a fungus such as Trichoderma viride which is capable of consuming the X campestris cells. Since the fungal cells of Trichoderma viride are larger, it was easier to filter out the fungal biomass than the microbial cells of X campestris. By this method more efficient clarification of the fermentation broth could be achieved[83,86].

RHEOLOGY OF POLYSACCHARIDE GUMS[I78]

The study of the flow behaviour of viscous aqueous solution including that of polysaccharide gum is called rheology. The polysaccharides are used because of their ability to thicken or to gel. All gums have a thickening viscosity producing effect, but only a few of them have the capacity to form gels, There is a close relationship between the viscosity and gelling characteristics of many gums. Some gums can be used as thickening agent at lower concentration and as a gelling agent at higher concentration.

The degree of thickening varies among gums, some gums at higher concentration give low viscosity, but most of the gums at very low concentration give high viscosity. The type of viscosity of gums varies form one another.

All common viscous solutions can be described by six basic rheological systems (Fig,5): (I) In Newtonian system, the shear stress is directly proportional to the rate of shear. For example, solutions of small molecules such as sugar syrups. Many polysaccharide gums at very low concentration and viscosity exhibit Newtonian behaviour. In Non-Newtonian system, the viscosity of the polysaccharide changes as the shear

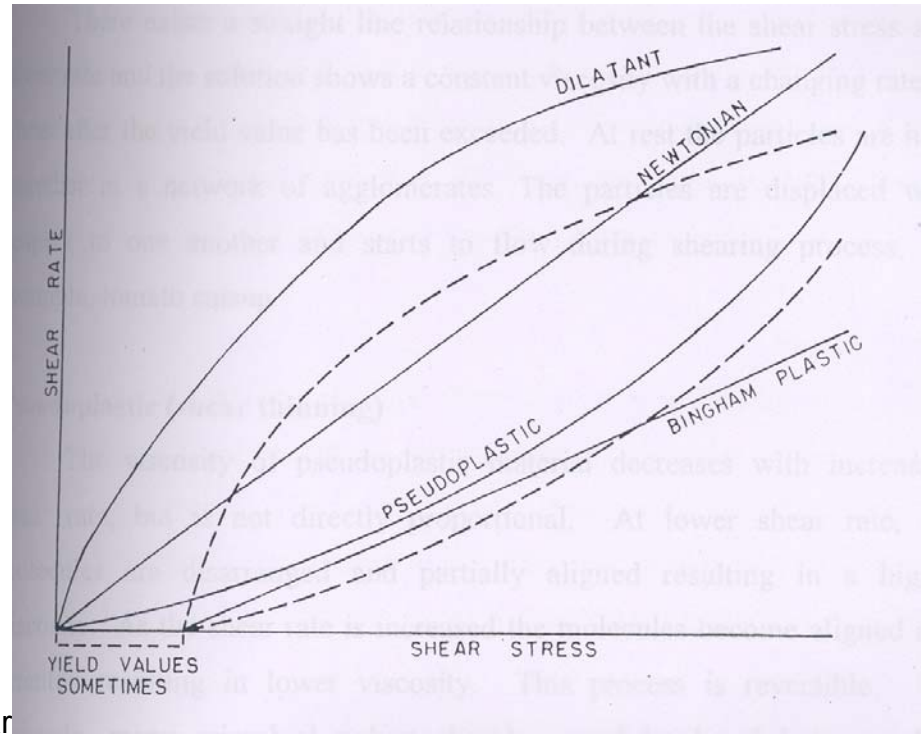


Fig.5: Basic rheological systems
(Ref: Glickman, M., 1982)[178]

rate changes. Non-Newtonian systems are: Bingham plastic, pseudoplastic, thixotrophic, dilatant and rheopectic;

Bingham plastic

There exists a straight line relationship between the shear stress and shear rate and the solution shows a constant viscosity with a changing rate of shear after the yield value has been exceeded. At rest the particles are held together in a network of agglomerates. The particles are displaced with respect to one another and starts to flow during shearing process, for example, tomato catsup.

Pseudo plastic (shear thinning)

The viscosity of pseudoplastic material decreases with increasing shear rate, but is not directly proportional. At lower shear rate, the molecules are disarranged and partially aligned resulting in a higher viscosity. As the shear rate is increased the molecules become aligned and oriented resulting in ,lower viscosity. This process is reversible. For example, many microbial polysaccharides, work-hardened butter, gelled desserts and pudding.

Dilatancy

The viscosity of dilatant materials increases with an increasing shear rate and reaches the point where the fluid becomes solid. At rest, the constituent are most closely packed. As the shear force is applied, flow commences, the particles in the system separate. The voids between them.32 are enlarged and filled immediately with the continuous liquid phase, covers he particles and dry out. Example: behaviour of sand at seashore.

Thixotropic flow (To change by the touch)

This system is time dependent. The viscosity of thixotropic material iecreases by agitation and regains to its original viscosity after a period of rest. This type of system shows a hysteresis effect in its flow curve which is obtained by increasing the rate of shear and then without stopping, decreasing the rate. Example: mayonnaise, catsup, sauces.

Rheopexy

The viscosity of rheopectic material increases with increasing shear. The system is shear thickening and the structure solidifies as the shear rate/shear force increases. This is reverse manner to thixotropy and with time, the system will regain its original liquid state if a change of phase/chemical change has not taken place. Example: beating of egg white/whipping creams.

Parameters affecting rheological flow

The viscosity and concentration, are almost interdependent. At low concentrations, some gums will show Newtonian behaviour but as the gum concentration increases the rheological property becomes Non-Newtonian.

Temperature: Majority of gums are Newtonian at low concentration, the effect of increase in temperature can bring change in the flow property from non-Newtonian to Newtonian.

Molecular weight: The molecular weight distribution of the polymer is one of the important factors which affects rheological flow. Non-Newtonian behaviour has been shown to occur only when the polymer chains are long enough to entangle and when molecular weight is of critical size.

GELATION[178]

Graham was the first person to coin the word “gel”. Gelation occurs because of the association/cross linking of long polymer chains to form a 3-dimensional continuous network which traps and immobilises the liquid within it to form a firm, rigid structure that is resistant to flow under pressure.

Mechanism of gel formation

Initially there is a gradual decrease in brownian movement of the colloidal particles which is caused by the exertion of long-range forces between the molecules. This results in hydration and coherence of the particles. The viscosity increases as gelation proceeds and the solvent is absorbed by the swelling solute and is gradually immobilised. As the process continues, a three dimensional network containing enmeshed portions of the liquid is gradually built up. The various fragments of the gelling polymer continue to react and finally form one large continuous structure [86].

Different types of gelling mechanisms

Gelatin is a protein extract having distinctive elastic property. It is used as a gelling agent which forms aqueous gels with water at any pH and without the need of additive cations or sugars. It is a thermoreversible gel.

The phenomenon of gelatin gelation comprises of a series of events having distinctive characteristics. Upon cooling a gelatin solution, it partially forms aggregates which link together to form a weak network gel. On further cooling by keeping the temperature constant, the strength of the gel increases. If the gel is heated, it melts to forms a solution containing the gelatin aggregates which then separate into individual gelatin molecules.

Mechanism

As shown in Fig.6, in gelatin the polypeptide chains link to form 5/6 crystalline regions/molecule. These principal cross-links probably form salt bonds between the amino and carboxyl groups of side chains. In between the links/strands, the molecule can move and give a flexlibility to the gel. These primary bonding links are stabilized further by secondary (HV bonding between the peptide groups with or without the participation of water. In addition, some disulfide cross-linkages may also occur since the gel melts so readily, it is believed that the links in the network involve only secondary forces and not covalent bond.

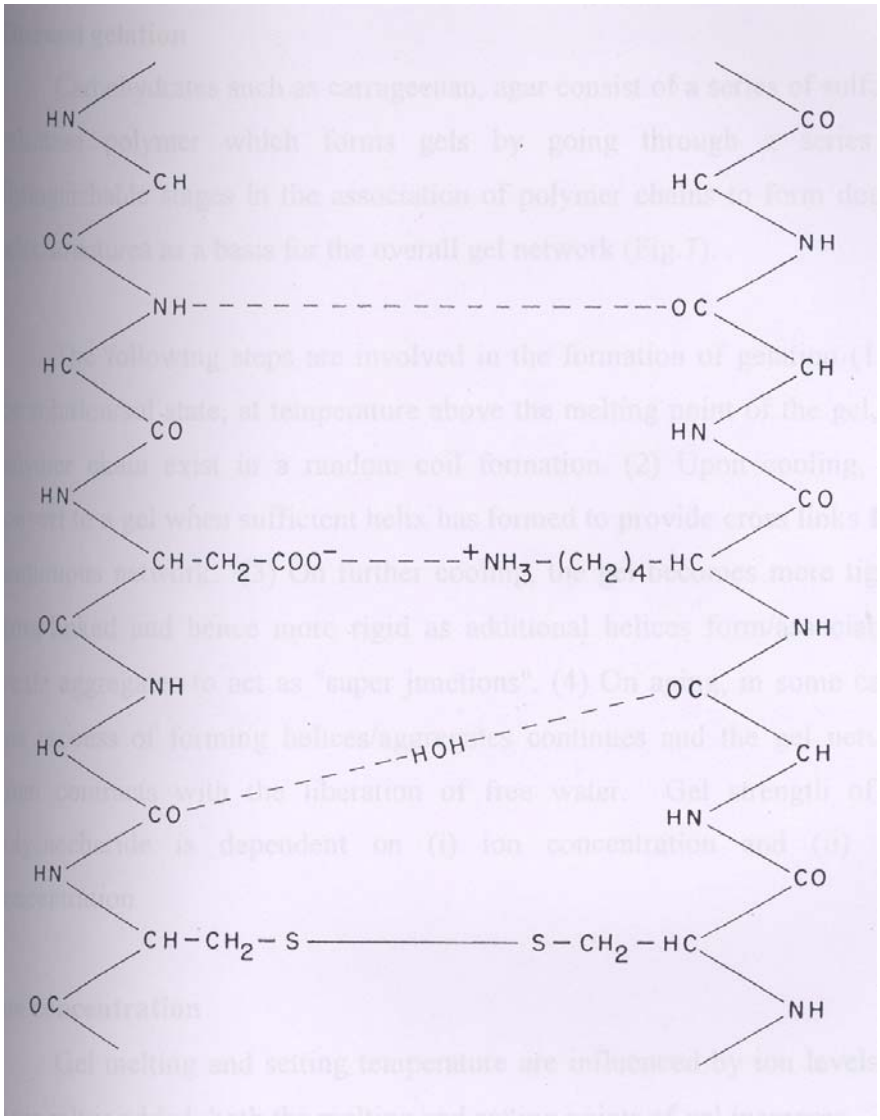


Fig.6: Types of bonding in gelatin gelation

(Ref: Glicksman, M., 1982)[178]

Thermal gelation

Carbohydrates such as carrageenan, agar consist of a series of sulfated galactose polymer which forms gels by going through a series of distinguishable stages in the association of polymer chains to form double helix structures as a basis for the overall gel network (Fig.7).

The following steps are involved in the formation of gelation (I) In the solution/sol state, at temperature above the melting point of the gel, the polymer chain exist in a random coil formation. (2) Upon cooling, this convert to a gel when sufficient helix has formed to provide cross links for a continuous network. (3) On further cooling, the gel becomes more tightly cross-linked and hence more rigid as additional helices form/associate to create aggregates to act as "super junctions". (4) On aging, in some cases, this process of forming helices/aggregates continues and the gel network often contracts with the liberation of free water. Gel strength of the polysaccharide is dependent on (i) ion concentration and (ji) gum concentration.

Ion concentration

Gel melting and setting temperature are influenced by ion levels. As more salt is added, both the melting and setting points of gel increases. With salt levels below those required for optimal gel strength, the gel melts below 100°C. With higher salt levels, the gels do not remelt even after autoclaving. Setting temperature of gels is usually in the range of 30-40°C. In general

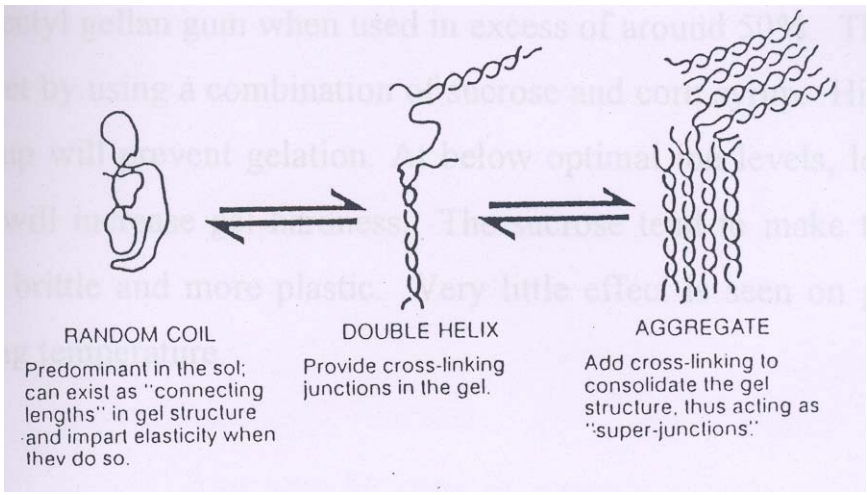


Fig.7: Gel formation by polysaccharide aggregation

(Ref: Glicksman, m., 1982)

monovalent cations give gels with high setting temperature than those prepared with divalent ions.

Sucrose

Low molecular hydrophilic materials such as sucrose will prevent gelation of low acetyl gellan gum when used in excess of around 50%. This effect can be offset by using a combination of sucrose and corn syrup. High level of corn syrup will prevent gelation. At below optimal ion levels, low level of sucrose will increase gel hardness. The sucrose tend to make the gels slightly less brittle and more plastic. Very little effect is seen on gel melting and setting temperature.

pH

Low acetyl gellan gum are fairly stable between pH 3.5-8. Changes in pH, effects melting temperature of gel. Gel prepared with low levels of monovalent ions melt around 70°C. At pH 3.5, the melting temperature is slightly increased. The same trend is not seen for the divalent ions/higher levels of monovalent ions.

Gum concentration

For a given gum concentration, hardness increases through a maximum as ionic strength is increased and similar hardness is obtained with different ions. Divalent ions "calcium and magnesium" provide maximum gel hardness at molar concentrations around one twenty fifth of those required for maximum gel hardness. With the monovalent ions Na

and K, maximum gel hardness is obtained at approximately the same ionic strength and gel hardness increases with gum concentration.

STRUCTURE AND COMPOSITION OF EXOPOLYSACCHARIDE

From the literature reports it is clear that the number of monosaccharide component present in polysaccharide is fairly limited. D-Glucose, D-galactose, D-mannose, L-fucose, L-rhamnose and D-glucuronic acid are most common sugars present in polysaccharide. In addition to sugars, acyl groups such as a-acetyl and pyruvate ketals, succinic acid and inorganic groups of sulphate/phosphate are present in polysaccharide.

Microbial polysaccharides consists of long chains of monosaccharides, ranging from one hundred to many thousands of units. The molecular weights usually range from 30,000-4,000,00,000 Da.

Although relatively a few monosaccharide types are commonly found in microbial polysaccharides, the various permutations available for glycosidic linkage between the monomer units is large, when the further variations of sugar conformation and glycosidic linkage configuration are considered, it is clear that the variety of possible structures for carbohydrate polymer is enormous.

Based on chemical structure, polysaccharides secreted by microorganisms can be divided into homopolysaccharides and heteropolysaccharides. Homopolysaccharide consisting of a single structural unit can be divided into (1) linear homopolysaccharide (bacterial cellulose) and (2) branched homopolysaccharide (levan). Most of the heteropolysaccharides are composed of an oligosaccharide repeating unit varying in complexity from a disaccharide to a hexasaccharide and rarely larger than an octasaccharide. Usually the oligosaccharide from which these polysaccharides are constructed possess a short side chain but the essential feature of the molecule is its regular repeating structure.

Alginates differ nom other heteropolysaccharides in their lack of a regular structure. Alginates are formed nom two-structural units, D mannuronic acid and L-glucuronic acid. They are composed of (a) Blocks of 1-4 linked □-D-mannuronic acid; (b) Blocks

of 1-4 linked □-L-glucuronic acid and (c) Mixed blocks of alternating mannuronic acid and glucuronic acid residues.

Relation of properties to polymer structure

Structural properties of polysaccharide was studied only to know the chemical sequence of it. Increase in demand for polysaccharide has created tile need for explanation on the solution properties at molecular level. There are various physical methods such as NMR, CD, DSC and OR to determine order-disorder transitions of polysaccharide in solution. These techniques correlate not only to the solution properties of polysaccharide with their molecular structure but also helps in screening of new polysaccharide for potential useful properties.

Xanthan is composed of a cellulose backbone with trisaccharide branches of alternating D-glucopyranosyl unit. In both dissolved and solid states, the branches appeared to be aligned with the main chain and provide a high degree of rigidity to a single or multi stranded right-handed helix. The stable complex between trisaccharides stubs and the main chain appears, to result in a shielding of the glycosidic linkages to provide extraordinary stability to strongly acid and alkaline conditions and enzymatic degradation by preventing the formation of enzyme-substrate complexes.

Gellan gum is a linear polysaccharide with a tetrasaccharide repeating unit. Welan has the same repeating unit as gellan but with a single glycosyl side chain substitute,90. The side chain unit can be either an □-L-rhamnopyranosyl/ a-Lmannopyranosyl unit linked (1->3) to the 4-0 sllbstituted □-D-glucopyranosyl unit in the backbone[91]. The presence of this ring unit side chain transfonns the properties from a gelling polysaccharide to a polysaccharide having excellent compatibility with divalent cations at temperature up to 135^{o}C.

Inter molecular association among polymer chains are known to result in the formation of a complex network of entangled rod like molecules. These weakly bound aggregates are progressively disrupted under the influence of shear, thus resulting in the pronounced pseudoplasticity of xanthan solutions. The network of

entangled stiff molecules accounts for the high yield value of xanthan, which gives rise to its suspending power for particles and emulsions. A low concentration of electrolyte appears to stabilize the helical conformation of xanthan, reducing the electrostatic repulsion between carboxylate anions.

Rhamsan has the same repeating back bone unit as that of gellan but with a disaccharide side chain on 0-6 of the 3-0 substituted β-D-gluco-pyranosyl unit. The side chain is a β-D-glucopyransyl (1->6) a-D-glucopyranosyl unit92. Rhamsan has similar rheological properties but because of some difference in its stability and compatibility it finds different industrial application[93,94]. A fourth member of the same series, S-88 has a similar repeat unit and different properties because of small differences, in the side chain[95].

Solubility of polysaccharide

The physical property of polysaccharide are derived from the interaction of polysaccharide molecules between themselves and with the water molecules[96]. For example, structurally, linear molecules will dissolve slowly compared to branched molecules but will form solutions at equal concentrations of branched molecules having same molecular weight. Linear molecules gyrate in solution, they sweep out greater volumes of space. It gives higher viscosity even at low concentrations of additive.

Branched molecules will dissolve readily to form solution. They entangle if the concentration is high enough which results in 'gel'. Extensive association is not possible for the bush like molecules. These molecules are stable and do not undergo spontaneous precipitation, partially because of entangling with one another in solution. Branched polysaccharides can produce tacky pastes if concentrations are sufficiently high. When solutions of branched polysaccharides are dried, the molecules still do not form strong il1termolecular association, hence if water is again added to the dry coating, the polysaccharide easily redissolves/reconstitutes, softening quickly to a tacky layer. Many of the branched polysaccharides are used as re-moistening gums/adhesives. Highly branched polysaccharide form brittle films and have no application as self supporting sheets.

Chemical modification

The solubility of linear molecules can obviously be improved by changes in the molecule that would reduce or prevent the fit by introducing charges which by Coulombic repulsion would facilitate solubility of the molecules and while in solution prevent their extensive attraction resulting ill a precipitation. When the cellulose molecules are derivatized to make carboxymethyl cellulose/ methylcellulose, the product is water soluble because the substituent groups fit between chains and prevent association.

Pseudo plastic rheology

Most of the polysaccharides in solution show pseudoplastic nature. The viscosity of the polysaccharide gum decreases with increasing shear rate, but is not directly proportional. As the shear rate is increased, the molecules become oriented and aligned, thus resulting in a decrease of inner friction indicating lower viscosity. This process is reversible as the molecules disorient themselves after the shearing force is stopped, and the system returns to its original viscosity. Similarly, as the shear rate is decreased, the molecules are disarranged and only partially aligned resulting in higher viscosity.

GENERAL FUNCTIONS AND APPLICATION OF POL YSACCHA-RIDE GUMS

Polysaccharides have many functional properties that make them useful in food industrial applications. These properties vary from adhesiveness to whippability and are all derived from the basic major prosperty of (a) Viscosity or thickening and (b) Gelling. The degree of thickening varies among polysaccharides, with a few polysaccharides giving low viscosities at fairly high concentrations, but most polysaccharides giving high viscosity at very low concentrations, usually lower than 1 %. The flow behaviour or rheology of gum solutions, as well as liquid food systems, can be related to the organoleptic properties of the solution and there by to the acceptability of the product. In thjs way the rheological characteristics of a gum solution are useful and to the selection of the proper gums for a specific food or industrial application.

Dietary fibre (dietetic food)

Pullulan can be used as a low-calorie food ingredient in bakery products such as artificial rice noodles by replacing a portion of wheat flour/starch as it can be slightly depolymerised by digestjve enzymes.

Pullulan and scleroglucan, are versatile, oil resistant, antistatic, transparent, polysaccharides which are readily soluble in water and have low permeability. Pullulan is used in packaging of coffee, soup, curry /soy sauces powder, freeze dried vegetables etc. It is used in packaging of meat to preserve or retain flavour/ appearance for a prolonged period and contents can be cooked without opening the wrapping of package.[98,99,100].

Binding agent

Xanthan is combined with locust bean gum and other gel forming components to produce homogenous gelled productsl01. In canned gravy-based pet foods it prevents undesirable gelation 102. In fermented protein-based pet foods it acts as a gellable binder[l03].

Adhesive

Pullulan is used in food pastes as binder for the preparation of novel snack foods in sticks/sheets made from fish, beef/pork. [104]

Crystallization inhibitor

Xanthan and dextran are used in ice cream to minimize the growth of ice crystals and to obtain high quality product. In bakery icings however it is desirable to prevent the disappearance of the visible sugar crystals by dissolution into the water phase of the icing system.[105]

Thickening agent

Xanthan and pullulan can build up viscosity in dry mix products such as desserts, gravies, sauces, beverages and dressings as they are easily dispersed in either hot or cold water[109]

Clarifying agent

Sodium alginate and carrageenan assist the process of clarification by coagulating soluble proteins in the beer which would otherwise stabilise the suspension of yeast

and other particulate matter[110]. This suspending material can then be sedimented using traditional fining agents such as icinglass.

Clouding agent

Xanthan and dextran are used as clouding agents for fruit flavoured dry beverage mixes to provide the opacity and mouth-feel of natural fruit juices. In beverages they contribute pleasing mouth-feel, rapid and complete solubility at low pH, excellent suspension of insoluble ingredients and compatibility with a broad range of components including alcohol [111].

Suspending agent

Carrageenan and dextran when used in pet chocolate milk, interact with milk proteins and form gel network in the product which keeps the cocoa particles suspended but which is not apparent on pouring or in the mouth. [112]

Encapsulating agent

Carrageenan and alginate are used to encapsulate flavours for inclusion in cake mixes, chewing gum, confections, soluble coffee and others. Encapsulation of oil and fat components for dried soups and salad dressings were considered as a method for separating reactive ingredients in dry mix products.

Foam stabilization

Dextran, agar and carrageenan are used in dairy products where the butter and milk protein have been completely replaced by vegetable oil and non-milk protein. It is necessary to use an emulsifier to disperse the fat and a stabilizer to stabilize the foam system.

Syrlergistic effect

In synergistic phenomenon, mixture of two or more gums are used. In ice cream, the primary stabilizer, locust bean gum performs as a thickening or water holding agent, while a comparatively small amount of the secondary stabilizer such as xanthan serves to prevent the tendency of the whey to separate in the mix.

Emulsifier

Xanthan is known to be an extraordinary effective emulsion stabilizer.[112] Dressings containing 0.25-0.3% xanthan gum are shelf stable and eating qualities are enhanced by the clean mouth-feel imparted by xanthan gum. High degree of pseudoplasticity, also keeps the dressing on the top of salad. Freeze and thaw stable spoonable dressing formulations can be prepared using 0.25-0.3% xanthan gum along with starch.

Fabricated/restricted food

Artificial cherries are prepared by dropping colored and flavored alginate sugar solution droplets into a soluble calcium salt coagulating solution. An insoluble calcium alginate skin is formed immediately around the surface of the droplet and upon soaking in the calcium salt bath, the calcium ions would diffuse into the droplet to gel the interior/core to form "Molded cherry balls".

OTHER APPLICATIONS

Enhanced oil recovery

Xanthan is used as mobility control agent in enhanced oil recovery with the aim of increasing sweep efficiency. The polymer solution displaces oil which would otherwise remain in situ, being by passed by injected water flood. The basic requirement is for a polymer yielding a high viscosity solution, minimally affected by high salt concentration, high temperature, high pressure and the high shear forces expected in pumping operations.

Paper industry

Scleroglucan is used as binder in paper industry to give greater strength or as coating to provide a smooth, glossy surface. [114]

Textile printing and dying

In textile printing, addition of xanthan pastes as thickeners, controls the rheological properties of the paste during application and to produce sharp, clean patterns by preventing migration of the dye. The quality of the finished print is dependent on control of dye migration.

Cleaners

Xanthan can be formulated in cleaners as it gives good viscosity, stability in strong acids and alkalies. The pseudoplasticity of gum solutions produce cleaners that have high viscosity at low shear rates, allowing the cleaners to cling to inclined surfaces for extended period of time.

Explosives

Xanthan is used to thicken and to gel blasting agents and explosive slurries. The gum is also used as water blocking agent in dry cartridge explosives.

Ceramic

Scleroglucan and xanthan are used in water based paints, printing inks, porcelain and ceramic glazes, extruded refractory products, liquid animal feed concentrates and as ceramic binder.

Cosmetics

Scleroglucan and xanthan, may be used in formulations for hair sprays, various skin care preparations, creams, protective lotions, emollients,

demulcents and antisoilants, The polysaccharides increase the viscosity, assist in imparting spreading, add a smooth feel, to the skin and form a protective coating.[113,114]

Pharmaceutical

Scleroglucan and pullulan are used in tablet coatings, ophthalmic solutions, injectable antibiotic suspensions and calamine lotions,[I13,114]

Agriculture

Scleroglucan is used as anti-settling agent for photosanitary products, it facilities the preparation of spraying mixtures and particularly improves contact of the droplets sprayed onto leaves, It may also be used in pesticide, defoliate sprays and seed coatings.[I14]

Agar substitute

Gellan gum is used in agricultural application pertaining to plant tissue culture where the concentration needed is only 1/4 that of agar, The clear and transparent gellan gum facilitates observation and photography of root growth and detection of microbial contamination gellan gum can also be used as agar substitute in various microbiological media preparation [15,87,122].

OBJECTIVES AND SCOPE OF INVESTIGATION

OBJECTIVES AND SCOPE OF INVESTIGATION

Polysaccharides obtained from plants and seaweeds make up most significant quantity of the natural gums of commercial importance. A number of microbial polysaccharides such as dextran, xanthan, gellan etc., with varied properties have been discovered or commercialised.

Properties that make a microbial polysaccharide commercially attractive which are not found in other gums are

1. Compatibility with a number of salts
2. High viscosity
3. Gelling properties
4. Stability over wide range of temperature and pH
5. Synergism with other gums
6. Uniformity in the product quality, etc

With regard to microbial polysaccharides it is possible to work out productive cost through economic production, down stream processing for their use as a food or non food additive.

Gelling polysaccharides such as agar, alginate etc., are obtained from seaweeds. Production of these polysaccharides are localised to certain areas, the yields are fluctuating and based on environmental conditions, the collection and extraction are labour intensive. In contrast to this, gelling polysaccharide can also be produced by microorganisms having diversified rheology, structure and functions, the quality and quantity of which can be maintained by fermentation techniques. Species of Bacillus, Pseudomonas,Alcaligenes, Arthrobacter, Rhizobium, Enterobacter etc., can also produce gelling polysaccharides. Gellan produced by Pseudomonas elodea is currently the only microbial polymer which is commercially produced and considered as an agar substitute. The gel strength of gellan is reported to be greater than that of agar. But this gum requires monovalent or divalent cations for the promotion of gelation. Because of the diversity found amongst the microorganisms, there is still scope for developing new polysaccharides with better production or properties superior to those of the existing polysaccharides.

The objectives of the present work were:

1. Isolation of microorganisms (producing polysaccharides) from soil, water, decaying materials.
2. Screening of isolated pure cultures for production polysaccharide.
3. Identification and characterization of the promising culture.
4. Basic studies on the nutrition and culture conditions and optimization of these conditions for optimum polysaccharide production.
5. Studies on isolation of the polysaccharide and its physico-chemical and structural characters.
6. Utilization of the polysaccharide (as agar substitute in microbiological media emulisifier, thickening agent).

Materials and Methods (General)

MATERIALS

The materials used in the study are listed here. This includes media ingredients, ready made media, organic and inorganic chemical substances and solvents.

Following materials such as media ingredients, ready made media, carbohydrates, aminoacids, dyes were obtained from Himedia laboratories Mumbai, India.

Media ingredients

Malt extract, Yeast extract, peptone, meat extract, beef extract, casein, agar, tryptone.

Ready made media

Potato dextrose agar, Nutrient agar.

Carbohydrates

Dextrin, glucose, cellulose, mannose, sorbose, sorbitol, mannitol, maltose, trehalose, sucrose, lactose, xylan, xylose, arabinose, melizitose.

Amino acids

Lysine, □-cysteine HCl, cystine, alanine, asparagine, phenylalanine, arginine, proline, valine, leucine, methionine, aspartic acid, threonine, tryptophan, histidine, glutamic acid.

Dyes: Bromocresol purple, phenol red.

Solvents: Isopropanol (SRL, India), ethanol.

Chcmicals (A.R., SRL, India):

Sucrose, NH_4C1, NH_4NO_3, urea, $(NH_4)_2S0_4$, Na_2HPO_4, KH_2PO_4, K_2HPO_4, K_2SO_4, NaCI, $CaCl_2$ $2H_2O$, $MgSO_4$ $7H_2O$, KNO_3, $NaNO_3$, sodiumthiosulfate, lactose. Other materials used are listed in the respective chapters.

METHODS

General methods used during experimentations are given here. Specific methods are dealt with in the respective chapters.

Cultivation methods

Sterilization of media

Media used in various experiments were sterilized in flasks or tubes plugged with absorbent cotton, at 15 lbs pressure and 121°C for 20 minutes.

Glass ware sterilization

Glass wares such as petriplates, flasks, test tubes and pipettes were sterilized by dry heat at 180°C for 1 Y2hours, pipettes were lightly plugged at pipetting end with cotton and placed in copper container for sterilization.

Maintenance of cultures

Isolated bacteria were streaked on slants of malt extract yeast extract agar, incubated at 30°C for 24h and then refrigerated at 4°C for further use, Subculturing was done once in a month.

For long time storage, actively growing cells were collected under sterile conditions after centrifugation of culture broth, suspended in sterile skim milk and lyophilized in vials, sealed and stored (4°C).

Inoculum

Actively growing cells from a freshly prepared slant culture (24h) were transfered to 100ml sterile inoculum medium contained in 500ml Erlenmeyer flasks. Flasks were placed on rotary shaker (250rpm) at ambient temperature of 28° -30°C for 24h and used.

Cultivation of bacteria

Inoculated flasks were incubated on rotary shaker (250rpm) upto 120h at ambient temperature of 28°-30°C.

Analytical Methods

Measurement of pH

The pH of medium or broth was measured using a pH meter (Control Dynamics Ltd. Bangalore).

Measurement of viscosity

Viscosity of fermented broth was measured using Brookfield meter (HBT model, USA). Unless otherwise mentioned all the readings were taken with spindle 2 at speed 20. The reading obtained was multiplied with a factor of 160 to express the viscosity in centipoise (cPs) with that spindle and speed.

Estimation of polysaccharides

Polysaccharides present in the fermented broth was precipitated using 2.5 volumes of absolute ethanol or isopropanol. Polysaccharides which separated along with biomass as a cottony mass was passed through Whatman No.1 filter paper circle of known weight. Material was dried to a constant weight at 75^{o}C. The crude polysaccharide weight was represented as g% (w/v) of fermented broth.

For the estimation of pure polysaccharide, the broth was diluted with distilled water in I: 1 0 ratio and homogenized with homogeniser at 8000 rpm (Universal motor, RQ-127, Remi motors, Bombay) for 5min: The mixture was then centrifuged (Sorvall, RC-SB; USA) at 10,000rpm for 60min. The clarified supernatant was mixed with 2.S volumes of absolute ethanol or isopropanol to precipitate the polysaccharides. The polysaccharide was rehydrated, homogenised, centrifuged and reprecipitated, three

more times and dried to a constant weight (at 70°C). The preparation was free from biomass.

Estimation of biomass

Biomass was sedimented from diluted broth by centrifugation as mentioned above. The sedimented biomass was washed with water by recentrifugation and was finally transferred onto a previously weighed aluminum foil. The material was dried at 70°C to a constant weight. Weight of biomass was represented as g% (w/v) of fermented broth.

Estimation of residual sugar

Residual sugar present in the fermented broth was estimated by DNS method8. For this, sucrose present in the broth was inverted by the addition of 10 ml of 1:1 HCI to 50ml of diluted broth. The mixture was set aside overnight for hydrolysis. The pH of the hydrolysate was adjusted to 7.0 by using 40% NaOH solution and diluted to 100ml in a volumetric flask. One ml of this sample was used further with or without dilution for the estimation of reducing sugar.

Reagent: 100ml of reagent (ONS) contained 1.0g of 3.5-dinitrosalicylic acid, 20ml of 2N NaOH and 30g of Na-K-tartarte. For the estimation of reducing sugar 2ml of ONS reagent was added to 1 ml of t sample, the tube was kept in boiling water bath for 5min. The tube was cooled and 20ml of distilled water was added. The orange yellow color developed was measured at 540 nm in spectrophotometer (Shimadzu, UV 160, Japan) against reagent blank. Glucose was used as standard. Sensitivity of the method is upto 2000 g.

Total cell counts

Total number of cells present in the samples were calculated by using haemocytometer (Neubauer, Hellige, Germany). It had 16 chambers with 25 in each chamber. Total number of squares were 400 and each square measured 1/400 mm^2. Overall area of the chamber was 1 mm^2 and depth and it could hold 0.001 cc of sample. Diluted sample was placed on haemocytometer and total number of cell counts was taken through microscope. Total cell number was calculated by using the formula:

Dilution x No. of cells (in 16 chambers) x 10,000 = No. of cells/ml

Viable plate count

Total plate count or viable cell count of fermenting broth was estimated by plating diluted broth on nutrient medium. One ml of the broth was serially diluted ten times in test tubes containing 9 ml of sterile water and 0.1 ml of 10^{-6}, 10^{-8} and 10^{-10} dilutions were plated in duplicates on nutrient medium contained in petri dishes. Plates were incubated at 30^{o}C for 48-72h and the number of colonies developed were counted. Taking into consideration the dilution factor, the number of cells were calculated and represented as cell forming units (cfu) or viable cell counts/ml.

Estimation of moisture

Moisture was estimated by drying the sample to a constant weight in a previously weighed silica crucible at 105^{o}C.

Estimation of Ash

Sample used for the estimation of moisture was placed in muffle furnace and ashed at 500°C for 3-4h. The silica crucible was cooled in a desicator over fused CaCl2 and weighed. Weight of ash was calculated as g% of the sample used.

Estimation of protein

Protein was estimated using Lowry's method[115]. The procedure followed was.

Reagent-A: 2% Na2CO3 in O.IN NaOH -

Reagent-B: 0.5% CUSO4 2H2O in 1 % sodium or potassium tartarate

Reagent-C: 50ml of A was mixed with 1 ml of B

Reagent-D: Falin and Ciocalteu phenol reagent (AR, 2 normal, SRL, Bombay) was diluted with water (1 ml reagent + 2 ml water).

To 0.5 ml of sample, 5 ml of Lowry's reagent (C) was added and incubated at room temperature for 10 min. Then 0.5 ml of diluted reagent (D) was added and incubated for 20 min. OD at 620 nm was measured against reagent blank. Bovine serum albumin was used as standard (upto120 □g).

Clarification of polysaccharide by physical methods

Filtration

Fermentation broth containing polysaccharide was diluted with water M 1:10 to 1:20 ratio, depending on initial viscosity. The mixture was heated to 80^{o}C on a boiling water bath. The mixture turned watery and was filtered under vacuum while hot through buchner funnel containing Whatman No.3 filter paper pulp of 1 inch thickness. The filtrate was clear and polysaccharide contained in it was precipitated by 2.5 volumes of isopropanol/absolute ethanol. The precipitate was collected and dried at 70°C to a constant weight and powdered.

Centrifugation

Polysaccharide containing broth obtained after fermentation was diluted with distilled water depending on viscosity in 1:10 to 1:20 ratio. Diluted broth was homogenised well up to 8000 rpm for 5 min using Universal Homogenizer (Remi motors, RQ-127, Bombay, India). Biomass was removed by centrifugation at 10000 rpm for 60 min (Sorvall Centrifuge, RC-5B, USA). Supernatant hence obtained was free from biomass and was clear.

Preparation of pure polysaccharide for analysis

Polysaccharide present in the above clarified supernatant was further purified by dialysis. The sample was transferred to dialysis bag and the sealed bag was placed in distilled water for dialysis. Water was changed once in 2 h and at the end of 10 h, polysaccharide was precipitated using 2.5 volumes of isopropanol/absolute ethanol. The precipitate which separated, cottony mass was resuspended in triple distilled water, homogenized bifuged and reprecipitated as mentioned above. The process was three more times and the final precipitate was dissolved thoroughly in tripple distilled water and freeze dried (lyophiliser FD-3, Denmark).

Modification of polysaccharide

Polysaccharide broth was diluted with water in 1:1 0 ratio, pH was increased to 10, using 4N NaOH. Temperature of the both was increased to 80^{o}C by keeping the sample in hot water bath and the temperature was maintained for 10 min. Sample was cooled, pH was adjusted to 7 with dilute 804 and the polysaccharide was separated from biomass by filtration as mentioned above. Polysaccharide was

precipitated using 2.5 volumes of isopanol/absolute ethanol. The precipitate was collected and dried to a constant weight at 70°C.

CHAPTER – I

ISOLATION, SCREENING AND CHARACTERIZATION OF POLYSACCHARIDE PRODUCING BACTERIA

1.0 INTRODUCTION

Polysaccharides are ubiquitous, renewable resources, which present a wide range of potential products of use to man due to their unique physical and constant chemical properties. In ancient days, polysaccharides (gums) were obtained from plants and seaweeds by man but later there was a surge in demand because of the various new industrial applications they were put into. However the constant raise in demand could not be met with limited availability and consequently the very high price provoked to obtain polysaccharide by alternative method such as microorganisms. In natural environment such as soil, water and air there are a number of microorganisms which produce different types of polysaccharides which posses varied rheological characters compared to plant polysaccharides. Due to microbial diversity it is always possible to obtain cultures having better properties. Hence attempts were made to isolate gel producing bacteria. This chapter deals with isolation, screening, purification and characterization of bacterium isolated locally that produces gelling polysaccharide.

1.1 MEDIA

Various media were prepared and used for screening of bacterial isolates, culture maintenance, preparation of inoculum and production of polysaccharide. The composition of basal medium for screening and polysaccharide production is as follows.

Basal Medium

Ingredients	g/100ml of distilled water
Sucrose	3.0
Na_2HPO_4	0.5
K_2SO_4	0.1
KH_2PO_4	0.3
$MgSO_4.7H_2O$	0.025
CaCb,$2H_2O$	0.001

The ingredients were solubilized in distilled water and made up to 100 ml. Nitrogen sources used (g% w/v) in basal medium were (a) peptone (0.25g) or (b) tryptone (0.25g) and yeast extract (0.19) or (c) KNO_3 (0.3g). The pH of the medium was

adjusted to 7.0, dispensed in 100ml volumes into 500ml Erlenmeyer flasks and sterilized.

Sterilization

Liquid and solid media used for growth production, characterization and maintenance were sterilized at 15lbs (121°C) for 20min and used.

Inoculum medium

Composition of inoculum medium was similar to basal medium with tryptone and yeast extract but it contained lesser amounts of sucrose (10g/1)

Maintenance medium

Bacterial isolates were maintained on malt extract yeast extract agar (MY A) of following composition:

Ingredients	g/l of distilled water
Yeast extract	15
Malt extract	15
Agar	20

The culture could also be cultivated and maintained on polysaccharide production medium. Final pH of the medium was 7.0 and the medium was dispensed into test tubes, sterilized and allowed to solidify in slanted position.

Gelatin agar

Gelatin (20 g/l) and agar (20 g/l) were added to polysaccharide production medium (basal medium) sterilized in test tubes and used to test growth of bacteria.

Mannitol agar

Polysaccharide production medium containing peptone was substituted with mannitol (2 g/l) and agar (20 g/l) and used for bacterial growth.

Pepone agar

Growth of bacteria was also tested on polysaccharide production medium containing peptone (2.5 g/l) and agar (20 g/l) but sucrose was not added to tile medium.

Nutrient agar

Nutrient agar slants used in experiment were prepared as follows.

Ingredients	g/l distilled water
Peptone	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15
(pH 7.0)	

Potato dextrose agar (PDA)

Bacterial cultures were also grown on PDA in growth experiments. The composition of PDA is mentioned below.

Ingredients	g/l distilled water
Potato (Sliced, boiled till soft and extract used)	200
Glucose	20
Agar	15
(pH 6.0)	

Tripple sugar Iron medium

The medium contained following ingredients:-

Ingredients	g/l distilled water
Casein	10
Peptone	10
Glucose	10
Lactose	10
Sucrose	10

Ferrous ammonium sulphate	0.2
NaCl	5
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	13
pH (7.0)	

METHODS

Isolation of polysaccharide producing bacteria

Soil, water and decaying materials were collected from local areas. Samples (1 g) were dispersed in sterile water (9ml) homogenized and serially diluted 5 times and plated (0.2 ml) on MYA plates. The plates were incubated at 30°C for 24 h. The mucoid colonies were isolated onto MYA slants. Isolated cultures were purified by repeated streaking on MYA contained in petriplate and pure colonies were again transferred on to MYA slants. Microscopic observations were made to confirm purity of culture.

Standard culture

After the identification of test culture as that of Agrobacterium sp. Standard culture of Agrobacterium tumefaciens maintained at Department of food Microbiology, CFTRI, Mysore was used for comparison.

Screening of cultures for polysaccharide production

Inocula of purified cultures were prepared and inoculated (10% v/v) in sterile basal medium (100ml) containing peptone. The flasks were placed on rotary shaker at 250 rpm at ambient temperature of 28°-30°C. After 96h of growth the viscosity of the broths were measured.

Gel test

Cultures which showed viscosity during screening were tested for gel formation. The broth was heated in boiling water bath in the presence of 0.1% CaCl2H2O. Increase in viscosity or gelling after heating and cooling indicated the possible production of gelling polysaccharide and such cultures considered for further work.

Softening of vegetables by bacterial culture

Vegetables such as potato, carrot, beans, tomato and cabbage were sliced, washed with water and held in 70% ethanol (v/v) for 2 min. The vegetables were washed with sterile water and immersed into 0.2% $HgCl_2$ solution for a minute. By these treatments bacterial contaminants present on surface were removed. Residual $HgCl_2$ was washed off thoroughly by using sterile water 5 times and the surface sterilized vegetables were transferred into sterile 250ml Erlenmeyer flasks. The samples were inoculated with 2 ml (20%) suspension of 24h old slant of bacterial test culture and A.tumefaciens. Flasks were incubated at 300c up to 120h. Samples were observed daily for growth and softening of the tissues and degree of softening was compared with uninoculated control.

Phytopathogenicity test

Test culture was inoculated onto injured stems and roots of tomato plants. The plant was allowed to grow in previously sterilized soil for 8 weeks and observed for phytopathological changes.

Nodule formation

Possibility of root nodule formation was tested by inoculating bacterial cells onto roots of leguminous plant such as beans. The plant was grown in previously sterilized soil.

Effect of temperature on the growth

To find out optimum temperature for growth, the cultures were inoculated on MY A (slants) and incubated at 10, 25, 30, 37 and 50°C for

Effect of pH on growth

The effect of medium pH on the growth of bacteria was tested by adjusting pH of polysaccharide production medium containing peptone between 3-11. The inoculated media were incubated at 30°C for 48h and observed for growth.

Characterization of gelling polysaccharide producing culture

Gelling polysaccharide producing strains (Isolate No.PS.18 and PS.19) were characterized by using morphological and biochemical methods as described below116,118.

Growth on various agar media

Various agar media (gelatin agar, nutrient agar, PDA, Mannitol agar MYA, peptone agar and triple sugar Iron agar) slants were streaked with the bacterial culture and allowed to grow at 30°C for 48h.

Gram stain

Reagents

1. Aqueous ammonium oxalate solution (1 %) was prepared and kept overnight. One volume of saturated alcohol solution of crystal violet was added to 4 volumes of ammonium oxalate solution and filtered.
2. Potassium iodide (2 g) was dissolved in 300 ml of water. Iodine (1 g) was solubilized in this KI solution.
3. Ethanol (95%, v/v) was prepared in distilled water.
4. Saturated alcohol solution of safranin (100 ml) was prepared and the volume was made upto 1 litre with distilled water.

Method

A thin smear of the culture was made on a slide and the cells were heat fixed by passing over Bunsen burner, quickly. A drop of a crystal violet reagent was added onto the cells and allowed for 30 sec. Excess of stain was rinsed with water and cells were allowed to react with iodine solution for 30 sec and rinsed again with water. This was followed by 95% ethanol treatment for 10-30 sec. After rinsing the slide with water, safranin solution was added and allowed to act for 10 sec. The slide was washed and then observed under the microscope. Retention of crystal violet indicated that the bacterium was gram positive and uptake of pinkish safranin counter stain by cells was considered gram negative[II6].

Capsular stain

Cells were dried over a slide to which Indian ink was added and then washed off with methanol. Methyl violet (0.5g in 100ml water) was added to the cell smear and allowed to stand for a few seconds. The stain was decanted and the cells were

observed under microscope, where in capsule if present stained pink and bacteria appeared blue[116].

Cell size

Cell size was measured by using occular micrometer (one scale division = 1/10mm (1 00 □) and stage micrometer (scale division= 1/100mm) in the microscope. First the occular micrometer was calibrated by using stage micrometer as follows.

If “a” divisions of scale of the stage micrometer are covered by "b" divisions of the scale of occular micrometer, micrometer value is ax 10/b, e.g,20 division of stage micrometer are covered by 53 of occular micrometer, micrometer value is 20x10/53=3.773, that is each division of the scale of occular micrometer corresponds to 3. 773 □ with the combination of eye piece and stage micrometer in question. The length of object covering 5 divisions of occular micrometer will thus be 3.773 x 5 = 18.865□.

Aerobicity and motility

This was studied in the semisolid medium of Hugh Leifson (HiMedia, Bombay), The medium contained (gil): peptone 2, NaCI 5, K2HPO4 0.3, bromothymol blue, 0.05, glucose 10, agar 2.0 and pH was 7.0. The medium was sterilized in test tube and allowed to cool in an erect position. The agar was stabbed with a loopful of bacterial cells so as to traverse till the bottom of the slant in the center. Another tube was prepared similarly and covered with sterile paraffin oil at the top. The tubes were incubated at 30oc up to 72h, spreading of the growth along the stab and side ways indicated motility. The medium changed color from green to yellow if the organism has the

capacity to ferment glucose. Surface growth on the slant without paraffin oil indicates aerobic nature of culture and presence of growth if present in paraffin oil covered culture would indicate anaerobic nature of cells.

Flagella staining

Reagents: Following reagents were prepared to stain the bacterial flagella

1. Fixative contained a mixture of the ethanol, chloroform and formalin (6:3:1)

2. Mordant was prepared by mixing 20% tannic acid with 5% ferric chloride in the ratio of 3:1 .
3. Silver stain was prepared by adding 10ml of ammonia solution to silver nitrate-containing water.
4 5% sodium thiosulphate was dissolved in 100 ml distilled water.

Growing young cells were held in 3-4 drops of fixative on a glass slide, air dried and washed with distilled water. The sample was covered with mordant for 5-6 min, washed with distilled water and dried. Cells were covered with silver stain for 15-20 min and finally washed with water. The above step was repeated until a golden brown color was observed microscopically. For the above slide 5% sodium thiosulphate was added, washed after 2 min and observed until golden brown/black colored flagella visible under mi croscope[117].

Staining for poly-□-hydroxybutyrate

Reagents

Following reagents were prepared to stain bacteria for any possible presence of poly-□-hydroxybutyrate.

1. 0.3g of Sudan black was dissolved in 100ml Ethylene glycol.
2. 0.5% aqueous safran in was prepared in distilled water. Heat fixed film of the specimen present on the slide was immersed in solution of sudan black for 20min. The slide was washed and counter stained for 5-10 sec with aqueous safranin. Finally the slide was washed, blot dried and examined macroscopically. Presence of blackish granules in the cells is the positive test for the presence of poly-□-hydroxybutyrate[116].

Nitrate reduction test

Reagents

Solution A: 1-Napthyl-ethylenediamine diHCl (0.02g) was dissolved in 100ml 1.5N HCl solution by gentle heating in a fumehood.

Solution B: Sulfanilic acid (1 g) was dissolved in 100ml of 1.5NHCl by gentle heating in a fumehood.

Procedure: Basal medium was supplemented with 0.1% potassium nitrate and 0.17% agar, sterilized and inoculated. Cultures were periodically examined for gas bubbles, which were presumptive evidences for denitrification. To 24hrs old culture

1ml of solution A and 1ml of solution B were added. A pink or red color would indicate the presence of nitrite. In the absence of red color formation, the culture would be mixed with 5mg zinc powder per ml of the mixture. If the nitrate in the medium had not been reduced, the zinc would reduce it chemically to nitrite and red color would appear. In the absence of color development the organism is considered to have reduced all the nitrite and denitrification has probably occurred. Control culture grown without nitrite should not exhibit gas [118].

Catalase test

Nutrient agar slant was inoculated with the culture and incubated overnight at 30°C. One ml of 3% H2O2 was trickled down the slant. The slant was examined for evolution of bubbles, the presence of which indicated a positive test[118].

Indole production

Filter paper strip was dipped in a warm saturated solution of oxalic acid and allowed to cool. The strip containing crystals of oxalic acid was dried and inserted aseptically into the inoculated culture tube. The strip was held in position with the help of cotton plug. Pink or red color formation on the strip during the growth of organism indicated positive test for indole production 118.

Urease production

Medium containing (g/l) peptone,1; glucose, 1; NaCl, 5; KH2PO4, 2; yeast extract 0.18; urea, 20; phenol red, 0.012 and agar 20, of pH 6.8 was sterilized in small test tube and the slant was inoculated and incubated. Reddish violet color indicated a positive testl18.

Hydrolysis of gelatin

Nutrient broth was supplemented with 12% gelatin, inoculated and fed at 30°C. The tube was observed for hydrolysis or loosening of solid medium[118].

Citrate utilisation

Medium containing (g/l) sodium citrate, 3; glucose, 0.2; yeast extract, 0.5; phenol red, 0.012; cysteine HCl, 0.1, ferric ammonium chloride, 0.4; KH2PO4, 1; NaC1, 5; sodium thiosulphate, 0.08; and agar 20 of pH 6.8 was sterilized in small test tubes

and slanted. The slant was inoculated and incubated. Development of reddish color indicated a positive reaction[118].

Ammonia production from arginine

Arginine medium was prepared by dissolving (g/l) tryptone, 10; yeast exact, 5; NaCl, 5; and arginine-HCl, 3. The pH was adjusted to 7, sterilized and inoculated with test culture. A medium lacking arginine was prepared similarly and inoculated. After incubation for 2-3 days, the cultures were spot tested with Nessler's solution. Yellow or orange color development compared to control indicated a positive test[l18].

Oxidase test

A filter paper strip was moistened in 1% solution of N, N, N1, N1,- tetramethyl-p-phenylenediamine-dihydrochloride. The growth of the test culture from a slant was placed on the paper. Development of purple color within10 sec was due to positive test for oxidase [118].

Hydrolysis of casein

Sterile skim milk was mixed with an equal volume of sterile double strength nutrient agar medium at 50°C and plated. The plate was streaked with test culture and incubated. Clear zone around the colonies indicated hydrolysis of casein[118].

3-Ketolactose from lactose oxidation

Initially the organism was grown on a medium containing (g/l) yeast exact, 10; glucose, 20; CaCO3, 20 and agar 15. The growth from the slant was transferred onto a plate of lactose agar. The composition of lactose agar medium was (g/l) lactose, 10; yeast extract, 1 and agar 20. After 1-2 days of incubation the plate was flooded with Benedict's reagent. A deposit of yellow ring of cuprous oxide around the growth in 2 h indicated a positive test[118].

Triple sugar iron reaction

The medium, the composition of which is already mentioned earlier was inoculated with test culture. On incubation at 30°C three types of results could be obtained.

(a) Formation of acid butt, alkaline slant (yellow butt, red slant), for fermentation of glucose but not sucrose/lactose; (b) acid butt, acid slant (yellow butt, yellow slant) for fermentation of sucrose and lactose; (c) alkaline butt, alkaline slant (red butt, red slant) where in neither glucose, lactose nor sucrose has been fermented[118].

Gas production

In the above slant gas production was indicated if present, by bubbles the butt with agar broken or pushed forward.

H2S production

This can be observed in the above slant, if H2S was produced by the organism, blackening of the butt occurred due to reaction of H2S with ferrous ammonium sulphate to form black ferrous sulfide.

Hydrolysis of cellulose

Nutrient agar medium without carbohydrate was sterilized along with strip of Whatman No.1 filter paper. Inoculated culture would grow and hydrolyze the paper if it contained cellulases leading to disintegration of the paper[118].

Acid production from carbohydrates

Nutrient medium of pH 7.0 containing 5 mg % bromocresol purple and 2g% carbohydrate was inoculated with fresh culture of the test organism. Carbohydrates used were glucose, galactose, lactose, xylose, sucrose, mannose, trehalose, cellobiose, fructose, rhamnose, arabinose, mellibiose, raffinose, melizitose, sorbose, dextrin, mannitol, sorbitol, maltose, xylan, starch, carboxy methyl cellulose, cellulose and mesoinositol. Change in color from purple to yellow indicated acid production by the bacterium in the medium[118].

Utilisation of substrates as carbon sources

Polysaccharide production medium (with peptone) was used in the experiment. Sucrose was replaced by 2% of sodium citrate, sodium acetate, calcium lactate, sodium propionate, tannic acid, galacturonic acid, glucuronic acid, sodium formate, tartaric acid, malic acid, maleic acid, oxalic acid, furmaric acid, casein, casaminoacid and mannitol: Media (100 each) were prepared in 500 ml capacity Erlenmeyer flasks.

Inoculated media were incubated on rotary shaker at 250 rpm at ambient temperature of 25-30^{o}C for 96 h. growth was assessed visually by biomass build up.

Utilization of substrates as N-sources

Polysaccharide production medium was substituted with 0.1 % of KNO3, NaNO3, NaNO2, NaCI, NaN03, (Na)2S04, peptone, com steep liquor, urea and diammonium phosphate as N-Source.

Scanning electron microscopy

Centrifuged bacterial cells (24h) were fixed in 2.5% glutaraldehyde, for 1h. The cells were separated by centrifugation at 8000 rpm for 5min. This pellet was washed thrice in phosphate buffer and centrifuged each time for 15 min at 4°C. The cells were dehydrated using acetone. To avoid initial osmotic damage cells were successively suspended in acetone gradient of 30, 70, 80, 90, 95 and finally in 100% of acetone. All these steps were carried out at 4°C. The cells were air dried and stored in vacuum dessicator. Dessicated cells were placed on aluminium stubs (35 nm thick) and scans were taken by using scanning electron microscope.

Differential characteristics of the species

Some of the characteristic tests mentioned in Table 12 were carried out to find out differential characters of the species of the genus Agrobacterium.

1.3 RESULTS

Isolation and screening of bacteria producing polysaccharides

About 150 mucoid bacterial cultures were isolated from various sources such as soil, water -and decaying plant materials. On cultivation in polysaccharide medium it was found that only 23 isolates produced viscosity after growth (Table-3). Viscosity produced by the culture varied from 80- 5800 cP. Out of 23 viscous cultures only 6 cultures formed gels on heating with 0.l% CaCI2. 2H2O and cooling. Cultures PSI8 and PSI9 gave stronger gels compared to others. As the cultures later showed similar characters only

Table-12: Differential characteristics of the species of the genus Agrobacterium (According to Bergey's Manual – 9)

Characteristics	A. radiobacter		A.rhizogenes	A. rubi	A. timefaciens		
	Biovar 1	Biovar 2	Biovar 2		Biovar 1	Biovar 2	Biovar 3
Growth							
At 3S^{o}C	+	-	-	+	-		
On medium of Scroth et at.	+	-	-	+	-	-	
On medium of New and Kerr	-	+	+	-	+	-	
In resence of 2% NaCl	+	-	-	+	-	+	
3-Ketolactose roduced	+	-	-	-	+	-	-
Acidic reaction produced from							
Meso-E britol	-	+	+	+	-	+	-
Melezitose				+	-	-	
Ethanol	+	-	-	-	+	-	-
Alkaline reaction roduced om							
Sodium malonate	-	+	+	+	-	+	+
Sodium L-tartarate						+	+
Soidum ro ionate						-	-
Simmon's citrate with 0.0005% east extract	-	+	+	-	-	+	-
Reaction in litmus milk							
Alkaline	+	-	-	+	+	-	+
Acidic	-	+	+	-	-	+	-
Formation of pellicle in ferric ammonium citrate solution	+	-	-	-	+	-	

Growth factor requirements							
Biotin and/or glutamic acid	-	+	+		-	+	
L-Glutamic acid and yeast extract	-	-	-	+	-	-	
Phytopathogenicity							
Tumors produced on wounded stems of e.g. tomato plants, Helianthus annuis, Nicotiana tabacum and/or on discs of Daucus carota	-	-	+	+	+	+	
Roots produced on discs of Daucus carota	-	-	+	-	-	-	-

PS-18 was used for further studies. In was designated as PS- 18 (CFTRI) till further identification. The slant and plate culture of PS I 8, a single colony and SEM of cells are shown in plates 1-3. The agar culture was creamy, growth was mucoid and filiform. Isolated colony has cells in the center surrounded by polysaccharide material.

Characterization of PSI8 culture

Various morphological and biochemical tests such as growth on various media, gram stain, capsular stain, motility, cell size, aerobicity, catalase test, production of urease, indole, ammonia from arginine, H2S and gas, hydrolysis of cellulose, casein, starch and gelatin, oxidase test, utilisation of citrate, utilisation of various substrates as C and N sources, acid production from various carbohydrates, softening of vegetable tissue by bacterial growth, etc, were studied using PS I 8 culture. The results are recorded in Tables-4 to 11.

The bacterial culture grew well on nutrient agar, PDA, mannitol agar and MY A. Growth on these media was mucoid, thick white to creamish. Only peptone agar without carbon source did not support good growth.

PSI8 cells were about 1.2 to 2.4 μ in size, Gram -ve, small rods and cells were found singly or in pairs. It did not form spores and the younger cells were motile. Cells were aerobic, did not hydrolyse starch, gelatin or cellulose. It produced H2S. It was catalase, oxidase and urease positive. It did not produce gas, indole or 3-ketolactose from lactose. It produced

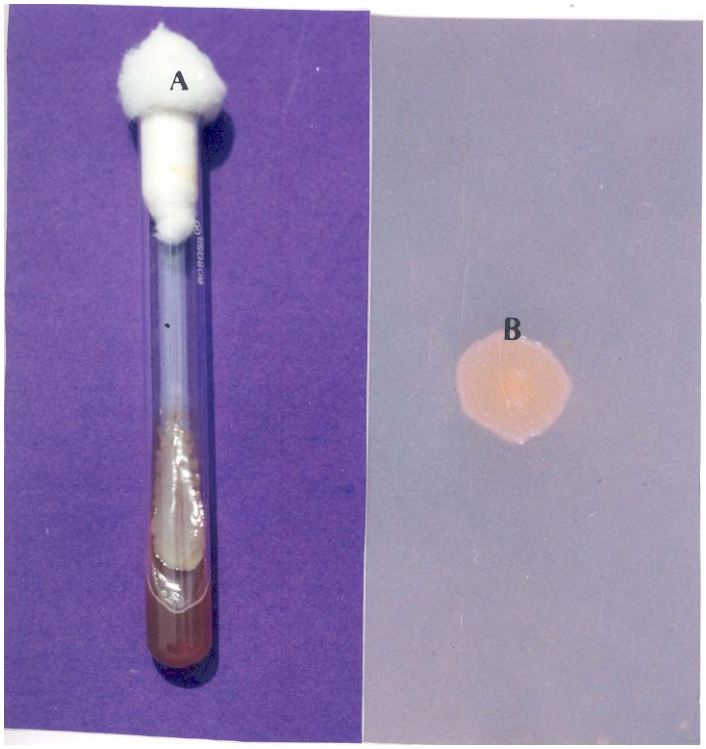


Plate-1: Slant cultur (A) and isolated colony (B) of PS18 culture

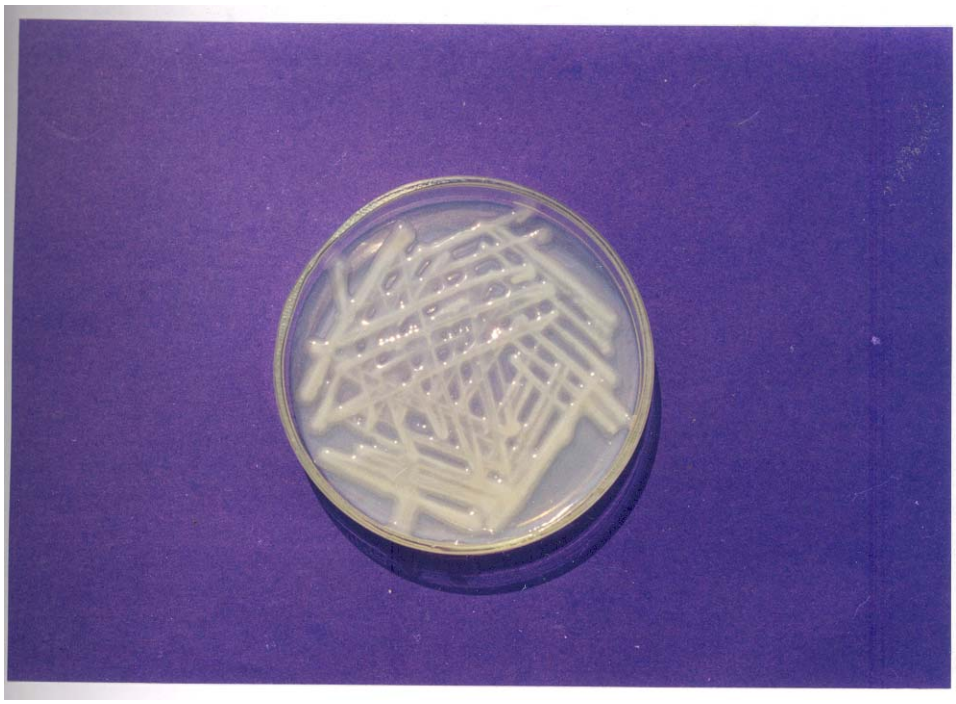


Plate-2: PS18 culture on MYA plate

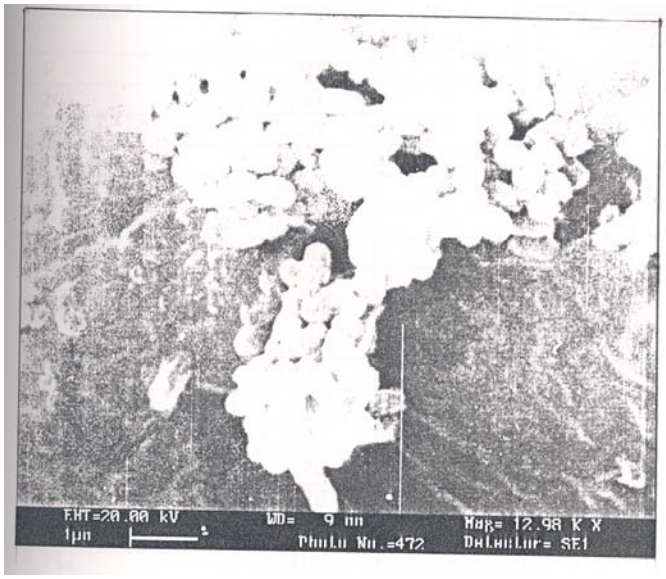


Plate-3: SEM of PS18 culture

Table – 3: Screening of muscoid bacterial colonies for viscosity and gel formation

Culture Number*	Viscosity (cP)	Gel test**
1	780	-
2	180	-
3	3000	+
5	2800	+
PS-18	5800	++

PS-19	3800	++
25	4100	+
38	750	-
40	920	-
41	1200	-
56	1800	-
60	950	-
75	860	-
82	320	-
91	120	-
100	80	-
111	2100	-
125	3010	+
127	270	-
128	120	-
136	80	-
148	850	-
149	1100	-

* Result of cultures which produced viscosity (out of 150 cultures) are given
* Viscous broth was heated to 80°C in the presence of 0.1% CaCh.2H2O and cooled for gelling

Table – 4: Growth of PS18 culture on various agar slants

Medium	Growth		
	Overall growth	Colour	Type
Gelatin agar	Medium	Cream	Mucoid
Nutrient agar	Good	Creamy	Glistening
Potato dextrose agar	Good	Cream	Glistening
Mannitol agar	Profuse	White	Mucoid
Malt extract – yeast extract agar	Profuse	White	Mucoid
Peptide agar	Poor	White	--
Tripple sugar iron agar	Medium	Yellow	Glistening

Table-5: Growth of PSI8 culture at different temperature and pH

Temperature (°C)	Growth
10	-ve
25	+ve (Mucoid)
30	+ve (Highly Mucoid)
37	+ve (Mucoid)
50	-ve
pH	Growth
3.0	-ve
4.0	-ve
5.0	Poor
6.0	Good (Mucoid)
7.0	Optimum (Highly mucoid)
9.0	+ve
11.0	+ve Sparse

Table 6: Characteristics of PSI8 culture at different temperature and pH

Test	Result
Gram stain	-ve;
Colony	Circular, entire
Morphology of cells	Small roads in pairs/single
Mutility	Younger cells motile
Capsule	Present
Spores	Nil
Size	1.2 to 2.4 □
Growth on agar slant	Filiform
Aerobicity	Aerobic
Hydrolysis a. Starch b. Geltan c. Cellulose	 -ve -ve -ve
H_2S production	+ve
Catalase	+ve
Gas from glucose	-ve
Ammonia from arginine	+ve
Urease	+ve
Oxidase	+ve
Indole production	-ve
Milk	No curdling, brownish
3-ketolactose from lactose	-ve
Nitrate reduction	+ve
Utilization of a. Citrate b. Alcohols c. Nitrate d. Nitrite	 +ve -ve +ve -ve

Table 8: Utilisation of organic acids (salts) and other substrates for growth of PSI8 culture as C-source

Organic acid and other compounds	Growth	Morphology of cells
Sodium citrate	++	Small rods
Sodium acetate	++	Small rods
Calcium lactate	+++	Long rods
Sodium propionate	-ve	--
Tannic acid	-ve	--
Galacturonic acid	+	Short plum rods
Glucuronic acid	+	Coccoid like
Sodium formate	+	Small rods
Tartaric acid	+	Small rods
Malic acid	Weak	Medium rods
Maleic acid	-ve	--
Oxalic acid	+	Small rods
Fumaric acid	+	Plum rods
Casein	+	Normal rods
Casamino acid	+	Normal rods
Mannitol	++	Normal rods

Table 9: Effect of different nitrogen sources on growth of PSI8

Nitrogen sources	Growth
Potassium nitrate	+++
Sodium nitrate	+++
Sodium nitrite	-
Ammonium Chloride	+
Ammonium Nitrate	+
Ammonium Sulphate	+
Peptone	+++
Trytone	+++
Corn steep liquor	++
Urea	+
Diammonium phosphate	+

Table 10: Utilisation test of amino acids by PS 18 culture as a source of nitrogen/carbon

Amino acids	Growth	
	As N-source	As C-source
Arginine	+	+
Histidine	+	+
Lysine	+	+
Cystine	-ve	+
Cysteine	-ve	+
Tyrosine	-ve	+
Alanine	-ve	-ve
Phel!ylalanine	-ve	+
Proline	-ve	-ve
Valine	-ve	+
Leucine	-ve	+
Methionine	-ve	-ve
Aspartic acid	-ve	-ve
Threonine	-ve	-ve
Tryptophan	-ve	-ve.
Glutamic acid	-ve	-ve

Table 11: Softening of vegetables by the growth of PS 18 and A tumefaciens (standard)

Vegetable	Effect of growth			
	Growth		Softening	
	a	b	a	b
Carrot	Nil	+	Nil	+
Beans	+	-	Nil	-
Tomato	++	-	Soft	Nil
Cabbage	Nil	-	Nil	Nil
Potato	++	+	Soft	+

a = By PS 18

b = By A.tumefaciens

ammonia from arginine. It could utilise citrate and nitrate for growth. It grew well between 25-37oC and optimum for polysaccharide production appeared to be 30^{o}C wherein the growth on slant was highly mucoid. Even though optimum pH for growth was at 7.0, the bacterium could grow between pH 6 to 11.

The isolate produced acid from carbohydrates such as sucrose, galactose, lactose, mannose, glucose, trehalose, cellobiose, fructose, dextrin, and starch. Weak reaction was obtained for mannitol, sorbitol, and maltose. There was no acid production on rhamnose, melibiose, mesoinositol, cellulose, xylan, arabinose, melizitose, and sorbose.

PSI8 utilised many substrates such as sodium citrate, sodium acetate, calcium lactate, galacturonic acid, glucuronic acid, sodium formate, tartaric acid, oxalic acid, fumaric acid, casein, casamino acid, mannitol, arginine, histidine, lysine, cystin, phenylalanine leucine, cysteine, and valine as C-sources for growth. But it could utilise only a few amino acids such as arginine, histidine, lysine as N-sources for growth. It grew well in nitrates, peptone and tryptone.

The organism grew well on tomato and potato and softened the tissues completely (Plate-4). But it did not produce any phytopathogenicity on tomato plants. It failed to produce root nodules in leguminous plants.

Plate-4: Growth of PSI8 culture on various agar slants

The bacterium could grow at 35°C, gave alkaline reaction in litmus milk did not require growth factor and formed pellicle in ferric ammonium citrate solution.

1.4 DISCUSSION

Isolation of bacteria from soil and decaying material indicates that various strains that are capable of synthesising exopolysaccharides Occur in large numbers in nature. But the strains that produce gelling polysaccharide are apparently less.

Based on morphological and biochemical tests, isolate 18 was classified according to Bergey's Manual as belonging to class Schizomycetes, order Eubacteriales, family Rhizobiaceae, genus Agrobacterium and species radiobacter[120].

Gram negative, aerobic small rods as found in the case of PS 18 was assigned to family Rhizobiaceae because the cells were without endospores, rod shaped, glucose was utilized without appreciable acid formation. This family contains three genera namely Rhizobium, Chromobacterium, and Agrobacterium. The isolated culture was not capable of fixing free nitrogen or producing violet chromogene which would otl1erwise indicate that the culture was Rhizobium or Chromobacterium. Agrobacterium is most likely to be confused with Rhizobium to which it is closely related.

Typical characters found in Agrobacterium which are different from Rhizobium are; growth on glucose-peptone agar, production of H2S, absence of polyhydroxy butyratel19 and failure to nodulate in leguminous plants.

The bacterium possessed the main characters of Agrobacterium. In this study standard culture of A. tumefaciens was used for comparison. Typical characteristics of Agrobacterium which were present in isolate 18 were: small short rods (0.6 to 1.0 x 1.5 to 3.4 µm) occurring singly or in pairs, non spore forming, gram negative, motility by peritriochous flagella, aerobic, colonies convex circular, smooth, non pigmented, absence of gas production or sufficient acid in ordinary culture media, absence of gelatin liquefaction, inability to fix free N2, utilization of nitrates and ammonium salts for growth, catalase positive, urease positive, oxidase positive, chemoorganotroph utilizing a wide range of carbohydrates, salts of organic acids and

amino acids as carbon source, optimum growth temperature between 20o-30oC, and soil habitat.

Further tests were carried out to confirm the characteristics of the species Agrobacterium (Table 12). Confirmatory results obtained were: Isolate grew at 35°C, reaction in litmus milk was alkaline, did not require growth factors, did not produce alkaline reaction in salts of organic acid and phytopathogenicity was absent.

With the exception of A. radiobacter other species of Agrobacterium invade the crown, root and stem of dicot. The isolated bacterium was not capable of causing infection to dicot plant (e.g. Tomato). It was isolated from soil, grew well on potato. Hence it was concluded the culture may be A. radiobacter.

CHAPTER – 2
OPTIMISATIONOF MEDIUM AND CULTURAL PARAMETERS FOR POLYSACCHARIDE PRODUCTION (TRADITIONAL METHOD)

INTRODUCTION

In nature there are various microbial strains that produce polysaccharide abundantly which would lead to the possibility of discovering new polysaccharides with unique or superior properties. During the last two decades hundreds of potential microorganisms have been screened and processes have been developed for a few of them to produce polysaccharide on a commercial scale.

After selecting the potential microorganism optimization of nutritional and cultural conditions of fermentation for growth and production of polysaccharide are necessary for process formulation. The important parameters that affect fermentation include type and concentration of carbon source, nitrogen source, mineral salts, pH, temperature, agitation, aeration and inoculum level.

In this context results of studies carried out on optimization of various fermentation parameters on growth and polysaccharide production by traditional method in batch cultures are presented in this chapter.

2.1 MEDIA

Screening of media

Various media reported in literature for polysaccharide production by bacteria (Table 13, Media No.1 ,4,5,6) and formulated in the lab (Media No. 2 and 3) were used for the growth and polysaccharide production by A.radiobacter.

Table-13: Screening of media for viscosity production (72 h) by A.radiobacter culture.

Nutrients	Concentration of nutrient (g/l, w/v) in various media					
	1	2	3	4	5	6
Na2HPO4	5.0	5.0	5.0	-	-	-
KH2PO4	3.0	3.0	3.0	-	-	-
K2HPO4	-	-	-	0.5	0.5	0.5
K2SO4	1.0	1.0	1.0	-	-	-
NaCl	1.0	1.0	1.0	-	0.2	0.1
MgSO4 7H2O	0.25	0.25	0.25	0.25	-	-
CaCl2 2H2O	0.02	0.02	0.02	0.02	-	-
NaMoO4	-	-	-	-	0.05	-
CaSO4	-	-	-	-	0.1	-
Tryptone	2.5	-	-	-	-	-
Casamino acid	-	-	-	-	-	0.5
NH4NO3	-	-	-	-	-	0.9
Peptone	-	2.5	-	2.5	-	-
Yeast extract	1.0	-	-	-	-	-
KNO3	-	-	3.0	-	1.0	-
Sucrose	30	30	30	30	30	30
Viscosity (cP)	2040	2000	4240	1080	520	540

Media for optimization

Various media were prepared and used for determination of optimum carbon source, nitrogen source, and mineral salts for viscosity and polysaccharide production by A.radiobacter. For the selection of best C-source, experiments were carried out using lab formulated basal medium of PH7, the composition of which is given under Chapter 1. Instead of source various other carbon sources were supplemented (equivalent to 1.2 g% of carbon, w/v) to the medium. Similarly, for the selection of optimum nitrogen source, different N-sources (equivalent to 0.0 4g%. Nitrogen, w/v) were supplemented to the medium. For selection of suitable mineral salts, nutrient limitation technique was used, where in basal medium devoid of individual mineral salt was tried (Table 14).

Inoculum medium

The medium used for inoculum preparation was similar to basal medium described in chapter 1 but it contained 1g% sucrose (w/v), with peptone (0.25g%, w/v) as N-source.

2.2 METHODS

All the experiments detailed below were carried out in duplicates using 100 ml medium contained in 500ml Erlenmeyer flasks. The sterile media were inoculated with 10% (v/v) of 18h old inoculum. The flasks were incubated at 250 rpm on a shaker at ambient temperature of 28^{o} –30^{o} C for

Table 14: Viscosity and polysaccharide production by A.radiobacter culture in media lacking various individual mineral salts.

Components (g%, w/v)	Medium								
	1	2	3	4	5	6	7	8	Control
Na2HPO4 (0.5)	-	+	+	+	+	+	+	+	+
KH2PO4 (0.3)	+	-	+	+	+	+	+	+	+
Mg SO4 7H2O (0.025)	+	+	-	+	+	+	+	+	+
K2SO4 (0.1)	+	+	+	+	+	+	+	+	+

NaCl (0.1)	+	+	+	-	+	+	+	+	+
CaCl2 2H2O (0.002)	+	+	+	+	+	-	+	+	+
KNO3 (0.3)	+	+	+	+	+	+	-	+	+
Sucrose (3.0)	+	+	+	+	+	+	+	-	+
Viscosity (cP)	500	7040	50	6560	7440	6960	Nil	Nil	7000
Polysaccharide (g%, w/v)	0.20	1.65	0.49	1.48	1.70	1.10	Nil	Nil	1.70

72h-96h. Viscosity and polysaccharide contents were analysed as mentioned in chapter on materials and methods,

Effect of various C-sources on polysaccharide production

Glucose, raffinose, sucrose, galactose, lactose, fructose, maltose, mellibiose, xylose starch, arabinose, cellobiose and manose were tested as carbon substrates for polysaccharide production by A.radiobacter. Individual C-source (equivalent to 1,2 g% carbon, w/v) were incorporated to basal medium containing KNO3 as N-source, dispensed (100 ml) into 500 ml Erlenmeyer flasks and sterilized (15/bs, 20min), Viscosity and polysaccharide production were measured after 72hr of growth at 250 rpm and 28-30^{o}C.

Effect of various N-sources on polysaccharide production

Ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrate, ammonium nitrate, peptone, tryptone, comsteep, liquor, urea, and diammonium phosphate were tested as N-sources for polysaccharide production. Individual nitrogen sources were incorporated (equivalent to 0.04g% nitrogen, w/v) into basal medium, Varying concentrations of KNO3 (0.1-0.5%) was also used for polysaccharide production. Media were dispensed (100ml) into 500ml Erlenmeyer flasks, sterilized (15 lbs, 20 min), cooled and inoculated with 10% (v/v) of 18h old A.radiobacter inoculum. Cultivation was carried upto 72h at 250 rpm and 28-30oC, followed by measurement of viscosity and estimation of polysaccharide.

Effect of individual mineral salts on polysaccharide production

The effect of different mineral salts such as disodium hydrogen phosphate, potassium hydrogen phosphate, magnesium sulphate, sodium chloride and calcium chloride, on the production of polysaccharide by A.radiobacter was worked out using nutrient limitation technique, Mineral salts were individually deleted from basal medium (Table-1 4), and these media were dispensed in 100ml volume into 500ml Erlenmeyer flasks, sterilised (15 lbs, 20 min) and inoculated (10% inoculum, w/v) with A.radiobacter. The culture was grown for 96h at 250rpm and 28-30oC and analysed for viscosity and polysaccharide production.

Effect of mineral salts concentrations and cultural parameters on polysaccharide production

Mineral salts such as Na2HPO4, and MgSO4. 7H2O which were found essential for polysaccharide production were used at different concentrations in basal medium containing KNO3 as N-source. Different pH, incoulum levels and temperature were also tested as follows.

Disodium hydrogen phosphate

Different concentrations of Na2HPO4 (0.1-1 g% w/v) were incorporated into KNO3 containing basal medium, sterilized, inoculated and incubated for 96h as mentioned above.

Magnesium sulphate

Magnesium sulphate was used between 0.025-1 % (g%, w/v) in basal medium containing KNO3. Fermentation (96h) and sample analysis were carried out as detailed above.

Medium pH

Effect of initial pH on polysaccharide production by A. radiobacter was rested by adjusting pH of basal medium between 3-9 and carrying out fermentation (96h) as mentioned above.

Temperature

Effect of incubation temperature on the production of polysaccharide by A.radiobacter was tested by incubating the inoculated flask containing sterile basal medium at 10,25, 30, 35 and 40°C at 250 rpm for 96h.

Inoculum level

Different concentration of incoulum of A. radiobacter, varying from 1 to 10% (v/v) were added to 100ml sterile basal medium. Flasks containing inoculated media were incubated for 96h at 250 rpm and 28°-30°C.

Kinetics of polysaccharide production

Sterile basal medium (100ml) with tryptone (0.25 g% w/v) and yeast extract (0,1%) or KNO3 (0.3%) contained in 500ml Erlenmeyer flasks were inoculated with A.radiobacter inoculum (10%, v/v) and bacterium was allowed to grow upto 96h at 250 rpm and 28-30oC. Biomass, pH, viscosity, ell counts, polysaccharide weight and residual sugar were estimated as mentioned under chapter on materials and methods at the end of 24, 48, 72 and 96h of cultivation.

Polysaccharide production in optimised medium

Optimised medium having (g% w/v) Na2HPO4 (0.4%) MgSO4 7H2O (0.1%), sucrose (3%) and KNO3 (0.3%) was adjusted to pH 7.0 sterilised and inoculated with 1% of inoculum (v/v). This experimental optimised set was compared for polysaccharide productions with standard medium (pH 7.0) set which contained (g%, w/v) Na2HPO4 (0.5%); K2SO4 (0.1%); KH2PO4 (0.3%); MgSO4.7H2O (0.025%); CaCb.2H2O (0.001%); sucrose, (3%); KNO3 (0.3%) and inoculated with 10% (v/v) inoculum. Cultivation was carried out for 96h at 250 rpm and 28-30^{o}C.

2.3 RESULTS

Screening of media

As shown in table 13, formulated media (No.2 and 3) containing peptone and KNO3 as N-sources gave maximum viscosity compared to other media and hence was used for further experimentation.

Standardisation of nutritional and cultural parameters

Effect of C-source

Results recorded in table 15 show that A.radiobacter produced high viscosity in the presence of sucrose (4800 cP) followed by galactose (4160cP) and lactose (3360 cP). Viscosity produced was low in medium containing glucose, mellibiose etc., yield of polysaccharide was also maximum (1.85 g%, w/v) in the presence of sucrose compared to other carbon sources.

Effect of N-source

Amongst various N-sources tested (Table-16) A. radiobacter produced maximum viscosity in organic nitrogen source namely tryptone (6400 cP) followed by KNO3 (4800 cP) as inorganic N-source. Sodium nitrate was also utilized for good viscosity (4480 cP) and polysaccharide production (1.65% g%). Low viscosity was obtained for ammonium salts (200-2600 cP). The final pH of fermented broth containing ammonium salts was low compared to other N-sources.

Using different C/N ratio it was found that a ratio of 29 gave optimal values for viscosity polysaccharide production and conversion (Table-16a). In the presence of increased N-level, the biomass build up was more but it was not favourable for polysaccharide production.

Effect of individual mineral salts

Data in table 14 indicate that Na2HPO4, MgSO4 7H2O, nitrogen source (KNO3) and carbon source (sucrose) significantly influence the viscosity and polysaccharide production by A.radiobacter. This is because very poor viscosity (0-500 cP) and polysaccharide concentration (0-0.49 g%) were obtained in media lacking these nutrients. Absence of K2SO4, NaCl, CaCI2. 2H2O and KH2PO4 indicated marginal differences in viscosity (6560-7440

Table –15: Polysaccharide production (72 h) by A.radiobacter in the presence of various C-sources

C-Source (Equivalent to 1.2g% carbon, w/v)	Viscosity (cP)	Polysaccharide (g%, w/v)
Sucrose	4800	1.85
Galactose	4160	1.55
Lactose	3360	1.50

Fructose	3040	1.35
Maltose	3040	1.42
Raffinose	3000	0.98
Glucose	2400	1.25
Mellibiose	1800	1.24
Xylose	1120	0.98
Starch	-	-
Arabinose	-	-
Cellobiose	-	-
Mannose	-	-

Table-16: Polysaccharide production (72 h) by A.radiobacter in the presence of various N-sources in 3g% sucrose (equal to 1.2g% carbon, w/v) containing medium

N-Source (Equivalent to 0.042g % N, W/V)	Viscosity (cP)	pH (Final)	Polysaccharide (g% w/v)
Potassium nitrate	4800	7.29	1.85
Sodium nitrate	4480	7.12	1.65
Ammonium nitrate	2600	5.90	1.12
Ammonium sulphate	1000	5.07	0.78
Ammonium chloride	200	4.90	0.10
Peptone	4160	6.80	1.50
Tryptone	6400	6.40	1.85
Corn steep liquor	2800	6.54	1.04
Urea	720	6.35	0.80
Diammonium phosphate	200	4.00	0.58

Table-17: Polysaccharide production (72 h) by A.radiobacter using different concentrations of KNO3 and sucrose (3g%), after 72 h of fermentation

Concentration of KNO3 (g%, w/v)	C/N ratio	Viscosity (cP)	Polysaccharide (g%, w/v)	Biomass (mg%)	Conversion (%)
0.1	85	3520	0.96	40	48
0.2	43	4160	1.10	180	60
0.3	29	4960	1.44	120	72
0.4	22	4080	1.38	160	58
0.5	18	3920	1.00	200	50

CP) and polysaccharide production (1.1-1.65 g%) compared to respective values (7000 cP and 1.7 g% yield) obtained for control medium.

Concentration of Na2HP04

Results compiled in table 17 show that by increasing the concentration of Na2HPO4 from 0.1 to 0.4%, viscosity of the broth also increased from 5600 to 6720 cP and polysaccharide yield from 1.36g% to 1.80g%. Viscosity as well as polysaccharide production decreased by further increase in Na2HPO4 concentration beyond 0.5g%.

Concentration of MgSO4 7H2O

By varying the concentrations of MgSO4 7H2O incorporation into medium from 0.025 to Ig% it was observed (Table 18) that A.radiobacter gave optimum values for viscosity (6860 cP) and polysaccharide (1.9g%) at 0.1g% concentration compared to other levels.

Media pH

Data obtained for polysaccharide and viscosity production (Table 19) show that the bacterium grew in pH 5-9. However optimum pH for viscosity (7040 cP) and polysaccharide formation (1.7g%) was around pH 7.0.

Table-17: Effect of various concentrations of Na2HPO4 on polysaccharide production by A.radiobacter.

Na2HPO4 (g% w/v) in basal medium	Viscosity (cP)	Polysaccharide (g%, w/v)
0.1	5600	1.36
0.2	6400	1.60
0.4	6720	1.80
0.5 (Standard)	6000	1.70
0.6	6160	1.70
0.8	5760	1.63
1.0	5600	1.11

Table – 18: Effect of various concentrations of MgSO4 7H2O on polysaccharide production by A.radiobacter culture

MgSO4 7H2O (g, w/v) in basal medium	Viscosity (cP)	Polysaccharide (g%, w/v)
0.025	5920	1.69
0.1	6860	1.94
0.2	6860	1.91
0.4	4880	1.80
0.6	4080	1.61
0.8	1840	1.55
1.0	1280	1.13

Table-19: Polysaccharide production by A.radiobacter at different initial medium pH

Initial pH	Viscosity (cP)	Polysaccharide (g%, w/v)
3	-	-
4	-	-
5	5040	1.52
6	5350	1.60
7	7040	1.76
8	6800	1.70
9	6000	1.65

Inoculum concentration

Utilization of lower concentrations of inoculum itself (2% v/v) supported maximum viscosity (6120 cP) compared to higher inoculum concentration (Table 20).

Temperature

Results in Table-21 show that the culture of A. radiobacter grew well between 25°-35°C. Optimum temperature for polysaccharide production was around 30°C as indicated by maximum viscosity (7040 cP) and polysaccharide production (1.8g%). There was no growth at 10°C.

Kinetics of polysaccharide production

Results in Fig.8 show that in tryptone medium A.radiobacter cells grew till 48h ($2.2x10^8$ cells/ml) and were viable upto 96h. Maximum viscosity achieved was 5200 cP in 96h with polysaccharide yield of 1.1 g%. The pH of the medium which was set to 7.0 initially did not alter much during fermentation. In KNO3 as N-source (Fig.9) maximum cell counts of $3x10^8$ cells/ml was recorded at 96h, however cell growth was low upto 48h. Viable cell counts were maximum at 72h (6xJ.06 cells/ml) and viscosity obtained was 6200 cP in 96h with a polysaccharide yield of 1.9g%.

Polysaccharide production in optimised medium

In optimised medium A.radiobacter produced 7600 cP viscosity and 1.9% polysaccharide after 96h fermentation (Table 22).

Table - 20: Effect of inoculum concentration on viscosity production by A. radiobacter

Inoculum (%, v/v)	Viscosity(cP)
1.0	5800
2.0	6120
4.0	6000
6.0	6020
8.0	6000
10	6040

Table 21: Polysaccharide production by A.radiobacter under different temperatures

Temperature (^{o}C}	Viscosity (cP)	Polysaccharide
10	-	-
25	6800	1.70
30	7040	1.80
35	5200	1.10
40	3000	0.80

Table - 22: Polysaccharide production by A.radiobacter in medium optimised by traditional method

Medium	Viscosity (cP)	Crude polysaccharide (g%, w/v)
Optimised	7600	1.90
Control (standard)	7120	1.75

2.4 DISCUSSION

Kinetics of polysaccharide production

The rate of polysaccharide produced by bacteria varies at different stages of growth. It is reported that in Pseudomonas aeruginosa the polysaccharide production occurs throughout the growth phase and cessation of growth and polysaccharide production occur simultaneously and the specific rate of exopolysaccharide production increases with increased specific growth rate121. This differs from the exopolysaccharide production by Azotobacter vinelandii, Xanthomonas campestris and another species of Pseudomonas in which the specific rate of exopolysaccharide synthesis is independent of growth rate.

In Sphingomonas paucimobilis, Aureobasidium pullulans Z. ramigera, the secretion of polysaccharide takes place only in the late exponential or in the stationary phase of growthI22-125. Data in fig 8 and 9 indicate that the exopolysaccharide production in A. radiobacter is similar to that of X. campestris and occurs throughout the growth phase.

Role of nutritional and cultural parameters on polysaccharide production

Polysaccharide producing bacteria require specific quality and quantity of carbon source for viscosity and polysaccharide production. Most of the microorganisms can utilize a wide variety of carbon substrates but some species require specific carbon substrates for polysaccharide synthesis. For example starch and dextrin are essential carbon substrates for

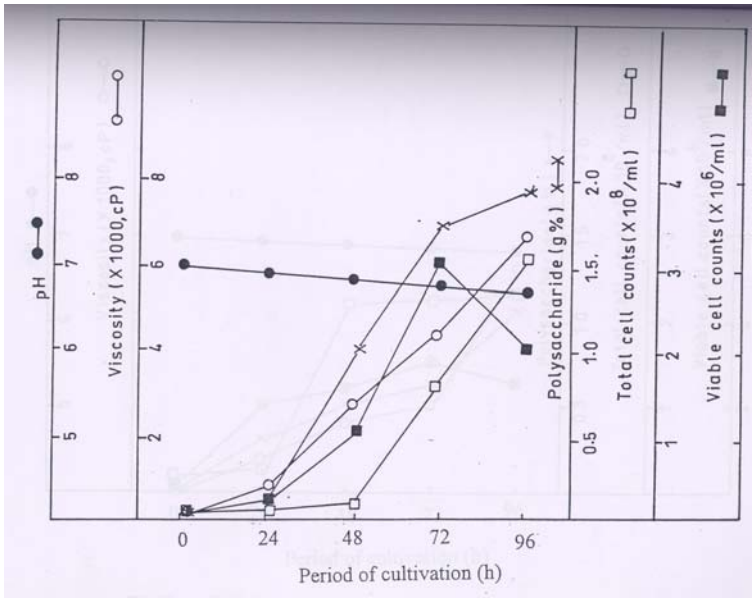


Fig.8. Growth of PS18 in the presence of KNO_3 as N-source

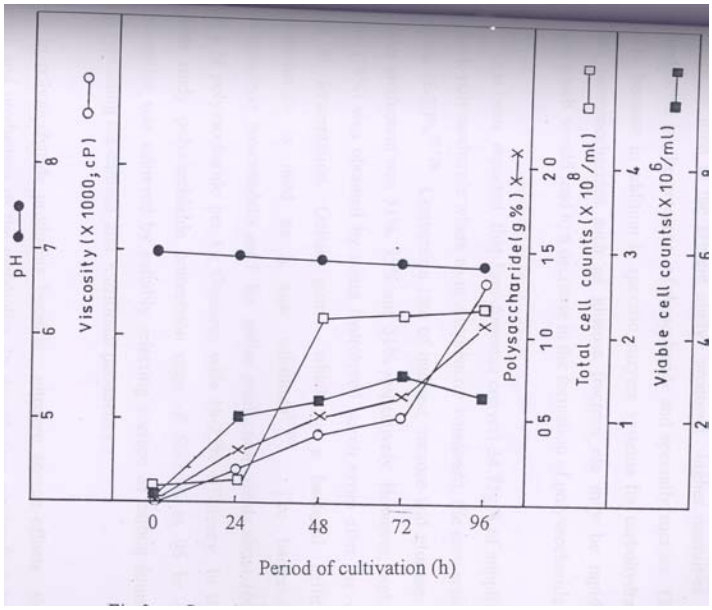


Fig.9. Growth of PS18 in the presence of tryptone as N-source

the production of polysaccharide by Achromobacter mucosum[29]. Sucrose is the specific substrate for the" production of dextran by Leuconostoc Spp.30. Rhizobium hedysari and R.meliloti can grow well on mannitol followed by glucose and galactose but growth is poor on lactose and maltose. A.Radiobacter used in the present study produced higher quantities of polysaccharide in the presence of disaccharide and specially sucrose. This may be because in addition to specific enzyme systems for carbohydrate uptake, monosaccharides such as glucose fructose etc may be rapidly utilized, which would lead to a decrease in the formation of polysaccharide.

It has been reported that Pseudomonas convert 34.75g% of supplied carbon to polysaccharide where as in Azotobacter vinelandii, the conversion rate was 16-30%35,128. Conversion rate of maltose, sucrose and glucose in pullulan an production was 51 %, 35% and 31 % respectively. However, higher yields (76%) was obtained by using hydrolysed starch syrup after seven days of fermentation. Gellan gum which is a bacterial gelling polysaccharide is used as a agar substitute 1 5,86. The bacterium Sphingomonas paucimobilis used for gellan production yields about 160-250 g of polysaccharide per kg C-source with 16-25% efficiency. In the present study polysaccharide conversion rates of 50-70% in 96 hr of fermentation was achieved by suitably selecting sucrose as carbon source and optimising the cultural and nutritional parameters.

In polysaccharide producing bacteria, nitrogen source effects the growth and production of the metabolite. In Azotobacter vinelandii it has been observed that increasing the concentration of KNO3 beyond 0.5 gl^{-1} did not result in concomitant increase in growth and polysaccharide production whereas sodium nitrate when used in the medium decreased the lag phase and generation time and thus the fermentation period41. Utilization of NH4Cl at 0.1gl^{-1} gave the best yield and increase in its concentration resulted in better growth but polysaccharide recovery was poor. In the presence of urea growth and polysaccharide production similar at all were concentrations. In contrast to this, Rhizobium hedysari required organic nitrogen source like lysine for polysaccharide production42. In this study A.radiobacter gave more viscous polysaccharide in organic N-source such As tryptone. It could also give good yields on nitrates but ammonium salts did not support much of polysaccharide production. One of the reason for this may be

because of rapid fall in pH of ammonium salts containing media. Complex materials such a peptone, tryptone, yeast extract, in addition to providing buffering to medium, also supply essential growth factors for the bacterial cells. Nitrogen is a major constituent of proteins nucleic acids and coenzymes. Protein hydrolysates serve as a source of amino acids. Purines, pyramidines and vitamins present in yeast extract act as substrates for new protoplasm, as coenzymes of essential metabolic reaction, building blocks for DNA and RNA and also as precursors of nucleotide, for functioning in energy transferring and synthetic reaction of the bacterial cell.

Carbon and nitrogen ratio (C/N) has a pronounced effect on the bacterial polysaccharide production. In Pseudomonas sp the formation of extracellular polymer was promoted by high C/N ratios 127. The highest yield has been reported at C/N ratio of 40 129. A. radiobacter gave optimum yield of polysaccharide at a C/N ratio of 28.

Macroelements required for bacterial growth include nonmetals like nitrogen, carbon, sulphur and metals such as magnesium. Magnesium acts as an enzyme cofactor and is also found in cell walls and ribosomes. It has been shown that in Azotobacter vinelandii alginic acid production is dependent on the initial phosphate concentration. At low phosphate concentration the buffering capacity of the medium decreased so that during the course of the fermentation the pH fell to a point at which polysaccharide formation was suppressed [131]. In cultures where the pH decreased because of accumulation of acidic metabolites, addition of Na2HPO4/CaCO3 is required for high yield. Similarly in A. radiobacter fermentation also absence of Na2HPO4 in the medium resulted in a very poor viscosity. In addition to nitrogen, carbon and Na2HPO4 A. radiobacter also required MGSO4 7H2O for optimum fermentation. It has been shown that low Mg2+ concentration inhibited the growth of Azotobacter vinelandii. It has also been shown that Mg2+ ions are required for activating enzymes leading to EPS synthesisI33. It can be concluded that magnesium and phosphate limited conditions results in poor polysaccharide production and lower polysaccharide accumulation as they affect over all cell metabolism.

Other cultural parameters such as pH and temperature are to be optimized for greater activity of cell enzymes involved in polysaccharide biosynthesis. The optimum pH for polymer synthesis depends on the individual species, but it is near neutrality for most bacteria 114. It has also been shown that the bacterial cells producing curdlan form the polymer from sugars even at low concentration of inorganic salts provided the medium has a pH value of 6-7. Optimum pH and temperature for A.radiobacter growth and polysaccharide production was 7 and 30°C, respectively.

CONCLUSION

Using traditional method carbon, nitrogen, mineral salts, pH, temperature and inoculum level for A.radiobacter fermentation was optimised. Maximum polysaccharide yield (2.1g%) and viscosity (7600 cP) were produced aerobically in medium containing Na2HPO4 (O.4g%) MgSO4 7H2O (0.lg%), KNO3 (0.3g%) and sucrose (3%) at pH 7.0 and 30°C.

CHAPTERS - 3

OPTIMISATION OF FERMENTATION PROCESS FOR POLYSACCHARIDE PRODUCTION (RESPONSE SURFACE METHODOLOGY)

3.0 INTRODUCTION

Optimization of medium for the growth and production of polysaccharide by traditional method is an elaborate process which requires more time since it depends on many factors and interaction that affect the desired response. Under such circumstances response surface methodology (RSM) is considered to be an effective tool for optimising the fermentation process[136] RSM is a statistical method that uses quantitative data from appropriate experimental design to determine and simultaneously solve multivariate equation. It usually uses an experimental design such as central composite rotatable design (CCRD) to fit a first or second order polynomial by least significant technique. An equation is used to describe how the test variable affect the response, determine the interrelationship among the test variables, and desired combined effect of all the test variables in the response[137]. The contour plots can be usefully employed to study the response surfaces and locate the optimum.

In this context study was carried out on optimization of various fermentation parameters of growth and polysaccharide production by A.radiobacter using Response surface Methodology.

31. MEDIA

Experiments (in triplicates) were carried out using basal medium the composition of which is given in chapter 1. Variations in concentrations of sucrose (O.5-5.5g%) and pH (4-8) were made for different experiments. Ratio of KNO3: sucrose was maintained at 1 :10 for all the experiments

Inoculum

The composition of medium was similar to basal medium except that sucrose (lg% w/v) and peptone (0.2Sg%) were used as carbon and nitrogen sources.

3.2 EXPERIMENTAL DESIGN

A Central Composite Rotatable Design, CCRDI67 with 3 variables was used to study the response pattern and to determine the optimum combination of variables. The variables optimised were sucrose concentration (0.5-5.5g%, w/v), inoculum concentration (1-1S%, v/v) and pH of fermentation (4-8) each at 5levels: -1.682, -1, 0,1, and 1.682 KNO3: sucrose ratio was maintained at 1 :10. Table 2 3 shows the symbols and levels of nutrients used in the experiments.

The CCRD shown in Table 24 was arranged to allow for fitting an appropriate regression model using multiple regression programs. The CCRD combines the vertices of the hypercubes whose co-ordinates are given by 2n factorial design to provide for the estimation of curvature of the model [138]. Six replicates (treatment 15-20) at the center of the design were used to allow for estimation of pure error sum of squares. Experiments were randomised in order to maximise the effects of unexplained variability in the observed responses due to extraneous factors.

Table-23: Variables and their levels for CCRD

Variables	Symbols		Levels				
	Coded	Uncoded	-1.682	-1	0	1	1.682
Sucrose concentration (g%)	X1	X1	0.5	1.5	3.0	4.5	5.5
Inoculum concentration (% V/V)	X2	X2	1	3.84	8	12.16	15
PH	X3	X3	4	4.8	6	7.2	8

Where X1=(X1-3.0)/1.5, X2=(X2-8)/4.16 and X3=(X3-6)/1.2

Table - 24: Treatment schedule for a 3-factor CCRD and the response in terms of viscosity and yield of polysaccharide

Exp. No.	Variable levels			Viscosity (mPas)	Yield (g%)
	Xl	X2	X3		
1	-1	-1	-1	75.50	0.67
2	-1	-1	-1	33.50	1.40
3	-1	-1	-1	52.00	0.38
4	-1	-1	-I	60.10	1.25
5	-1	-1	1	58.50	1.45
6	-1	-1	1	43.50	1.20
7	-1	-1	1	25.50	0.60
8	1	1	1	60.00	0.49
9	-1.682	0	0	51.00	0.50
10	1.682	0	0	44.50	1.22
11	0	1.682	0	105.0	2.26
12	0	1.682	0	88.50	1.40
13	0	0	-1.682	19.50	0.19
14	0	0	1.682	6.90	0.16
15	0	0	0	81.10	1.25
16	0	0	0	82.00	1.37
17	0	0	0	84.30	1.35
18	0	0	0	86.50	1.28
19	0	0	0	85.20	1.33
20	0	0	0	84.20	1.27

Statistical analyses

A second order polynomial equation was used to fit the experimental data given in Table 24. The model proposed for the response (Y1 and Y2) $Y1=a_0+a1X1+a1X2+a3X3+a1X^2l+a_{22}X^22+a_{33}X^23+a_{12}X1X1+a13X2\ X3+\Sigma$ where Yi (i=1 and 2) is the predicted response for viscosity and yield, respectively, a_0 is the value of the fitted response at the center point of the design ai, aii, aiii the linear, quadratic, and cross product terms, respectively. Optimization of fitted polynomials for viscosity of the culture broth and yield of yield of polysaccharide was performed by numerical techniques using mathematical optimizer procedure of the Quatro pro software package (Quatro pro, version 4.0, Borland International Inc., USA)168. Further in order to deduce workable optimum conditions, graphical techniques were used [146] [169] by fixing one variable at predetermined optimum

condition. The optimum condition was verified by conducting experiments at those conditions. Responses were monitored and results were compared with model predictions.

The fitted polynomial equation was expressed as surface and contour plots using STATISTIC A program in order to visualize the relation between the response and experimental levels of each factor and to deduce the optimum conditions.

Production of polysaccharide

Erlenmeyer flasks (500 ml cap) containing sterile basal medium (100 ml) with varying Sucrose concentration (0.5-5.5g%), and pH (4-8) were inoculated with various inoculum concentrations of A.radiobacter (Table 23). The inoculated flasks were placed on rotary shaker at 250 rpm at ambient temperature of 28°-30°C for 96 hr.

3.3 ANALYSIS

All the experiments were done in triplicates, and polysaccharide yields were estimated (see chapter on materials, and methods) in the 96h broths.

Viscosity of the culture broth was measured after 96h of fermentation using Haake viscometer (Model # RT 10) with a coaxial cylinder attachment. The ratio of the external diameter of rotating bob to internal diameter of stationary cylinder was 0.954. The viscosity of the culture broth was deduced at 400 8-1 shear rate from the shear rate, shear stress curve generated at 25±0.l^{o}C with the experimentation time of 400 see.

RESUL TS AND DISCUSSION

Diagnostic checking of the models

Two responses were measured in the experiments namely: viscosity (Y1) and yield (Y2). The coefficients for the actual functional relatives for predicting (Y I) and (Y 2) are presented in table 25. The four responses under different combinations as defined in the design (Tables 23 and 24) was analysed using the ANOV A appropriate to the experimental design. The ANOVA for the data obtained using CCRD is presented in table 26. It is evident from the data presented that the first and second (quadratic and cross product) order terms were found to be significant and lack of fit was not

Table - 25: Estimated coefficient for the fitted second order polynomial representing the relationship between the response and process variables.

Coefficient	df	Estimated coefficient for Viscosity	Estimated coefficient for yield
a0	1	83.8705***	1.3085***
a1	1	-1.8548*	0.1795***
a2	1	-3.0131**	-0.2523***
a3	1	1 -4.0117***	-0.0008
a11	1	-12.6876***	-0.1592***
a22	1	4.6323***	0.1836***
a33	1	-24.8998***	-0.4014***
a12	1	12.4500***	0.035*
a13	1	6.6750***	-0.2450***
a23	1	-2.4500*	-0.1400***

***Significant at 0.1 % **Significant at 1.0%, *Significant at 5.0%.

Table – 26: Analysis of variance (ANOVA) for the fitted second order polynomial model and lack of fit for viscosity and yield as per CCRD

Source of variation	d.f	Viscosity (mPaas)			Yield		
		Sum of squares	Mean sum of squares	F-value	Sum of squares	Mean sum of squares	F-value
Regression							
First order terms	3	390.799	130.266	32.325*	1.3096	0.4365	186.8109*
Quadratic terms	3	11403.681	3801.227	943.264*	3.3358	1.1119	475.8602*
Cross products	3	1644.485	548.162	136.025*	0.6468	0.2156	92.2675*
Total	9	13438.965	1493.218		5.2922	0.5880	
Residual							
Lack of fit	5	35.871	7.174	1.780	0.0123	0.0025	1.0561
Pure error	5	20.149	4.030		0.0117	0.0023	
Total error	10	56.020	5.602		0.0240	0.0024	
Grand total	19	13494.986			5.3162		

*Significant at 5% level

significant. The lack of fit measures the failure of the model to represent data in experiment: domain at points which are not included in the regression. The high values of coefficient of determination (R 1 also suggest that the model is a good fit. The R 2 is proportion of variability in response values explained or accounted for by the model[139,140].

Response surface plotting

The effect of sucrose concentration, inoculum concentration and pH responses on viscosity and yield of the polysaccharide are recorded in Table-25.

The response surfaces based on these coefficients are shown in Fig.l0 (a and b) and Fig 11. (a and b) with one variable kept at optimum level and varying the other two within the experimental range. In general, exploration of the response surfaces indicated a complex interaction between the variables.

Effect of sucrose concentration and inoculum concentration on viscosity and yield of polysaccharide.

At the respective optimum level of pH, maximum viscosity and polysaccharide yield was at lowest inoculum concentration. (1 %) and sucrose concentration of 1.6% to 3.3%, respectively (Fig 10 a and b).

At the lowest level of inoculum concentration (1 %), viscosity changed insignificantly at first and then decreased with an increase in sucrose

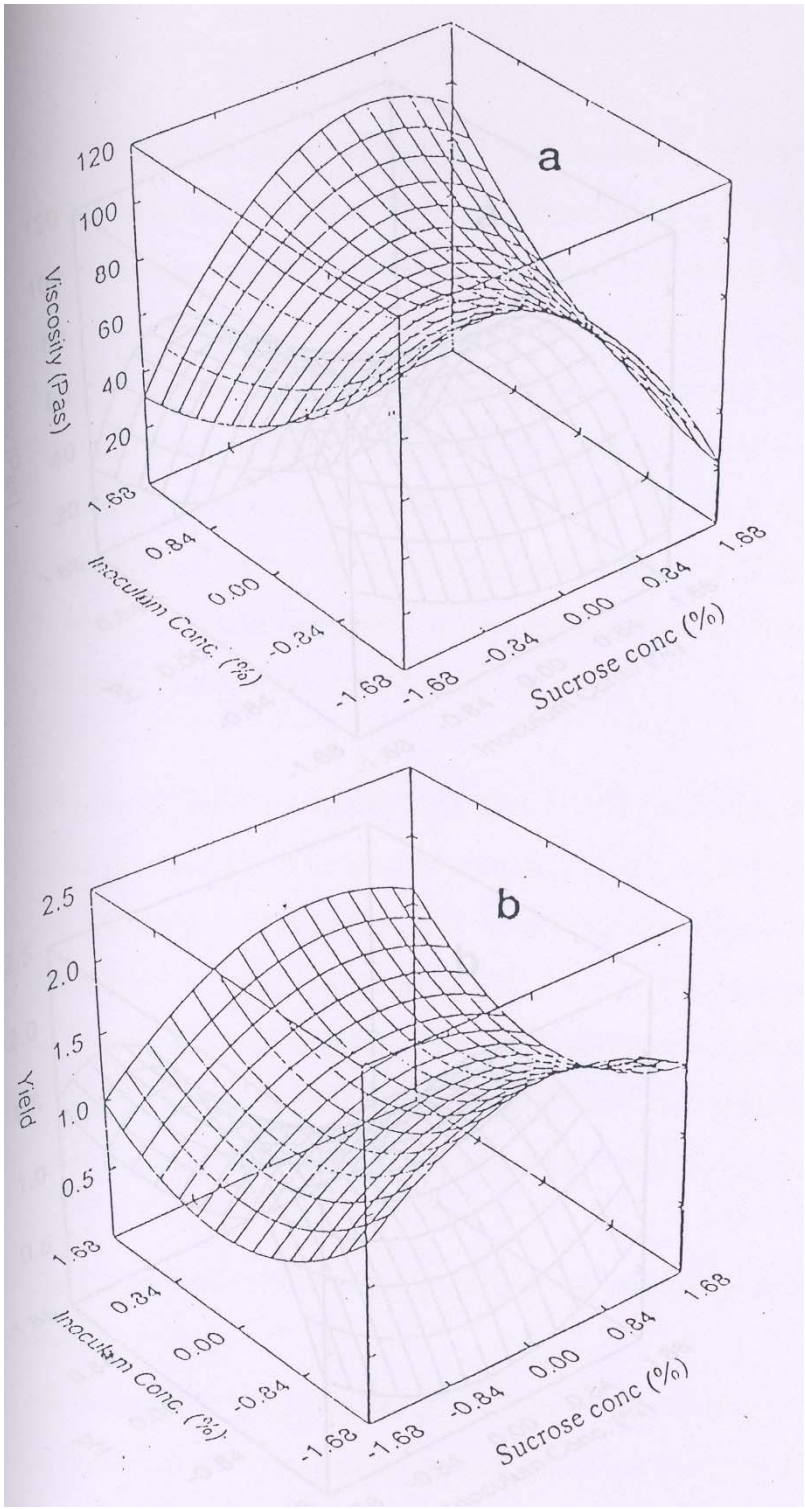


Fig.10: Response surface showing effect of inoculum an sucrose concentration on (a) Viscosity (b) Yield of polysaccaharide

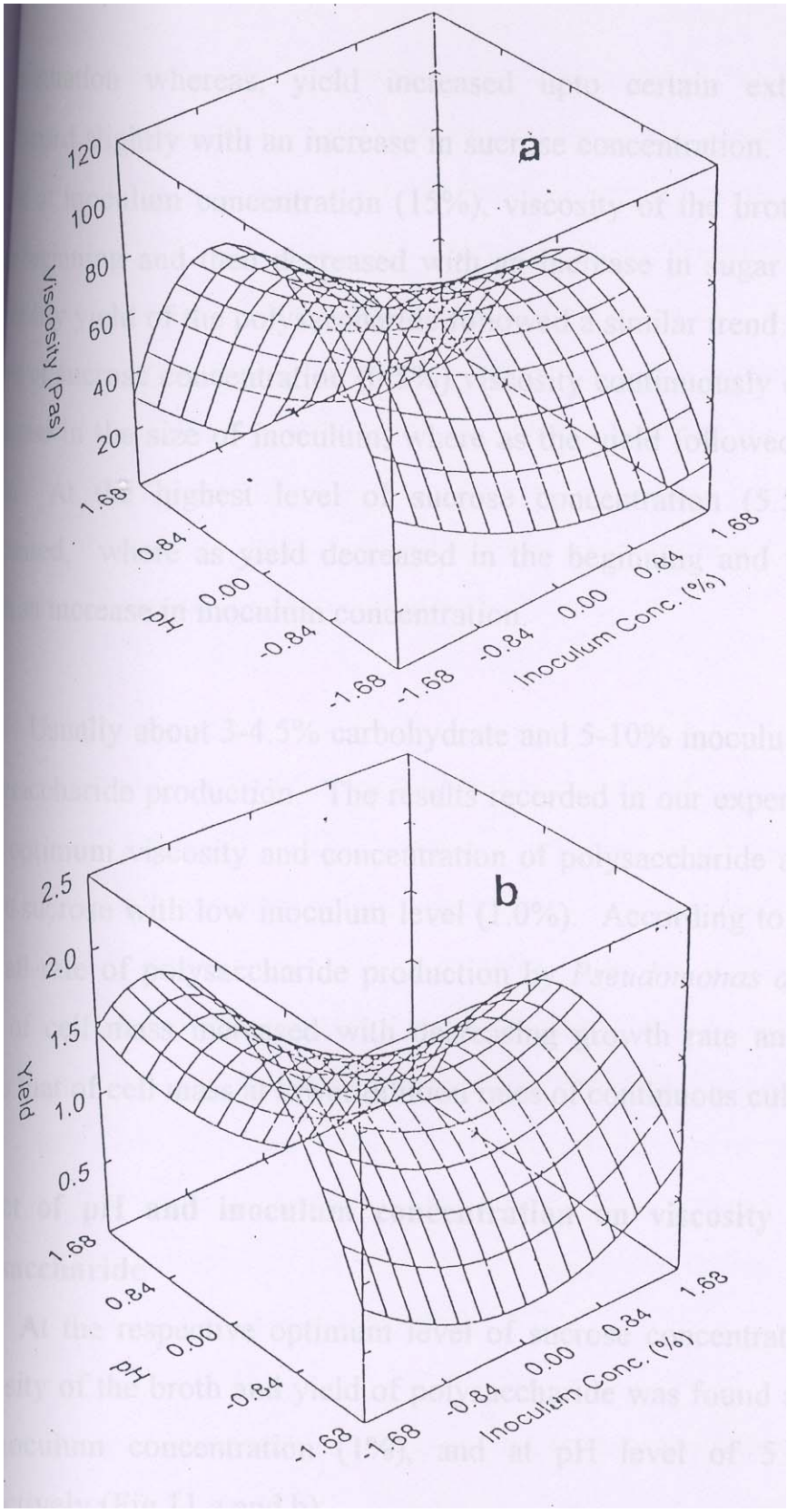


Fig.11: Response surface showing effect of pH and inoculum concentration on (a) Viscosity (b) Yield of polysaccharide

concentration whereas, yield increased up to certain extent and then decreased slightly with an increase in sucrose concentration. At the highest level of inoculum concentration (15%), viscosity of the broth increased in the beginning and then decreased with an increase in sugar concentration. Similarly yield of the polysaccharide followed a similar trend. At the lowest level of sucrose concentration (0.5%) viscosity continuously decreased with increase in the size of inoculum, where as the yield followed the parabolic path. At the highest level of sucrose concentration (5.5%), viscosity increased, where as yield decreased in the beginning and then increased with an increase in inoculum concentration.

Usually about 3-4.5% carbohydrate and 5-10% inoculum are used for polysaccharide production. The results recorded in our experiment indicate that optimum viscosity and concentration of polysaccharide are obtained at 3.3% sucrose with low inoculum level (1.0%). According to Oeavin et al[57] overall rate of polysaccharide production by Pseudomonas aeruginosa per unit of cell mass increased with decreasing growth rate and was several times of cell mass at lower dilution rates of continuous culture.

Effect of pH and inoculum concentration on viscosity and yield of polysaccharide

At the respective optimum level of sucrose concentration maximum viscosity of the broth and yield of polysaccharide was found at lowest level of inoculum concentration (1%), and at pH level of 5.85 and 6.28, respectively (Fig 11 a and b).

At the lowest level of inoculum concentration (1.0%) viscosity and yield followed the parabolic path with an increase of pH. Same trends were observed for broth at highest level of inoculum concentration (15.0%). At lowest level of pH (4.0), viscosity decreased in the beginning and then remained constant with increase in inoculum concentration. At the highest level of pH (8.0), viscosity of the broth decreased up to a certain extent with an increase in inoculum concentration and further increase, neither resulted in any development of viscosity nor any yield of the polysaccharide. In bacterial fermentations, pH of about 7.0 has been observed to influence polysaccharide production than cell growth 143.

Optimization

The optimum conditions' for viscosity and yield of polysaccharide are presented in Table-27. The viscosity of the broth and yield of polysaccharide are optimum at the lowest level of inoculum concentration (1% or -1.682 in coded level). Hence, in order to deduce workable optimum conditions graphical optimisation technique was adopted. This technique drastically reduces the amount of time effort required for investigation of multi factor, multi response systems. It also provides comprehensive and informative insight of the system, which leads to fast process optimisation. The specifications necessary for each of the response were first set and these also served as a constraint in optimisation [144-146].

An acceptable compromise was made based on the following criteria: Y1 ≥100cp and Y 2 ≥2.2%. The contour plots for the response were

Table – 27: Optimum conditions for maximum viscosity and yield

Parameters	For maximum viscosity (112.64 mPas)		For maximum yield (2.29%)	
	Coded Values	Actual values	Coded Values	Actual values
Sucrose concentration (g%)	-0.9306	1.60	0.2008	3.30
Inoculum concentration (v/v, %)	-1.6820	1.00	-1.6820	1.0
PH	-0.1225	5.85	0.2311	6.28

generated which are shown in fig 12 a and b and compared visually. The contour plots were superimposed and the regions that best satisfies all the constraints were selected as the optimum conditionsl41. Super imposed contour plots for each response are shown in Fig 13. A combination of optimum working conditions can be selected from this shaded area Sucrose concentration of 2.62g%, inoculum concentration of 1.0% (v/v) and pH of 6.24 can be recommended as practical optimum.

Verification of results

The suitability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions were determined to be optimum by response surface methodology optimisation approach and was also used to validate experimentally and predict the value of the responses using model equations. The experimental values were found to be in agreement with the predicted ones (Table 28). The polysaccharide had good viscosity and showed pseudoplastic flow behaviour. It gelled at 2% (W/V) to give elastic gels.

3.5 CONCLUSION

The viscosity of the broth and the yield of the polysaccharide are the complex functions of bacterial cells which are dependent on C/N concentration, inoculum level and pH.

Low inoculum concentration (1 %) with 2.62% sucrose and O.26g% (w/v) KNO_3 and pH 6.4 were found to be most favourable optimum

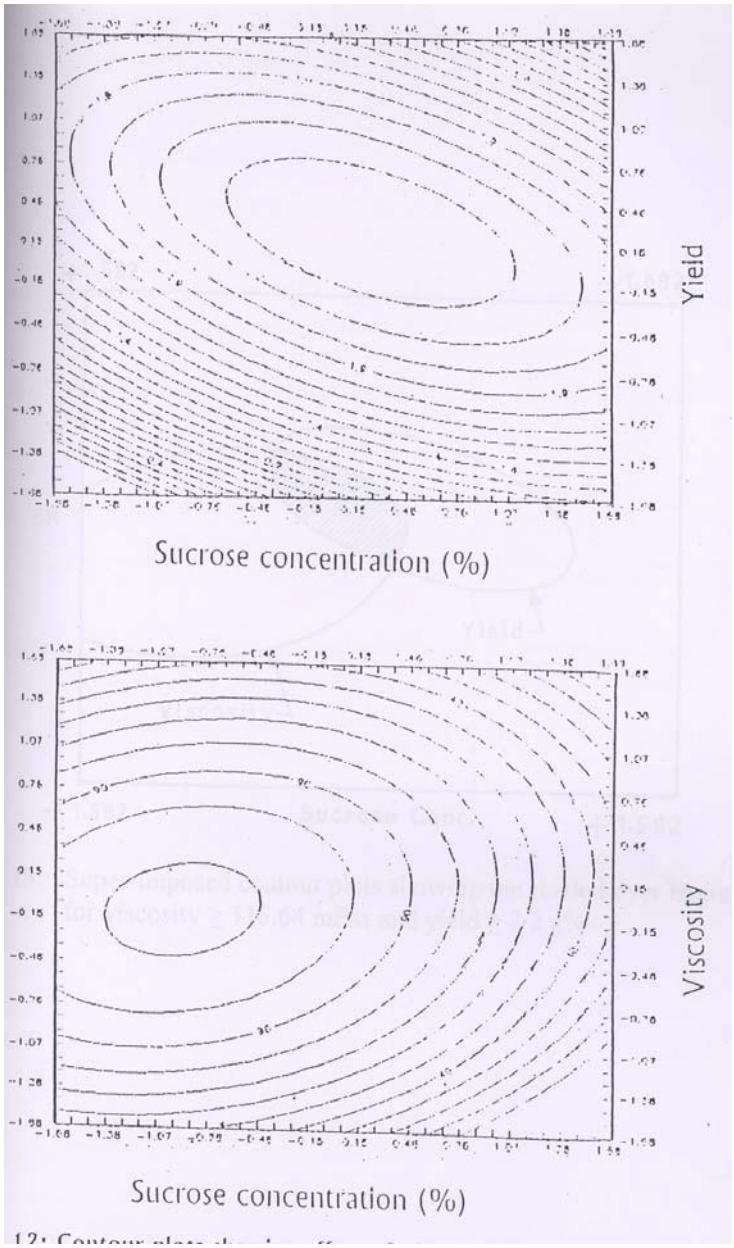


Fig.12: Contour plots showing effect of pH and sucrose concentration on yield and viscosity

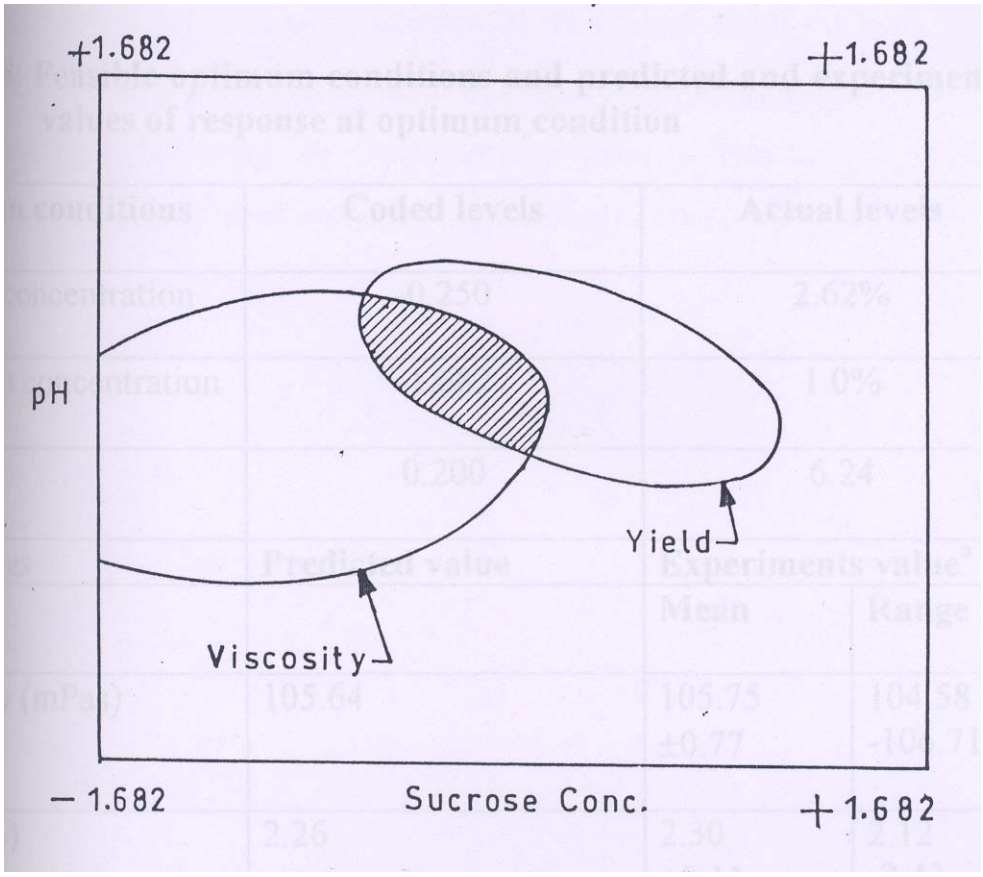


Fig.13: Super-imposed contour plots showing the shaded over lapping area for viscosity Ions for maximum viscosity and yield of polysaccharide by ≥115.64 mPas and yield ≥2.2 g%.

Table – 28: Feasible optimum conditions and predicted and experimental values of response at optimum conditions

Optimum conditions	Coded levels	Actual levels	
Sucrose concentration	-0.250	2.62%	
Inoculum concentration	-1.682	1.0%	
Ph	0.200	6.24	
Responses	Predicted value	Experiments value[a]	
Viscosity (mPas)	105.64	105.75 ±0.77	104.58 -106.71
Yield (%)	2.26	2.30 ±0.11	2.12 -2.43

Mean value of five determinations.

conditions for maximum viscosity and yield of polysaccharide by A.radiobacter using response surface methodology. The RSM provides an insight into the interaction and identifies the optimum combinations of variables for maximization of viscosity and yield with the help of a relatively small number of experiments. The experiment consists of three variables and five level experimental design which amounts to (5x5x5) 125 combinations, but only 20 combinations were sufficient to predict the optimum conditions, thus reducing the time and cost of study.

CHAPTER – 4
LARGE SCALE PRODUCTION OF POLYSACCHARIDE AND ITS RECOVERY

INTRODUCTION

Scaling up of lab scale microbial extracellular polysaccharide production is essential to establish a viable industrial technological process. Operating conditions for polysaccharide productions which exist in lab scale shake flask or submerged cultures are different compared to that of the bioreactor, For the large scale or commercial production, standardization of media, sterilization, mixing and aeration, control of process variables, fermentation systems, and forms of fermenters and product recovery are required for commercial viability of the process[130].

Down stream processing or post fermentation process or isolation and purification of the product are carried out with the following aims

1 Extraction of the polysaccharide in solid form which enables to purify the product, increase the storage life and easy handling and transportation.

2. To purify the polysaccharide from the fermentation broth. This helps in removal of media components, other metabolites, cells, which imparts turbidity, colour, and decreased functionality to polysaccharide.

3. To increase the functionality of .the polysaccharide or to modify it by chemical methods. A number of methods are available to achieve these steps and the steps adopted depend upon the end use of the polysaccharide and the cost.

Polysaccharide can be purified by various methods[130]:

Physical treatment

Polysaccharide broths are usually diluted or heated to reduce viscosity prior to filtration or centrifugation to remove the insoluble material such as biomass. Because of enormous volume of water used in dilution, there is a concomitant increase in the volume of material to be filtered or centrifuged. The dewatering process requires more organic solvent for precipitation of the polysaccharide, and hence the process becomes energy intensive.

Chemical pretreatment

Chemicals are used to coagulate or disintegrate the bacterial cells before solvent recovery. Chemical modification of the polysaccharide at the pre-recovery stage may sometimes be essential as in the case of gellan gum. Here deacetylation by alkali treatment causes changes in the polysaccharide and increases the gel strength.

Enzyme treatment

Polysaccharide broth can be clarified by enzymatic digestion of the cells. This can be achieved by proteases or by crude enzymatic preparations[148].

Various recovery methods are used based on type of polysaccharide and their application:

Solvent precipitation

Ethanol, methanol, isopropanol etc., are used in 1:2 volumes to precipitate the polysaccharide from the fermentation broth.

Salt precipitation

Polysaccharide can be precipitated as quaternary ammonium complex, metal on salt, or fatty amine complex at acidic or alkaline pHs.

Precipitated polysaccharides are usually dried 'under vacuum and milled to a known mesh before packing.

4.1 MEDIA

Optimised medium for polysaccharide production by A.radiobacter containing (g%, w/v) Na2HPO4 (0.4), MgSO4 7H2O (0.1), KNO3 (0.3) and sucrose (3) at pH 7.0 was used for fermentor trials and comparative shake flask experiments.

The composition of inoculum medium used is the same as described Chapter 1.

4.2 METHODS

Large scale fermentation

Fermentation was carried out in 10 liter fermentor (New Brunswick Scientific, USA) having all the accessories. Medium (7 liter) was transferred to the fermentor jar, and all the air entry points in the lid were

plugged with cotton. The jar was placed for sterilization at 15 lbs for 20 min. After cooling, it was connected to the main body and air inlet. Medium was inoculated with 1% (v/v) inoculum of A.radiobacter under sterile conditions. The fermentation was carried out for 72h at 300C and 1000 rpm with 1, 1.5 and 2 VVM of air: Samples were drawn aseptically once in 24h of growth and analysed for biomass, polysaccharide weight, pH, viscosity, residual sugar as mentioned in chapter on materials and methods.

Simultaneously fermentation was also carried out in 500ml conical flasks with 100 ml of above-mentioned medium. The flasks were inoculated with 1% inoculum and placed on shaker for 72 hr at 28°-30°C at 250rpm Flasks were removed in duplicates at an interval of 24h and analysed as mentioned above.

Purification of the polysaccharide

Fermented broth of A. radiobacter was processed by physical and enzymatic methods for the removal of biomass.

Physical method

Exopolysaccharide containing fermented broth of known biomass concentration was diluted in 1: 1 to 1 :20 dilutions and homogenised (Remi Motors, Type Ro 127 with adjustable speed upto 8000 rpm). The solutions were centrifuged at 10,000 rpm for various periods (30, 45 and 60 min). The supernatant was decanted and the polysaccharide was precipitated using 2 volumes of isopropanol. Pelleted biomass was dried at 70°C to a constant weight.

Enzymatic method

Effectiveness of macerating enzyme to degrade the bacterial cells contained in polysaccharide broth was tested by diluting the fermentation broth (1:10) and. subjecting it to the following treatments:

1. Addition of 0.1-0.3g% of multienzyme digestant (sanzyme, Sankyo company limited Tokyo, Japan).
2. Incubation of sample (pH 7.0) containing 0.lg% enzyme at 30, 40, 50 and 60°C.
3. pH of the sample was adjusted to 5-9 and incubated with 0.1 g% of enzyme at 50°C. Unless otherwise specified the samples (pH 7.0) were incubated at 50°C upto 24h and O.D was measured at 620 nm against water as blank

Enzyme activity: The enzyme utilized for cell degradation contained glucanases (140 u/mg) and proteases (125 u/mg) in addition to other enzymes (not assayed).

Modification of the polysaccharide

Various grades of the A. radiobacter polysaccharide were prepared to find out the rheological properties of the preparations. The method of preparations are as follows.

1. Fermented culture broth (96h) was precipitated directly to recover the polysaccharide by using 2 volumes of isopropanol. The precipitate was collected and dried at 70°C to a constant weight (product A).
2. Clear gel could be obtained by the removal of biomass. The fermented broth (96h) was diluted 5 times of its original volume and heated to 80°C. At this temperature the solution lost its viscosity and turned watery. The hot solution was filtered under vacuum through cellulose pad of 1 cm thickness. The filtrate was clear and the polysaccharide present in it was precipitated and dried as mentioned above. (Product B).
3. An attempt was made to modify the gel using alkali treatment. In this process pH of the diluted broth was increased to 11 and the resulting solution was heated to 80°C and maintained at that temperature for 15 min. Solution pH was decreased again to 7 and filtered while hot, polysaccharide was recovered and dried as mentioned above (product C).

4.3 RESULTS

Large scale fermentation

Initial experiments using I, l.5 and 2.0 VVM of air resulted in 1.5, 2.1 and 1,9 g% polysaccharide in 72h of fermentation and hence l.5 VVM was selected for detailed studies. In both the fermentor and shake flask, cultivation (Plates 5 and 6), the production of polysaccharide occurred throughout the fermentation period. Data in table 29 and Table-30 indicate that the increase in biomass was noticed upto 48 hr of fermentation. Maximum viscosity and polysaccharide produced was more in fermentor (4000 cP and 2.04g%) compared to shake flask cultures (2200 cP and] .25 g%) after 72 h of fermentation. The broth became very viscous after 72 h of cultivation and could not be mixed properly in the fermentor because of the increased viscosity. However conversion of sugar to polysaccharide was more in fermentor (80%) compared to shake flask (65%).

Purification of the polysaccharide

Physical method

Data obtained on clarification of polysaccharide of A. radiobacter by dilusion and centrifugation are recorded in Table 31. Results indicate that the broth having viscosity of 6000 cP is to be diluted 10 times or 20 times and centrifuged for 60 or 30 min, respectively, to obtain 97% removal of biomass

Plate – 5: Culture broth obtained after growth under shake flask cultivation: A: medium; B: broth

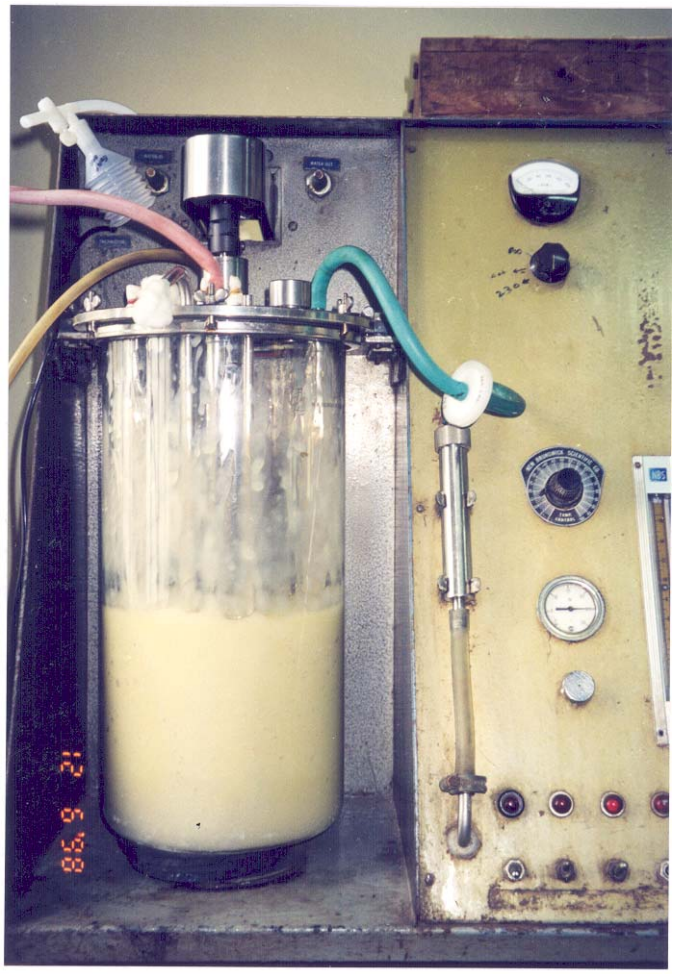


Plate – 6: Cultivation of bacterium in fermentor

Table-29: Kinetics of polysaccharide production in 10 litre fermentor using optimised medium at pH 7.0, 30^{o}C and 1.5 vvm of aeration

Period (h)	pH	Viscosity (cP)	Biomass (g%, w/v)	Residual sugar (g%, w/v)	Polysaccharide (pure, g% w/v)	Conversion (%)
24	7.0	900	0.18	1.70	0.74	57
48	7.0	2000	0.33	1.30	1.37	80
72	7.4	4000	0.36	0.45	2.04	80

Table.30: Kinetics of polysaccharide production in shake flask culture using optimised medium at pH 7.0, 28-30^{o}C and 250 rpm

Period (h)	pH	Viscosity (cP)	Biomass (g, w/v)	Residual sugar (g%, w/v)	Polysaccharide (pure, g% w/v)	Conversion (%)
24	7.0	400	0.11	1.9	0.7	64
48	7.0	960	0.26	1.6	0.81	58
72	7.6	2200	0.26	1.1	1.25	65

Table- 31: Clarification of polysaccharide using physical method (centrifugation)

Dilution (broth:water)	Viscosity (cP)	Centrifugation at 10,000 rpm (min)	Biomass removed (%)
1:1	1200	30	28
1:1.5	800	30	33
1:3.5	400	30	37
1:7.5	Nil	30	62
1:10	Nil	10	50
1:10	Nil	20	80
1:10	Nil	30	90
1:10	Nil	45	93
1:10	Nil	60	97
1:20	Nil	30	97

Enzymatic method

Data recorded in Figs.14-16 indicate that the multienzyme digestant used for the degradation of biomass is more active at 0.3g% (w/v) concentration at pH 7 and 50°C. Clarification of various concentration of rehydrated polysaccharide solutions at 50°C, pH 7.0 and 0.3g% enzyme concentration resulted in 57% degradation of the bacterial cell (Table-32). Precipitation and rehydration of the polysaccharide in original volume of water gave 80% clarity compared to untreated control. (Plate 7).

Modification of the polysaccharide

Different grades of A.radiobacter polysaccharide obtained (see above) were compared for recovery. The yields (g%, w/v) obtained were 2.18g of product A, 1.82 g of product Band 1.31 g of product C. Properties of these gels are dealt under chapter 5.

4.4 DISCUSSION

Fermentation

Mixing, aeration, dissolved oxygen, pH and temperature play important role in the production of the polysaccharides. Decrease in pH during fermentation will be due to formation of organic acids and also due to acidic functional groups contained in the polysaccharide. If the pH falls below 5, growth and polysaccharide production may decrease. In such cases it is important to control the optimum pH by the use of buffer or addition of alkali during the process. In A. radiobacter fermentation, pH change during fermentation was marginal which may be because of good buffering

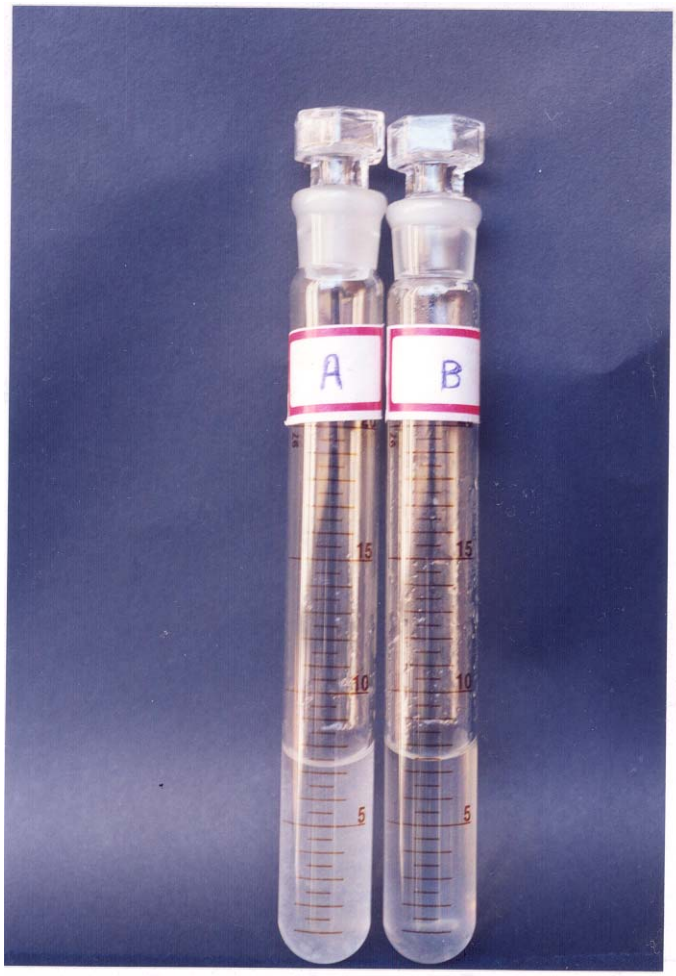


Plate - 7: Degradation of biomass (enzymatic) for clarification: A: control; (B) treated

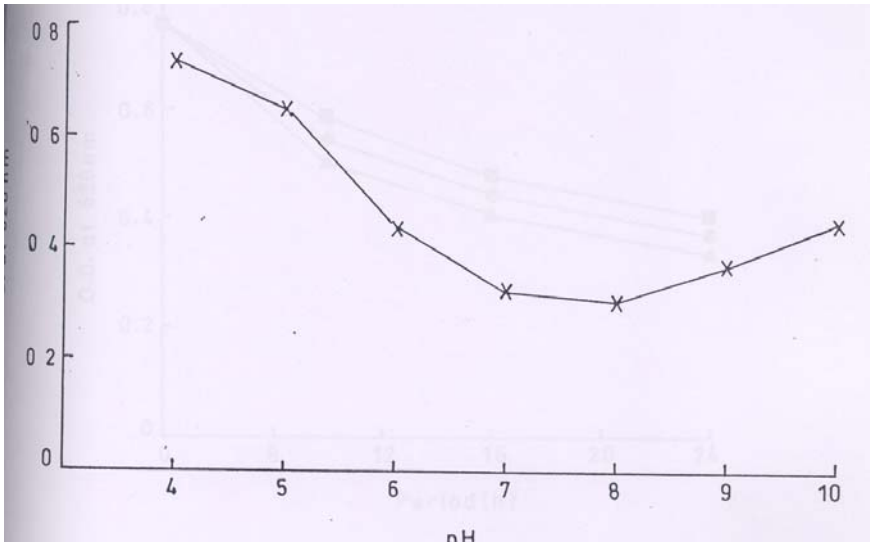


Fig.14 Clarification of culture broth at different pH

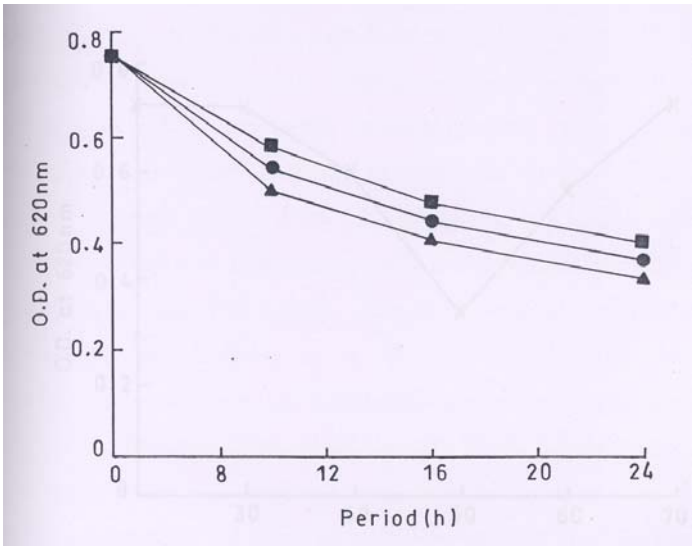


Fig.15 Effect of enzyme concentration on clarification of culture broth Enzyme concentration

0.1%▲–▲

0.2%●–●

and 0.3%■–■

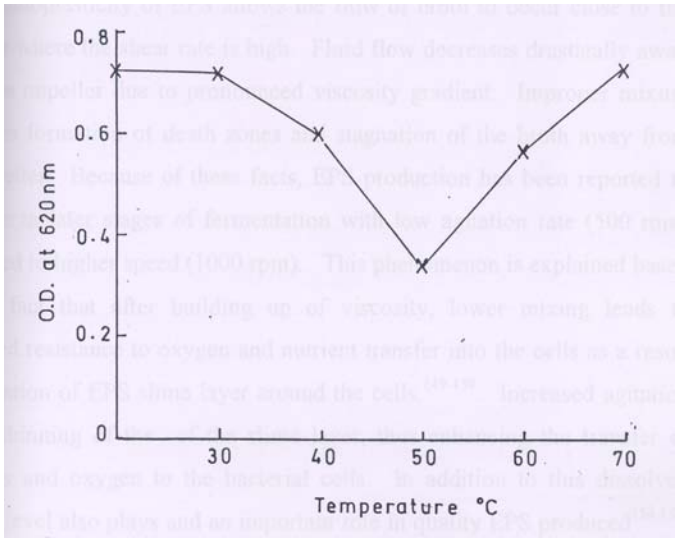


Fig.16 Effect of temperature on clarification of culture broth

provided by disodium hydrogen phosphate and quality of expolysaccharide (EPS) produced.

Mixing of highly viscous broth is an important aspect which affects the EPS fermentation. Mixing results in transport of oxygen from gas phase to the microorganisms and promotes homogenous blending of the nutrients. The pseudoplasticity of EPS allows the flow of broth to occur close to the impeller where the shear rate is high. Fluid flow decreases drastically away from the impeller due to pronounced viscosity gradient. Improper mixing promotes formation of death zones and stagnation of the broth away from the impeller. Because of these facts, EPS production has been reported to decrease in later stages of fermentation with low agitation rate (500 rpm) compared to higher speed (1000 rpm). This phenomenon is explained based on the fact that after building up of viscosity, lower mixing leads to increased resistance to oxygen and nutrient transfer into the cells as a result of formation of EPS slime layer around the cells. 149-159. Increased agitation causes thinning of the of the slime layer, thus enhancing the transfer of nutrients and oxygen to the bacterial cells. In addition to this dissolved oxygen level also plays and an important role in quality EPS produced[154,155]. The increase in agitation mayor may not increase the biomass production and may affect polysaccharide production only as observed in the synthesis of pullulan [157-159]. This would mainly depend on the aerobicity or microaerophillic nature of the microorganism that is involved in EPS production. Results obtained with A. radiobacter indicate that biomass as well as EPS produced and conversion rate was more in fermentor compared

to shake flasks. This could be due to better mixing in fermentor leading to availability of nutrients and oxygen to the cells. EPS production phase is also reported to be affected by critical oxygen levekl[160-163].

Purification

Bacteriolytic enzymes isolated from microorganisms are highly specific in degrading only the gram positive bacteria or gram negative bacteria[165-177]. This is because

1. Nature of cells walls found in these two groups.
2. Enzymes of gram negative bacteria cannot lyse other gram negative cells since the enzyme secreting bacteria might themselves to damaged by the lytic enzyme they secrete.

Multienzyme digestant used in the experiment contained glucanases and proteases in addition to other enzymes (not assayed). This could not lyse the live A.radiobacter cells tested at various incubation temperature, or pH. This may be because of outer resistant macromolecular capsular layer. The cells became susceptible once they were killed by heat treatment or during alcoholic precipitation of the polysaccharide. The cell wall of Agrobacterium sps is known to contain glutamic acid, alanine, diaminopimelic acid and aminosugars. In addition to this large amount of leucine, phenylalanine, serine and aspartic acid is also present. The lipopolysaccharide envelope contains glucose, fucose and 2-keto-3-deoxycotinic acid, and fatty acids such as 3-hydroxy tetradeconic acid, hydroxy hexadecane acid, phosphatidyl choline, phosphatidyl ethanolamine phosphatidyl N-methylethanolamine and phosphotidyl glycerol. Because of the molecular complexity involved, cell wall cannot be lysed by protease, or glucanasesases etc., individually, and it could be lysed by multienzyme system.

The clarity of enzyme treated sample increased by precipitation and resuspension of po]ysaccharide. This may be because lysed material which caused turbidity leached out into solvent system during precipitation step.

In physical method of biomass removal for purification of the polysaccharide, high degree of dilution followed by centrifugation are required. This would increase the production cost of pure polysaccharide preparation. Compared to this, enzymatic cell lysis and polysaccharide purification appear to be relatively less energy consuming and economical.

CHAPTER – 5
CHARACTERIZATION AND UTILIZATION OF THE POLYSACCHARIDE

5.0 INTRODUCTION

The world market for polysaccharides are presently dominated by plant and seaweed gums, Only a very few microbial polysaccharide such as xanthan, dextran and gellan have been commercialized so far, Commercialization of new polysaccharide will depend upon factors such as economics and availability, It also depends on properties of the polysaccharides, which are not found in others, They include better viscosity, gel formation, film formation, compatibility with a variety of salts, synergism with other gums, resistance to a wide pH, temperature, solubility etc. These properties depend on the microorganisms used, cultural and nutritional parameters adopted for cultivation, composition of the polysaccharide, rheology and molecular weight.

In addition to the utilization of polysaccharide as viscosity, suspender agent, coating, binding, clarifying, and film forming agents they also find use as gelling agents97,103-112. They impart texture and thickening properties in foods and also in non-food products, Summarized data in Table 33 indicate the conditions required for gelling of the existing polysaccharides, texture obtained and their utilization, Most of these require special conditions for thermal gelation to form soft, elastic or brittle gels.

In the present study, physico chemical characterization and utilization of the polysaccharide produced by A.radiobacter have been attempted.

Table-32: Enzymatic clarification of gelling polysaccharide using multienzyme digestant

Polysaccharide concentration (g%, w/v)	Percentage degradation of biomass (mg%, w/v)			
	Control	Enzyme treated		
	24 h	2 h	4 h	24 h
0.25	Nil	1.81	26.4	30.90
0.50	Nil	37.70	43.24	56.97
1.00	Nil	23.70	27.65	43.55
1.50	Nil	14.18	21.64	28.91
2.00	Nil	10.14	12.31	21.01

Table-33: Gelling polysaccharides (plant, algal, and microbial organism)

Polysaccharide	Gelling mechanism			Gel texture	Applications
	Thermal	Chemical	Conditions		
Agar	X	-	-	Brittle	Confectionery, canning, microbiological media
Carrageenans	X	-	Requires Ca or K ions	Elastic to brittle	Desserts, canning, pet foods
Furcellaran	X	-	Requires K ions	Brittle water gels	Desserts
Alginate	-	X	Require calcium	Brittle	Milk products
Gelatin	X	-	-	Soft	Desserts, confectionary
Pectin	X	Requires sugar and acidity	Soft	Jams and jellies	
Starches	X	-	-	Soft	Desserts, puddings
Xanthan + galacto-mannan	X		Does not gel individually	Elastic	-
Gellan	X	-	Requires mono or divilant actions	Brittle	Agar substitute in cultural media, plant tissue culture
Curdlan	X		Alkaline, calcium ions	Elastic	Pharmaceutical
Bacterial alginates	X		Calcium salts	Controllable	Desserts, textile industry

5.1 MEDIA

Media mentioned below were prepared as slants for experiments on comparative utilization of A.radiobacter gelling polysaccharide and agar for growth of various fungal and bacterial species.

Nutrient medium

Nutrient agar medium was prepared as standard medium for bacterial growth as mentioned in chapter 1. In another set agar was replaced from the medium with A.radiobacter polysaccharide (purified; 2g% w/v).

Potaoto dextrose medium

Potato dextrose agar was prepared for the cultivation of fungi (see chapter 1) for comparative study in another set of medium agar was replaced by purified A.radiobacter polysaccharide (2g%, w/v).

5.2 METHODS

Characterization of the polysaccharide

Purified gelling exopolysaccharide isolated from Agrobacterium radiobacter (see chapter on materials and methods) that was cultivated for 96^{th} in KNO_3 medium (Chapter 1, basal medium) was used for physicochemical characterization.

Physical properties of the polysaccharide

Viscosity

Viscosity of the polysaccharide samples was recorded using different spindles and speeds in Brookfield viscometer (Chapter: Materials and methods).

Viscosity of heat solubilised pure polysaccharide was compared with that of agar at 0.5g% (w/v) at various shear rates using Haake viscometer (Model R13M, Germany).

Effect of temperature on gel

To find out the effect of temperature on melting of the gel, pure polysaaccharide preparation was hydrated in water (1 g%, w/v). Sample (200 ml) taken in beaker (250ml capacity) was boiled to solubilise the polysaccharide and cooled to 10°C. The beaker was placed in water bath (temperature controlled) and the temperature was allowed to increase slowly upto 100°C. Viscosity of the suspension was simultaneously measured and recorded at various temperatures. Agar (1%) was used for comparison.

Effect of pH and temperature on gel

Pure polysaccharide was prepared and heated as mentioned above except that pH of the polysaccharide solutions were adjusted to 2.5, 6.5 and 11.5 before measurement of the viscosity.

Measurement of gel strength

Polysaccharide solutions were autoclaved for 20min at 15lbs and gel strengths were measured using Instron (Universal Testing Machine, Model 4301, UK) with 10 mm plunger at a speed of 10mm/min and load used was 5kn. Gel strength was calculated by using the following formula:

$$\text{Gel strength (g/cm2)} = \frac{\text{Load}}{\text{Area}}$$

$$\text{Where area} = \frac{\Pi d^2}{4}$$

Load= Compression or load utilized (at 80% compression).

Measurement of gel strength after autoclaving cycles

Polysaccharide was dissolved in water (100ml) at 2g% (w/v) concentration in 100ml beaker. Agar was used for comparison. Beakers were covered tightly with aluminum foil and autoclaved at 15lbs for 20min. Gel strength of the cooled sample was measured as mentioned above. Autoclaving and measurement of the gel strength during each cycle was calculated compared to 0h value.

Effect of polysaccharide concentration on gel strength

Polysaccharide was dissolved in water at 2 and 3g% (w/v) level, autoclaved at 15lbs for 20min and gel strength was measured as mentioned above.

Effect of salts on gel strength

Salts such as KCl, NaCl, MgCl26H2O, and CaCl2H2O were added at 0.1g% (w/v) to purified polysaccharide (2g%). Samples were autoclaved at 15 lbs for 20lnin, cooled and gel strengths were measured.

Elasticity

One hundred ml of A.radiobacter polysaccharide solutions (2g%, w/v) were autoclaved in beakers (100ml capacity at 15lbs for 20min. cooled and elasticity was measured by texture profile analysis technique[l06], using Instron (Universal testing machine, model 4301 UK). The gel of known height was compressed twice in succession using circular dia plunger (10 mm) at a cross head speed of 100mm/min. Agar (Himedia, Bombay, India) and gellan (Kelco, USA) were used (2g% solutions) for comparison. Elasticity (mm) was calculated by finding out the distance between the beginning and completion of second bite graph.

IR Spectra

Purified polysaccharide of A. radiobacter, agar and gellan were solubilized in DMSO and subjected to IR spectroscopy using KBr windows at 400-4000 cm-l wave number (Brucker IFS 25, Germany).

Scanning Electron Microscopy

Dehydrated polysaccharide, and also autoclaved (15 lbs 20 min) and solubilized samples of agar and A. radiobacter polysaccharjde were successively dehydrated by using 30% to 100% acetone and placed in vacuum. Dried sample was sputter coated and scans were taken in scanning electron microscope (Leo43S, UK).

Composition of the polysaccharide

The polysaccharide was analyzed for moisture and ash content according to the methods described in the chapter on Materials and Methods.

Total carbohydrates

Total carbohydrates was estimated by Phenol-sulfuric method[105,142]. Here sample (1 ml) was mixed with 1 ml of 5% phenol (distilled) followed by addition of 5ml of concentration. H2SO4. The contents of the tubes were cooled and intensity of the colour formed was measured against reagent blank at 480 nm. Concentration was calculated by comparing standard graph.

Chromatography

Hydrolysis of polysaccharide

Purified polysaccharide (20 mgs) was hydrated in water (1 ml) in a test tube. Trifluroacetic acid (TFA; E, Merck, Germany) of 4N concentration was added (1 ml) to the sample and the tubes were sealed. Sample was hydrolysed at 10SoC for 6h. TF A was removed by evaporation under vaccum (Rotary evaporator, Buchi, with B-160 vacobox and 461-water bath, Switzerland). The dried residue was suspended in about 5ml of water and evaporated again. The process was repeated several times to remove the residual TFA. Final residue was dissolved in 0.2ml triple distilled water and used for analysis.

Paper chromatography of carbohydrates

Carbohydrates present in the above polysaccharide hydrolysate was analyzed by descending paper chromatography. Various standard sugar solutions (1 %, w/v) such as rhamnose, xylose, mannose, galactose, fucose, arabinose, glucose, glucuronic acid, galacturonic acid, and manuronic were spotted individually (10ul) and in mixtures along with polysaccharide hydrolysate on Whatman No.1 filter paper sheets. The chromatograms were run by descending method in (a) n-butanol: ethanol: water (10:1 :2) (b) n-butanol: pyridine:

water (6:4:3) for separation and identification of neutral, sugar and in (c) ethyl acetate: pyridine: acetic acid: water (5:5:1:3) uronic acids. The papers were developed twice (24h each time)[179].

Aniline-hydrogen phthalate reagent

Phthalic acid (1.66g) and distilled aniline (0.93g) were dissolved in water saturated with n-butanol (100ml). The papers were dipped in the reagent, air-dried and placed in an oven at 10Soc for 10min. Characteristic red spots were observed for pentoses, brownish spots for hexoses and purple colour for uronic acids[180].

Gas liquid Chromatography

Polysaccharide solution hydrolyzed by TFA (the pH of which was neutral) was taken in a stoppered test tube, solid $NaBH_4$ (15-20mg) was added and allowed to stand overnight. Excess BH_4 was removed by addition of 2 drops of 2N acetic acid once in 30 min till neutralized and was distilled to dryness.

O-Acetylation was carried out by adding pyridine-acetic anhydride (0.5ml, 1:1 v/v) and heating on boiling water bath for 1 h. Excess reagents were destroyed by codistillation with water (1 ml, twice) and toluene (1 ml, twice) and dried.

Organic acids

Polysaccharide (100mg) was suspended in 4ml of 2NH2SO4. The tube was sealed and kept for hydrolysis at 100oC for 4hr. The sample was extracted with diethyl ether and concentrated by vacuum evaporation at 40^{o}C. Organic acid present in the sample was detected by using descending paper (Whatman No.1) chromatography. Solvent system used was n-butonal-acetic acid-water (4: l: 5). The organic acid spots were identified by spraying the paper with bromocresol green. Pyruvic acid, succinic acid and acetic acids were used as standards. Organic acid was also assayed colorimetrically[132-135].

Molecular weight

A. radiobacter was cultivated in KNO3 medium and purified was prepared from 24, 48, 72, 96 and 120 h old fermentation broth. Molecular weights were determined by gel permeation chromatography as follows.

Preswollen and washed Sepharose 2B (Pharmacia, Sweden) was packed in glass column (1.3 x 90 cm) at a flow rate of 35 ml/h. The column was equilibrated by washing with 0.1 M NaCl. The column was precalibrated with dextrans of various molecular weights (T 10, T 40, T 500, T 2000). Hydrated polysaccharide solution (5mg/ml) was loaded on to the column and eluted with 0.1M NaCl at a flow rate of 18ml and the fractions were collected. Fractions were analyzed for polysaccharide by phenol sulfuric acid reagents.

Structural analysis

Purified polysaccharide obtained after the growth of A. radiobacter in KNO3 medium for 96h was used for the structural analysis.

Methylation analysis[85,129]

Preparation of methyl sulfinyl carbanion (MSC): Sodium hydride (500mg) taken in a reaction vial was washed with petroleum ether (5 ml x 4) and dried. Dry DMSO (5 ml) was added in small portions (0.2-0.5 ml) over a period of 2hr. This reaction mixture was kept at 37°C for 12-14 hr with the occasional release of hydrogen that was liberated. Thus prepared MSC gave characteristic blood-red color with triphenyl methane.

Methylation: The polysaccharide (5mg/ml of DMSO) was completely dissolved in a reaction vial under stirring and/or occasional ultrasonification. Methyl sulfinyl carbanion (1 ml) was added to the polysaccharide solution. The mixture was stirred at room temperature for 3-4hr. A small drop of excess reagent was tested with triphenyl methane and confirmed that the test was positive. Methyl iodide (lml) was then added to the reaction mixture at ice-cold temperature, with the help of a syringe. The reaction mixture was stirred for 6-7 hr.

Purification of methylated polysaccharide

Sephadex LH-20: The methylated polysaccharide was extracted with chloroform (10 ml) and washed with water (10ml x 5). The organic extract was dried over anhydrous Na2S04 and concentrated. This was loaded onto a Sephadex LH-20 column (1.5x25cm), pre-equilibrated with a mixture of CHCl3: Methanol (1: 1 v/v) and eluted with the same mixture. Fractions (2 ml) were collected and tested on silica gel TLC strips, by charring with 5% H2SO4 in methyl alcohol. The fractions giving positive test were pooled and concentrated.

Hydrolysis of methylated polysaccharide

TIle methylated polysaccharide were hydrolyzed with formic acid (90%, 2 ml) at 100°C for 2hr. Excess acid was evaporated by codistillation with methylalcohol (lml, x 4). The resulted dry material was hydrolysed again with tirfluoro acetic acid (2M, 2 ml) in sealed tubes at 100°C for 4 hr

After the hydrolysis, the sealed tubes were opened, and the acid was evaporated by co-distilling with methyl alcohol (1 ml, x 4).

Conversion of alditol acetates[181]

The hydrolyzed sample was reduced with sodium borodeuteride (10 mg) in stoppered test tube overnight. Addition of dilute acetic acid (2N) destroyed the borohydride. Boric acid formed was removed by co-distillation with methanol. Glycitols were acetylated with pyridine and acetic anhydride (0.5ml, 1:1 v/v) in boiling water bath for 60min. Excess reagents were removed by successive evaporations with water and toluene.

Operating conditions of GLC

Shimadzu GLC (Model-CR4A) fitted with flame ionization detector was used for analysis. OV -225 was the column used with column temperature injector and detector block temperatures maintained at 170, 250 and 50°C respectively. Nitrogen (40 ml/min) was used as carrier gas.

GLC-Mass Spectrometry[85]

GLC-MS analysis was carried out on Shimadzu (Model 2P 5000) using Sp 2380 capillary column. A temperature gradient of 180-200oC with an increase of 4^{o}C/min was maintained for the analysis. Ionization potential was 70ev and mass range (m/z) was 40-400. Helium was the carrier gas used.

Utilization of the polysaccharide

Polysaccharide isolated from 96h of A. radiobacter cultivation was used as agar substitute in microbiological media preparation, as an emulsifier and as a thickening gent.

Microbiological media

Purified polysaccharide was used as gelling agent in the preparation of media (see 5.1) for the growth of fungi (potato dextrose medium) and bacteria (Nutrient medium). Bacterial cultures such as Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas sp and Acromobacter sp drawn from the stock culture of Food Microbiology Department of CFTRI Mysore were streaked on to nutrient agar slants and nutrient medium with A radiobacter polysaccharide slants were incubated at 37^{o}C.

Fungal cultures such as Aspergillus sp, Aspergillus niger, Pleurotus 37^{o}C. Trichoderma viride, Rhizopus oligosporous etc also obtained from source mentioned above were inoculated on to potato dextrose agar slants and potato dextrose with A. radiobacter polysaccharide. The slants were incubated at 30°C. The cultures were known to produce certain enzymes or metabolites as follows:

Fungal culture	Enzyme/metabolite
Aspergillus ustus (1131)	Cellulases, hemicellulases
A. niger (18)	Citric acid
A. carbonarius (1047)	Amyloglucosidase
Trichoderma sps (99)	Proteases
Sporotrichum pulverulentum (1106)	Lignases
A.niger (104)	Tannase
A.oryzae (1058)	Amylase
Polyporus squamosus (1134)	Single cell protein
A.oryzae (1120)	Amylase
Fusarium Sp (1128)	Aflatoxin
Rhizopus oligosporus (1104)	Protease
Coriolus versicolor (1109)	Cellulase

Stability of the gels and growth of microorganisms were observed after 48-l20h of growth.

Estimation of growth

Nutrient agar and nutrient medium with polysaccharide (2g%w/v) were autoclaved (15 lbs, 20min) and poured into sterile petri plates. The plates (in duplicates) were spread with 0.2ml of bacterial cultures (mentioned earlier) which were serially diluted six times. Plates were

incubated at 37°C. Total plates counts were taken after 24-48h of incubation

Emulsification

Emulsification property of isolated polysaccharide was tested according to the method of Cooper and Goldenberg (1987)[107] groundnut oil, olive oil, kerosene, castor oil, benzene, xylene and hexane (3 ml each) were mixed with 2ml of polysaccharide solutions (0.25g%, w/v) and homogenized using cyclomixer for 1-2 min. The heights of emulsion phase and aqueous phase formed were measured (mm) at 0 hr. The samples were kept undistributed at room temperature and measurements were made again after 24h and one month of standing. Emulsification index was calculated by the formula.

$$\text{Emulsification index} = \frac{\text{Volume of emulsion layer}}{\text{Total volume}} \times 100$$

Food additive

Purified polysaccharide of A.radiobacter was incorporated into milk beverage. This was prepared by addition of rose essence, colour sucrose (10%) and polysaccharide (1 g%) to boiling milk. Heating was continued for 10min, cooled and refrigerated.

Water desert gel was prepared by solubilizing polysaccharide (2g%) sucrose (10%) sodium citrate and flavour in boiling water. The mixture was cooled and refrigerated. Jam could also be prepared by using 1-15% of the polysaccharide preparation.

5.3 RESULTS AND DISCUSSION

Physico-chemical properties of the polysaccharide

Isolated exopolysaccharide of A. radiobacter produced viscous solution and after heat treatment it formed elastic gel. Using different spindles viscosity readings, of fermentation broth could not be measured with spindle and spindle 7. This is because the sample was very viscous to be measured with spindle 1 and less viscous for spindle 7. Viscosity recorded for spindle 2 and 6 are shown in Fig.17. Viscosity of A.radiobacter heat solubilised polysaccharide was more compared to agar (Fig.18), Data in Figs.17 and 18 indicate that the polysaccharide is non-newtonian and pseudoplastie in nature and exhibits shear thinning property. At low shear rates it had more viscosity indicating that it can perform better in stabilizing suspensions.

As shown in Fig 19 the polysaccharide had a higher setting point (50^{o}C) compared to agar (40°C). Agar gel is known to be unstable at highly acidic and alkaline conditions whereas A. radiobacter polysaccharide remained stable over a pH range of 2.5 to 11.5 (Fig 20) indicating wider area of application of its usage.

Crude preparation of A. radiobacter gel and agar showed 26 and 31 % decrease, respectively in gel strength after 5 autoclaving cycles (Table-34).

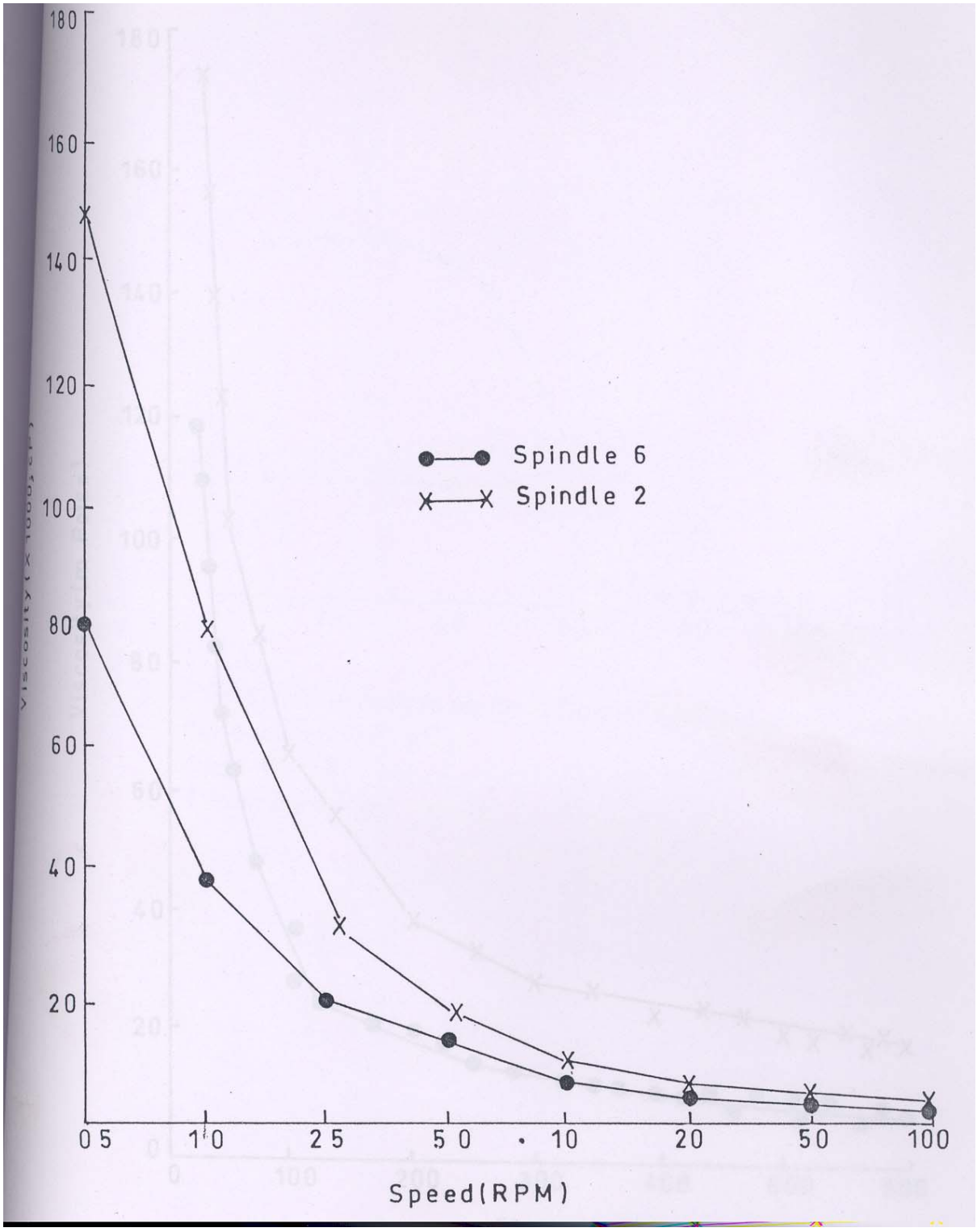


Fig.17 Viscosity of polysaccharide broth measured with different spindles and speeds

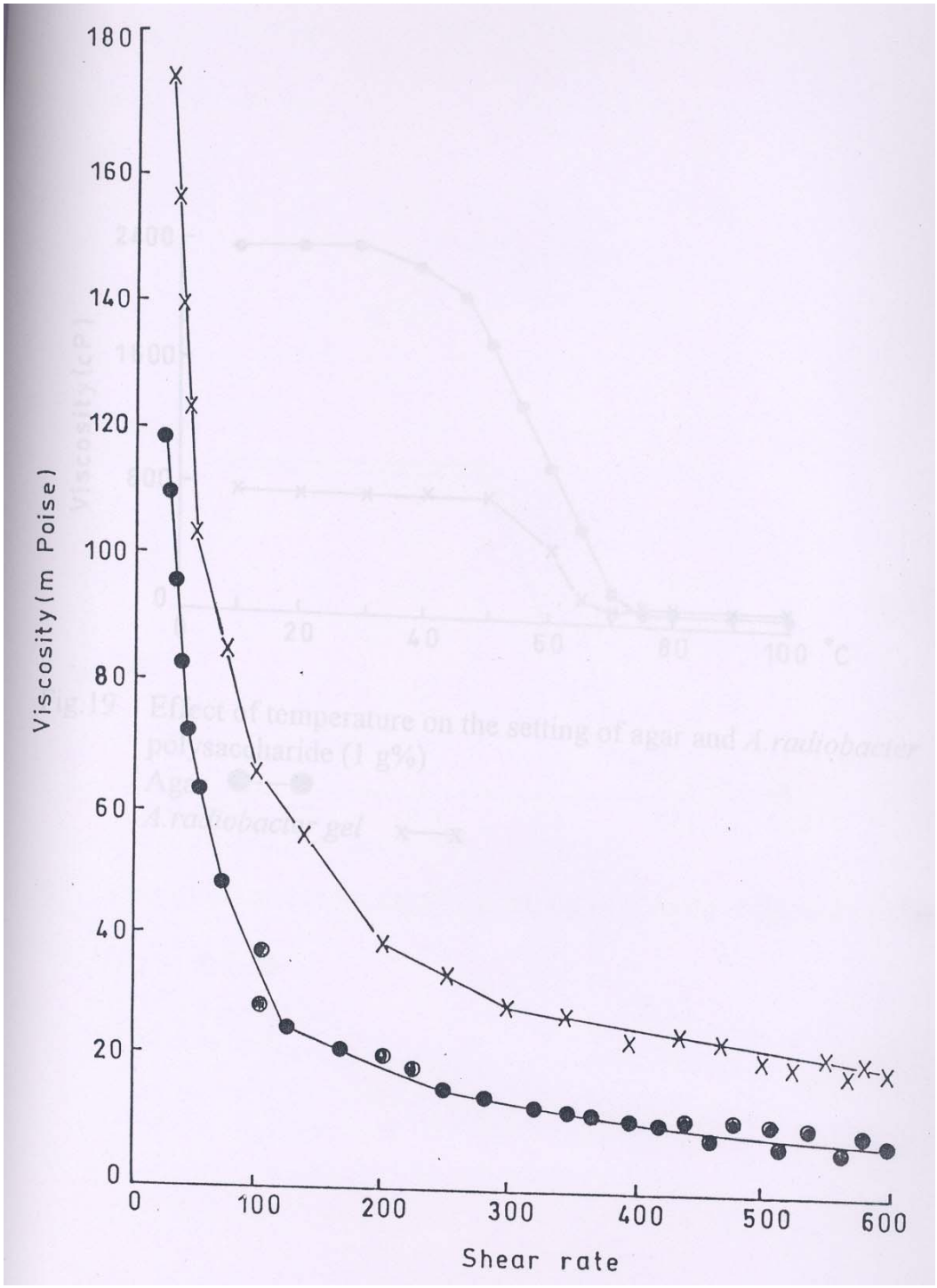


Fig.18 Effect of shear rate on viscosity of agar and A. radiographer polysaccharide (0.5 g% w/v) solubilised at 40-50oC, using Haake viscometer

Agar ●—●

A.radiobacter polysaccharide x—x

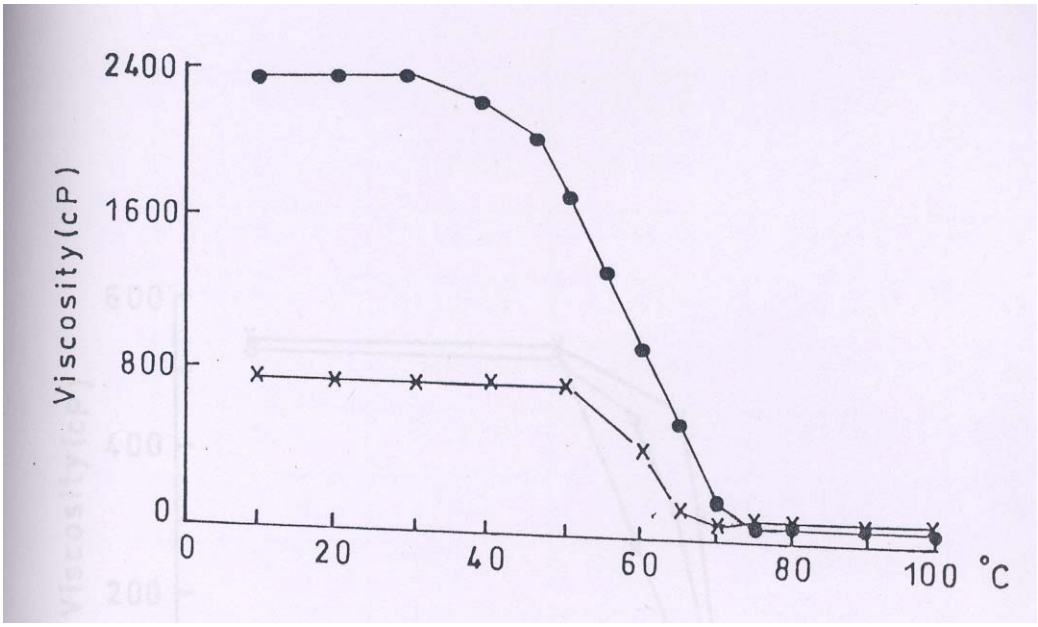


Fig.19: Effect of temperature on the setting of agar and A.radiobacter polysccharide (1 g%)

Agar ●—●

A. radiobacter gel x—x

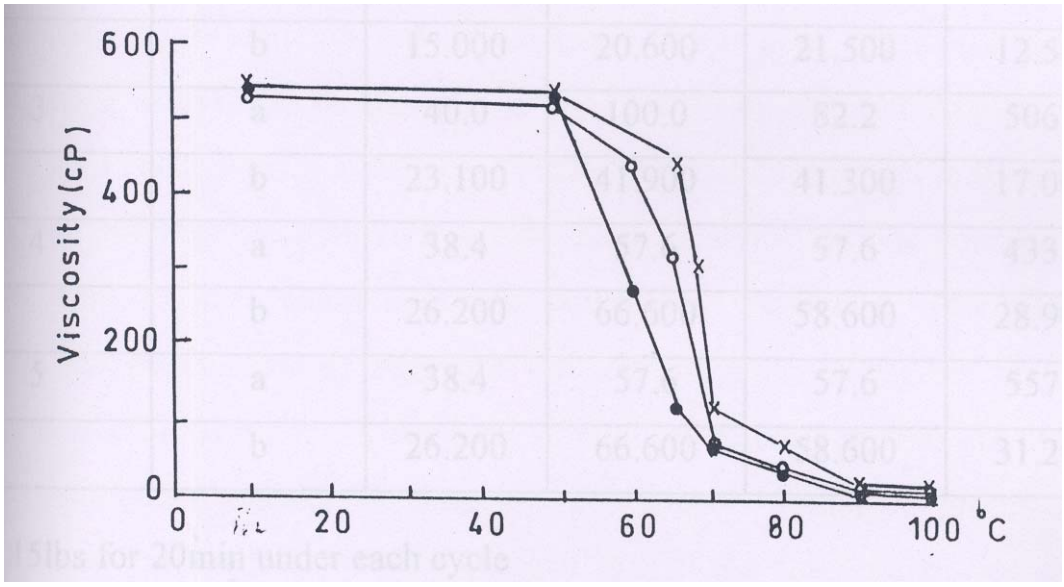


Fig.20 Effect of pH and temperature on setting of A. radiobacter gel (1 g%) at

pH 2.5 ●—●

pH 6.5 x—x

pH 11.5 O—O

Table – 34: Gel strengths of A.radiobacter gels and agar (2g%, w/v) measured using Instron during 5 autoclaving cycles

Autoclaving cycles*	Parameter	Gel strength (g/com^2)			
		A	B	C	Agar
0	a	52.0	172.0	140.0	610.0
1	a	51.2	170.8	136.6	604.5
	b	1.600	0.700	2.500	0.900
2	a	44.2	136.6	110.0	533.7
	b	15.000	20.600	21.500	12.500
3	a	40.0	100.0	82.2	506.3
	b	23.100	41.900	41.300	17.000
4	a	38.4	57.6	57.6	433.7
	b	26.200	66.600	58.600	28.900
5	a	38.4	57.6	57.6	557.3
	b	26.200	66.600	58.600	31.200

* At 15lbs for 20min under each cycle

a: Gel strength g/cm2

b: % change compared to "0" value-where polysaccharide was heat solubilized and cooled.

A. radiobacter polysaccha ride:

A. Directly processed from fermented broth (with biomass)

B. Pure polysaccharide (free from biomass)

C. Pure polysaccharide (free from biomass) treated at pH 10, 80°C for 10 min.

The gel was stable to heat, but the gel strength of crude polysaccharide (2 g%) was less (52gicm2). This is because the polysaccharide concentration of crude preparation was less due to the presence of biomass compared to purified product (140-1 72g/cm2). Purified product without any additional nutrients gave decreased gel strength by 58-68% after 5 autoclaving cycles. Stability of the gel increased by suspending the polysaccharide in basal medium (See chapter I) as the decrease in gel strength after 5-7 auto calving cycle was 34% (Table-35). Gel strengths of polysaccharide was enhanced by 32-62% by brining up gel concentration from 2 to 3% (Table-36). Enhancement could also be brought up by 23-42% by supplementation of 0.1% of KCl NaCl, MgCl and CaCl (Table-37). KCI contributed maximum increase in gel strength compared to other salts. Cations such as potassium ions as well as inter chain hydrogen bonds and interaction with water stabilize the polysaccharide structure for gel formation. Calcium ion is known to exert maximum affect on gel strength of gellan gum and gel does not remelt under normal sterilization condition. Reactive group of carbohydrate link with positively charged group and their molecules are brought together by ionic bonding followed by hydrogen bonding which builds aggregates and immobilize water.

Typical texture data that could be obtained by Instron is shown in Fig.21. Texture of modified A.radiobacter gel was more elastic, cohesive than brittle and hard. Texture profile obtained was different compared to other polysaccharides (Figs.22-24). Elasticity values for agar, gel Ian and A.radiobacter gel were 9.23,23 and 42.5 (mm), respectively.
Corresponding

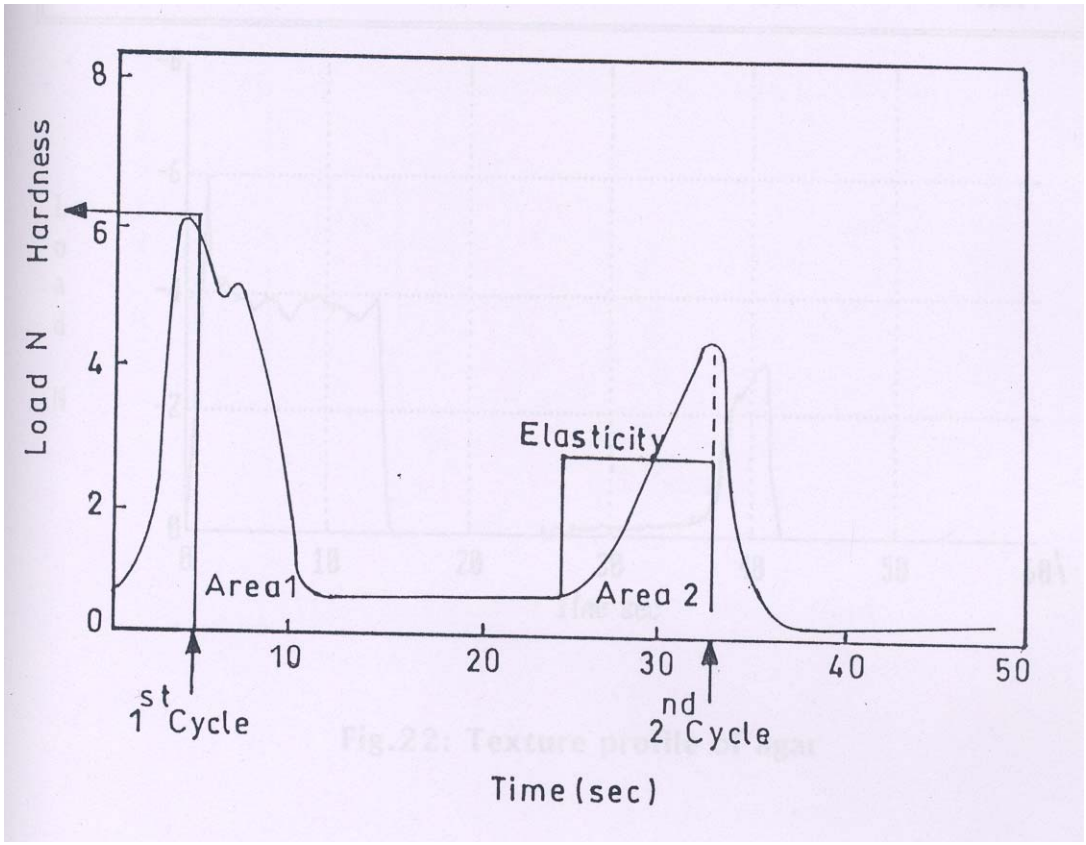


Fig.12 Texture profile analysis

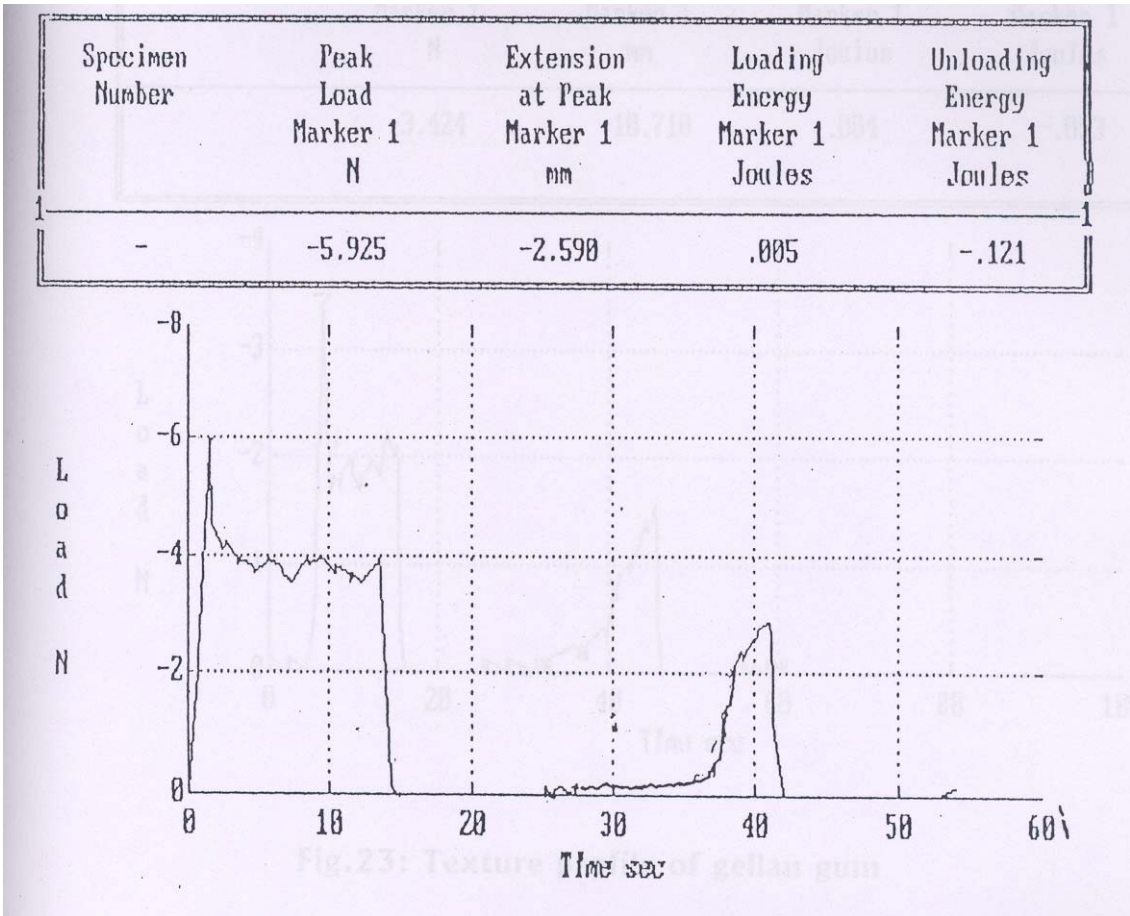


Fig.22: Texture profile of agar

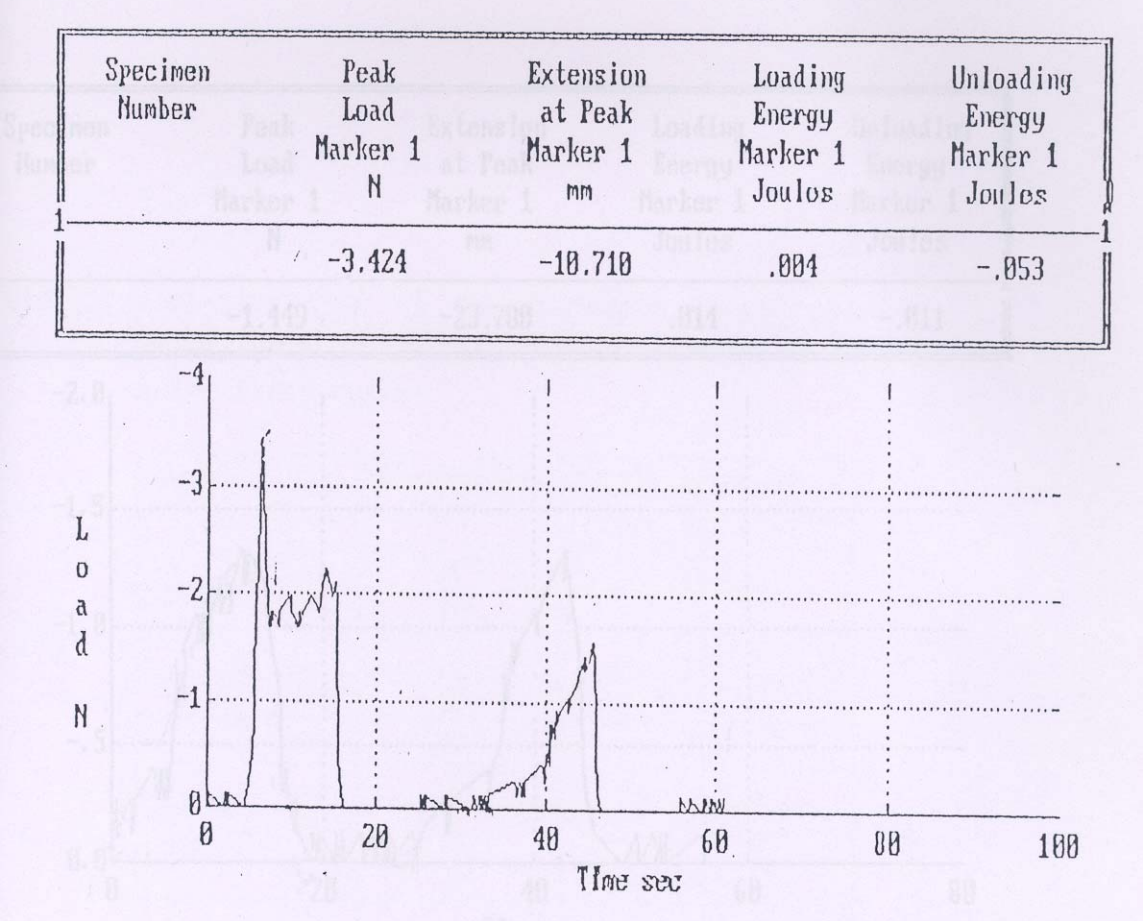


Fig.23: Texture profile of gellam gum

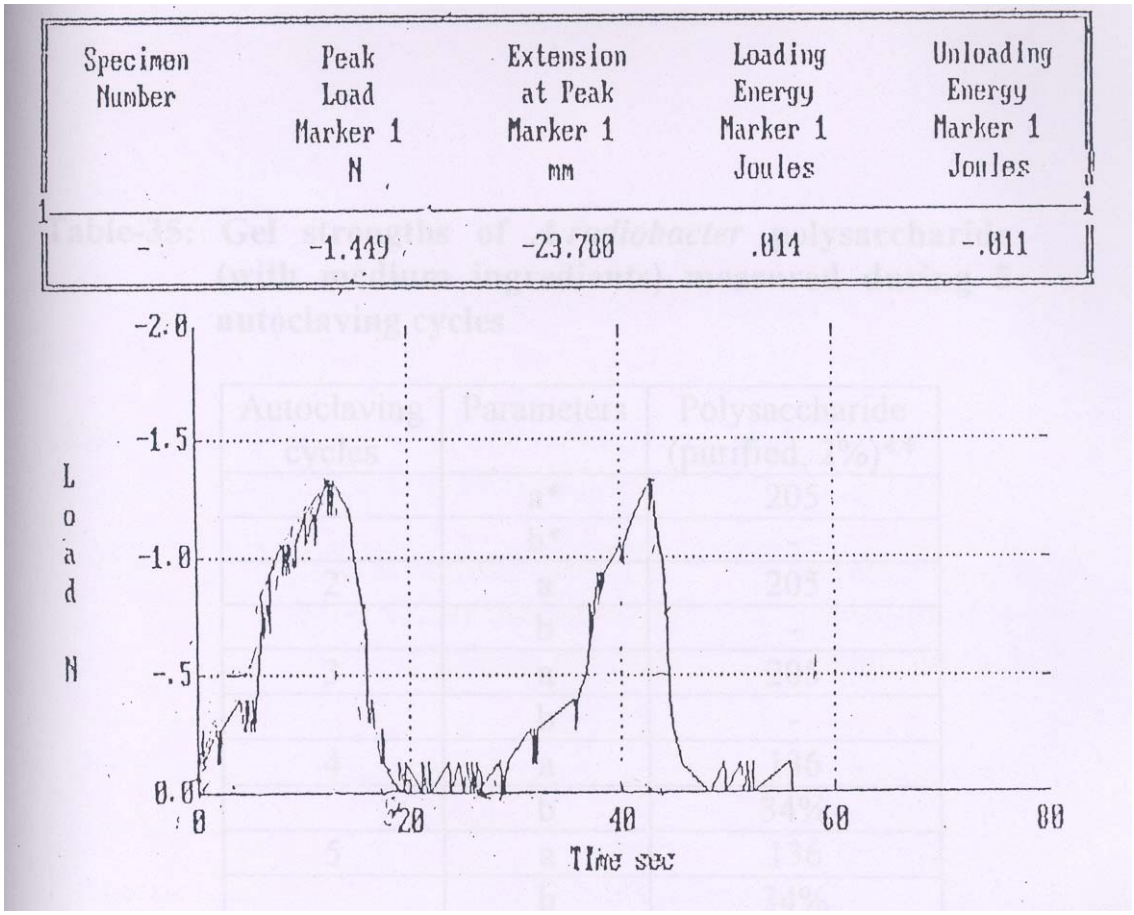


Fig.24: Texture profile of A.radiobacter gel

Table-35: Gel strengths of A.radiobacter polysaccharide (with medium ingredients) measured during 5 autoclaving cycles

Autoclaving cycles	Parameters	Polysaccharide (purified, 2%)**
1	a*	205
	b*	-
2	a	205
	b	-
3	a	205
	b	-
4	a	136
	b	34%
5	a	136
	b	34%
6	a	136
	b	136
7	a	136
	b	34%

* As in Table-34

** Prepared in basal medium (see Chapter 1)

Table-36: Gel strengths of different grades and concentrations of A.radiobacter gel

Gel	Gel strength (g/cm) at different concentrations (g%, w/v) of polysaccharide		Increase in gel strength of B compared to A
	A(2%)	B(3%)	
A*	52	136	62%
B*	172	375	55%
C*	140	205	32%

* As described in Table-34

Table-37: Effect of salts (0.1(-10, w/v) on gel strength of A.radiobacter gel (Pure gel, 2g0A), w/v)

Salt	Gel strength (g/cm^2)	Increase in gel strength over control
KCl	290	42.0%
NaCl	222	23.1%
MgCl2.2H2O	222	23.1 %
CaCl2.H2O	239	28.6%
Control	170	-

results for stress (kg/mm2) required to break at first compression was 0.075,0.043 and 0.018. The stress values for agar and gellan reduced to about half the value for second compression where as the value remained constant for A. radiobacter gel (Figs 22-24).

IR spectra of polysaccharides are shown in Figs.25-29. Spectrum of polysaccharide of A. tumefacines (Fig.27) was different compared to that of A.radiobacter (Fig.28). Spectrum characteristics between 3500 to 4000 wave number indicated that hydrophilic groups are more in modified A. radiobacter polysaccharide (Fig 29) compared to unmodified polysaccharide (Fig.28).

Scanning electron microscopy of polysaccharide (Plates 8-12) revealed that after pressure cooking A. radiobacter formed a gel where in the components appeared to be and randomly held as a matrix where as it appeared to be parallely placed in agar gel (Plate 12). The plates indicate that the gel matrix formed is not uniform which may because of formation of aggregates with lesser number of bonding sites.

The polysaccharide contained (Table-38) low concentration of ash and protein. Carbohydrate which formed major component of the purified material was made up of glucose, galactose and rhamnose as analysed by paper chromatography (Plate-13). Further analyses of the hydrolysate by GLC also confirmed the results (Fig.30). Glucose amounted to major concentration (88.9% of carbohydrate) compared to galactose (10.44%) and

rhamnose (0.66%). Succinic acid was detected by paper chromatography and amounted to 0.5%.

Standard calibration graph obtained for gel permeation chromatography is given in Fig.31. The results obtained for A.radiobacter polysaccharide prepared from 24-120 hr fermentation are represented in Figs.32-36. Data in Table 39 indicate that the molecular weight of polysaccharide produced was in the range of 3.16x107 to 5.0x109. Higher molecular weight polysaccharide was observed after 96h of fermentation and its concentration was more after 120h. It has been noticed that in polysaccharide broth though the yield is more, the viscosity will be less. This would depend mainly on the change in molecular size of the polysaccharide produced under various conditions. The molecular weight of polysaccharide produced in KNO3 medium increased stepwise upto 120h indicating that the conditions were more conducive for increase in molecular size and the chains were not detached from cell surface earlier, which otherwise would happen under higher nitrogen concentration etc.

On the basis of Methylation analysis by GLC and GLC-MS the polysaccharide was found to contain mainly 2,3,4,6-Me4-Glucose, 2,4,6, Me3-glucose and 2,4,-Me2-Glucose (Table-40). From these studies the polysaccharide was found to contain a glucan backbone in 1,3 linkages with branches through 1-6 linkages. 2,3,4,6-Me4-glucose was the terminal sugar.

Many bacteria are known to produce exopolysaccharides composed of glucose as a major component. This includes neutral polysaccharide such as dextran, pullalan, curdlan, scleroglucan, cellulose, levan etc., which are commercially important, Various strains of Azotobacter produce EPS composed of glucose with a combination of rhamnose, galactose, glucuronic acid and fucose[166-169,26,27,35,41,42]. Pyruvic acid, acetic acid and succinic acids are found in various EPS[171-179]. Pyruvic acid is found very commonly in EPS produced by many species of bacteria such as Rhizobium, Azotobacter, Xanthomonas, Pseudomonas, Cornybacterium, Agrobacterium etc [43,44,50,51,64,69,84,147,170]. Agrobacterium Sp rare an mostly found in Alcaligenes sp and Agrobacteria[97]. Agrobacterium Sp are also found to contain pyruvic acid and acetic acid in addition to succinic acid[88,89].

Curdlan, which is a gelling polysaccharide, is a □ 1-3 glucan and it is produced by Alcaligenes Sp and also by Agrobacterium radiobacter. This EPS is insoluble in water and gel is formed by dialysing alkaline solution or by adding calcium ions to weak alkaline solution. A.radiobacter also, produces water soluble EPS of succinoglucan type. This constitutes an economically interesting group of hetropolysaccharide which can be efficiently used as thickeners, stabilizers and emulsifiers.A, radiobacter polysaccharide generally contains 76-83% glucose, 9.3-12% galactose, 4,9-6.3% pyruvic acid, 0.3-1,5% acetic acid and 4.8-7,4% succinic acid. Glucans produced by this species contains □ (1-3), □ (1-4) and □ (1-6) linked D-glucose and □ (1-3) linked D-galactosel82. It is also reported that the capacity of stock cultures of Alacaligenes sps and Agrobacterium sps to produce polysaccharide is unstable because of self mutations88,89. Wild strain of A. radiobacter isolated in the present study was very stable, high yields and consistant results were obtained through out the experiments. polysaccharide produced contained succinic acid, glucose and galactose similar to other A. radiobacter polysaccharide but it differed from others in having rhamnose, and with branches through 1-6 linkages. It formed clear gel even in the absence of cations (Plate 14) and elasticity of gel increased by alkali treatment and supplementation of cations.

Utilization of the polysaccharide

All the bacterial and fungal cultures streaked on nutrient medium and potato dextrose medium, respectively containing A. radiobacter polysaccharide as gelling agent grew well in these media (plates 15-16). Various fungal cultures that produce hydrolases grew equally well on this medium compared to agar containing standard medium (Table-41). The gel formed a good support and was not hydrolysed after fungal growth. Viable counts obtained

for various species of Bacillus were also similar compared to agar medium (Table 42). Microbial polysaccharide such as gellan has been efficiently used as agar substitute. A. radiohacter polysaccharide also can serve as an excellent matrix for supporting growth of microorganism. A. radiobacter polysaccharide also gave good emulsion with castor oil (Table 43) compared to other plant or microbial gums. The emulsion index increased to 100 by increasing the gum concentration (Table-44). The emulsifying property of EPS compared well with other emulsifiers. Nonionic emulsifiers and polysaccharide stabilize emulsions by forming an interfacial film barrier126,148,113, which prevent the coalescence of dispersed drop lets.

Utilization of A. radiobacter polysaccharide in water gels and milk based beverage gave good consistency suspension and good mouthfeel to the products. Even though it can used as an efficient additive, intense research activities are required to prove the grass status of the product.

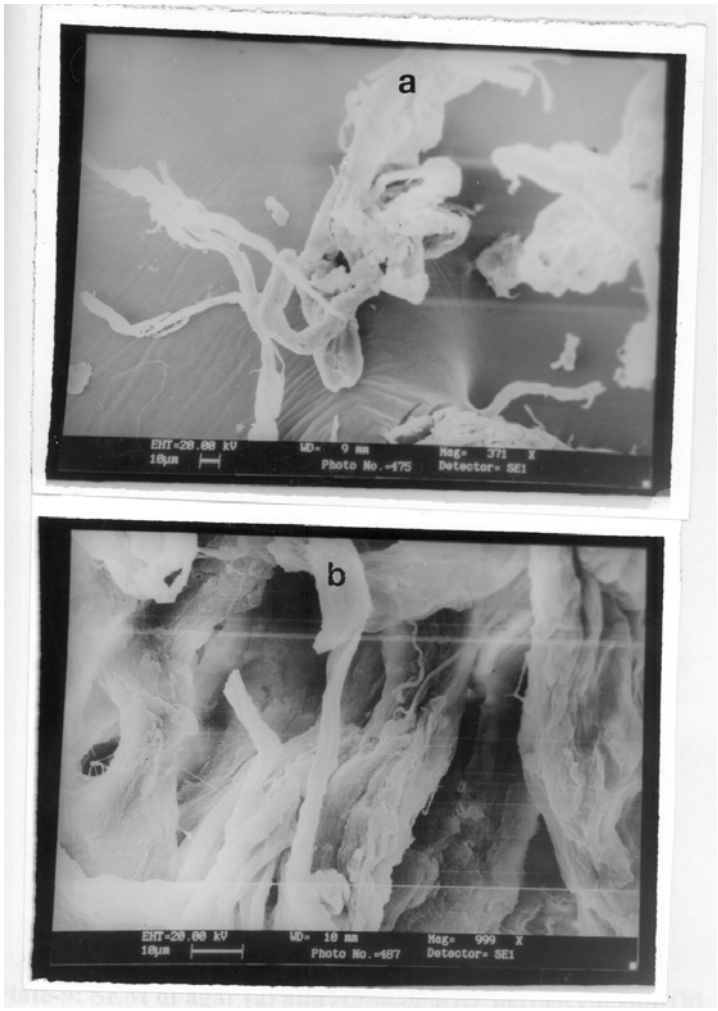


Plate – 8: SEM of gellan (a) and carrageenan (b) powders

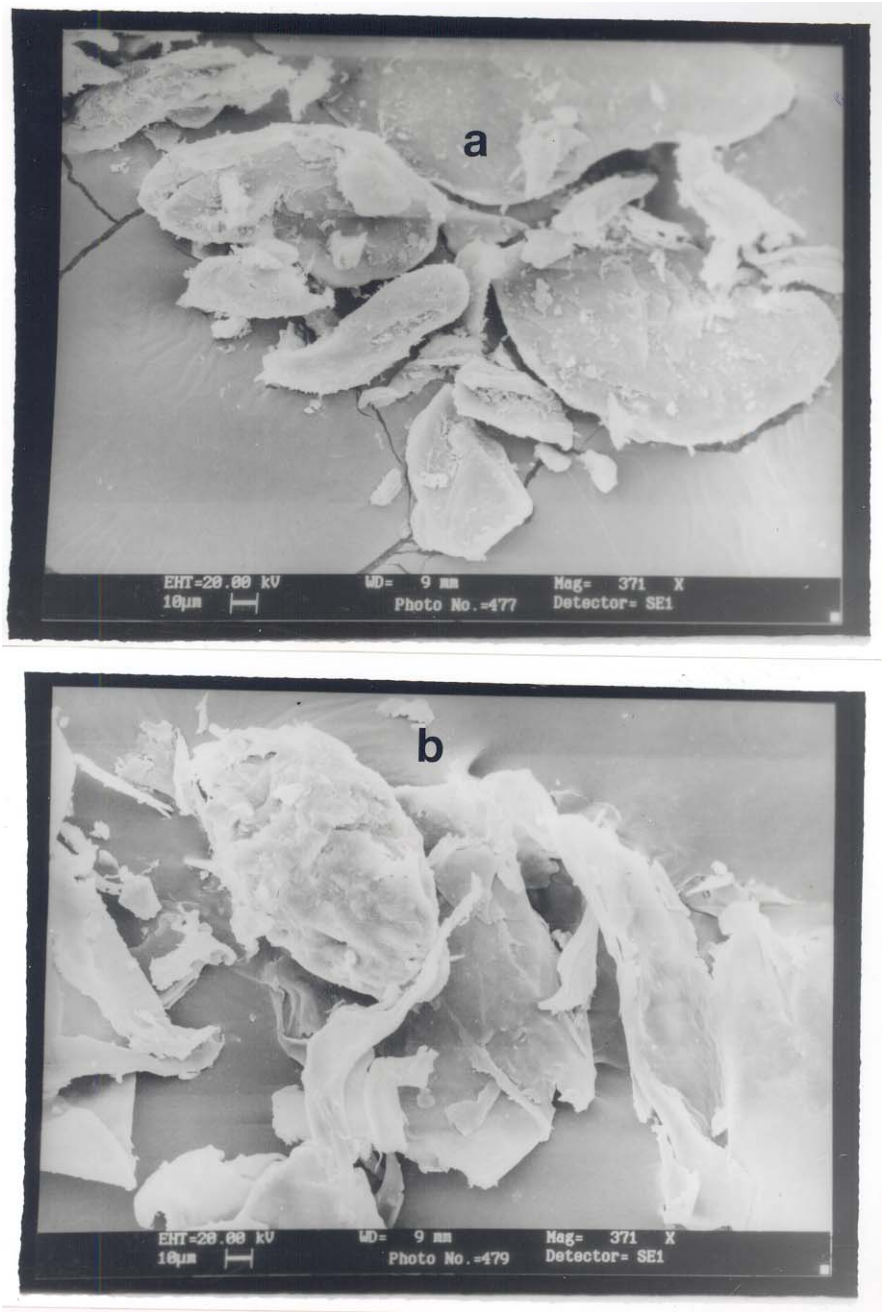


Plate –8: SEM of agar (a) and A. radiobacter polysaccharide (b)

Plate – 10: SEM of solubilised A.radiobacter polysaccharide

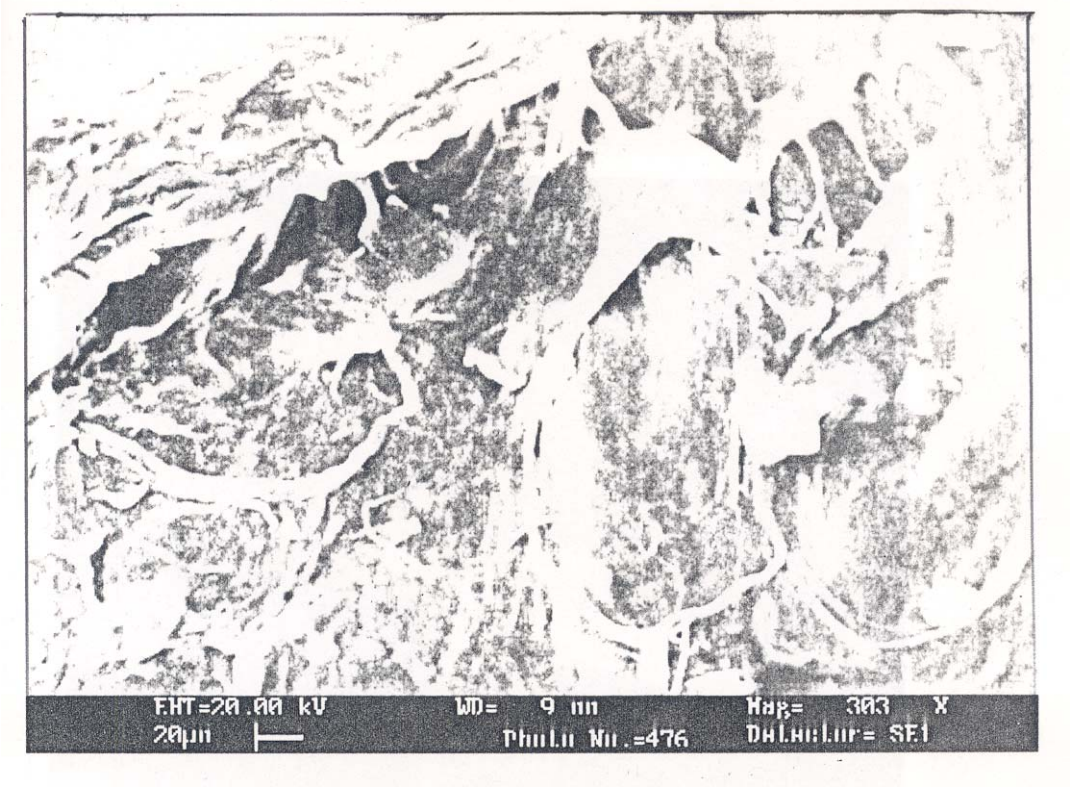
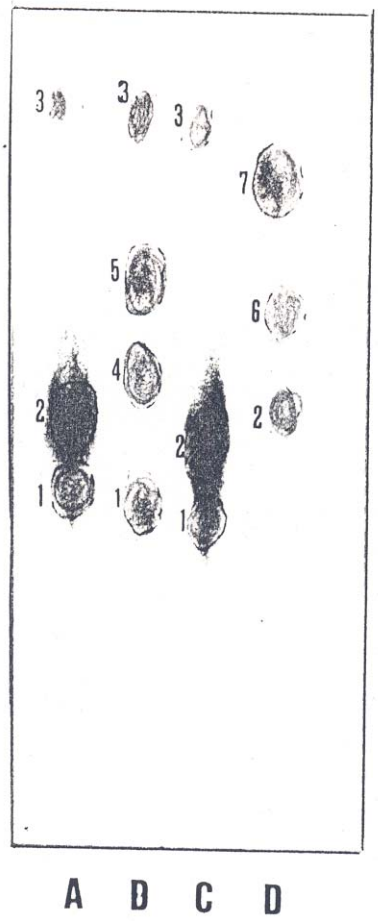


Plate – 11: SEM of pressure cooked A.radiobacter gel

Plate – 12: SEM of pressure cooked agar



.Plate-13: Paper chromatography of polysaccharide hydrolysate

A: Hydrolysate; B: Standards; C: Hydrolysate; D: Standards;

1: Galactose; 2: Glucose; 3: Rhamnose; 4: Mannose;

5: Xylose; 6: Fructose; 7: Arabinose

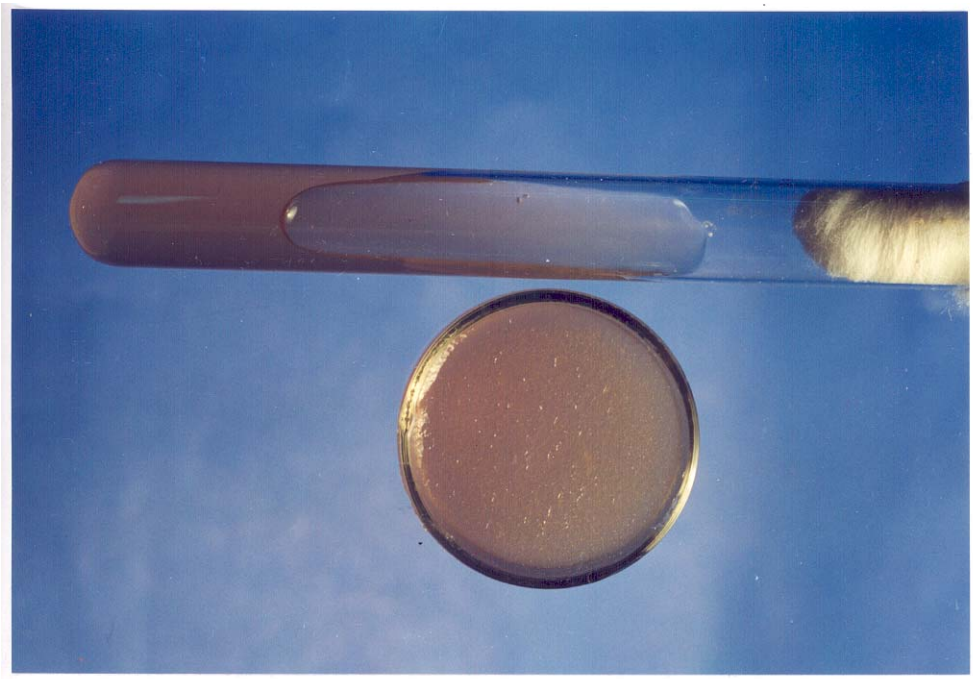


Plate – 14: Solid nutrient medium containing A.radiobacter polysaccharide as a gelling agent

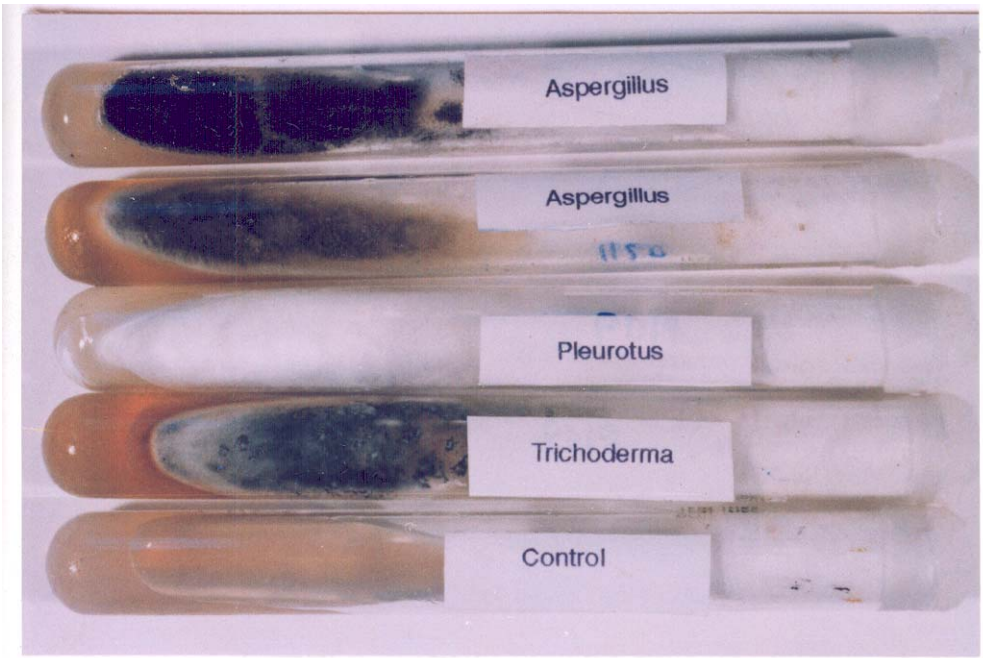


Plate – 15: Utilisation of A. radiobacter polysaccharide as agar substitute for the growth of fungal cultures

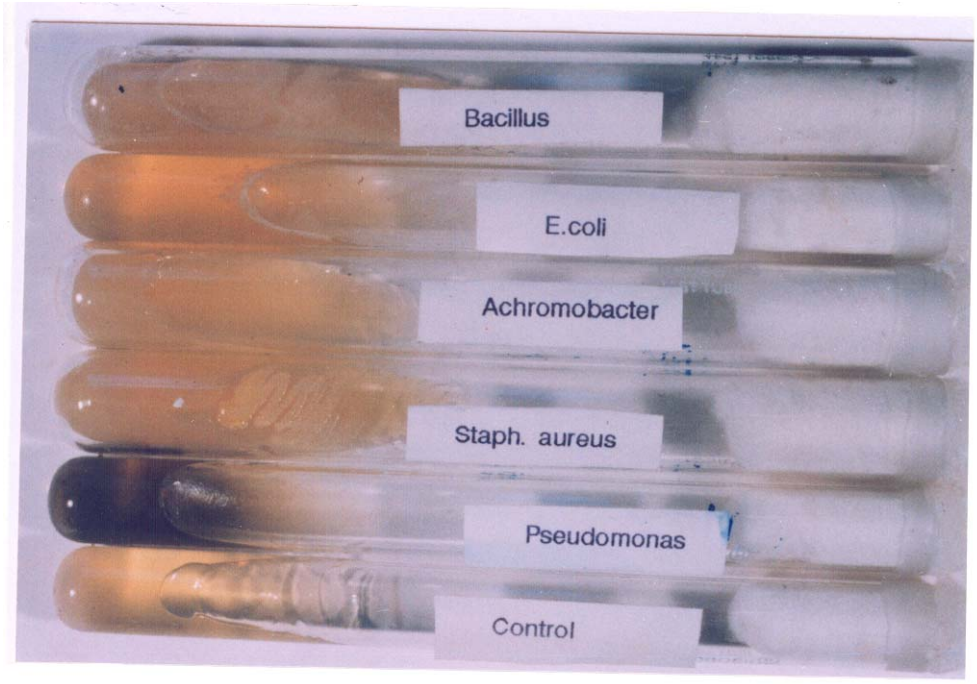


Plate – 16: Utilisation of A.radiobacter polysaccharide as agar substitute for the growth of bacteria

Plate – 17: Milk based beverages with A.radioba

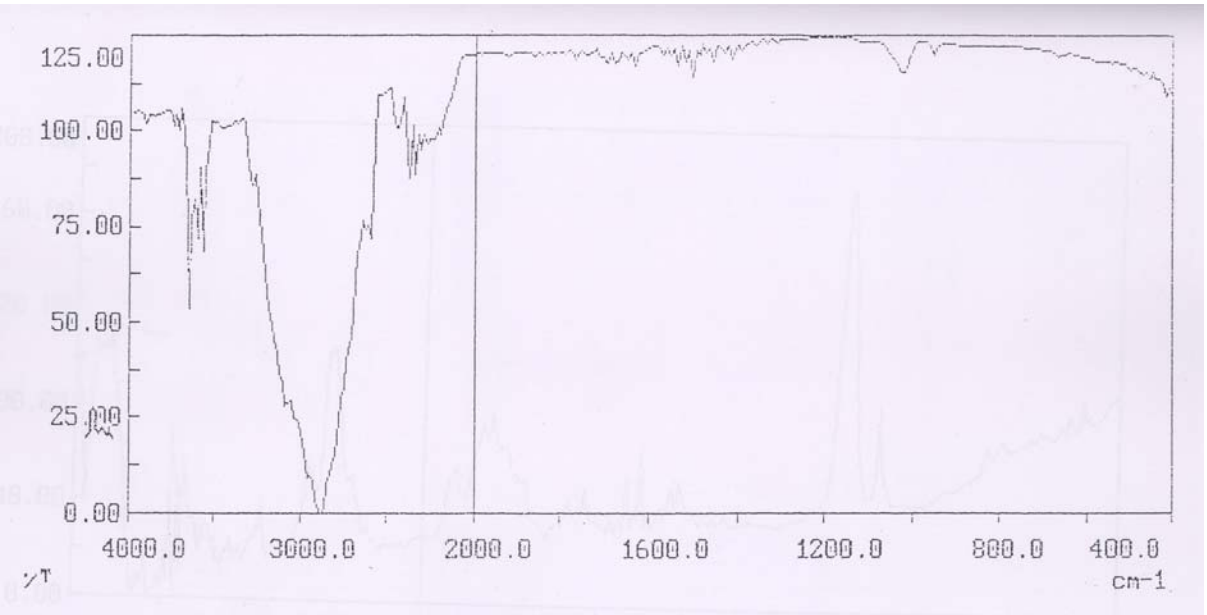


Fig.25: IR spectrum of agar

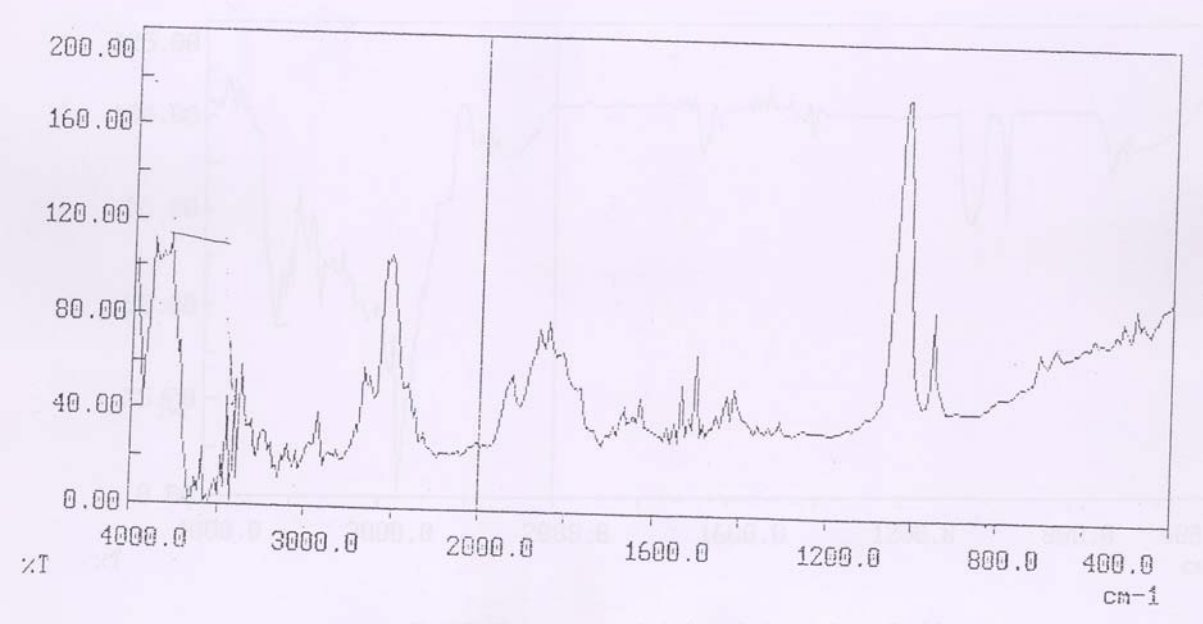


Fig.26: IR spectrum of gellan

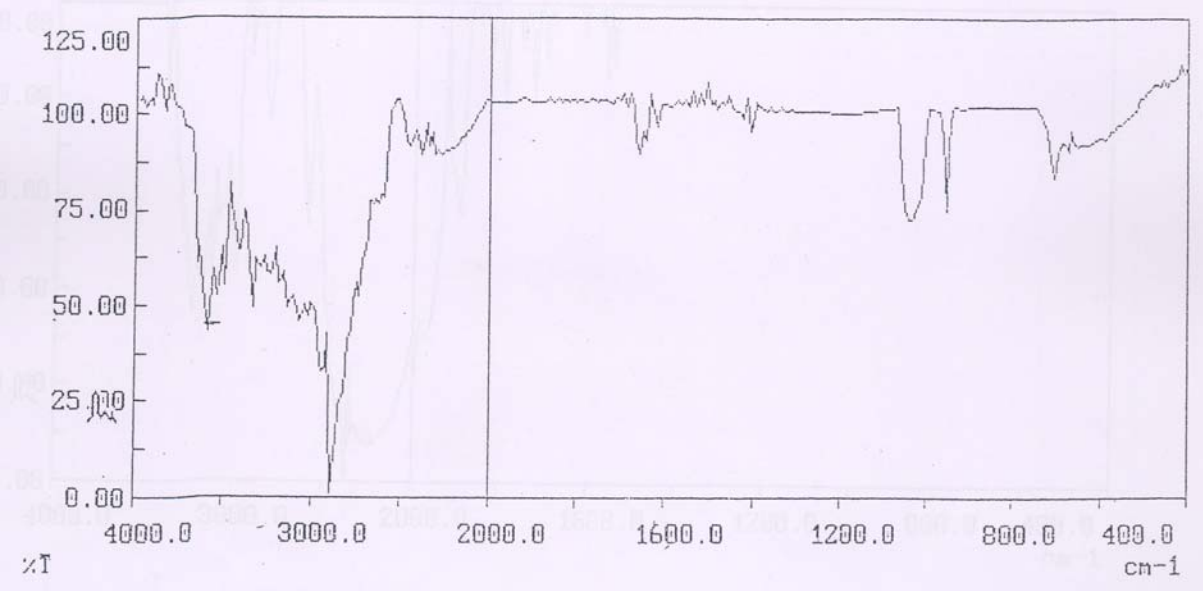


Fig.27: IR spectrum of A-tumefaciens polysaccharide

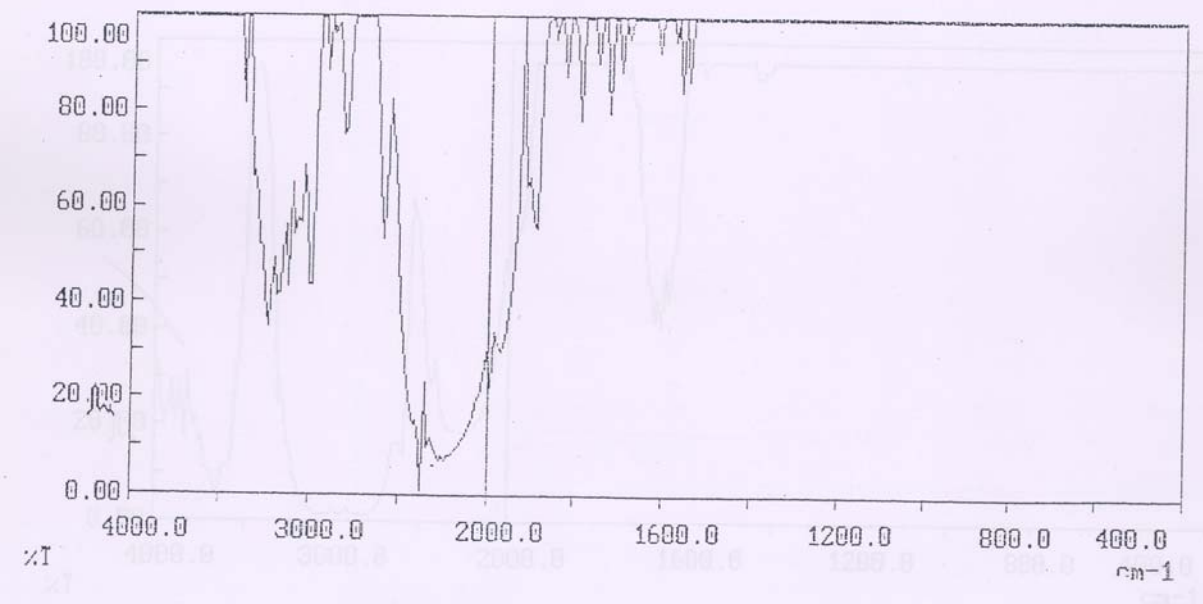


Fig.28: IR spectrum of A-radiobacter polysaccharide

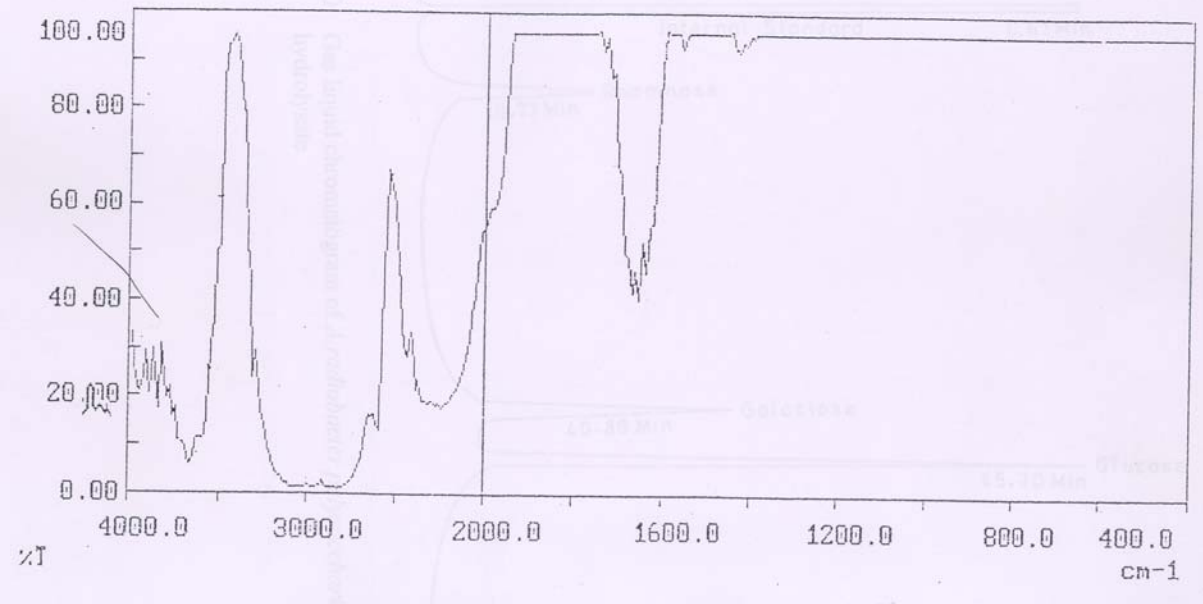


Fig.29: IR spectrum of modified A. radiobacter polysaccharide

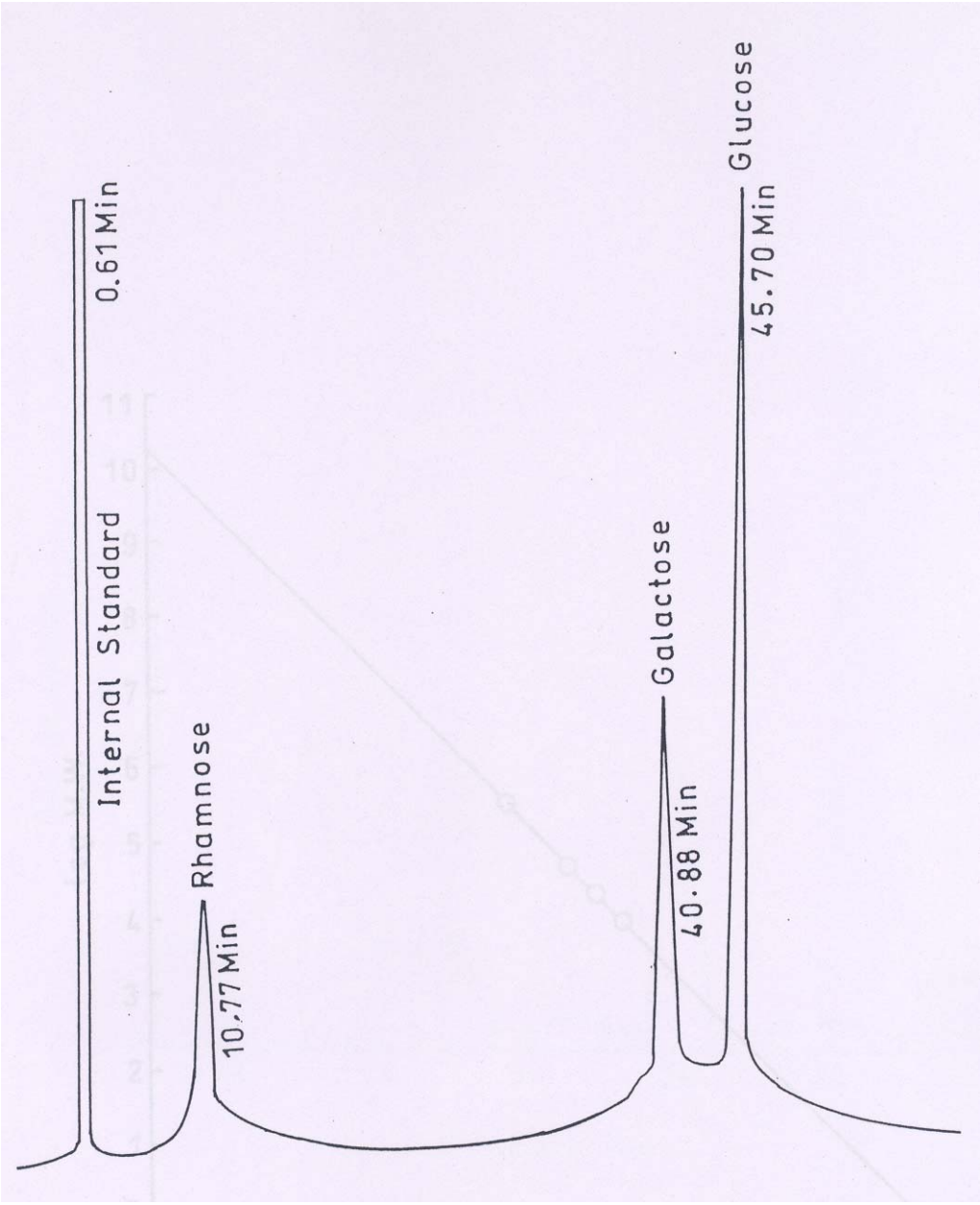


Fig.30: Gas liquid chromatogram of A. radiobacter polysaccharide hydrolysate

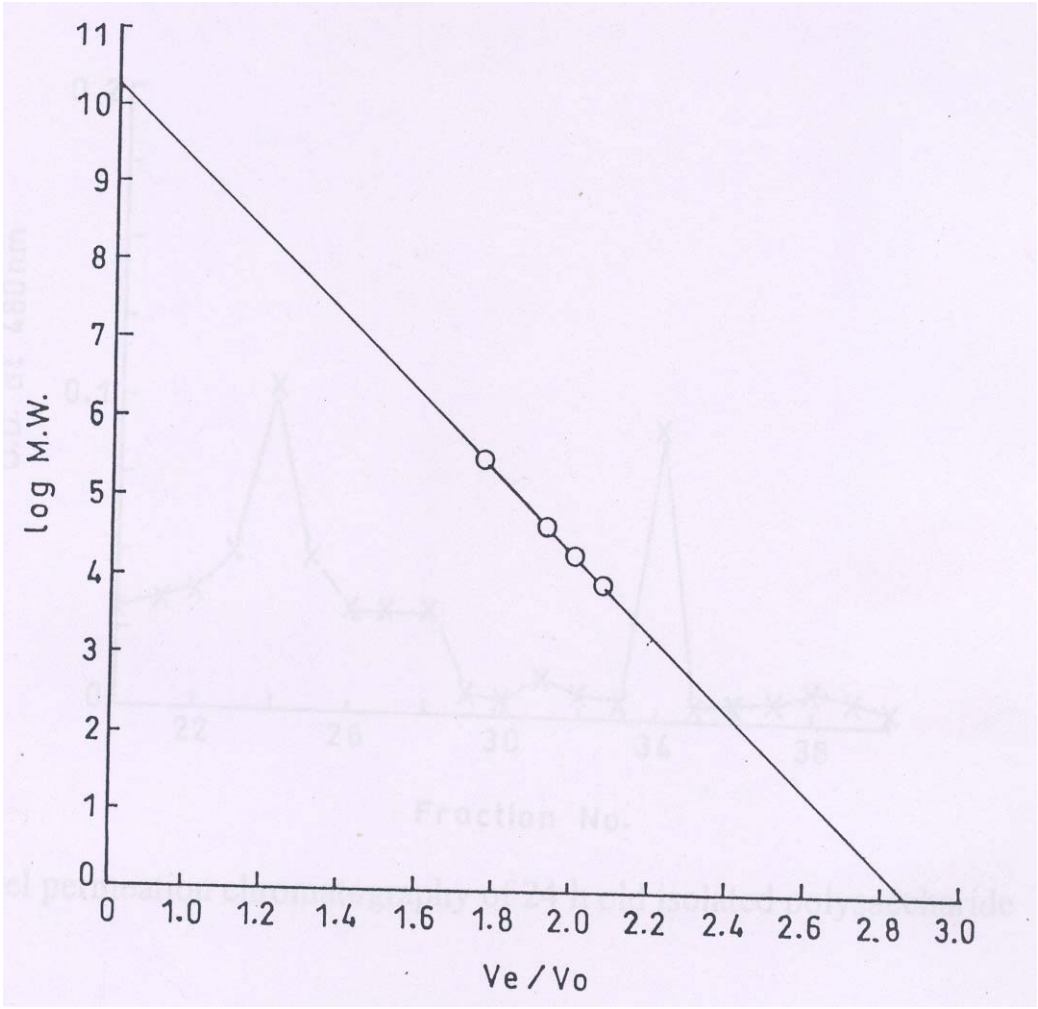


Fig.31: Calibration graph for gel permeation chromatography (standard dextran)

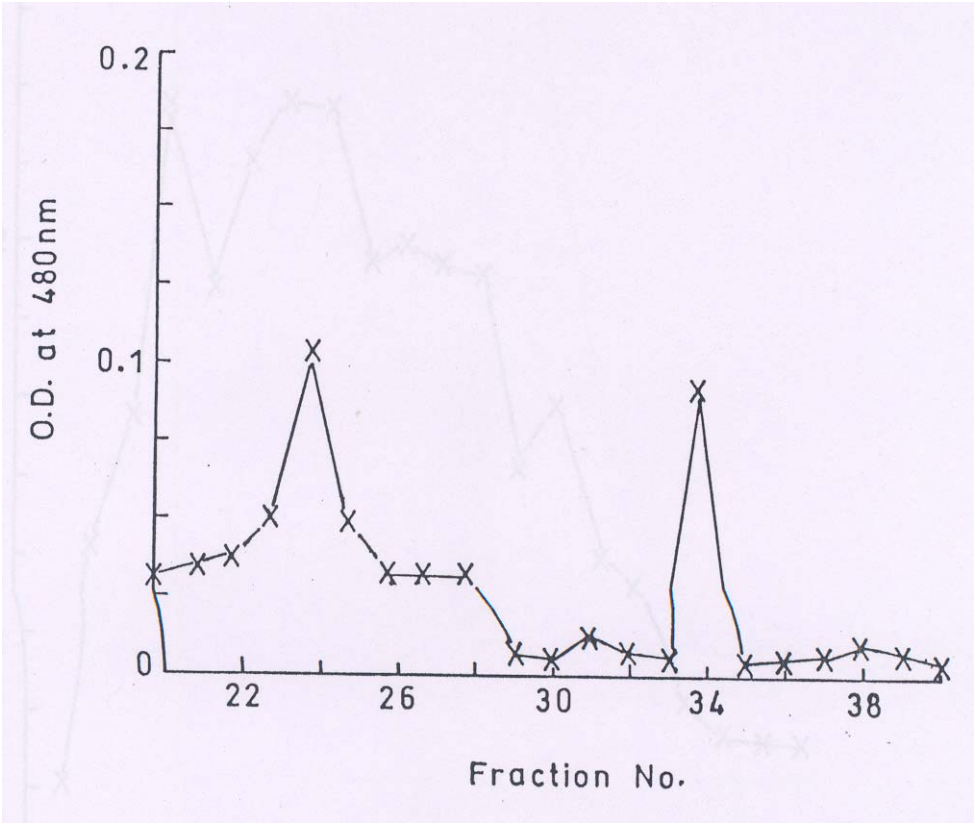


Fig.32: Gel permeation chromatography of 24 h old isolated polysaccharide

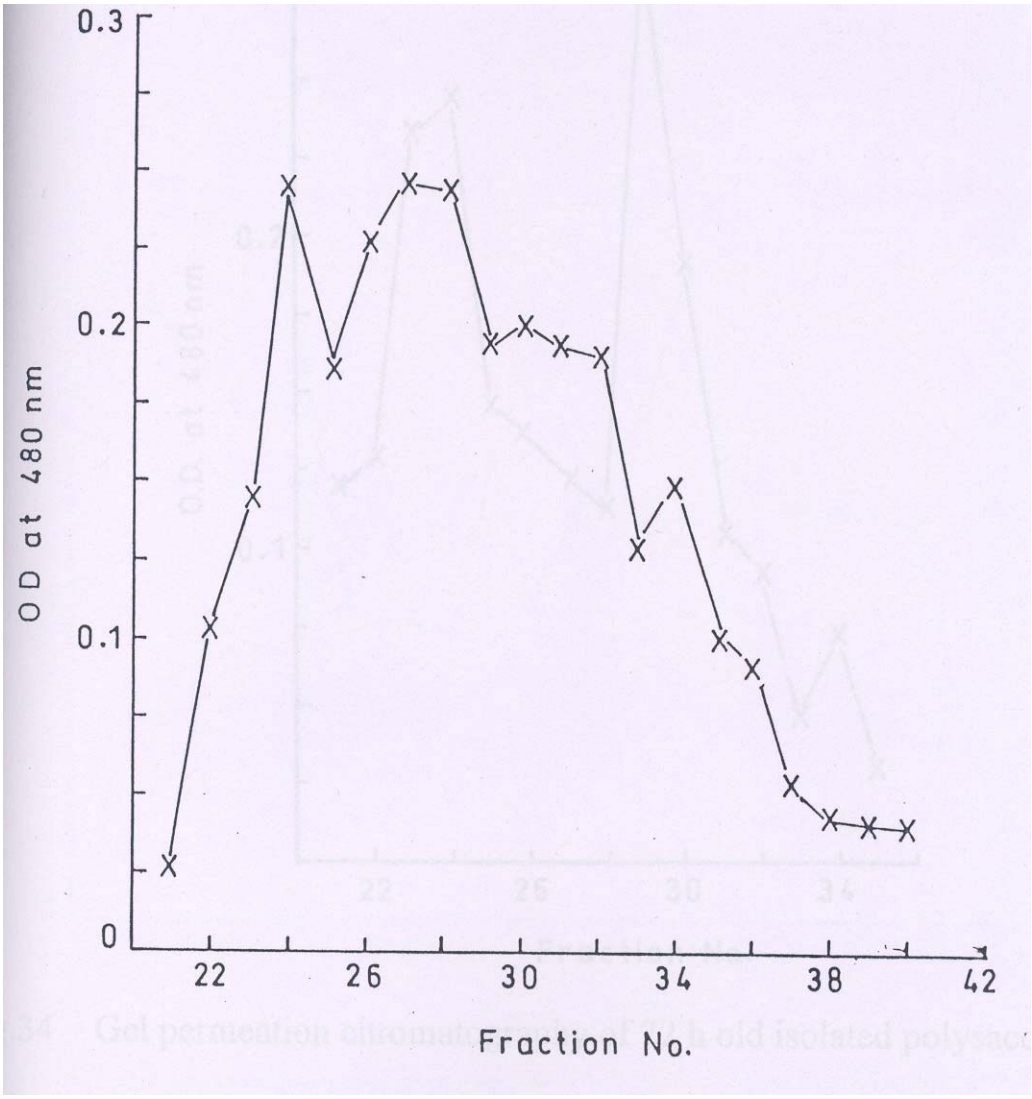


Fig.33: Gel permeation chromatography of 48h old isolated polysaccharide

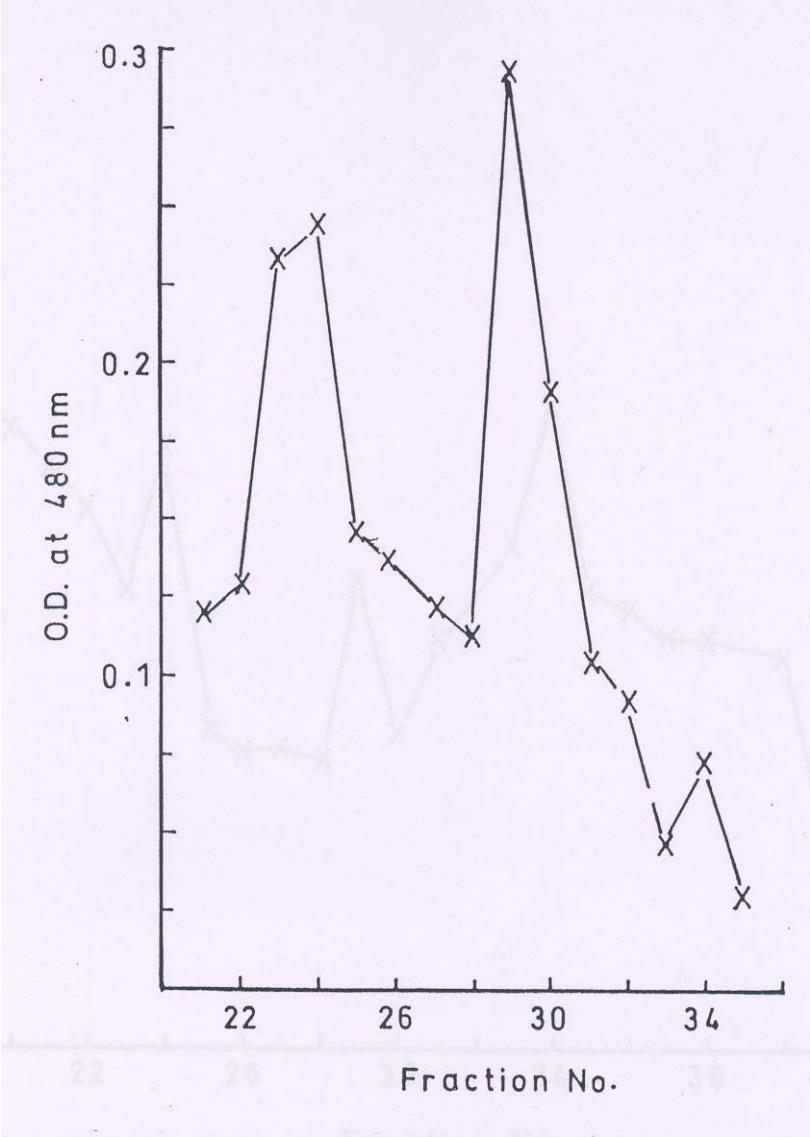


Fig.34: Gel permeation chromatography of 72 h old isolated polysaccharide

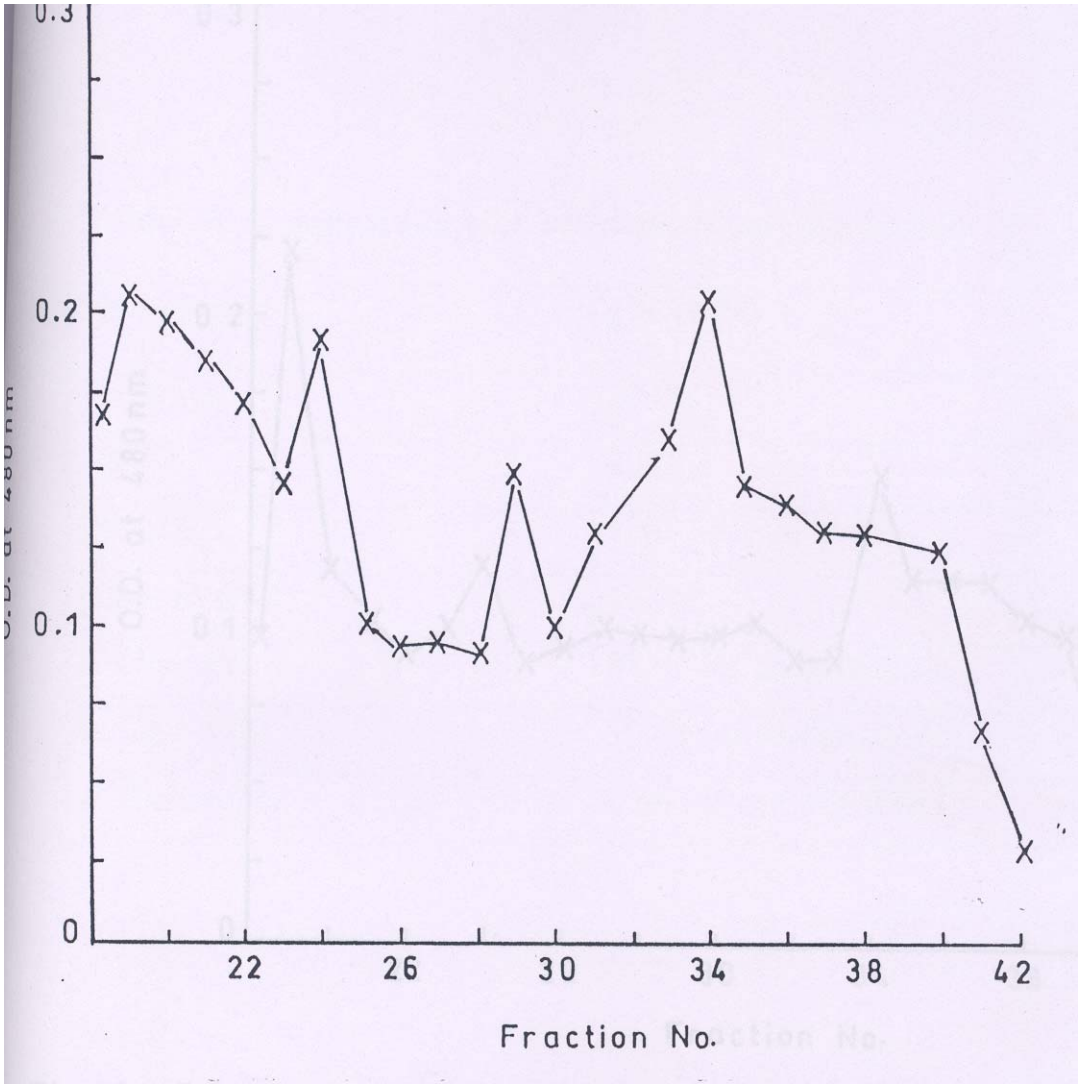


Fig.35: Gel permeation chromatography of 96 h old isolated polysaccharide

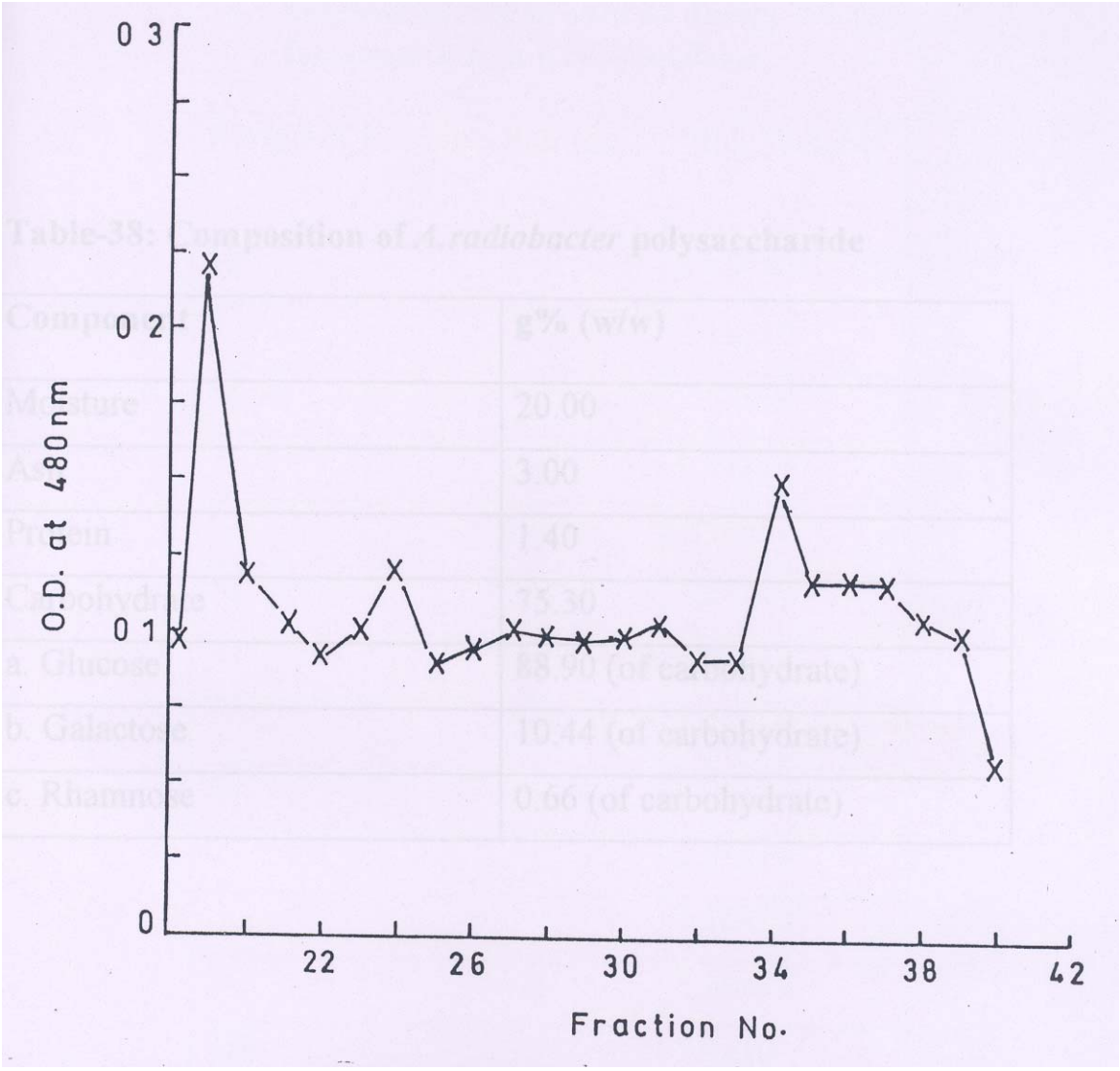


Fig.36: Gel permeation chromatography of 120 h old isolated polysaccharide

Table-38: Composition of A.radiobacter polysaccharide

Component	**g% (w/w)**
Moisture	20.00
Ash	3 .00
Protein	1.40
Carbohydrate	75.30
a. Glucose	88.90 (of carbohydrate)
b. Galactose	10.44 (of carbohydrate)
c. Rhamnose	0.66 (of carbohydrate)

Table-39: Molecular weights of A.radiobacter polysaccharide produced during 24-120h fermentation in KNO3 medium

Period of fermentation (h)	Mol. Weights
24	$3.16x10^{9}$
	$16x10^{7}$
48	$.51x10^{9}$
	$6.31x10^{8}$
	$3.16x10^{7}$
72	$2.51x10^{9}$
	$3.16x10^{8}$
	$3.16x10^{7}$
96	$5.0x10^{9}$
	$2.51x10^{9}$
	$3 .16x10^{8}$
	$3.16x10^{7}$
120	$5.0x10^{9}$
	$2.5lxl0^{9}$
	$3.16xl0^{7}$

Table - 40: Methylation analysis of the polysaccharide isolated from A. radiobacter

Peak No	0-methyl ether	T_{TMG}	Diagnostic mass fragments, M/Z	Mode of linkage	Relative conc
1	2,3,4,6,-Me_4-glucose	1.0	205,161,145,129, 117,101,87,43	Glc→1	0.7
2	2,4,6-Me_3-glucose	1.7	234,161,129,118, 101,43	3→Glc → 1	1.0
3	2,4-Me^2-glucose	3.3	234,189,160,139,1, 29,118,101,43	3→Glc→1	0.8

Table-41: Comparative growth of fungi on potato dextrose agar and potato dextrose medium with A.radiobacter polysaccharide

Fungal cultures	Growth after 120h	
	Potato Dextrose agar	Potato dextrose A.radiobacter polysaccharide
Aspergillus ustus	Sporulated	Sporulated
Aspergillus niger	Sporulated	Sporulated
Aspergillus carbonarious	Sporulated	Sporulated
Trichoderma hazarianium	Moderate	Moderate
Sprotrichum pulvarulentum	Medium	Medium
Aspergillus niger	Sporulated	Sporulated
Aspergillus oryzae	Sporulated	Sporulated
Polyporous squamosus	Medium	Medium
Aspergillus oryzae	Sporulated	Sporulated
Coriolus versicolor	Complete	Complete
Fusarium	Good	Good.
Rhizopus oligosporous	Sporulated	Sporulated

Table - 42: Growth of Bacillus sp on nutrient agar and on nutrient medium with A.radiobacter gelling polysaccharide

Bacillus sp	Cfu/ml			
	Agar medium		A.radiobacter polysaccharide media	
	24h	48h	24h	48h
B. coagulans	$8.8x10^{10}$	$lx10^{11}$	$6.25x10^{10}$	$6.4x10^{11}$
B. Circulans	$7.5x10^{4}$	$7.5x10^{11}$	$7.5x10^{4}$	$7.5x10^{11}$
B. lorevis	$5x10^{10}$	$5x10^{10}$	$lx10^{11}$	$1x10^{11}$
B. laterosporus	$1.5x10^{10}$	$1.5x10^{4}$	$2x10^{11}$	$2x10^{11}$
B. licheniformes	$5.5x10^{4}$	$3.5x10^{4}$	$6.5x10^{10}$	$8x10^{11}$

Table - 43: Emulsification of polysaccharides in the presence of solvents

Polysaccharide	Solvents	Emulsion index	
		0 hr	24 hr
Arabic	Benzene	26.07	17.84
Tragacanth	Benzene	29.59	24.23
Guar gum	Benzene	25.55	19.23
Xanthan	Benzene	35.7	35.7
Gellam	Benzene	9.94	9.0
A. radiobacter polysaccharide	Benzene	28.56	20.00
Arabic	Xylene	24.94	11.42
Tragacanth	Xylene	61.21	58.00
Guar gum	Xylene	35	27.5
Xanthan	Xylene	40	40
Gellam	Xylene	1.8.14	19.28
A. radiobacter polysaccharide	Xylene	A40	40
Arabic	Kerosene	1.8.85	18.85
Tragacanth	Kerosene	45.7	45.7
Guar gum	Kerosene	46.78	46.78
Xanthan	Kerosene	41.73	41.73
A. radiobacter polysaccharide	Kerosene	32.5	32.5
Arabic	Hexane	21.42	12.49
Tragacanth	Hexane	15	12.5
Guar gum	Hexane	38.5	32.14
Xanthan	Hexane	50	50
Gellam	Hexane	-	-
A. radiobacter polysaccharide	Hexane	42.84	42.84

Table-43a: Emulsification of polysaccharides in presence of oils

Polysaccharide	Oil	Emulsion index		
		0 hr	1 day	30 days
Arabic	Ground nut	55	35	35

Tragacanth	Ground nut	50	21.49	21.42
Guar gum	Ground nut	64.28	39.28	39.28
Xanthan	Ground nut	50	37.5	37.5
Gellam	Ground nut	53.56	42.84	42.84
A. radiobacter polysaccharide	Ground nut	42.5	42.5	42.5
Arabic	Olive oil	-	-	-
Tragacanth	Olive oil	40.3	11.6	-
Guar gum	Olive oil	43.92	8.52	-
Xanthan	Olive oil	40	40	40
Gellam	Olive oil	-	-	-
A. radiobacter polysaccharide	Olive oil	40.1	40.1	40.1
Arabic	Castor oil	40	28.56	-
Tragacanth	Castor oil	32.14	28.56	-
Guar gum	Castor oil	75	30	-
Xanthan	Castor oil	46.42	46.42	46.42
Gellam	Castor oil	50	30	30
A. radiobacter polysaccharide	Castor oil	65.7	50	50

Table-44: Effect of A.radiobacter polysaccharide on emulsification in the presence of castor

Concentration of polysaccharide (g% w/v)	**Emulsion index**		
	0 hr	**1 day**	**30 days**
0.2	70	40	40
0.4	75	75	75
0.6	100	100	100
0.8	100	100	100
1.0	100	100	100

SUMMARY AND CONCLUSION

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Pectin, carrageenans and agar have been used as gelling agents for about a century. Metabolic rate and product formation by microorganisms can be enhanced considerably using fermentation techniques which give economic appeal to the process compared to plant gums. Reports exist on the production of gel forming microbial polysaccharides such as curdlan, gellans and bacterial alginates by spores of Bacillus, Enterobacter, Alcaligenes, Arthobacter, Rhizobium, Pseudomonas etc. These polysaccharides impart texture and thickening properties to foods, cosmetics, pharmaceutical products etc and hence have a wide range of applications. Economic production, good gel strength, improved clarity of gel, stability to temperature, salts and different pHs are important criteria for the production and commercialization of these gums. Gellan produced by Pseudomonas elodea gives excellent clear gel at low concentrations but yield is relatively low compared to other industrially important polysaccharides. This causes higher production costs and price for gellan gum. The polysaccharide is to be treated and modified for gelation and gel formation is dependent on cations. Owing to microbial biodiversity that occurs in nature it is possible to find bacteria having different rheological properties and better growth rates. Hence isolation of bacteria producing gelling polysaccharide, its characterization and studies connected with it were taken up.

About 150 mucoid cultures were isolated from various sources such as Soil, water and decaying plant materials. On screening it was found that 23 isolates produced polysaccharides and only 6 were of gelling type. One of the cultures was selected for further work.

Based on morphological and biochemical tests the culture was classified according to Bergey's Manual of Determinative Bacteriology as belonging to class Schizomycetes, order Eubacterials, family Rhizobiaceae, genus Agrobacterium and species radiobacter. Gram negative, aerobic small rods as found in this case was assigned to family Rhizobiaceae because the cells were without endospores, rodshaped and glucose was used without appreciable gas formation. This family has three genera namely Rhizobium, Chromobacterium and Agrobacterium. The isolated culture was not capable of fixing free nitrogen or producing violet chromogenes which would other wise indicate that the culture was Rhizobium or Chromobacterium. Agrobacterium is most likely to be confused with Rhizohium to which it is close1y re1ated. Typica1 characters found in Agrobacterium which are different from most Rhizobia are: growth on glucose peptone agar, production of H2S, absence of polyhydroxybutyrate and failure to nodulate in leguminous plants. Confirmatory tests of species (as radiobacter) were growth at 350C, alkaline reaction in litmus milk, absence of growth factor requirements, and absence of alkaline reaction in media containing

salts of organic acid. With the exception of A. radiohacter, other species of Agrobacterium invade the crown, root and stem of dicot. The isolated bacterium was not capable of causing infection to dicot plant. It was isolated from soil, grew well on potato and it was concluded that the isolated culture may be Agrobacterium radiobacter.

Various media were screened under shake flask conditions for optimal polysaccharide production. Optimal production medium was further tested using various C and N substances. The bacterium grew and produced higher concentration of polysaccharide in the presence of tryptone or KNO3 as N-source, sucrose or galactose as C-source. It required Na2HPO4 and MgSO4 7H2O only as other medium ingredients. The bacterium grew well at pH 7.0, 30oC and with low inoculum conc. (1% v/v). In optimised medium containing KNO3 as N-source and sucrose as C-source, maximum viscosity of 7600 cP and crude polysaccharide yield of 1.9 g% were obtained. Conversion rate of carbon source to polysaccharide achieved was 60-70% with C/N ratio of 28 in 96h of fermentation under optimised cultural and nutritional conditions.

Using traditional method, medium was optimised polysaccharide production and it contained (g% w/v): Na2HPO4 (0.4). MgSO4 7H2O (0.1), KNO_3 (0.3), and sucrose (3). Required initial pH for aerobic fermentation was 7.0 and temperature was 30°C for a period upto 96 h.

Optimization of medium for growth and production of polysaccharide by traditional method is an elaborate process which requires more time since it depends on many factors and interactions that affect the desired response. Under such circumstances response surface methodology (RSM) is considered to be all effective tool for optimising the fermentation process. RSM is a statistical method that uses quantitative data from appropriate experimental design to determine and simultaneously solve multivariate equation, Three variables (sucrose, inoculum, pH) and five level experimental design amounting to 125 combinations are required for experimentation but only 20 combinations were sufficient to predict the optimum conditions thus reducing the cost and time of study. Experiments carried out showed that low inoculum concentration (1%, v/v), 2,62 g% sucrose 0.3 g% KNO3, 0.4 g% Na2HPO4 and 0.1 g%, MgSO4 7H2O at pH 6.4, 30°C, were most favorable optimum conditions for maximum viscosity and polysaccharide yield by A. radiobacter. Scaling up of lab scale microbial exopolysaccharide production is essential to establish a viable industrial technological process. Operating conditions which exist in lab scale shake flask cultures are different compared to that of the bioreactor. Fermentor of 10 litre capacity was used for experimentation. Initial experiment using 1, 1.5 and 2 wm of air resulted in 1,5,2.1 and 1.9 g% of crude polysaccharide in 72h of fermentation and hence 1.5

wm was selected for kinetic study. The polysaccharide was produced by the bacterium throughout the fermentation period in optimized KNO_3 medium with sucrose as C-source. Maximum viscosity and polysaccharide produced was more in fermentor (4000 cP and 2.04 g%) compared to shake flask cultures (2200 cP and 1.25 g%) after 72h of fermentation. Conversion of sugar to polysaccharide was more in fermentor (80%) compared to shake flasks (65%). Mixing, aeration, dissolved oxygen, pH and temperature play important role in the production of polysaccharide. In A. radiobacter fermentation pH change during fennentation was marginal which may be because of good buffering provided by Na2HPO4 and the quality of polysaccharide produced. Proper mixing results in transport of oxygen from gas phase to the microorganisms and promote homogenous blending of the nutrient. Increased agitation causes thinning of the slime layer around the cells and enhances the transfer of nutrients and oxygen to the cells. Due to proper mixing and agitation of medium, polysaccharide production was more in the fermentor compared to shake flask.

Post fermentation process for isolation and purification of the product would lead to increased fimctionality of the polysaccharide, better shelf life and transportation, etc. Presence of biomass imparts turbidity to the product. This can be removed by physical, chemical or enzymatic method. In physical method of biomass removal for purification of the polysaccharide, high degree of dilution (1:10 to 1:20) of the broth followed by high speed centrifugation (10,000 rpm) for long duration (30-60min) were required to get 90-97% biomass removal. Such an operation would increase the production cost of pure polysaccharide preparation. A. radiobacter cells could be lysed by multienzyme system having glucanases, proteases etc. Clarification of polysaccharide broth at 50°C, pH 7-8 with 0.1 g% enzyme for 24h resulted in 80% degradation of the biomass. Compared to physical method, enzymatic method is relatively 1ess energy consuming and economical.

In addition to economics of production and availability, commercialization of the polysaccharide also depends upon physicochemical properties. This includes high viscosity, gel formation, compatibility witl1 a variety of salts, synergism with other gums, resistance to a wide pH, temperature etc. A. radiobacter polysaccharide was non-newtonian and pseudoplastic and showed shear thinning property. At low shear rates it had more viscosity indicating that it can perform better in stabilizing suspensions. It had a higher setting point (50°C) compared to agar. It formed gel at 2 g% level even in the absence of cations and was resistant to acidic and alkaline conditions (PH 2.5-11). Agar gel on the other hand is not stable at very low or very high pH. Gellan which is an excellent microbial gelling polysaccharide forms gel only in the presence of monovalent or divalent cations and its yield is low compared to A.radiobacter polysaccharide production. These properties indicate a possible wide area of application of A.radiobacter polysaccharide.

Elasticity values for agar, gellan gel and A.radiobacter gel were 9.23, 3 and 42.5 (mm) respectively. This indicated that A.radiobacter polysaccharide had a higher degree of elasticity. Corresponding values for the stress (kgimm1 required to break at first compression was 0.075, 0.043 and 0.018. The stress values for agar and gellan reduced to half the values in second compression by 2-bite analysis using Instron, whereas the A.radiobacter gel regained its original position and the value remained constant for second bite also. The gel was elastic and cohesive than brittle and hard. Crude preparation of A.radiobacter gel (with biomass) and agar showed 26 and 31 % decrease respectively, in gel strength after 5 heating cycles. Due to presence of biomass the gel strength was low (52g/cm2) compared purified polysaccharide (140-172g/cm2). But purified polysaccharide gel lost 58-68% of gel strength after 5 heating cycles. However by suspending the polysaccharide in mineral medium the stability of the gel increased and decrease in gel strength after 5-7 heating cycle was 34% and this compared well with agar.

Gel strength required for microbiological medium is 200-300g/cm^2. This could be achieved by -

1. Enhancing the gum concentration from 2 to 3%
2. In the presence of salts like KCl
3. After addition of polysaccharide to nutrients containing medium.

The polysaccharide contained moisture (20%) low cone of ash (3%) and protein (1.4%) and high conc of carbohydrate (75.3%). Paper chromatography and GLC it showed that the polysaccharide is made up of glucose (88.9%) galactose (10%) and traces of rhamnose (0.66%). The molecular weight of polysaccharide produced by A. radiobacter ranged from $3.16x10^7$ to $5.0x10^9$ during 24-120h fermentation.

The highest molecular size was found only after 96h of growth and its concentration was more at 120h. On the basis of methylation analysis followed by GLC and MS, polysaccharide was found to contain mainly

glucose backbone of □-1,3 linkages with branches through 1-6 linkages and 2,3,4, 6-Me4-glucose was the terminal sugar. Various Agrobacterium sp are known to produce many kinds of □-glucans which contain □ (1-2) □ (1-3) and other linkages. Curdlan which is a □-glucan is also reported to be produced by strains of A. radiobacter. Curdlan is also a gelling polysaccharide which is insoluble in water and gel is formed by dialyzing alkaline solution or by adding calcium ions to weak alkaline solution. The polysaccharide produced by A. radiobacter in this study was different and it gelled even in the absence of above mentioned conditions.

The polysaccharide produced was successfully utilized as an agar substitute in microbiological media preparation to grow various species of fungi (Aspergilli, Trichoderma, Pleurotus, Rhizopus, Fusarium, etc) and bacteria (Species of Bacillus, E.coli, Staphylococcus, Pseudomonas etc). The gel was not hydroslysable after microbial growth and gave comparative growth as found on agar slants.

A. radiobacter polysaccharide also gave good emulsion with castor oil compared to other gums. Nonionic emulsifiers and polysaccharides stabilize emulsions by forming an interfacial film barrier which prevents the coalescence of dispersed droplets.

Overall it can be concluded that the bacterial culture isolated from soil and characterized as Agrobacterium radiobacter can produce highly viscous broth under optimised conditions. The culture was a stable producer of polysaccharide unlike other Agrobacterium sp.

A.radiobacter can be optimally cultivated in a simpler medium in large scale and the exopolysaccharide can be purified by physical or enzymatic method. The yields obtained are high (upto 80% conversion) and it forms clear elastic gel at 2 g% concentration. The gel sets at high temperature (50°C). Due to pH stability and better thermal tolerance it can find wider application. It can be utilized as an agar substitute in microbiological media preparation as a thickner and as an emulsifier.

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