PREPARATION AND CHARACTERISATION OF POLYSACCHARIDE-BASED BIODEGRADABLE FILMS

THESIS

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In

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By

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September 2000

... to My Dear Parents

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22nd September 2000

CERTIFICATE

I certify that the thesis entitled ***PREPARATION AND CHARACTERISATION OF POLYSACCHARIDE-BASED BIODEGRADABLE FILMS*** submitted to the University of Mysore, Mysore, for the award of degree of ***DOCTOR OF PHILOSOPHY in BIOCHEMISTRY*** by Ms. Saroja,N. is the result of work carried out by her in the Department of Biochemistry and Nutrition, CFTRI, Mysore, under my guidance during the period 1995-2000.

Rn. Thara... 22.9.2000
(R.N. THARANATHAN)

DECLARATION

I declare that the thesis entitled *PREPARATION AND CHARACTERISATION OF POLYSACCHARIDE-BASED BIODEGRADABLE **FILMS*** submitted to the University of Mysore, Mysore, for the award of degree of *DOCTOR OF PHILOSOPHY IN BIOCHEMISTR Y* is the result of work carried out by me under the guidance of Dr. R.N. THARANATHAN, Scientist, Department of Biochemistry and Nutrition, CFTRI, Mysore, during the period 1995-2000.

I further declare that the results of this work have not been submitted for the award of any degree or fellowship.

Mysore
22nd September, 2000

(SAROJA,N.)

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N.SAROJA

CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

PS	Potato starch
CS	Cassava starch
PS-g-PAN	Potato starch-graft-polyacrylonitrile
CS-g-PAN	Cassava starch-graft-polyacrylonitrile
AN	Acrylonitrile
AM	Acrylamide
AC	Acrylic acid
Am	Amylose
Ap	Amylopectin
GC	Gas liquid chromatography
GPC	Gel permeation chromatography
SE-HPLC	Size exclusion-high performance liquid chromatography
NMR	Nuclear magnetic resonance
IR	Infrared spectroscopy
SEM	Scanning electron microscopy
DSC	Differential scanning calorimetry
DP	Degree of polymerisation
GC-MS	Gas chromatography-Mass spectrometry
v/v	Volume by volume
w/w	Weight/weight
MW	Molecular weight
h	Hour(s)
min	Minute(s)
sec	Second(s)
θ	Theta
^0C	Degree centigrade
nm	Nanometer
OD	Optical density
~	Approximately
α-	Alpha
β-	Beta
mg	Milligram
g	Gram
SAN	Styrene-acrylonitrile
ABS	Acrylonitrile-butadiene-styrene

ml	Millilitre
µl	Microlitre
R_T	Retention time
ΔH	Enthalpy
RH	Relative humidity
%	Percentage
NH	Nitrile hydratase
rpm	Rotations per minute
mm	Millimeter
TSS	Total soluble solids
DMSO	Dimethylsulphoxide
CMC	Carboxymethylcellulose
HPS	Hydroxypropylstarch
HPC	Hydroxypropylcellulose
CMS	Carboxymethylstarch
DS	Degree of substitution

SYNOPSIS

Polysaccharides such as cellulose and starch are available in abundance from highly renewable agricultural resources. The use of such natural polymers in plastics as a partial replacement for synthetic monomers, helps in the biodegradation of the starch-synthetic polymer finished plastic to lose its integrity and reduce to particles small enough to cause minimal environmental pollution. Secondly, the incorporation of starch into plastics reduces the dependence on costly petrochemical derived monomers.

By suitable manipulation of the reaction conditions, a variety of polymeric derivatives from starch and cellulose has been prepared, which as films have excellent packaging characteristics. Graft copolymerisation of synthetic polymers onto starch provides an excellent method for preparing starch-polymer composites. An important advantage of graft copolymerisation is that the natural and synthetic components are held together by a covalent bonding rather than existing merely as a physical mixture.

Studies on the extent and mechanism of biodegradation of starch containing plastics in various environments are of current interest. Such an information helps in the development of ecofriendly biodegradable packaging films for a variety of applications.

The ability of *Gloeophyllum trabeum,* a brown rot to degrade a aliphatic polyether via extracellular one-electron oxidation using hydroquinone driven Fenton reaction is reported. Ligninases and peroxidases of *Phanerochaete* and *Streptomyces* species are reported to degrade the polyethylene component of starch-polyethylene composite as evidenced by a reduction in the overall molecular weight distribution. Only a scanty information is available on the enzymatic degradation of polyacrylonitrile fibres, which hold approximately 10% of the global synthetic fibre market, and which are used as copolymers in grafted starches. Starch-graft-polyacrylonitrile (S-g-PAN) has several applications in food packaging.

Therefore, it was felt desirable that a systematic study be initiated to evaluate the structural, biochemical and biodegradable characteristics of S-g-PAN. With these objectives in view, the present investigation on grafting of starch (potato and cassava) with a synthetic monomer (acrylonitrile) was under taken and the results obtained were consolidated in the form of a thesis having the following layout.

Chapter I presents a General Introduction of the subject with special reference to polysaccharides such as starch, cellulose and chitin, which are available in abundance and have excellent film forming properties that are useful for packaging applications. An overview of plastic packaging, its merits and demerits is given. Emphasis is given to alternate packaging materials, i.e. biodegradable packaging films, such as polyhydroxy-

alkanoates, hydrocolloids and bioplastics. Their film forming properties, packaging characteristics and biodegradability are also mentioned. The latter includes the products of microbial degradation, enzymes involved and their mechanism. Finally, the origin and scope of the present investigation are provided.

Chapter II describes the Materials and Methods used in the present study. Detailed methodology of each of the experiment is given with procedures and data computation.

Chapter III consolidates Results and Discussion of the work carried out in this investigation, and constitutes the major bulk of the thesis.

Carboxymethylation (CM) and hydroxypropylation (HP) of starch (S) and cellulose (C) were achieved with degrees of substitution 0.86 and 0.94, respectively. Biodegradable films from the blends of CMC with HPC and CMS were prepared by wet casting - air-drying method. The films were tested for barrier and mechanical properties. The water vapour transmission rate values for films of CMC+HPC and CMC+CMS were found to be 1100 and 1137 $gm^{"2}$. 24 $h^{"1}$, respectively at 65% RH. Then-tensile strength values were 62.12 and 14.61 M Pa, whereas the percentage elongation values were 15 and 10%), respectively. The gas permeability of these films was very high.

The effect of two composite coatings based on CMC+HPC and CMC+HPS in maintaining the quality and an extended shelf life of banana at 27°C was investigated and compared with commercial Waxol formulation. Coated fruits had maximum freshness, surface colour, texture, taste and were best even after 21 days of storage, while the uncoated control fruits blackened due to over ripening and fungal infection. These coatings significantly reduced respiration rates, however their effect on physiological loss in weight was low.

Heterogeneous graft copolymerisation of acrylonitrile onto potato and cassava starch using eerie ammonium nitrate as a catalyst gave derivatives having a grafting percentage of 75-80, as determined gravimetrically. The evidence for grafting was seen by Fourier transform infrared spectroscopy. Appearance of absorption peak at 2260 cm^{-1} due to nitrile groups and CH_2 deformation vibration at around 1460 cm^{-1} confirmed grafting.

Differential scanning calorimetry studies showed a clear endothermic peak at around 55-58°C, which corresponds to the gelatinisation temperature range for native potato starch. No sharp melting peaks for S-g-PAN were seen in the DSC thermogram indicating that the native crystallite originally present in starch was lost during graft copolymerisation.

X-ray diffraction measurements were characteristic of B- and V-type diffraction patterns in the native and grafted material. The latter was attributed to starch gelatinisation during grafting.

Further proof for the grafting was derived from C-NMR studies. The graft copolymer showed the presence of a nitrile (-ON) group at 119.8 ppm and an aliphatic carbon atom of acrylonitrile at 27.9 ppm.

To understand the extent of grafting, the polyacrylonitrile branches were cleaved from the grafted starch by subjecting it to acid hydrolysis. The detached PAN chains were solubilised in dimethylformamide and fractionated (SE-HPLC). The results revealed considerable heterogeneity of these chains with molecular weight values of 6.3×10^7, 1.5×10^6, 2.5 x 10 and 5.01 x 10 D. The peak having a molecular weight 2.5 x 10 D was the major species.

A reverse phase HPLC method for simultaneous determination of acrylonitrile, acrylamide and acrylic acid was developed and the results were compared with GC data. Acrylic acid, 10.5 and 11.0 μg g"[1] determined by HPLC and GC, respectively was the residual monomer found in grafted starch. Its content in the alcohol washings was 21.5 and 22.5 μg g"[1], respectively.

Various enzymatic hydrolyses of the starch moiety of S-g-PAN were tried to understand its biodegradation. The percent α- and β- amylolyses of grafted starch were 55 and 50 in comparison with 80 and 70 for native starches, respectively. Sequential degradation with α-amylase and glucoamylase showed -70% hydrolysis. The maltooligosaccharide profile by HPLC of the hydrolysate showed oligomers upto DP 3, whereas the native starch hydrolysate showed oligomers upto DP 7. Almost comparable results were obtained for both potato and cassava grafted starches. Further treatment of the maltooligosaccharides with *Bacillus cereus* cells showed the presence of very low molecular weight PAN chains grafted onto them. The SE-HPLC analysis indicated the amylose component to preferentially undergo graft copolymerisation reaction.

Bacillus cereus isolated from the soil, aerobically degraded S-g-PAN. The extracellular peroxidase activity reached a maximum after ~3 h and is probably initiated the depolymerisation of polyacrylonitrile chains of S-g-PAN to the free monomer, acrylonitrile. The conversion of acrylonitrile to acrylamide and acrylic acid was later catalysed by intracellular nitrile hydratase (NH) and amidase, respectively. The concentration of the former increased with the increased induction by acrylonitrile. Maximum degradation of added S-g-PAN (0.64%) and acrylonitrile (0.32%) to the culture broths occurred after ~6 h of bacterial growth, with the corresponding formation of acrylamide, 0.19 and 0.26% and acrylic acid, 0.13 and 0.19%, respectively. After 48 h of growth neither acrylamide nor acrylic acid could

be detected indicating their complete utilization. Extracellular α- amylase activity was maximal after 8 h of growth and the starch moiety was degraded to low molecular weight dextrin-type products.

The bioconversion of acrylonitrile to acrylamide by induced cells was maximum for cells induced at 0.4% acrylonitrile, in the presence of $FeS0_4.H_20$. With the increase in induction from 0.4 to 1% there was a decrease in cell growth as seen by dry weight of the biomass. The SDS-PAGE analysis of the cell-free extract of the induced cells showed varied protein profiles. The intensity of protein band increased with increase in induction upto -0.6% indicating an increase in the production of enzyme. The results showed that NH is an induced enzyme.

A colourimetric assay for the determination of acrylamide formed by the action of NH on acrylonitrile was developed. A linear curve with a regression coefficient of 0.992 was obtained for concentrations ranging from 20-100 µg. Acrylic acid, the product of amide hydrolysis did not interfere in the colour formation indicating this reaction to be amide specific. The specific activity of NH in the crude extracts was 5.49 ± 0.05 units mg^{-1} protein, and the initial velocity was directly proportional to total enzyme concentration [E_t]. The pH and the temperature optima were 7.0 and 4° C, respectively. The saturation kinetic data showed K_m 1.06 mM and V_{max} 5.8 µmoles min^{-1} mg^{-1} protein and with no substrate or product inhibition. Other amides such as benzamide and acetamide could also be quantitated by this method.

The salient features deduced from this study are listed as Summary and Conclusion in **Chapter IV.** Finally, citation of References made use of in consolidating this work is listed in the last section.

INTRODUCTION

1.1 POLYSACCHARIDES

Polysaccharides may be regarded as condensation polymers of monosaccharides resulting in the formation of glycosidic linkages by elimination of water. As components of almost all living organisms, they are most abundant in the higher order of land plants and in seaweeds where they constitute approximately three-quarters of the dry weight. They perform diverse roles in the physiology of plants, animals, and microorganisms. Foremost are, they serve as structural materials and as fuel reserves in plants. As surface material they partially protect tissues from desiccation and as gums they are exuded from plants to seal and protect wounds. As thickeners they serve a physical or mechanical role in animals and as specific substances they are of importance in blood group specificity and in other immunological reactions.

Polysaccharides containing only one kind of polymerised sugar unit (homoglycans) are more abundant than those which contain two or more kinds of sugar units (heteroglycans). Structural polysaccharides, of which cellulose is the prime example, are almost always linear molecules, while those which serve primarily as reserve foods are commonly branched or, in the case of starch, a mixture of linear and branched polysaccharides with the latter predominating. In general, branched polysaccharides are easily soluble in water and have immense thickening properties. Linear molecules, on the other hand, are excellent structural materials because they pack closely and form many intermolecular secondary valence attachments which make the structure strong, rigid and insoluble or at least difficultly soluble.

1.1.1 **Starch**

Starch, a polysaccharide of repeating glucose units, is a mixture of two polymers, amylose and amylopectin (Fig.l). Amylose is a predominantly linear (to lightly branched) polymer, comprised of (l->4) α-D linkages with number average molecular weight in the range of several hundred thousands. Amylopectin is highly branched, with intermittent

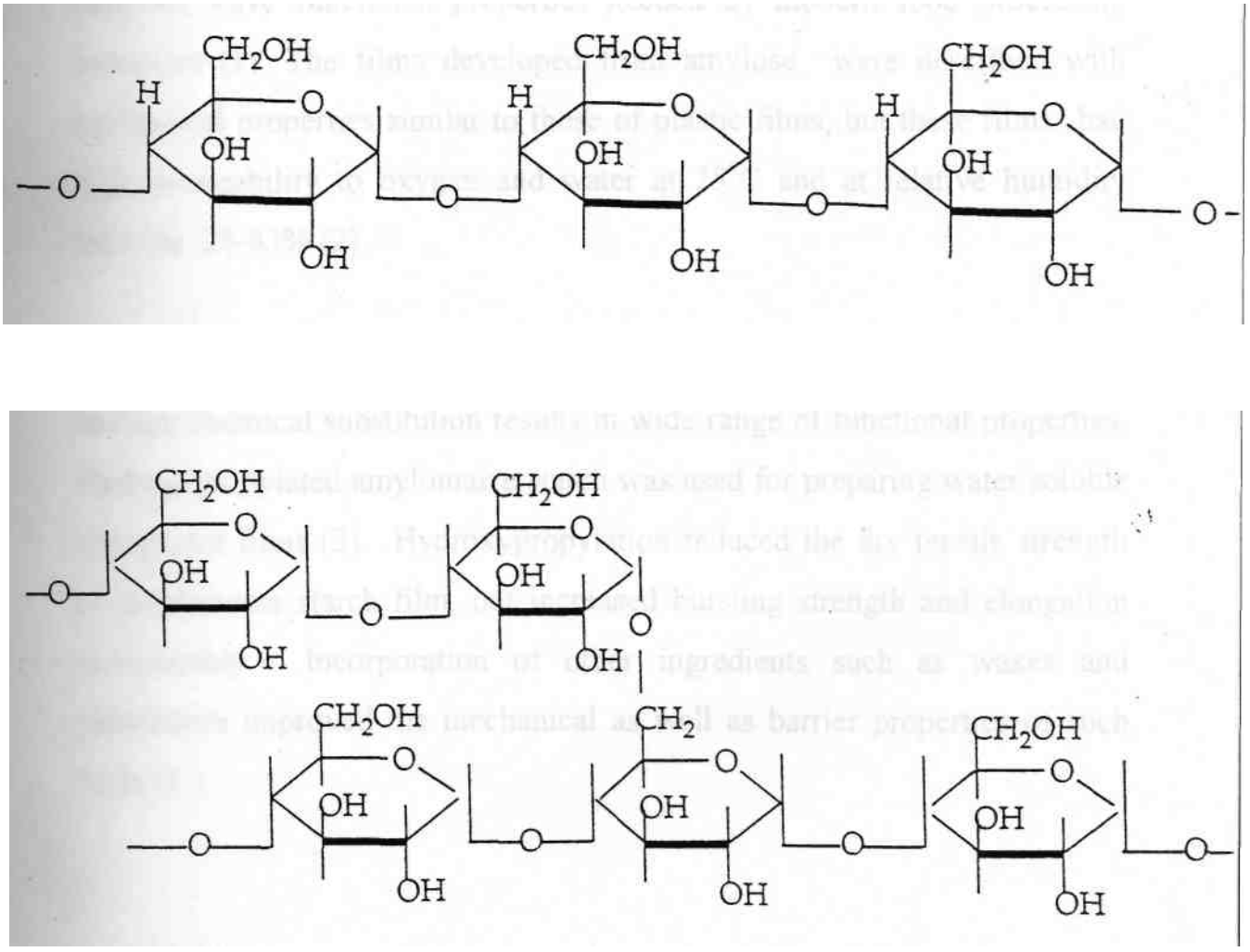

Fig.l. Structural representation of amylose and amylopectin

(l->6) links; its molecular weight is of the order of several millions, and can be as great as 50 millions. The structural difference in the two constituents of starch have a considerable effect on the properties of starch materials. Because of its branched structure, amylopectin generally has inferior mechanical properties relative to amylose.

Starch in its unmodified form has limited use in the food industry as it does not have functional properties needed by modern food processing industries (1). The films developed from amylose were described with mechanical properties similar to those of plastic films, but these films had high permeability to oxygen and water at 25°C and at relative humidity between 25-83% (2).

Modification of native starch by disruption of hydrogen bonding through chemical substitution results in wide range of functional properties. Hydroxypropylated amylomaize starch was used for preparing water soluble transparent films (3). Hydroxypropylation reduced the dry tensile strength of amylomaize starch film, but increased bursting strength and elongation considerably. Incorporation of other ingredients such as waxes and plasticizers improved the mechanical as well as barrier properties of such films (4).

1.1.2 Cellulose

Cellulose, a linear polymer of β-D-glucopyranose (see Fig.2), is the most abundant of all naturally occurring organic substances. It is the main constituent of the cell walls of land plants.

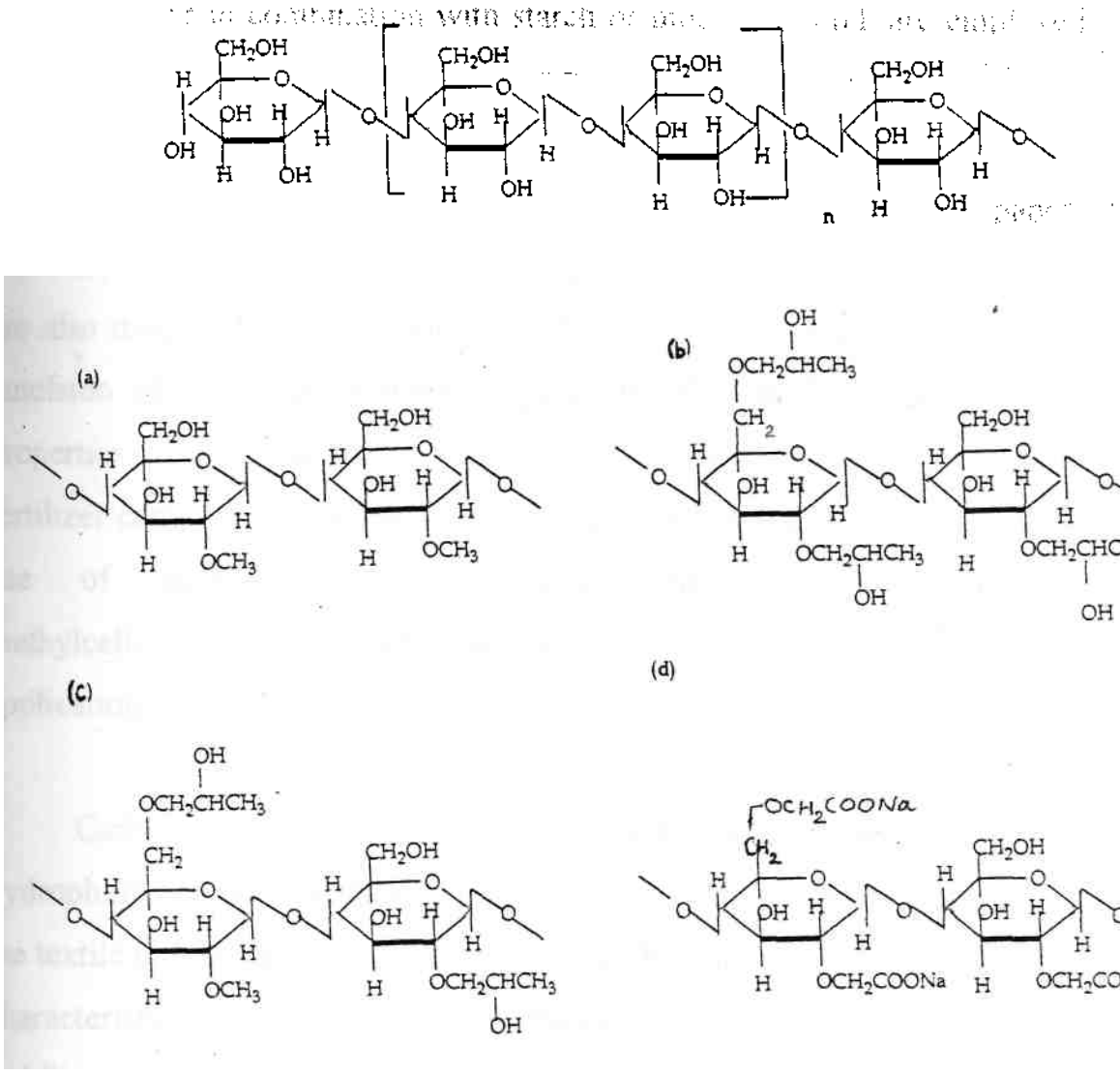

Fig.2. Structure of cellulose, and (a) methylcellulose, (b) hydroxypropyl cellulose, (c) hydroxypropylmethylcellulose and (d) Na^+ salt of carboxymethyl cellulose

Etherification of cellulose provides a broad spectrum of products that include low-substituted alkyl ethers that are insoluble in water and organic solvents. Alkyl ethers of intermediate substitution are water soluble, for example hydroxypropylcellulose and highly substituted ethers such as ethyl and methylcelluloses are soluble in organic solvents. Methylcellulose gums either alone or in combination with starch or modified starch are employed as wall paper adhesives (5). They are more resistant to microbial growth than are the starches and are often used as heavy-duty wall paper adhesives with papers that have low water permeability, such as vinyl-coated papers (6). They can be used as adhesives for bonding of leather and textiles. They are also used to thicken a variety of adhesives based on aqueous polymer emulsion of poly(vinyl acetate) and acrylate ester copolymers. Binding properties of methylcelluloses allow their use as a granulating agent for fertilizer compositions and as a medium to bind fertilizer to seeds (7). The use of methylcellulose, particularly the hydroxyalkyl-modified methylcelluose gums as thickeners for latex paints has been a major application.

Carboxymethylcellulose (CMC) is useful in application in which hydrophilic colloids are indicated . It has received considerable attention in the textile industry because of its ready solubility and excellent film-forming characteristics (8). It serves as an extrusion aid, acts as a binder, helps to stabilize emulsion, and retards sugar crystal growth. Because of its uniform quality, CMC, which is insoluble in stomach acid but soluble in alkaline intestinal fluids, is a good enteric coating for powders and tablets. It is a

mild but effective bulk laxative. It is used as a stabilizer for hand lotions and vitamin-oil emulsions and widely used as a binder in cosmetic products.

1.1.3 Chitin

Chitin, a poly-β-(l—► 4) linked N-acetyl-D-glucosamine (Fig.3), is a biopolymer widely distributed in nature. It is a polysaccharide of considerable interest because of its abundance and unusual combination of properties, which include toughness, biodegradability, and relative inertness, all of which contribute to making chitin an attractive speciality material.

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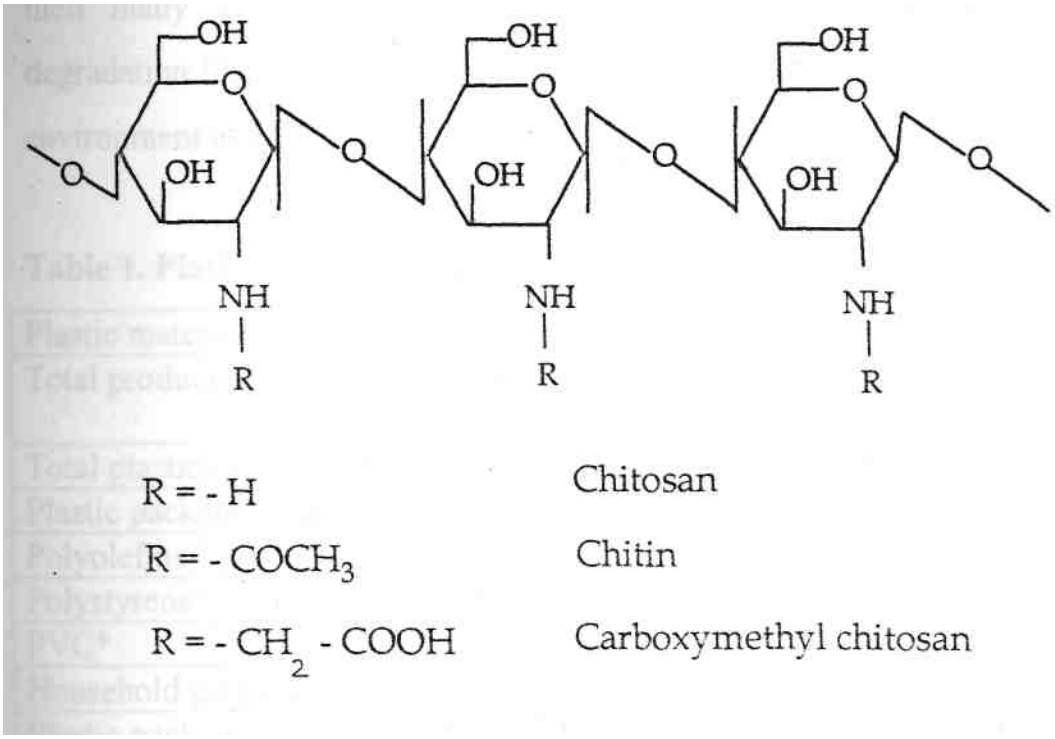


Fig.3. Structure of chitin , chitosan and carboxymethyl chitosan

However, because chitin is insoluble in water and most ordinary solvents, it cannot readily be fabricated into useful materials such as fibers or membranes, which has limited its use in many fields. The hydrophilicity of chitin can be increased by deacetylation of its N-acetyl glucosamine units in strong alkali. Chitosan, a derivative of chitin that has been maximally deacetylated, is readily soluble in dilute acidic solution and is easily fabricated into gels and films.

1.2 PLASTIC PACKAGING

Plastic materials have become an integral part of our life because of their many desirable properties including durability and resistance to degradation (Table 1) (9). These non-degradable plastics accumulate in the environment at a rate of 25 million tons per year (10).

Table 1. Plastic materials for packaging

Plastic materials	$x10^6$ tons
Total production of packaging materials	10.0
Total plastics consumption	6.6
Plastic packaging (40% food, 60% non-food)	1.4
Polyolefms * 70%	
Polystyrene* 15%	
PVC* 10%	
Household garbage	14.0*
Plastic packaging materials in household garbage.	0.7

*Disposal by incineration 33%, and landfill 66%

The 5% plastics in household waste adds to an increasing garbage mountain which is recognised as an ecological threat. Space for landfills is limited and additional incineration capacities require high capital investments. Further, incineration yields CO_2 which adds to the problem of green house effect and the liberated gases (NO, $S0_2$) and toxic degradation products like polycyclic hydrocarbons, pose additional environmental problems. In recent years there is a global awareness about the need to reduce the amount of plastic waste discarded in landfills. Although improved efforts to recycle discarded plastics would help accomplish this goal, recycling would be neither practical nor economical for certain end - use applications such as agricultural mulch films, planting pots and garbage bags. For such applications plastics are needed that will fragment or degrade into benign by-products under composting conditions.

The different strategies employed for plastic waste management are shown below.

1. **Prevention**
    a) Improved production processes
    b) Reuse of packaging materials, e.g. bottles
2. **Recycling (separation, selection, cleaning, reprocessing)**
    a) Pure polymers
    b) Polymer mixtures
3. **Thermic utilization**
    a) Incineration (heat of combustion)

4. Disposal in landfills/composting

5. Chemical utilization

a) Hydrocracking (hydrocarbon)

b) Hydrolysis (acids, amines polyolefins)

c) Pyrolysis (aromatic hydrocarbons)

6. Degradable polymers

a) Chemical modification of classical polymers

b) Starch containing polymers (polyethylene, polyethylene/ polyacrylate copolymers, polyvinylalcohol)

c) Thermoplastic starch

d) Biopolymers (polyhydroxybutyrate, polylactic acid)

1.3 ALTERNATE PACKAGING MATERIALS

Biodegradable polymers offer an interesting alternative to classical non-degradable polymeric films, especially for short term use such as agricultural mulches, beverage and fast food packages, refuse and retails bags. After disposal in landfills (compost) these materials are degraded by photodegradation, chemodegradation and biodegradation or a combination of the three into environmentally harmless substances or low molecular weight products which can be further metabolized by microorganisms. They can be broadly classified into -

a) Polyesters (biopolymers),

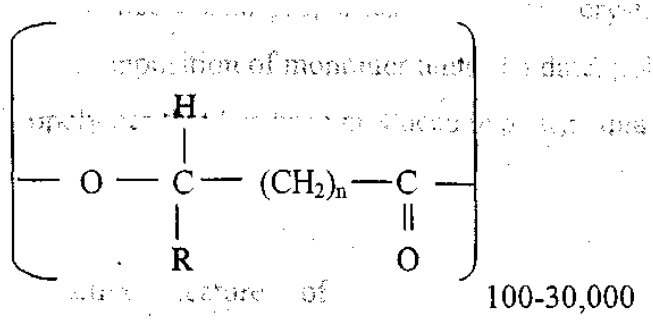
a) Edible coatings and films,

b) Starch as filler/ composites, and

c) Starch-graft-copolymers

1.3 (a) Polyesters (bacterial polyhydroxyalkanoates)

Polyhydroxyalkanoates (PHAs) are polyesters (Fig.4) synthesised (11) by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells.



When n = 1, R = hydrogen the polyhydroxyalkanoate is poly(3-hydroxypropionate)

Fig.4. General structure of polyhydroxyalkanoates

Numerous bacteria synthesize PHAs as a sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon source (12). When the supply of limiting nutrient is restored the PHA can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source (11). The molecular weights of polymers are in the range of 2 x 5^3 to 3 x 10^6 Da, depending on the microorganism and growth conditions (13).

To date, most of the studies on the physical and thermal properties of bacterial PHAs have been carried out with poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). The P(3HB) is 100%

stereospecific with all the asymmetric carbon atoms in the D(-) configuration. It is therefore highly crystalline. The crystallinity range from 55-80%. The glass transition temperature and the melting point of P(3HB) are approximately 5 and 175°C, respectively (14). The family of PHAs exhibits a wide variety of mechanical properties, from hard crystalline to elastic, depending on the composition of monomer units. To date, poly(3HB-co-3HV) is the only copolymer that has been produced in a large quantity.

Biodegradation of PHA

The most attractive feature of PHAs is their complete biodegradability. A number of aerobic and anaerobic PHA-degrading bacteria and fungi have been isolated from various environments which include *Acidovorax facilis, Aspergillus fumigatus* from soil, *Alcaligenes faecalis* and *Pseudomonas fluorescens* from activated sludge. These microorganisms excrete extracellular PHA depolymerases to degrade PHAs into water soluble monomers and oligomers, and later use them as a carbon source. The PHA depolymerases have a hydrophobic domain binding site to adhere to the surface and a catalytic domain containing the lipase specific sequence, Gly-X]-Ser-X_2-Gly (15). The rate of biodegradation is influenced by a number of factors including the microbial population in a given environment, the temperature and the properties of the plastic material to be degraded. It is shown that P(3HB-co-3HV) was completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water, respectively. Due to its complete biodegradation, PHA fits perfectly well in the ecosystem. PHAs have been drawing considerable industrial interest as

candidates for biodegradable and/or biocompatible plastics for a wide range of applications such as packaging films; bags and containers; biodegradable carrier for long term dosage of drugs, medicines, insecticides, herbicides; disposable items such as razors, utensils; starting materials for chiral compounds; surgical pens, sutures, staples and swabs; and wound dressing. Many companies world wide are developing products from PHAs.

However, there are two drawbacks to the commercial use of P(3HB) (16). Firstly, P(3HB) has a poor melt stability because it decomposes at approximately 200°C, which is close to its melting temperature. Secondly, P(3HB) becomes brittle over a period of several days upon storage under ambient conditions.

1.3 (b) Edible coatings and films

1. *From proteins*

The film-forming ability of several proteinaceous substances has been utilized in industrial applications. A number of proteins, both of plant and animal origin, have received attention for production of films and coatings. These proteins are corn zein, wheat gluten, soy protein, peanut protein, keratin, collagen, gelatin, casein, and milk whey proteins. The use of protein-based coatings on fresh produce of high moisture content has been restricted due to its limited water vapour resistance. The effect of corn zein coatings on tomatoes showed (17) shelf life extension by six days as evidenced by sensory evaluation. Casein and acetylated monoglyceride emulsion coatings were used in controlling moisture loss and oxidative

browning in cut, peeled apple pieces (18). Corn zein-based edible coatings extended the shelf life of nuts by retarding rancidity, staling and sogginess (19). Such coatings reportedly prolonged the shelf life of coated chocolate cubes, donuts and fig bars (20).

Glutenins are the high molecular weight proteins in the wheat gluten complex that are primarily responsible for dough viscoelasticity. It is believed that glutenins result from cross-linking of polypeptide subunits. Wheat gluten films can be produced by deposition and subsequent drying of wheat gluten dispersions in aqueous ethanol. Alkaline or acidic conditions are required for the formation of homogeneous film-forming solutions (21). Upon casting, disulfide bridges are reformed, linking together polypeptide chains, to yield firm structure. Reoxidation in air and sulfhydryl-disulfide interchange reactions are the mechanisms that contribute to reformation of disulfide bonds (22).

The brittleness of these films results from extensive intermolecular associations (23). The plasticizer addition to wheat gluten solution brings about modification in film flexibility (Table 2) (24). Plasticizer molecules mediate between polypeptide chains, disrupting some of these associations and decreasing the rigidity of the film structure. The advantages of protein films are excellent oxygen and carbon dioxide barriers at low relative humidities, whereas their resistance to water vapour transmission is limited. Their good gas barrier properties are drastically reduced in high humidity

Table 2. Tensile strength and elongation at breadth of protein-based edible films

Film	Tensile strength (MPa)	Elongation at break (%)
Wheat gluten-lactic acid (1:1)	0.01	75
Wheat gluten-lactic acid (1:1)	0.02	63
Wheat gluten-soy protein- glycerin (1.75:0.75:1)	4.4	233
Corn zein-glycerin (2.9:1)	3.9	-
Soy protein-glycerin (1.7:1)	4.3	78

environments because proteins are susceptible to moisture absorption and swelling. Improvement of resistance to water vapour remains one of the main objectives for protein films. Several chemical and physical treatments (i.e, tanning treatment with aldehyde, treatment at the protein isoelectric point) show some effectiveness in promoting cross linking and hardening of the protein structure and also improving film barrier and mechanical properties.

The disadvantage of edible protein-based films and coatings is the potential allergenic responses to specific protein sources by some individuals and allergy to food proteins is linked to several diseases (25). Incidence of gluten intolerance is known which is characterized by nutrient malabsorption as a consequence of gluten-dependent damage to the mucosa of the small intestine (26).

2. From lipids and resins

Lipid-based coatings are mainly used to prevent weight loss, slow down aerobic respiration and to improve appearance by providing glossy characteristics. They are also useful in reducing surface abrasion during handling operations of fruits and vegetables and as carriers of fungicides, antioxidants, antimicrobials and growth regulators. The edible lipid-based coatings include mainly neutral lipids of glycerides and waxes which are esters of long chain monohydric alcohols and fatty acids (27). Waxes such as carnauba, beeswax, paraffin, rice bran wax and candelilla are reported to be used in combination with resins or polysaccharide to coat fresh fruits and vegetables such as citrus and apples (28, 29, 30). Beeswax and vegetable oil extended the shelf life of stored raisin (31). Shelf life of eggs stored at low temperature (35°F) was extended by the use of paraffin wax (32).

The disadvantage of lipid and resin-based coatings is that they show poor flexibility and high degree of cohesiveness. This is minimized by the addition of plasticizers such as monoglycerides, phospholipids and ester derivatives of glycerol (33).

3. *From polysaccharides*

The development of coatings from water soluble polysaccharides has brought a surge of new types of coatings for extending the shelf life of fruits and vegetables, because of selective permeability of these polymers to $C0_2$ and 0_2. Polysaccharide-based coatings are thus utilized to modify the atmosphere thereby reducing fruit and vegetable respiration. They are of

significant importance to the food industry because they are abundantly available, usually are of low cost, and are non-toxic (34).

Water soluble polysaccharides are long chain polymers that dissolve or disperse in water to give a thickening or viscosity building effect. Cellulose is insoluble in water due to the high level of intramolecular hydrogen bonding in the polymer, whereas ethers of cellulose such as carboxymethyl cellulose (CMC) and hydroxypropyl cellulose (HPC) and hydroxypropyl methyl cellulose (HPMC) are water soluble and are good film formers. They are capable of yielding tough and flexible, transparent films owing to the linear structure of the polymer backbone (35). The films are soluble in water and resistant to fats and oils. Methylcellulose being the least hydrophilic of the cellulose ethers, produce films that have relatively high water vapour permeability.

The high quality film forming characteristics of HPC have been applied to retard moisture absorption and the development of oxidative rancidity in nutmegs (36) and to retard spoilage and moisture absorption in coated nuts and candies. Bilayer films, composed of HPMC and solid lipid such as beeswax, paraffin, hydrogenated palm oil, or stearic acid yielded water vapour permeabilities that were lower than that of low density polyethylene (37). A bilayer film consisting of stearic-palmitic acids and HPMC showed moisture transfer from the high moisture food (tomato paste) to the low moisture food. Formulations consisting of MC, HPMC and HPC (later named Nature Seal) resulted in a delay of ripening and browning and

increase in volatile flavour components of fresh commodities such as mangoes and banana (38).

The use of an aqueous slurry of an amylose-rich starch ether in gelatinised form as a protective coating for foods is reported (39). A gluten-dextrin coating was used to coat dry roasted peanuts prior to application of salt. Low-methoxyl pectinate as a coating agent is used for certain foods as it gives an attractive, non-sticky surface to foods (40). Carrageenan which is a complex mixture of several polysaccharides is used as food coatings to enhance the stability against the growth of surface microorganisms (41). A carrageenan-based coating applied on cut grape fruit halves resulted in less shrinkage and deterioration of taste after two weeks of storage at room temperature (42).

Chitosan-based films

Chitin on treatment with alkalie gives chitosan, a heterogeneous substance in various stages of deacetylation and depolymerization. Chitosan applications include coatings, flocculating agents, and ingredients for foods and feeds. Chitosan can form a semipermeable coating which can modify the internal atmosphere, thereby delaying ripening and decreasing transpiration rates in fruits and vegetables (43). It can inhibit the growth of fungi and phytopathogens (44).

A method for preparing chitosan derivatives with a wide variety of agricultural and industrial applications has been developed. N,0-Carboxymethyl chitosan (NOCC) (45) is water soluble, biodegradable and forms selectively permeable non-toxic films. Nutrisave, an NOCC-based formulation was reported to have some success as a post harvest edible coating for fresh fruits (46).

1.3 (c) Starch as filler/composites

Physical incorporation (as blends) of starch as a biodegradable element in classical polymers is reported (9). Starch (6-20%) is incorporated e.g., into polyethylene matrix without any chemical interaction. In these films the starch degradation is caused by the attack of microbial enzymes and thus the plastic film becomes porous and susceptible to further oxidative degradation. The amount of hydrophilic starch and the hydrophobic polyethylene determines the property and the performance of the film. The starch/polyethylene blend films (47) in the ratio 1:1 showed properties such as antiblocking behaviour, printability and water vapour permeability but on the other hand the mechanical properties of the films were reduced.

In the case of composites with starch, the starch content is as high as 50% (48) by weight and it forms a continuous phase with the hydrophobic synthetic polymer. The synthetic components used in composites are non-toxic and are of comparatively low molecular weight (500-5000 Da).The monomers used are hydrophilic and are able to create strong physical interactions by direct chemical linkages with the starch. Starch/polyvinyl

alcohol composite films as biodegradable agricultural mulches have been described (49). The water absorption behaviour of such composite films depends very much on the concentration of additives and on the process conditions. Their mechanical properties are between that of LDPE and HDPE, as far as elasticity modulus, shear modulus and elongation at break are concerned (Table 3) (9).

Table 3. Mechanical properties of starch composite films

	Starch composite	LDPE	HDPE
Elasticity modulus (Kg/cm^2)	1000-8000	1000-2800	4200-12000
Shear modulus (Kg/cm^2)	100-250	50-160	220-380
Elongation at break (%)	20-300	90-1000	20-130

Burial tests, $C0_2$ development and oxygen consumption by microorganisms showed that the biodegradability of these composite materials lie in between non-degradable polyethylene and fully degradable paper.

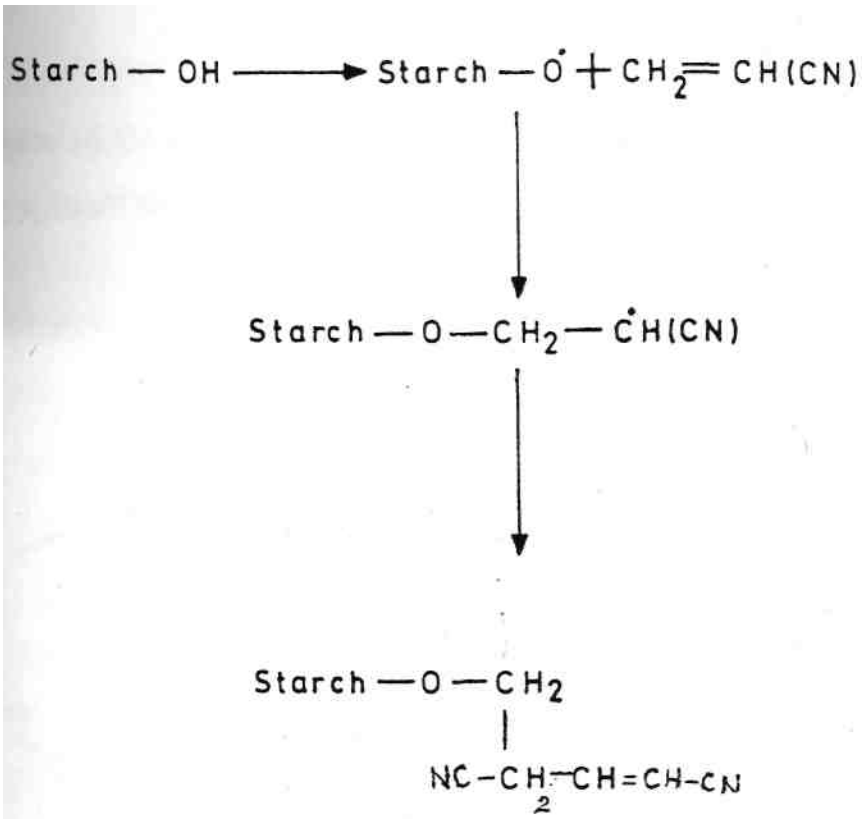
1.3 (d) Starch-graft-copolymers

The incorporation of starch into plastics to enhance their fragmentation and degradability in the environment has generated considerable interest (50). Starch is inexpensive, totally biodegradable and is available in large quantities from certain crops (i.e., corn, wheat and tubers). Replacement of petroleum-based plastics with starch is also

attractive from the standpoint of conserving our costly petrochemical resources. Two basic approaches are being pursued in the direction of biodegradable plastics, viz.

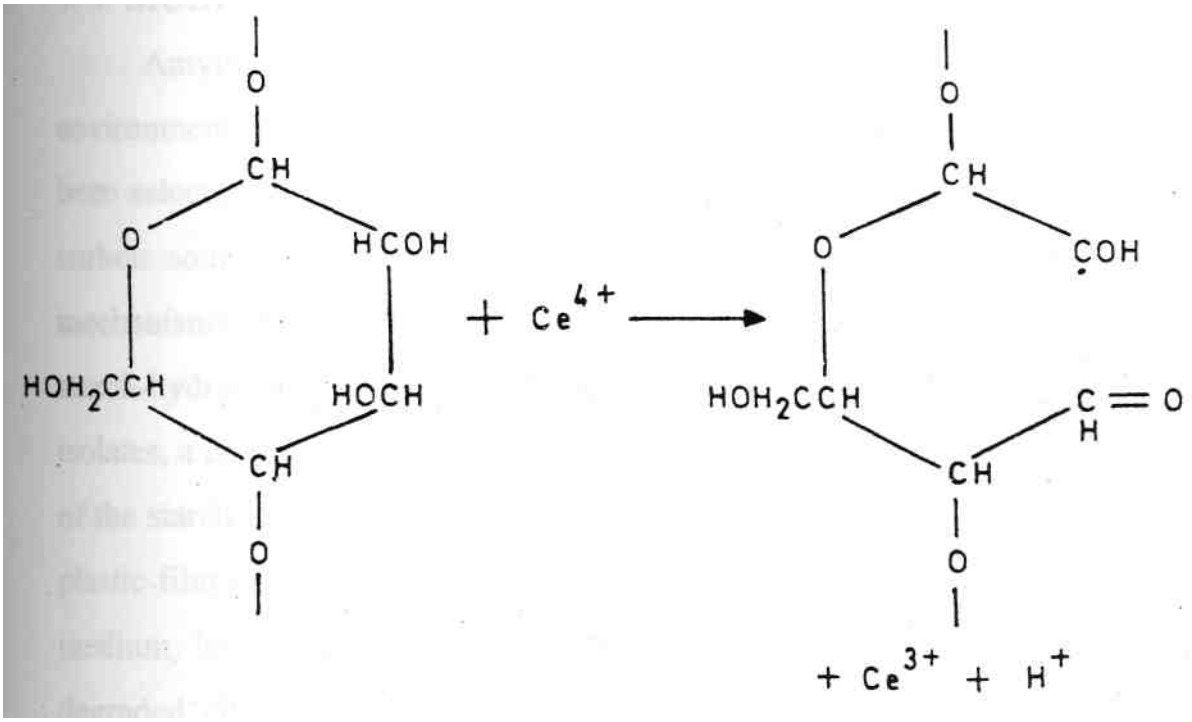
1. Blends of starch with other polymers especially biodegradable ones, are being compounded and formed into films or injection molded into articles, and
2. Starches grafted with thermoplastic chains are being synthesised and formed into films or injection molded items.

Grafting a synthetic polymer to a natural polysaccharide is a way of creating large molecules that have some of the characteristics of each individual polymer. Grafting is initiated by generating one or more free radicals on the polysaccharide chain and allowing them to react with polymerizable monomers that will constitute the grafted chain. Thus, if a polysaccharide is reacted, for example, with high energy radiation, such as X-rays and ultraviolet radiation, free radicals may be created in various ways but principally through hydrogen atom elimination. More often, free radical generators such as ferrous ion or eerie ammonium nitrate, along with hydrogen peroxide, is used. After formation the free radical is available to initiate a free radical polymerization as illustrated by the following pathway for reaction of starch with acrylonitrile.



Although not all radicals produced on a polysaccharide chain react with monomer to initiate growth of a synthetic polymer chain, polymerization efficiency can be fairly high, often exceeding 50%. Many different monomers subject to free radial polymerisation have been tried successfully, with those commonly used being vinyl acetate, acrylamide and methylmethacrylate. Evidence for the formation of free radicals in graft copolymerisation reactions has been obtained by electron spin resonance spectroscopy (ESR) (51). The reaction of eerie ion in aqueous solution with cellulose cleaves the anhydroglucose ring between C_2 and C_3 with the formation of short-lived radical on C_2 and oxidation of C_3 to a reducing group. Grafting occurs by reaction of the radical at C_2 with monomer. Radical termination occurs by reaction of C_2 with Ce^{4+} to yield Ce^{3+} and

oxidation of C_2 to a reducing group. The role of carbon C_2 and C_3 in the reaction could be reversed (52).



Oxidative depolymerization of cellulose also occurs and could lead to short-lived intermediate homopolymerisation. In case where an intermediate radical is not formed, the reactions could occur to form the initiating macrocellulosic radicals, which were followed by ESR spectroscopy. Graftcopolymerisation of methylmethacrylate (MA) onto either granular and gelatinized starch is reported (53) using eerie ammonium nitrate as initiator. Homogeneous graft copolymerisation of methylmethacrylate onto ethylcellulose using radical initiators such as ammonium persulphate,

potassium persulphate and benzoyl peroxide was carried out in benzene/ dimethylsulphoxide (1:1, v/v) mixed solvent system (54).

1. 4 BIODEGRADATION OF STARCH-GRAFT-COPOLYMERS

Amylolytic bacteria have been isolated from a wide range of environments and studied for starch-plastic biodegradation. Bacteria have been selected for their ability to utilize starch in S-g-copolymers as the sole carbon source in liquid culture media. In an effort to understand the mechanisms of microbial degradation of starch-containing plastics, several starch-hydrolysing bacterial isolates have been examined. One of these isolates, a consortium of bacteria designated as LD67, degraded upto to 80% of the starch in starch - polyethylene -ethylene-co-acrylic acid (S-PE-EAA) plastic film (originally 40% starch by weight) in 60 days in a liquid culture medium, leaving behind the non-starch components (55) of the film non-degraded. The loss of starch from the film was accompanied by concomitant loss in weight and tensile strength of the films, which may have contributed to further degradation of the film by mechanical forces. Laboratory studies with highly amylolytic *Arthrobacter* sp., revealed that in a liquid culture medium where starch containing films were the sole carbon source, bacteria readily metabolised starch. Progressive removal of starch from the S-PE-EAA film by the bacteria over 56 days of exposure was demonstrated by electron microscopy. It was noted that the bacteria bound significantly more densly to starch-g-poly (methacrylate) plastic film than to S-PE-EAA film, whereas starch was more readily hydrolysed in the latter. This

suggested that adhesion of bacteria to the film is not an adequate indication of degradability of starch within the film.

The *Lactobacillus amylovorus* bacterium, isolated from corn waste fermentation, secreted amylase that rapidly degraded starch granules in starch-graft films. Studies on microbial surface interactions between *L. amylovorus* and granular starch indicated that colonisation by these bacteria on corn starch granules was important for starch granule degradation (56).

At very low concentration of starch, only surface starch would be accessible to direct attack by microorganisms. The rate and extent of starch removal from starch plastic composites by several commercially available amylases were measured in cell-free systems. Amylases derived from animals, plants and microbial sources all hydrolysed starch rapidly. Pulverised S-PE-EAA plastic from injection molded specimens was 40-60% hydrolysed by these enzymes within several days.

Biodegradation of S-g-PMA was studied using three fungal suspensions such as *Aspergillus niger, Penicillium funiculosum* and *Tricoderma viride* (Table 4) (57). The percent degradation or starch utilization was determined by loss in tensile strength and scanning electron microscopy. With starch as a readily available carbon source, excellent growth and sporulation was observed in five days with *A.niger,* whereas

good growth but less sporulation was noted with *P. funiculosum* and *T. viride.*

Table 4. Weight loss and tensile strength properties of biodegraded S-g-PMA

Copolymer[a]	Inoculum	Weight loss, %	Tensile Strength, MPa
A	*P.funiculosum*	37.5	4.14
A	*A. niger*	40.0	8.41
A	*T.viride*	16.9	7.52
A	None[b]	10.2	10.10
A	Control	(100.0)	20.44
B	*P.funiculosum*	12.9	5.93
B	*A. niger*	16.0	5.34
B	*T.viride*	12.4	5.65
B	None[b]	1.0	8.03
B	Control	(100.0)	11.82

a. Copolymer A contains 50% starch, copolymer B 40% starch.
b. Incubated for 22 days at 25°C without inoculum.

1.5 BIODEGRADATION OF THE SYNTHETIC MOIETY OF STARCH COMPOSITE

The ability of lignin-degrading microorganisms to attack starch-polyethylene was investigated in pure shake flask cultures. The known lignin-degrading bacteria *Streptomyces viridosporus* and fungus *Phanerochaete chrysosporium* were used by following reduction in percent elongation and molecular weight distribution. It was found that ligninases of *S. viridosporus* and peroxidases of *P. chrysosporium* were involved in polyethylene biodegradation (10). But it was noted that these

microorganisms were unable to utilize starch, which was a component of the degradable plastic film. In another report it was shown that a brown rot fungus *Gloephyllum trabeum* used an extracellular oxidative system to degrade a recalcitrant polymer, polyethylene glycol (PEG). An extracellular metabolite, 2,5-dimethoxy-l,4-benzoquinone, produced by the fungus was reduced to 2,5-dimethoxy-hydroxybenzoquinone along with the reduction of Fe^{3+} to Fe^{2+} and with concomitant production of H_20_2 (Fig.5). These results provided evidence that *G. trabeum* used a hydroquinone-driven Fenton reaction to cleave PEG (58). Further investigation led to the conclusion that *G. trabeum* also cleaved polyethylene oxide (PEO) rapidly by an endo route. ^{13}C-NMR analysis of unlabled and perdeuterated PEOs recovered from *G.trabeum* cultures (59) showed that a major route for depolymerisation was oxidative C-C bond cleavage, a reaction for hydrogen abstraction from

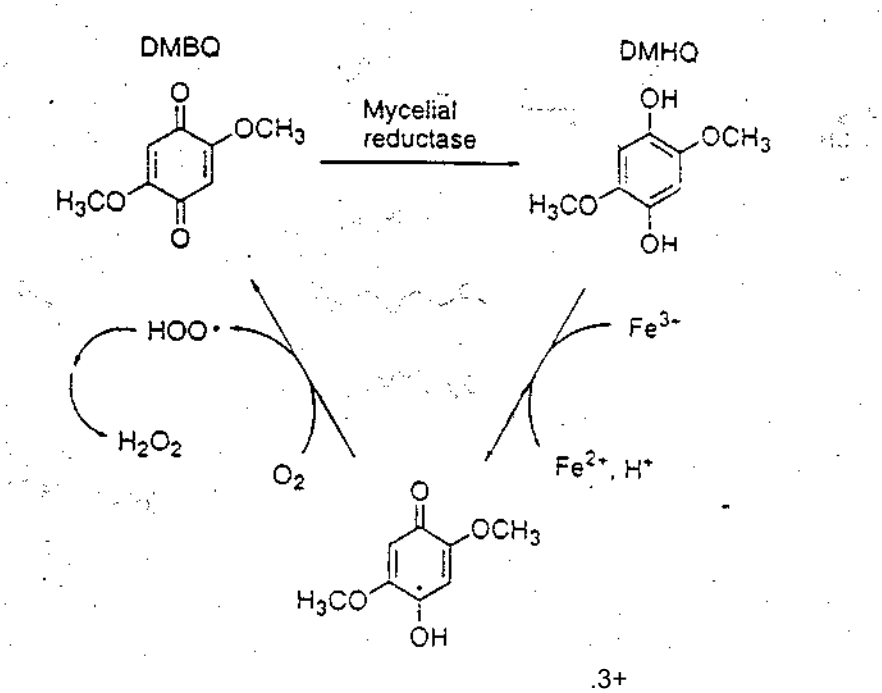


Fig.5. Proposed pathway for extracellular Fe^{3+} reduction and H_20_2 production by *G.trabeum*

a PEO methylene group by a radical oxidant, that is an extracellularly produced one-electron oxidant which cleaves PEO rapidly via β-scission reaction (59). In another report, the cleavage of labelled polyethylene oxide, [^{14}C] PEO, was demonstrated (60). The MW distribution of [^{14}C] PEO in *G. trabeum* cultures spread uniformly to lower values as degradation progressed. It was established that depolymerisation followed an endo rather than an exo route as there was no free ^{14}C released from PEO. Two likely routes (Fig. 6) deduced for endo PEO oxidation by *G. trabeum* were oxygen insertion between carbon and hydrogen in a methylene group and hydrogen abstraction from a methylene and carbon. Identification of new end groups in degraded PEO showed that *G.trabeum* produced a strong extracellular oxidant that leads to extensive PEO depolymerisation by abstracting hydrogens from the internal methylene

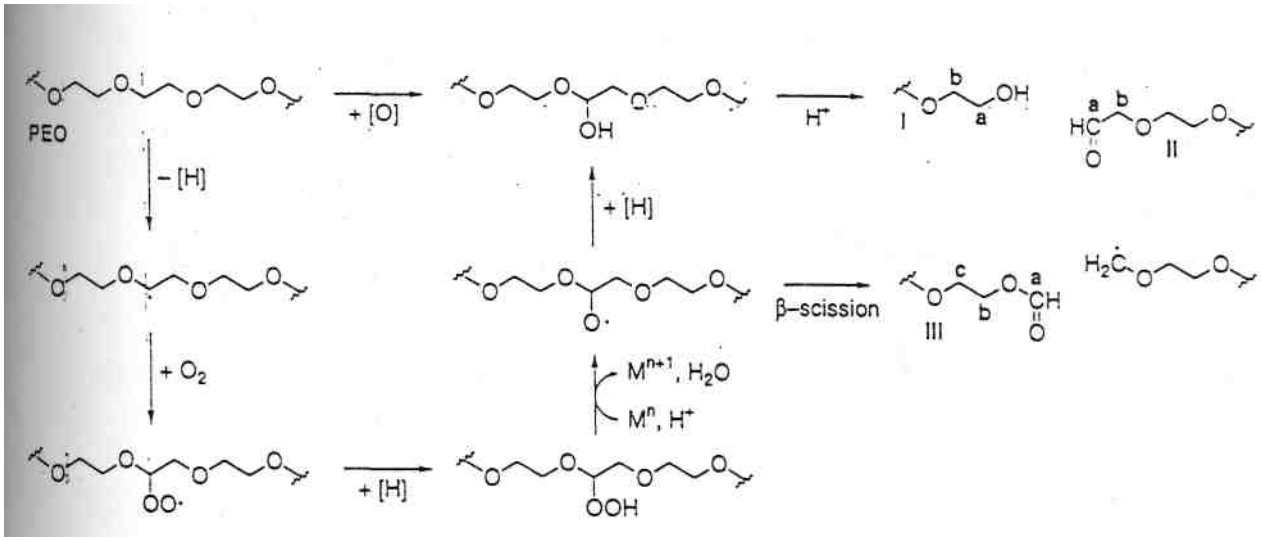


Fig. 6. Predicted pathway of PEO scission after hydrogen abstraction, M^n indicates a transition metal ion.

groups. The role of oxidant required for this reaction is played by H_2O_2 (61) which is produced by extracellular oxidoreductase of brown rot fungi (62, 63, 64).

1. 6 PRODUCTION OF FREE RADICALS BY THE ACTION OF LIGNINASE WITH H_20_2

Resting ligninase enzyme reacts with H_20_2 to produce the two-electron oxidised intermediate, compound I, which oxidises lignin substrate (RH) to yield the one-electron oxidized intermediate, compound II and a substrate radical. Compound II returns to resting enzyme by oxidising a second substrate molecule. The free radical (R) (Fig.7) (65) can undergo a variety of reactions mainly C-C bond cleavage, hydroxylation, phenol dimerization and demethylation (66). The chemistry of ligninase-catalyzed oxidation is

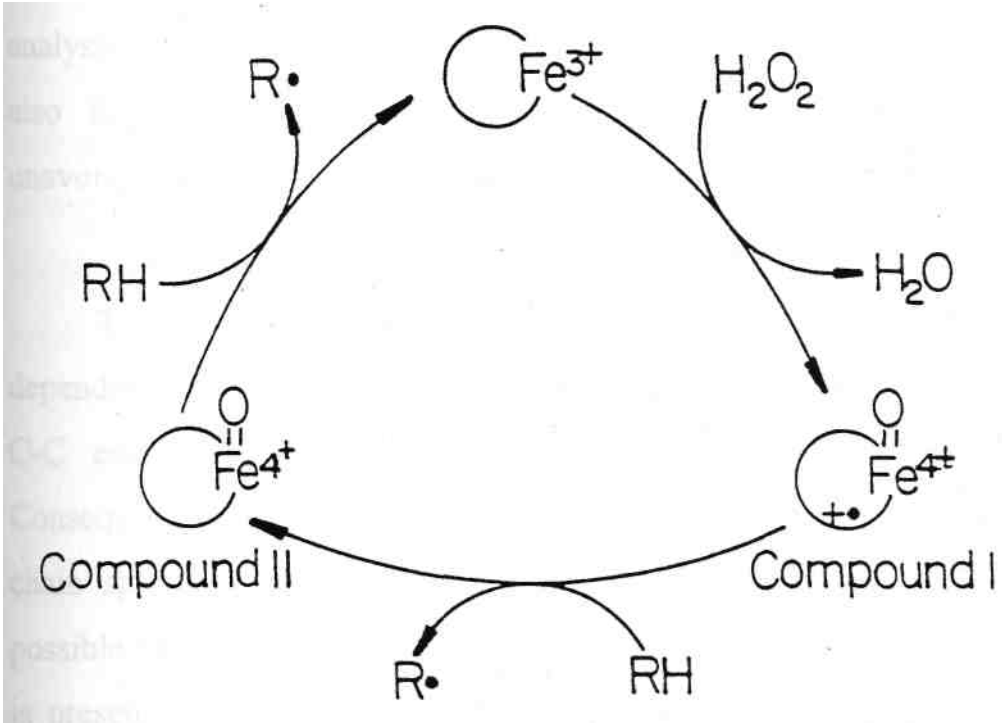


Fig. 7. Production of free radicals by the action of ligninase

very similar to one electron chemical (Fenton) oxidation. The product profiles, stoichiometries and kinetics for many of the lignin-like substrates are consistent with cation radical chemistry (67). If the results with the model compounds are extrapolated to other aliphatic polymers, one can envision depolymerisation occurring through radical-mediated cleavage of C-C bonds. The mechanism of lignin biodegradation is thus viewed as a process directed more by substrate chemistry than by enzyme chemistry (68).

1.7 OXIDATIVE AND MICROBIAL EFFECTS IN THE DEGRADATION OF POLYETHYLENE

Bioconversion of ^{14}C present in HDPE film to respiratory $^{14}C0_2$ during aerated cultivation with soil or with a fungus *Fusarium redolens* showed a weight loss of 0.16 % (69). On gel permeation chromatographic analysis it was found that the long polyolefin chains were degraded. It was also found that the autooxidative-progressive aging of the film was unavoidable, which had a cumulative effect on biodegradation.

The enzymatic degradation of a long, straight olefin chain is dependent on the cleavage of C-C bond by an endoenzyme such as n-alkane C-C endohydrolase, which does not seem to occur in nature (70). Consequently the polythenes can never stimulate the production of a long-chain splitting degradative enzyme by 'induction'. Such an induction is possible exclusively in a specific case when the genetic information [codon] is present in the cells of an attacking organism. A recent report on the

induction of a kind of oxidizing enzyme in *Pseudomonas sp.* by poly(vinyl alcohol) shows that the induced enzyme resulted in the production of H_2O_2, followed by the appearance of methyl ketones (71).

In view of current industrial and commercial interests in the basic molecular mechanisms of degradation it may be said that molecules above MW 1000 are inert to microbial utilization (72, 73). The long chain polymers are initially converted to short chain fragments by aging which is merely an autocatalytic pathway (74, 75). The cycles of both aging and biodegradation are complementary or rather synergistic with each other.

1. 8 ORIGIN AND SCOPE OF THE PROPOSED INVESTIGATION

Plastics, the synthetic polymers that are largely resistant to microbial degradation and deterioration, are accumulating in the environment in huge amounts. Their persistence either as litter or through landfill disposal of municipal solid waste has prompted a rethinking on their continuous use. Incorporation of natural polymers into plastics not only helps in their disintegration but it also reduces the dependence on the dwindling but expensive petroleum-derived monomers. Graft copolymerisation of synthetic monomers such as acrylonitrile, a precursor of acrylic fibres and plastics, onto starch provides an excellent method for preparing starch-graft copolymers, which on extrusion form films useful for packaging applications. Starch is inexpensive, totally biodegradable and available in large quantities. Therefore, it was felt desirable that a systematic study be initiated to understand the biodegradation of starch-graft-copolymers. Such

a study would help knowing the mechanism of biodegradation of synthetic monomer grafted to natural polymer (starch). The following programme of action was proposed.

1. Preparation of carboxymethyl- /hydroxypropyl- derivatives of starch and cellulose, and starch-graft-copolymers. Determination of the percentage grafting,

2. Detection of grafting by IR, NMR, DSC, and X-ray diffraction measurements. Determination of the molecular weight of grafted chains by size-exclusion GPC,

3. Determination of the residual monomers in starch-graft- copolymers by RpHPLC, and its comparison with GC,

4. Isolation of starch and acrylonitrile degrading microorganisms. *In vivo* degradation of S-g-PAN by the organisms and induction studies of acrylonitrile degrading enzyme nitrile hydratase. Identification of the degraded products by HPLC, GC and GC-MS,

5. *In vitro* degradation of starch moiety of S-g-PAN by various amylolytic enzymes and comparison of the degradation patterns with that of ungrafted starch, and

6. Development of a colourimetric assay for nitrile hydratase, and studying its kinetic parameters.

The results of this investigation have finally shown that both starch and polyacrylonitrile undergo biodegradation by *Bacillus cereus* through an enzymatic pathway.

MATERIALS AND METHODS

MATERIALS

All the chemicals, organic solvents and acids used were of analytical reagent grade.

Amylose, amylopectin sugar standards, dinitrosalicylic acid, O-dianisidine, cellulose, propylene oxide, catalase (E.C No. 1.11.1.7), bovine serum albumin, Coomassie brilliant blue R-250, pancreatic α-amylase (E.C.No.3.2.1.1.), peroxidase (E.C.No.3.2.2.4), β-amylase (E.C.No.3.2.1.2), pullulanase (E.C.No. 3.2.1.4.1), DEAE-Sephadex, and protein standards are from Sigma Chemical Co., USA. Sepharose CL-2B and dextrans (T-10, T-20,T-40,T-70,T-500 and T-2000) were from Pharmacia Fine Chemicals, Sweden. Acrylonitrile, acrylamide and acrylic acid were from Fluka, Switzerland. Cassava *(Manihot utilisima)* starch (CS) was a gift from Central Tuber Crops Research Institute (CTCPJ), Trivandrum. μ-Bondagel E-linear and E-1000 columns were from Waters Associates, USA. Maxil Cig and 5-NH_2 columns were from Phenomenox, U.S.A. Styrene-acrylonitrile and acrylonitrile-butadiene-styrene copolymers were obtained from Central Institute for Plastic Engineering and Technology (CIPET), Mysore. GC column containing PEGS, 20% on Chromosorb P was obtained from Perkin-Elmer, U.S.A. Etheral, AR grade, was purchased from Mysore Pure Chemicals, Mysore. Chitosan was supplied by CFTRI Regional Centre, Mangalore.

Fruits

Banana *(Musa robustana)* with 75% maturity, and optimally matured mango *{Mangifera indicia* cv Alphanso) grown in local farms were harvested and washed thoroughly with water. Fruits of uniform size, free of physical damage and fungal infection were used. Fruits were randomly distributed into groups, individual in the case of mango and groups (hands) of 8-10, in the case of banana. Each group or individual represented one replicate, and for each treatment, 6-8 replicates were used.

General

a) All results are the average of not less than three independent experiments.
b) Preparation of reagents (and determinations) were done using deionised double glass distilled water.
c) HPLC solvents were triple distilled, degassed and filtered through 2µ Millipore membrane.
d) Samples were weighed in a Mettler AE-100 digital balance.
e) Incubation with enzymes was done in a Julabo SW-20C thermostat shaking water bath.
f) Centrifugations were carried out using either refrigerated Hermle-Z 320K or Sigma 202-C bench top centrifuge.
g) Boiling water bath temperature was 97°C.
h) All rotary flash evaporations were done in a Buchi Rotavapor RE 120 under reduced pressure at a bath temperature of ~40°C.
i) Shimadzu UV-160A spectrometer was used to read the colour developed in all analytical determinations.

j) Samples were lyophilised in a Virtis Freeze mobile-12 lyophilizer at-60°C and~100μ vacuum.

METHODS

Isolation

Potato starch

Peeled potato (500 g) was kept in cold water for 30 min, crushed (wet grinder) into a fine paste and suspended in water. The slurry was centrifuged at 4000 rpm for 30 min. The residue was slurried with O.l N NaOH to pH 9.0, stirred continuously for -10 min and centrifuged. The starch was washed thoroughly with water to remove residual alkali. The sedimented starch was treated with 0.1N NaCl-toluene (10:1, v/v) for -20 min, centrifuged and the purified starch was thoroughly washed with water. Finally it was alcohol washed and dried at 60°C in an oven (76).

Acrylonitrile-degrading microorganisms

Acrylonitrile-degrading microorganisms were isolated from soil in a medium containing (g/1) NaCl, 1.0; KH_2P0_4, 1.0; K_2HP0_4, 1.0; $MgS0_4$, $7H_20$, 0.2; yeast extract, 3.0; malt extract, 5.0; peptone, 1.0; glucose, 10.0. After sterilisation, the pH of the medium was adjusted to 7.0 and acrylonitrile (0.4%) was added. The medium was inoculated with diluted soil samples. The liquid broth after 48 h of growth on a rotary shaker at 250 rpm and ambient temperature (±30°C) was streaked on nutrient agar plates containing acrylonitrile. The isolation medium mentioned above was used with 1.5%) agar and the bacterial cultures were restreaked, selected and

recultured in the above medium, supplemented with acrylomtrile (0.4% v/v). After 48 h of growth, the cells were centrifuged at 10,000 rpm for 20 min at ~4°C, the sediment was suspended in 100 mM phosphate buffer (pH 7.0) and the cells together with alumina were disrupted mechanically for 20 min at ~4°C and centrifuged. The cellular lysate was checked for nitrile hydratase activity and the culture having the maximum activity was selected. The colonies developed on agar medium containing acrylonitrile were examined microscopically, macroscopically and the purified isolate was identified according to Bergey's manual (77).

Preparations

Carboxymethyl derivatives of cellulose and starch

Starch and cellulose (4 g), NaOH (3.2 g) and monochloroacetic acid (4 g) were taken in a beaker, with 10 ml of water and the contents were subjected to continuous stirring to homogeneity. Subsequent reaction was allowed to proceed at 60 C for 2 h. The reaction products were precipitated with ethanol and washed alkali free and dried in an oven at 80°C for 3 h (78).

Hydroxypropyl derivatives of cellulose and starch

To a vigorously stirred slurry of 1 g of finely powdered cellulose or starch in 15.8 ml of 2-proρanol was added 1.9 g of 15.8% of NaOH over a period of 13 min at room temperature. The agitation was continued for 1 h after which 0.33 g of propylene oxide dissolved in 0.8 ml of 2-propanol was added. With continued stirring the reaction mixture was heated to about 55-

60°C for 5 h. The reaction products were precipitated with ethanol and washed alkali free and dried in an oven at 80 C for 3 h (79).

Crosslinking of CM cellulose and CM starch

Crosslinking of CM cellulose and CM starch was carried out either by homogeneous reaction by dissolving the derivatives in aqueous alkali (2-30% NaOH) and the desired amount of epichlorohydrin was added under continuous stirring for 1 h. After completing the reaction, the reaction mixture was neutralised with acetic acid and simultaneously rendered particulate by dispersal in a Warring blender. The slightly acidic product was filtered (on a glass filter, 10-15 μ), washed with water and acetone, and dried under vacuum at a temperature of 70°C to constant weight (80).

Starch-graft-polyacrylonitrile(S-g-PAN)

A slurry of starch (10 g in 200 ml of water) was heated (85°C, 30 min) while a slow stream of nitrogen gas was bubbled through. The gelatinised starch suspension was cooled to 25 C and acrylonitrile (15 g) was added, followed after about 30 sec by a freshly prepared solution of eerie ammonium nitrate (0.34 g in 3 ml of 1 N $HN0_3$). The mixture was stirred for 2 h at 25°C, neutralised (by adding 0.1 N NaOH) to pH 7.0 and ethanol (3 vol.) precipitated. The starch-g-copolymers (PS-g-PAN and CS-g-PAN) were filtered, washed with aqueous ethanol and dried (81).

Coating formulations

To prepare 100 ml of coating solution (2-3% total solids for banana and 1.5-2.5% for mango, w/v), 1.0-2.0 g of polysaccharide derivatives were dissolved in double distilled water and blended with either free fatty acids (palmitic and stearic) or glycerol monostearate or sucrose fatty acid ester. Tween-80, 0.2 ml was added to the solution to emulsify and to improve wettability. The above mixture was stirred for 30 min and the insoluble were removed by filtration. Si and S_2 refers to CMC + HPC and CMC + HPS, respectively.

HPLC standards and sample

The HPLC mobile phase consisted of 0.05M KH_2P0_4, pH 5.5. An isocratic elution at a flow rate of 1 ml min^{-1} was used at 30°C. A 10 µl volume of standard acrylonitrile, acrylamide and acrylic acid [1:50, 1:500 and 1:5000 dilution range in glass distilled water (v/v)] and samples were injected into column and eluted and detected at 220 nm using a detector range of 0.08 AUFS. Linear studies used appropriate detector range to ensure that all peaks remained on scale. The graft copolymers (1 g each) were washed with ethanol (100 ml) and 10 ml of this washing was used for analysis. The residual monomers in the EtOH washed graft copolymers were extracted with DMSO (1 mg ml^{-1}) and the diluted (1:10 with water) extract was injected into HPLC.

GC standards

A 40 µl of standard acrylonitrile (1:50) and acrylic acid (1:5000, v/v dilution in $CHC1_3$) was prepared and 0.4 µl injected into GC. The $CHC1_3$ washings of the copolymers were also injected. The residual monomers in a few industrial polymers styrene-acrylonitrile (SAN), 1 g and acrylonitrile butadiene-styrene (ABS), 1 g were $CHC1_3$ extracted 3 to 4 times (10 ml each time) and 10 µl injected into the GC.

Crude extract of *Bacillus cereus*

Acrylonitrile (0.4%, v/v) induced *Bacillus cereus* cells (200 mg) in peptone-malt-yeast-glucose medium were harvested after 48 h of growth and homogenised in 0.1M phosphate buffer (5 ml) containing 5 mM dithiothreitol, pH 7.0. The homogenate was centrifuged (10,000 rpm at 4°C for 30 min) and the supernatant was used as the source of nitrile hydratase.

Acrylamide standard curve

Acrylamide, ranging from 0-100 µg in 1.0 ml of distilled water was treated with 2.0 ml of freshly prepared hydroxylamine hydrochloride solution (2.3 M) and NaOH (3.5 N) (1:1 ratio), for 10 mm at 60°C. The red brown colour was developed by the addition of 4N HC1 (0.6 ml) and 1.23 M $FeCl_3$ in 0.O5N HC1 (1 ml) and read at 540 nm immediately (within 5 min) (82).

Enzyme assays

Nitrile hydratase activity (83) was determined spectrophotometrically. The assay was done by adding 1 ml of 50 mM acrylonitrile to 0.2 ml of 100 mM phosphate buffer, (pH 7.0) containing 100 µl of cellular lysate. The mixture was incubated at 4°C for 20 min. The formation of acrylamide was measured by an increase in absorbance at 235 nm (ε = 1106 $cm^{-1}M^{-1}$) and the activity was expressed as µ moles of acrylamide formed $min^{-1}mg^{-1}$ protein.

Amidase activity (84) was assayed in a reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 7.5), 10 mM acrylamide and 100 µl of cellular lysate. The ammonia formed was determined colourimetrically. The activity was expressed as µ moles of ammonia formed min^{-1} mg^{-1} protein.

α-Amylase activity was (85) determined by adding culture filtrate (0.5 ml) to 0.5 ml of 1% (w/v) soluble starch in 0.1 M phosphate buffer (pH 7.0) and incubating for 30 min at 4°C and the released reducing sugar was determined by the DNS method. An enzyme unit is defined as the amount of enzyme releasing 1.0 µg glucose equivalent from the substrate $min"^{1}$ at 40°C.

Peroxidase activity (86) was determined by adding the culture filtrate (100 µl) to a mixture of 100 µl of 1% H_20_2, 100 µl of 0.25% O-dianisidine and 700 µl of 50 mM phosphate buffer (pH 6.0). The buffer contained 1 mM NaN_3 which inhibited the catalase activity present in the culture filtrate.

The change of absorbance by oxidised O-dianisidine was measured at 460 nm. The activity of peroxidase was expressed as an increase in absorbance by 0.01 unit at 460 nm min^{-1} mg^{-1} protein.

Analytical methods

DS by acid wash method

To carboxymethyl starch (2 g), 40 ml of 7% HNO3 in methanol was added and shaken for 3-4 h and filtered on G3 sintered funnel. The residue was washed with 80% methanol at a very slow rate until the washings were free of acid and dried with anhydrous methanol. It was then transferred to a 250 ml Erlenmeyer flask (1 g) containing 10 ml of 70% methanol. The mixture was allowed to stand for 30 min and 100 ml of water and 25 ml of 0.5 N NaOH were added. The contents were shaken for 45 min and back titrated with 0.4 N HCl using phenolpthalein. Results were calculated according to the formula (87)

$$DS = \frac{0.162A}{(1-0.058A)}$$

where A= milliequivalents of NaOH required g^{-1}.

DS in hydroxypropyl starch

Hydroxypropyl derivatives (0.1 g) were weighed into a 100 ml volumetric flask containing 25 ml of IN H_2S0_4. The flasks were placed in a boiling water bath and heated until the samples were in solution. The contents were diluted to 100 ml and 1 ml of the solution was pipetted into a

25 ml graduated test tube with glass stopper and immersed in cold water, later 8 ml of cone. H_2SO_4 was added dropwise. The tubes were kept in boiling water bath, for exactly 3 min. Immediately the tubes were transferred to the bath and the solution was chilled and ninhydrin reagent (88) (3.6 ml) was carefully added and then placed in a 25°C water bath for 100 min. Absorbance was read at 590 nm. A factor 0.7763 was applied to convert mg of the glycol to hydroxpropyl group equivalent (88).

Film thickness

A constant-load micrometer (Testing Machines, Minneapolis) was used to measure the film thickness and the values are mean of six measurements after equilibration at 27°C and 65% RH.

Water vapour permeability

Water vapour transmission rates (WVTR) through the films were determined using aluminium test dishes according to the ASTM E-96-97 method (89). Four replicates of each sample type with 95 cm^2 of exposed area were tested at a_w gradients of 0-0.32 ($MgCl_2.6H_2O$),0-0.57 (NaBr) and 0-0.93 (KNO3) using the relevant saturated solutions at 27+1 °C (90), in a humidity cabinet (Laboratory Thermal Equipment, Glasgow, UK). The WVTR values were calculated applying least squares analysis and were then divided by the area of the film exposed. Water vapour permeance and permeability coefficients were calculated from WVTR data (91).

Oxygen transmission

Oxygen transmission rates through the films were determined using a volumetric permeability cell (Custom Scientific Instruments, New Jersey) according to the ASTM D-1434 procedure (92). Testing was performed at 27+l°C and 65% RH after the samples had been equilibrated for a minimum of 48h.

Mechanical properties

The tensile strength and percentage of elongation of the films were determined using an Instron Universal Testing Machine (Model 4301) with 5 cm jaw separation and a film width of 1.5 cm, according to the ASTM D-882 procedure with a strain rate of 17.5 cm min^{-1} (93). Tensile strength and elongation at break point were also noted.

Statistical analysis

Differences in the properties of the film samples were determined by Student's t-test using $p < 0.05$ level of significance (94).

Quality attributes

Quality of the fruits was assessed each week for banana and on the alternate days for mango. A sample of 4-5 fruits in total were randomly removed from each treatment and analysed. The presence of mold was evaluated visually. Cumulative physiological losses in weight (PLW) of the fruits were determined by difference in weight after 24 h at ambient temperature. Firmness was measured with Instron Universal Testing

Machine (Model 4301, Instron Corporation Conton, MA) and was expressed as kg force. Total soluble solids content (TSS) of the fruits was determined by Abbe's refractometer. Acids were titrated to phenolphthalein end point with 0.1 N NaOH and was expressed as % malic acid. Reducing sugar was determined by DNS method (95) after extraction with 80% hot alcohol and was expressed as mg of reducing sugar as glucose per gram of pulp. Respiration rate was determined on alternate days and expressed as mg of CO2 $kg^{-1}h^{-1}$). Sensory attributes like colour, texture, flavour and taste were assessed by a panel of six laboratory personnel familiar with banana and mango grades. The fruits were rated on hedonic scale of 1 to 10 (10 = excellent, 1 = poor). Data were evaluated by using ANOVA.

Total sugar

Total sugar by the modified phenol-H_2SO_4 method

a. Aqueous phenol solution: Phenol (5 ml) was mixed with distilled water (95 ml).

b. Standard aqueous glucose (0-25 µg) solution.

To 0.5 ml sample, 1.8 ml of cone. H_2SO_4 was added directly to the mixture with a wide tipped pipette and was shaken vigorously, and later transferred to ice cold water bath. Then 0.3 ml of 'a' was added and mixed. The tubes were allowed to cool at room temperature for about 20 min. The colour developed was measured at 480 nm (96).

Protein

Solution A: 2% solution of Na_2CO_3 prepared in 0.1 N NaOH

Solution B: Aqueous (1%) solution of $CuS0_4.5H_20$

Solution C: Mixture of 100 ml of solution A with 1 ml of solution B and 1 ml of aqueous (2%) sodium potassium tartarate solution.

Solution D: Folin-Ciocalteau reagent (1.0 ml diluted with 1.5 ml water).

To 1.0 ml sample (20-100 μg), 5.0 ml of solution 'C was added, mixed and incubated at room temperature for 10 min. Then 0.5 ml of solution 'D' was added, mixed immediately and again incubated at room temperature for 30 min. Absorbance of the colour was read at 660 nm (97).

Reducing sugar

a. DNS reagent : Dinitrosalicyclic acid (10 g) was dissolved in 16 g of NaOH solution (1 L) containing 300 g of sodium potassium tartarate.
b. Standard aqueous maltose (0-1000 μg) solution

To 1.0 ml of sample, 1.0 ml of DNS reagent was added, mixed and kept in a boiling water bath for 10 min. Then 10 ml of water was added and the absorbance of the colour developed was measured at 540 nm (95).

Determination of blue value

Starch (100 mg) suspension in water (1.0 ml), was carefully dispersed in IN KOH (20 ml). After 10 min the solution was diluted to 40 ml with water. From the above solution, 1 ml (-2.5 mg/ml) was taken and 0.5 N

HCl (1 ml), 0.1 g of potassium hydrogen tartarate and 0.5 ml iodine solution (2%) were added and diluted to 50 ml. The solution was mixed well and the absorbance was measured at 680 nm after 20 min. Blue value (B.V.) was determined according to the formula, B.V.= (Absorbance x 4)/C, where C is the carbohydrate content of starch (mg/dl) on dry basis (98).

Molecular weight determination

The molecular weights of isolated polyacrylonitrile branches were obtained with a Shimadzu HPLC system, consisting of an LC 6A pump equipped with RI detector on a E-linear and E-1000 μ Bondagel columns (30 cms x 3.9 mm id) connected in series with a guard column. The columns, eluted with dimethylformamide (filtered and degassed) at a flow rate of 0.2 ml min^{-1}, were maintained at 40°C. The RI attenuation was adjusted to 8 and chart speed was set to 5 mm min^{-1} . The void volume (using *Sesbanium mosaic* virus) and elution volume of standard dextran series were measured (99).

Differential scanning calorimetry

DSC analysis was carried out with DSC (+) Rheometric Scientific UK, instrument equipped with a thermal software ver 540. Samples (5-10 mg) were accurately weighed into small aluminium cups and water was added (1:3 and 1:5, w/w) to get approximately a water content of 50-80% (w/v). The cups were capped and reweighed, it was heated at 5° min^{-1} from 5-100°C for all defatted native starch and grafted starch samples. An empty pan was used as reference. Measurements in duplicate were performed for

each sample. Both gelatinization temperature and enthalpy values were corrected by using indium standard (100).

X-ray diffraction

X-ray diffraction patterns were obtained by using a EG-7G solid state germanium liquid N_2 cooled detector Scintag DS 2000 instrument equipped with a θ-θ goniometer, with the following operating conditions: 30 kV and 25 mA with CuK_α radiation at λ 1.5418 nm. Diffractograms were scanned from 2 to 40° at a diffraction angle of 2θ. The starch samples were powdered to pass through a 150 mesh sieve and kept for saturation with distilled water in a desiccator overnight (101).

Infrared spectroscopy

IR spectra were recorded in KBr discs on an Impact 410 Nicolet FTIR spectrometer under dry air at room temperature.

^{13}C- Nuclear magnetic resonance spectroscopy

For NMR, samples were prepared by dissolving (38.0 mg) in dimethylsulphoxide-d_6 (0.5-1 ml) under argon atmosphere at 60°C for 45 min in 5 mm quartz sample tubes. The spectra were recorded at 336 ± 1k on a Bruker WM 360 instrument at a carbon frequency of 90.1 mHz, with a special width of 32 k. The relaxation delay D_1 was set to 3 sec with a pulse angle of 60°. Under non-spinning conditions 18300 scans were accumulated and Fourier transformed after applying resolution enhancement using the spectrometer standard software. The spectra were normalised using the

carbon signal of dimethylsulphoxide-d6 at 39.50 ppm as an internal standard (102).

SEM studies of bacterial cells

Acrylonitrile induced, 48 h grown *B.cereus* cells were washed with 0.1M acetate buffer (pH 4.8) and suspended in 2.5% glutaraldehyde for 1 h. The pellet was washed thrice (15 min each) with 0.1M phosphate buffer (pH 7.0) and centrifuged. The cells were dehydrated with different percentages of acetone (30, 50, 70, 80 90 and 100%) each for 15 min at 4°C. The dehydrated cells were spread on a double sided conducting adhesive tape over a metallic stub and coated (-100 μ) with gold in a sputter coating unit and observed in a LEO 435 VP digital scanning electron microscope (103).

Gel electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a vertical mini slab gel electrophoresis unit (Balaji Scientific Services, Madras, India) with 0.8 mm thickness, 12.5% SDS-PAGE resolving gels and a 5% acrylamide stacking gel and stained with Coomassie brilliant blue R-250 (104).

Gas chromatography-Mass spectrometry (GC-MS)

Combined GC-MS analysis was carried out with a QP 5000 Shimadzu mass spectrometer - GC-17A Shimadzu gas chromatography equipped with an electron impact detector, operating at - injection temperature, 220°C; column temperature, 80 C; and detector temperature, 250°C. A capillary

column DB Wax (30 m x 0.25 mm, id) with a helium flow rate of 40 ml min^{-1} was used at an ionisation potential of 70 eV.

Fractionation of PS and CS and PS-g-PAN and CS-g-PAN

Native and grafted starches were fractionated by size exclusion chromatography (SEC) on a Sepharose CL-2B column (92 x 1.7 cms) to obtain amylose and amylopectin fractions. The void volume of the column was determined using blue dextran 2000 (MW = 2000,000). The starch samples (10 mg ml^{-1}) were dispersed by digesting in 85% (v/v) aqueous dimethylsulphoxide at 90 C for 40 min. The mixture was centrifuged (10,000 rpm, 30 min) to remove any insoluble residue (105). The solution (1 ml) was applied over the column bed and eluted with water containing 0.02% NaN_3 at a constant flow rate (18 ml h^{-1}). Fractions (3 ml) were analysed for total sugar (Pattabi) as well as starch-iodine blue colour (Gilbert).

Amylolysis

Native CS and PS as well as PS-g-PAN and CS-g-PAN (120 mg) suspended in water (5 ml) were separately gelatinized by boiling for 30 min and the aqueous suspensions were diluted to 10 ml with sodium phosphate buffer (0.02 M, pH 6.9). α-Amylase (0.4 ml, 200 units) was added and the mixture was incubated at 30 C for 1, 2, 4, 8, 12 and 24 h. The enzyme was inactivated by placing the tube in a boiling water bath for 10 min. The digests were then centrifuged to remove the precipitated enzyme. The supernatants were analysed for carbohydrate by the DNS method. Similar

reactions were carried out with β-amylase (0.4 ml, 200 units in sodium acetate buffer, 0.05 M, pH 4.8 at 37°C), glucoamylase (0.4 ml, 200 units in sodium acetate buffer, 0.1M, pH 4.6 at 37°C), and pullulanase (0.4 ml, 3.2 units in sodium acetate buffer 0.1M, pH 5.5 at 37°C) and the liberated sugars were analysed by the DNS method.

For sequential action of α-amylase and glucoamylase, the samples (120 mg) were separately gelatinized prior to hydrolysis, with water for 30 min and later were dispersed in 10 ml of sodium acetate buffer (0.1M, pH 6.9). α-Amylase (0.4 ml, 200 units) was added and the mixture was incubated for 2, 4, 6, 8, 12 and 24 h at 37°C. The enzyme was heat inactivated and the pH was reduced to 4.6. Glucoamylase (0.4 ml, 200 units) was added and the mixture was incubated for 2, 4, 6, 8, 12 and 24 h at 55°C. The enzyme was heat inactivated and centrifuged. The supernatants were precipitated with alcohol (3 vol.) to remove the enzyme protein. The clear filtrates were flash evaporated and oligosaccharides in them were analysed by HPLC using a Maxil 5-NH_2 column (250 x 4.6 mm, id), acetonitrile - water (75:25, v/v) at a flow rate of 1 ml min^{-1} at 37°C and RJ detector.

Hydrolysis of PS-g-PAN

PS-g-PAN (0.25g) was treated with 125 ml of 75% aqueous $ZnCl_2$ and kept for stirring overnight. An equal volume of 6N HC1 was added and stirred for 2 h. The contents were then heated for 4 h with constant stirring at 100°C and later the precipitate was washed with water and dissolved in N,N-dimethylformamide and oven dried at 60°C to constant weight (106).

Detection of grafted polyacrylonitrile chains in the hydrolysate

The hydrolysate (20 mg) was treated with a suspension of *Bacillus cereus* cells (isolated from soil, wet weight 10 mg ml"[1]) in a total volume of 10 ml in 100 mM phosphate buffer (pH 7.0) and the degradation was performed at 30°C and 250 rpm for 12, 24, 36 and 48 h. The reaction was arrested by centrifugation at 10,000 rpm for 30 min and the cell pellet was discarded. The degraded products were recovered by extraction with diethylether (3 vol.) and the solvent was removed and the products dissolved in water (1 ml), were analysed by Rp HPLC using a Maxil C_{18} column (25 cm x 1.5 mm, id) and Shimadzu HPLC system consisting of an LC-6A pump equipped with an UV-Vis spcetrophotometer detector and a Shimadzu (C-R 4A) Chromatopac integrator. The mobile phase consisted of KH_2P0_4 buffer (0.05 M, pH 5.5) at 1 ml min-^{1}, 30°C.

Biodegradation studies

Degradation of acrylonitrile and PS-g-PAN was performed using bacterial suspension *of Bacillus cereus* (15 mg ml-^{1} wet weight) in a total volume of 50 ml of 100 mM phosphate buffer pH 7.0 in a 250 ml conical flask. Concentrations of acrylonitrile monomer and PS-g-PAN was 0.4%. The solid S-g-PAN was ground in a pestle and mortar into a fine powder. Reactions were perfonned at 30°C and 250 rpm in a rotary shaker. Samples (25 ml) were taken at different intervals of time and the degradation halted by centrifugation for 10 min at 10,000 rpm. The cells were discarded and the degraded products were recovered from the supernatant by extracting with diethylether (3 vol.). The organic phase was collected, filtered through

Whatman No.l paper and rotary evaporated. Quantitative and qualitative analyses were performed by RpHPLC as before.

Induction of acrylonitrile degrading enzymes

This was performed by growing *B.cereus* cells in 100 ml growth medium with 0.4, 0.6, 0.8 and 1% (v/v) of acrylonitrile as the carbon source. Salts like $FeS0_4.7H_20$ and $CoCl_2$ (0.001% w/v) were incorporated into 0.4% (v/v) acrylonitrile containing growth medium separately according to the procedure of Nagasawa et al. (107). After 48 h of growth, the cells were harvested by centrifugation at 10,000 rpm for 30 min. The pellet was washed twice in 100 mM phosphate buffer (pH 7.0) and resuspended in the same buffer. The activities of cell free extracts were determined as described earlier and protein profiles were characterised by SDS-PAGE.

Coating application

The fruits, individually in the case of mango and hands comprising of 8-10 fruits in the case of banana, separately but simultaneously were dipped into the coating solutions as well as Waxol (positive control, 6% for banana and 3% for mango) and water (control). The excess solution was drained and the coated fruits were air dried. In addition to a antifungal paste, a 1 % chitosan solution was applied at the fascicle region of banana to curtail water loss and to prevent fungal growth. After drying, the fruits were stored at 27±2°C temperature with an RH of 65% for different lengths of time. At regular intervals the fruits were removed and analysed for weight loss, respiration rate and other quality parameters.

Film formation

Crosslinked CMC and HPC/HPS (1:1 w/w) were dissolved in 100 ml of water .The solution was stirred at room temperature for 45 min and filtered using a vacuum flask and aspirator to remove any undissolved material. Glass plates with an area of 20 x 20 cm were cleaned with ethylalcohol and levelled. The solution was poured into the centre of the plate and spread manually with a glass rod until the solution was evenly spread to the outside borders of the plates. Films were dried at ambient temperature for -30 h and peeled off.

RESULTS AND DISCUSSION

3.1 PACKAGING PROPERTIES OF POLYSACCHARIDE-BASED FILMS

Cellulose and starch on etherification give derivatives, which are good film formers. They are capable of yielding tough, flexible and transparent films owing to the linear structure of the polymer backbone. These films are soluble in water and resistant to fats and oils. Evaluation of edible bilayer films of methyl cellulose and polyethylene, methylcellulose and corn-zein have been documented in the literature (108). Studies on packaging with these films and the consequent extension of the shelf life of foodstuffs have been made with respect to cucumber and bell pepper fruits (109). Alternatively, modified starches have received great attention in the field of packaging. Water-soluble, transparent films have been produced from hydroxypropylated amylomaize starch having an amylose content of 71% **(3).**

These findings prompted us to take up further detailed studies on packaging films made out of modified cellulose and starch derivatives and evaluate their packaging application, as films and coating formulations, for shelf life extension of fruits and vegetables.

Various derivatives such as carboxymethyl-(CM), hydroxypropyl-(HP), and crosslinked CMC and CMS were prepared as per the reported procedures. As a prelude to understand this, potato starch and cellulose were taken for study. Crude starch was isolated from potato by the water steeping method in good yields (>80%). The purification was done by

successive treatments with dilute alkali and NaCl - toluene, and the purified starch was subjected to derivatization.

WVTR values of crosslinked CMC + HPC and cross-linked CMC + CMS films were 1100 and 1137 g $day^{-1}m^{-2}$ respectively at 25°C and 65% RH (Table 5). These values were comparable with those reported for low methoxyl pectin films (1400-4000 $gday^{-1}m\text{-}^{2}$ at 25°C and at differential RH of 31-81%) (110), but in variance with those of chitosan and crosslinked chitosan films, (111). LDPE and HDPE films showed much lower (0.079 and 0.02) WVTR values at 38°C and 0 - 90% RH (112) (Table 5). The hydrophilic nature of the polysaccharide and their chain length could be the reason for their very high WVTR.

Similarly, the tensile strength and percentage elongation of starch and cellulose based films were considerably different from those of LDPE, HDPE and chitosan films. The mean tensile strength value of the crosslinked CMC+HPC film was comparable to methyl cellulose film which showed a value in the range of 43.7- 70.2 MPa (113), but crosslinked CMC+CMS films had a much lower value, probably due to the low amylose chain length. The chitosan and crosslinked chitosan showed a high tensile strength of 70.3 and 93.8 MPa, as measured in machine direction (111).

Table 5. Physico-chemical characteristics of films

Films	Water vapour transmission rate $g\ day^{-1}m^{-2}$	Tensile strength MPa	Percentage elongation
Crosslinked CMC+HPC	1100	62.12	10
Crosslinked CMC+HPS	1137	14.61	15
Crosslinked Chitosan	290.1 (0-93%RH)	93.8	5.1
Chitosan	102.1 (0- 93%RH)	70.3	6.2
LDPE	0.079 90% RH	80-240	500
HDPE	0.023 90%RH	220-350	300

The mean percentage elongation of crosslinked CMC + HPC and crosslinked CMC+CMS were 10 and 15, much lower to LDPE and HDPE films but slightly higher than chitosan and crosslinked chitosan films. This is explainable on the basis of higher water holding capacity of starch films.

At 25°C and 65% RH, the starch and cellulose based films showed a very high permeability to oxygen (13,130 $cm^3.\mu m/m^2.d.KPa$) (114).

3.1.1 Applications

Crosslinked CMC+HPC films were ineffective in shelf life extension of climacteric fruits such as mangoes, as they absorbed moisture and lost the tensile strength. A limiting feature of these films is the poor barrier property at relative humidities above 0.85 (115). The ripening of capsicum was delayed to some extent by these films when compared to control (116) (117) but were not as superior as chitosan-based films in controlling the shriveling and carotenoids development.

3.2 POLYSACCHARIDE-BASED COATING FORMULATIONS

Water-soluble polysaccharide-based coatings are useful in extending the shelf life of fruits and vegetables as they modify the internal atmosphere, thereby reducing the respiration rate and physiological loss in weight. Hydrocolloid-based packaging films show selective permeability to O2 and $C0_2$. Both native and modified polysaccharides give in aqueous media a viscosity-building effect, which is of value for specific food applications. An edible coating mixture composed of sucrose fatty acid ester and sodium carboxymethyl cellulose (TAL-Prolong) produced a semipermeable modified atmosphere around fresh fruits after application (118). In another report, TAL-Prolong coating delayed ripening of bananas, but the colour and texture of the stored fruits were inferior compared to control (119). Nevertheless, the maturity of the fruit, variety of cultivar and permeability properties of the coating are all crucial factors to be taken into account in such applications. Two composite coating formulations based on CMC+HPC (S_1) and CMC + HPS (S_2) in maintaining the quality and

freshness of banana (Fig. 8) and mango were investigated and the results compared with Waxol formulation.

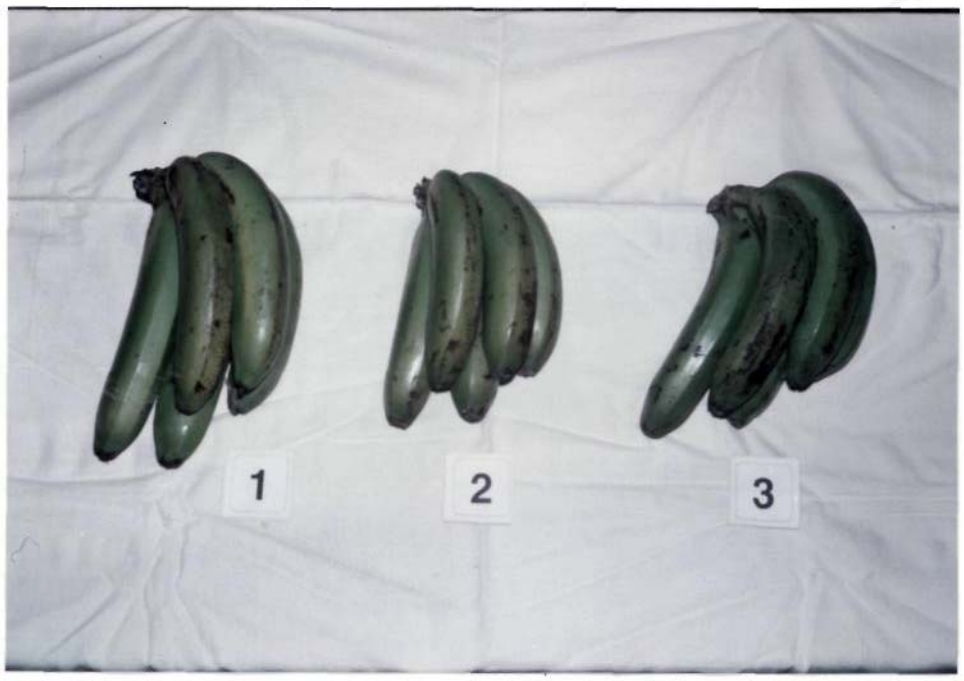


Fig. 8. Banana hands, uncoated control (1), Si coated (2) and S_2 coated (3)

3.2.1 Physiological loss in weight

Fig. 9 depicts weight loss of coated banana stored at 27 ± 2°C upto 12 days. Compared to control and Waxol coated fruits (positive control), the S_1 and S_2 coated fruits exliibited least reduction, <5% PLW. Waxol-coated fruits showed less weight loss than S_1 and S_2. Addition of a lipid component such as glycerol monostearate or palmetic acid or sucrose fatty acid esters significantly enhanced the effectiveness of these coatings, indicating that the presence of lipid component was essential to regulate the hydrophilic-

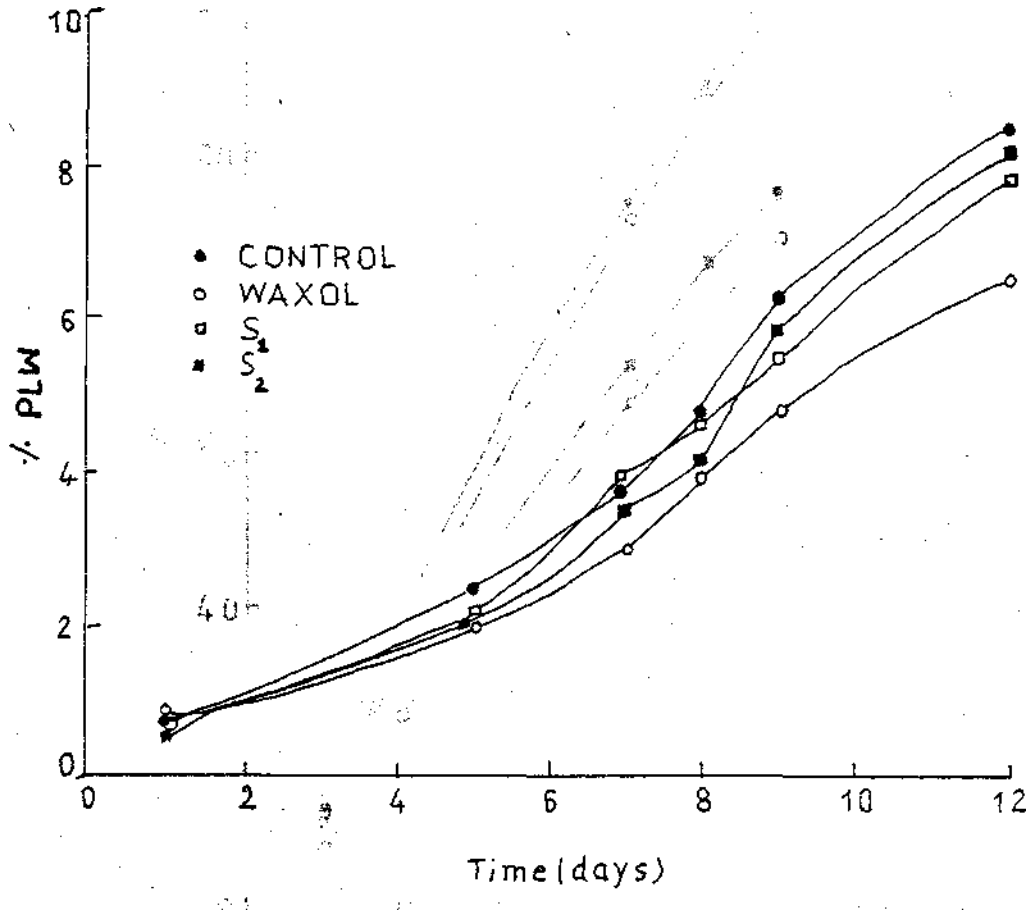


Fig. 9. Cumulative %PLW in coated banana stored at 27 ± 2°C **and 65-70% RH**

hydrophobic balance which could inturn restrict the water loss. Freshly harvested mature mangoes treated with the modified formulation, though showed significant reduction in PLW (Fig. 10) but in subsequent storage period, off flavour developed due to anaerobiosis, suggesting that these coatings have low air permeability and WVTR (120).

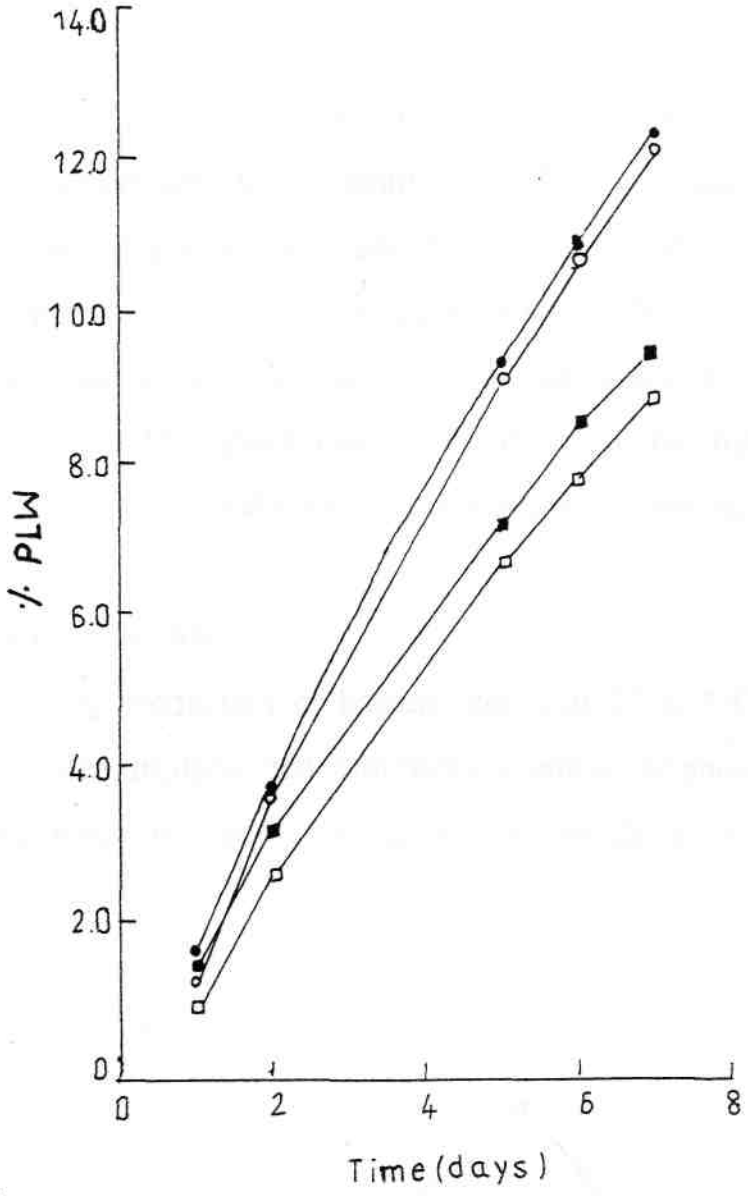


Fig. 10. Cumulative %PLW in coated mango fruits at 27± **2° C, 65% RH**

Formulations with a lesser total solid content of 2-2.5%, however were more effective in reducing the weight loss without causing any adverse effects on the quality of mango. Depending upon the type of fruit to be stored, the solid content of the coating formulations vary considerably. S_1 showed least weight loss, attributed to differences in the permeability

characteristics (121), which in turn could be due to qualitative and quantitative differences in the chemical nature of polysaccharide and lipid component and their relative concentrations. Overall, the use of above coating improved the sensory characteristics of banana and mango by reducing the water loss and maintaining dark green colour, with glassy shining and moist like appearance. When viewed under light microscope, a 'uniform coating with out cracks and pin holes was observed.

3.2.2 Respiration rate

The CO_2 production of banana stored at 27 ± 2°C is presented in Fig. 11. As seen, fruits treated with these formulations showed characteristic climacteric peak on day 8 for S_2, S_1 however did not show such a trend.

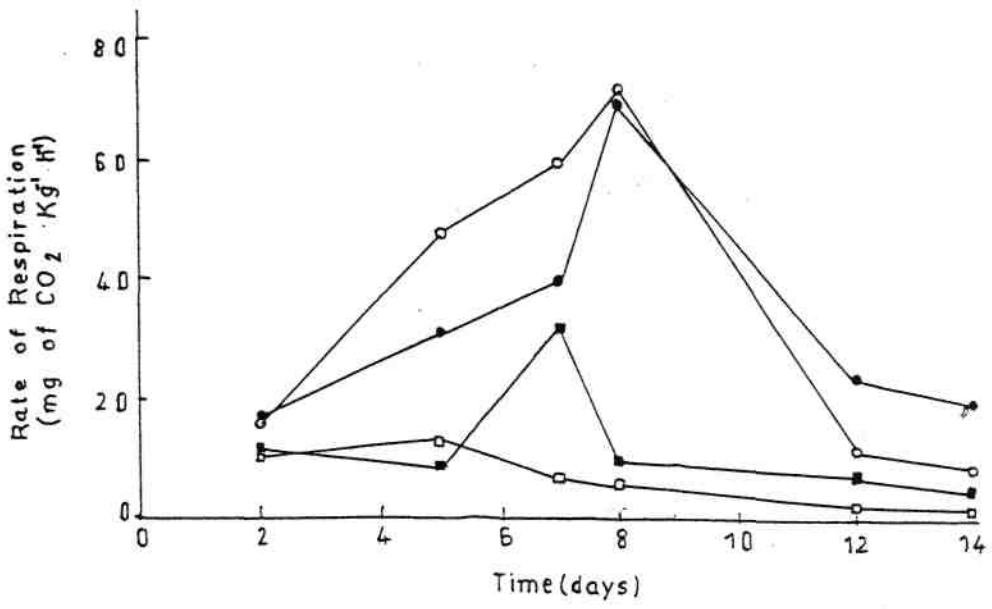


Fig.11. Rate of respiration of banana

Although the respiration pattern for S_2 treated fruits were similar to control, their CO_2 production rates were significantly lower than that of Waxol and control fruits at any stage of storage. In the case of control, $C0_2$ production rate increased rapidly from an initial value of 15 mg of $C0_2$ $kg^{-1}h^{-1}$ to a peak value of 75 mg of CO_2 kg^{-1} h^{-1} whereas S_2 showed a decreased CO_2 production rate of 35, 15, 10 and 8 mg of $C0_2$ $kg^{-1}h^{-1}$ on day 7, 8, 12 and 14, respectively. A similar effect was observed in the case of coated mango fruits (122), the control fruit showed a CO2 peak value of 102 mg on day 7, whereas S_1 and S_2 gave 80 and 86 mg of $C0_2$, respectively (Fig. 12). On the other hand, the Waxol coating had no influence on the rate of respiration

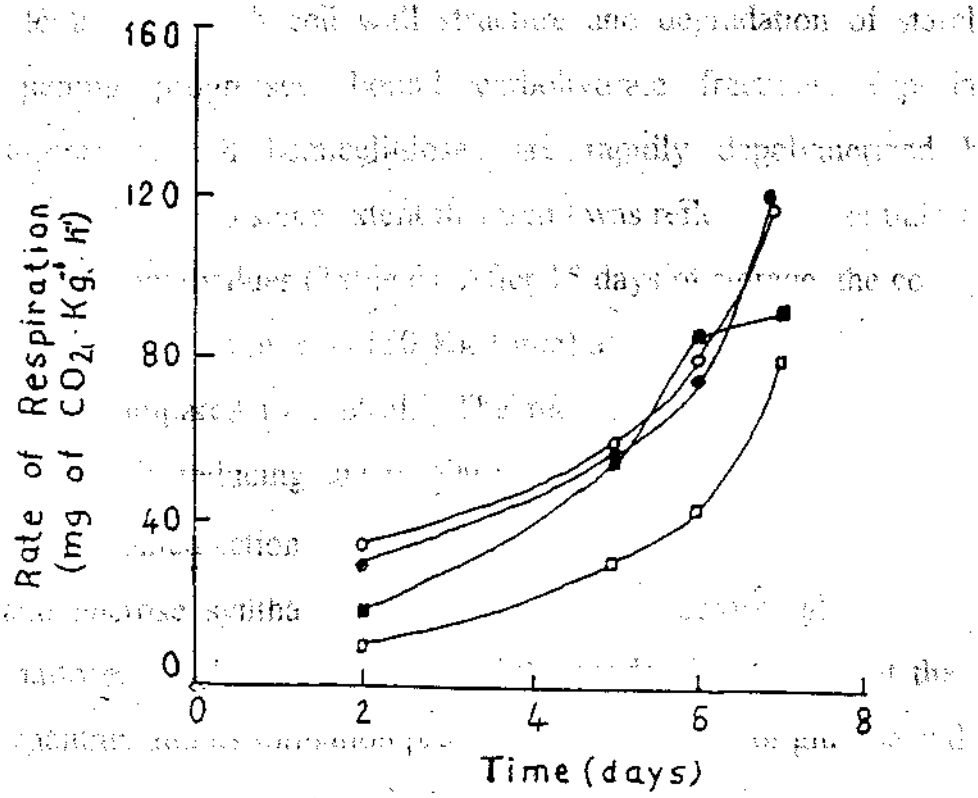


Fig.12. Rate of respiration of mango

of both banana and mango fruits. This suggested a dual effect of polysaccharide-based coatings having less air permeability in restricting CO_2 diffusion and causing the secondary physiological changes in the ripening process.

3.2.3 Quality attributes

The above coatings had beneficial effect on firmness, titratable acidity and reducing sugar content of stored banana (Table 6). Banana fruits treated with S2 were firmer, lesser in reducing sugar content and higher in titratable acidity than control and Waxol coated fruits, after 15 days of storage. The textural quality changes of coated banana during ripening results from alteration in both cell wall structure and degradation of starch. As the ripening progresses, bound carbohydrate fractions, especially pectic substances and hemicelluloses are rapidly depolymerized by various hydrolases. To some extent this trend was reflected in the pulp to peel ratio and firmness values (Table 6). After 15 days of storage, the coated fruits had higher firmness level (>150 Kg force) and lower pulp to peel ratios (<2.0), when compared to control. The reduced starch content was reflected in increase in reducing sugars. During ripening, starch is degraded rapidly by the combined action of amylases, starch phosphorylases, α-1,6- glucosidases and sucrose synthases to sugars such as sucrose, glucose, fructose, and maltose. In the pulp, sucrose is the predominant sugar at the start of the ripening, and its formation precedes accumulation of glucose and fructose.

Table 6. Effect of polysaccharide-based composite coatings on quality attributes of stored banana at 27+ 2°C, 65% RH

Coating	Pulp to peel Ratio	TSS Degree brix	Texture Kg force	Titratable acidity % malic aicd	Reducing sugar mg g^{-1} of pulp
Control	2.2	25.0	111.8	0.22	32.5
Waxol	1.7	25.0	150.0	0.18	25.5
S_1	1.6	7.5	138.9^{ab}	0.15^{a}	21.5^{ab}
S_2	1.5	4.5	157.7^{a}	0.15^{a}	22.0^{a}

[a, b] Mean within the same column are significantly different ($P<0.05$) between control and experimental.

The reducing sugar content and TSS of polysaccharide-based coated fruits were lower than control. This suggested that the coated fruits synthesized reducing sugars at a slower rate than the control (21.5-22.0 mg g"[1] as against 32.5 mg g"[1] for control). A similar trend was also observed in the case of mango. Retention of firmness, lower pulp to peel ratio and titratable acidity, and a slower rate of reducing sugar released demonstrate that these coatings slowed down metabolism and prolonged the storage life.

Coating fruits with semi-permeable film has generally been shown to retard ripening by modifying the levels of endogeneous $C0_2$ and 0_2 and ethylene. Further more, these fruits developed the normal yellow colour, and the coating neither affected appearance nor caused phytotoxity after 21

days of storage for banana and 8 days for mango. Nevertheless, etheral treatment after desired storage period was found to be advantageous for uniform colour development of stored fruits. In the case of banana, however, control fruits after 21 days of storage turned black with a collapsed structure due to over ripening and fungal infection . Control mangoes on the other hand, showed advanced ripening with very soft texture when compared to coated fruits. Application of an additional 1% chitosan coating to the fascicle region during storage however, significantly reduced the incidence of mold growth (123). The control of mold growth could be attributed to either fungistatic property of chitosan *per se* or its ability to induce defense enzymes (i.e., chitinase and β-l,3-glucanase) and phytoalexins in plants or a combination of both.

3.2.4 Sensory evaluation

Sensory evaluation of banana fruits coated with S_1 (Fig. 13) and S_2 (Fig. 14) revealed significant differences in colour, texture, flavour and taste compared to Waxol coated and control fruits ($p<0.05$). The former showed maximum freshness, surface colour, good texture and nice taste, and were best even after 21 days of storage, while those coated with Waxol had ripened to acceptable quality, but its texture was slightly inferior. Uncoated control fruits on the other hand blackened due to overripening and fungal infection (Table 7). Mangoes treated with these polysaccharide-based coatings also showed similar characteristics.

Fig. 13. Banana hands of uncoated control (1) and Si coated *($)* fruits after 12 days of storage at ~ 27°C, 65%RH

Fig. 14. Banana hands of uncoated control (1) and S_2 coated (4) after 12 days of storage at ~ 27°C, 65%RH.

Table 7. Effect of post harvest treatment on the sensory qualities (scored 1-10) of Robusta banana after 21 days of storage at- 27°C, 65% RH

Coating	Colour & appearance	Texture	Flavour	Taste	Overall quality
Control	2	5	3	3	3
Waxol	6	6	6	6	6
Si	3[a]	6[a]	6[a]	6[a]	6[a]
S_2	6[a]	7[a]	6[a]	6[a]	6[a]

[a] Mean within the same column are significantly different ($p < 0.05$) between control and experimental.

3.3 GRAFT COPOLYMERISATION

Starch is amenable for graft copolymerisation to incorporate desirable mechanical properties without sacrificing its biodegradable nature. Chemical modification of starch via grafting is one of the most effective methods for preparing starch graft polymer composites. In graft copolymerisation the natural and synthetic components are held together by a covalent bonding rather than existing merely as a physical mixture.

Due to its unique properties, eerie ammonium nitrate has been chosen as an initiator. Its preferential use as an initiator over other free radical initiators in grafting onto different polyols, is advantageous for the simple reason that it generates single electron transfer, forms free radicals on the back bone polymer, produces pure graft-copolymer and the reaction can be carried out at ambient temperature in aqueous medium (124). Literature

survey reveals that grafting of acrylamide and acrylonitrile onto starch has been extensively investigated (125,126) . So far nothing is known on the biodegradation, extrusion and the mechanical properties of such grafted-extruded films.

Potato starch-graft-polyacrylonitrile (PS-g-PAN) and cassava starch-graft-polyacrylonitrile (CS-g-PAN) samples obtained were pale brown coloured and hard. Though DMSO is a good solvent for pure starch and acrylonitrile, the grafted starches however, could only swell but did not dissolve even after a considerable time and heating. The graft-copolymers were also insoluble in N,N[1]-dimethyl formamide which is a good solvent for polyacrylonitrile. The insolubility of these copolymers may be the result of excessive crosslinking and chain association (127).

The graft copolymerisation was evidenced by an increase in the weight of the starch-graft after thorough extraction and washing (Table 8). Around 70-85% grafting was achieved for starch-graft-copolymer of AN. The grafting of AN onto holocellulose was reported to be 60-70% (128)

Table 8. Percentage of grafting of acrylonitrile onto starches

Starch	Initial weight $W_{Jíg}$	Final weight W_2,g	Grafting % W_2-Wι / $W_{2 \times 100}$
Potato	10	18.5	85
Cassava	10	17.8	78

and this was a half fold increase in comparison with the maximum grafting percentage found in grafted bamboo. These differences have been attributed to difference in the chemical composition *per se* of the raw materials used. Nevertheless graft copolymerisation of bamboo with AN was retarded considerably because of the presence of lignin. In another report, grafting of AN onto cellulose was shown to be more efficient than onto chitin (129). This was ascribed to hydrophilic groups playing an important role, most likely via the formation of complexes between tri-n-butylborane (initiator) and chitin during grafting. The starch-grafted samples did not fully gelatinise when heated in water, which is consistent with their crosslinking (127).

3.3.1 Infrared spectroscopy

Infrared spectrum is characteristic of an organic compound. This technique provides a spectrum containing a large number of absorption bands from which a lot of information can be derived about the structure of the compound. The absorption of IR radiations causes electronic excitations in a molecule, leading to stretching and bending with respect to one another. Graft-copolymerisation of a synthetic monomer onto any natural polymer can therefore be ascertained by the presence of characteristic IR absorptions. Appearance of absorption band (Fig. 15c) at 2260 cm^{-1} due to nitrile groups and $-CH_2$ deformation vibration at around 1460 cm^{-1} confirmed grafting of AN onto starch. Broad absorption bands at around 3400-3500 cm^{-1} were due to hydroxyl group of native starch (see Fig. 15a). It was found that due to homopolymerisation, the intensity of absorption band of-ON at 2260 cm^{-1}

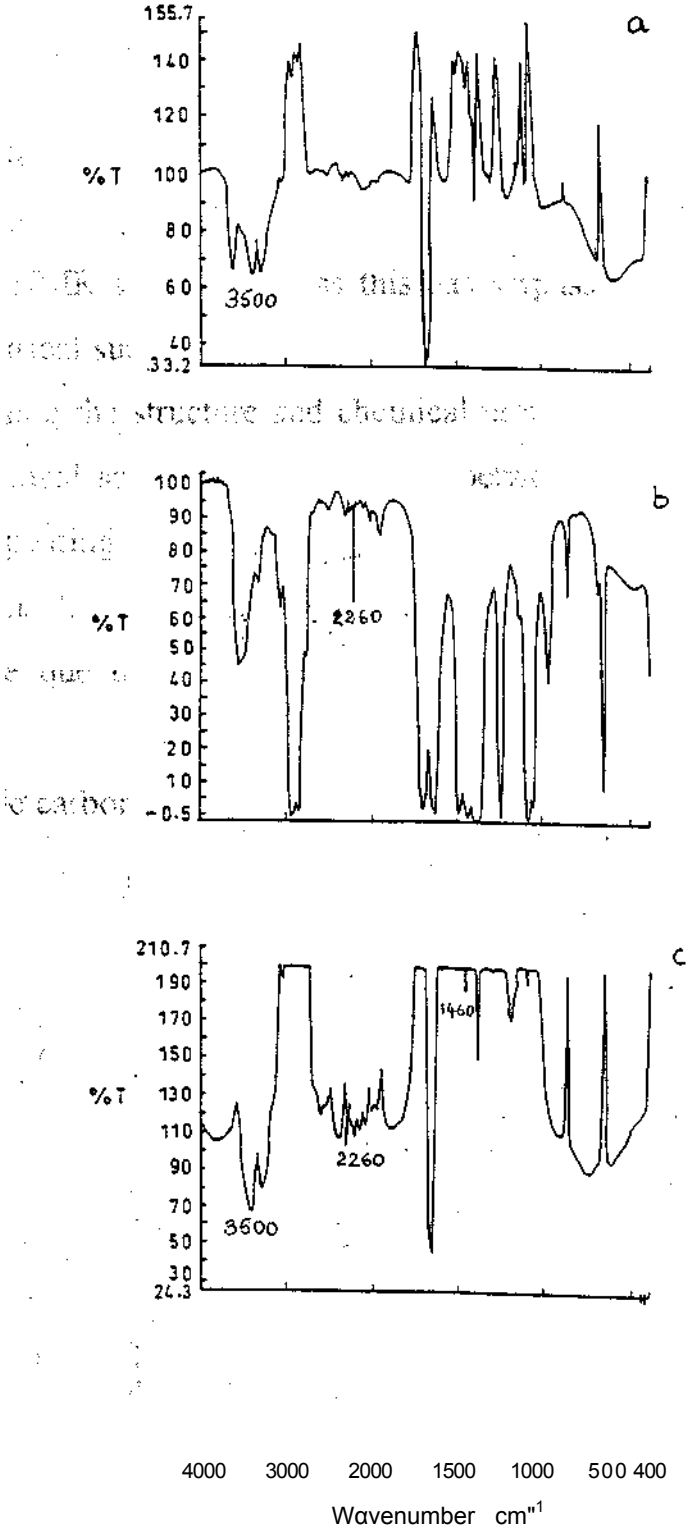


Fig.15. IR spectra of potato starch (a), acrylonitrile (b) and S-g-PAN (c)

was reduced when compared to that of free monomer (Fig. 15b). The spectral results were comparable with those obtained for grafted AN onto bamboo (130,131).

3.3.2 Nuclear magnetic resonance spectroscopy

Further confirmation of S-g-PAN was obtained by carrying out high resolution NMR spectroscopy as this has surpassed all other techniques in the geometrical survey of organic molecules which is of great importance in understanding the structure and chemical properties. In ^{1}H-NMR spectroscopy, chemical shift of protons appear between 0-10 ppm and number of signals appearing in the spectrum reveal the different types of equivalent hydrogen in the molecule, while in C-NMR spectroscopy, the resonance signals are due to C nuclei and are much more specific. The latter copolymers showed the presence of a nitrile (-ON) group at 119.8 ppm and an aliphatic carbon atom of AN at 27.9 ppm (Fig. 16b).

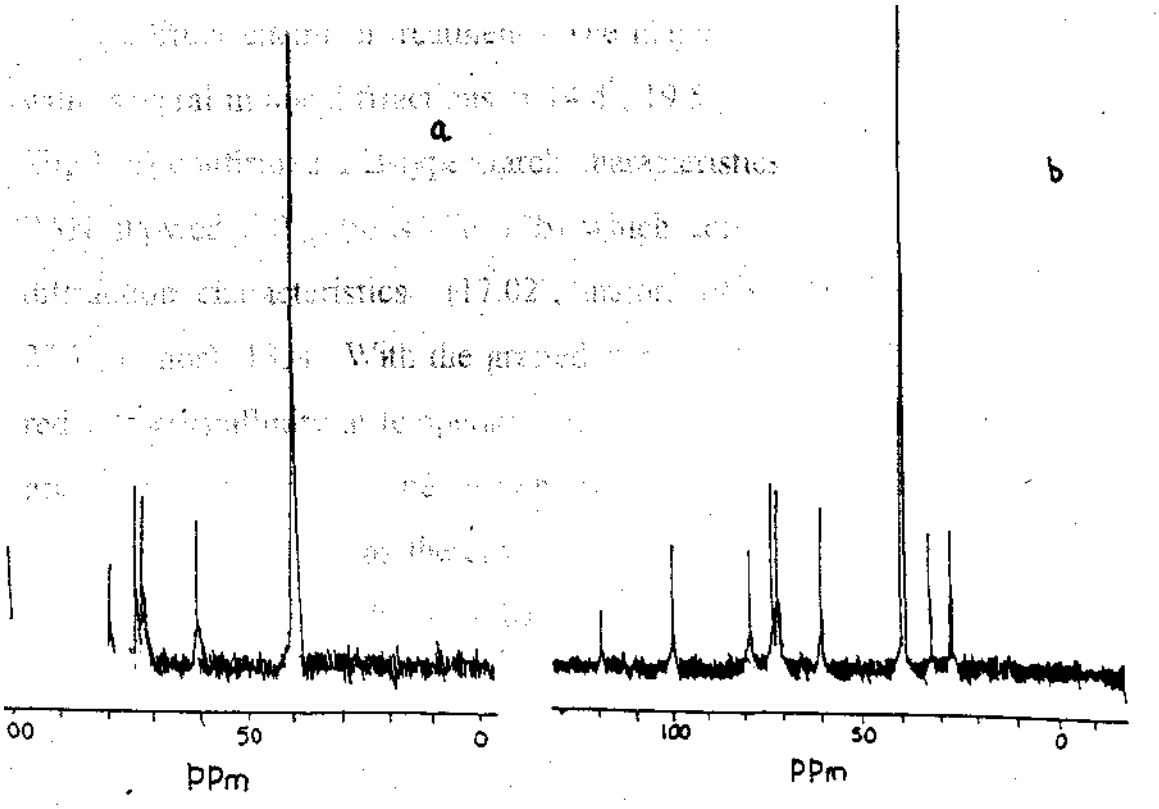


Fig. 16. ^{13}C- NMR spectra of starch (a) and S-g-PAN (b)

The presence of six resonance signals at 100.08, 78.90, 73.27, 72.09, 71.66 and 60.63 ppm, corresponded to C-1, C-4, C-2, C-3, C-5 and C-6 nuclei of starch glucose residues (Fig. 16a). The signals were comparable to published data (132).

3.3.3 X-ray diffraction studies

Crystalline and non-crystalline (amorphous) structures and their relationship are the major factors in determining starch properties. Structural changes that occur in starch during graft-copolymerisation have been studied by X-ray diffraction. Native potato starch granules exhibit a B-type diffraction pattern which is either partially or completely destroyed during further chemical treatment. The major 2θ diffraction at 17.2° along with several minor diffractions at 14.4°, 19.5°, 22.2°, 24.0° 26.3° and 34.4° (Fig. 17a) confirmed a B-type starch characteristics. On the other hand S-g-PAN showed diffractions (Fig. 17b) which corroborated well with V-type diffraction characteristics (17.02°, major; 19.5°, 21.7°, 22.6°, 23.6° and 27.1°, minor) (133). With the grafted starch, the diffraction pattern showed reduced crystallinity at temperature as low as 70°C. During the process of graft-copolymerisation the starch was gelatinised at ~85°C and the crystalline structure was therefore completely destroyed, hence the X-ray diffraction pattern was found to be typical of an amorphous state (134).

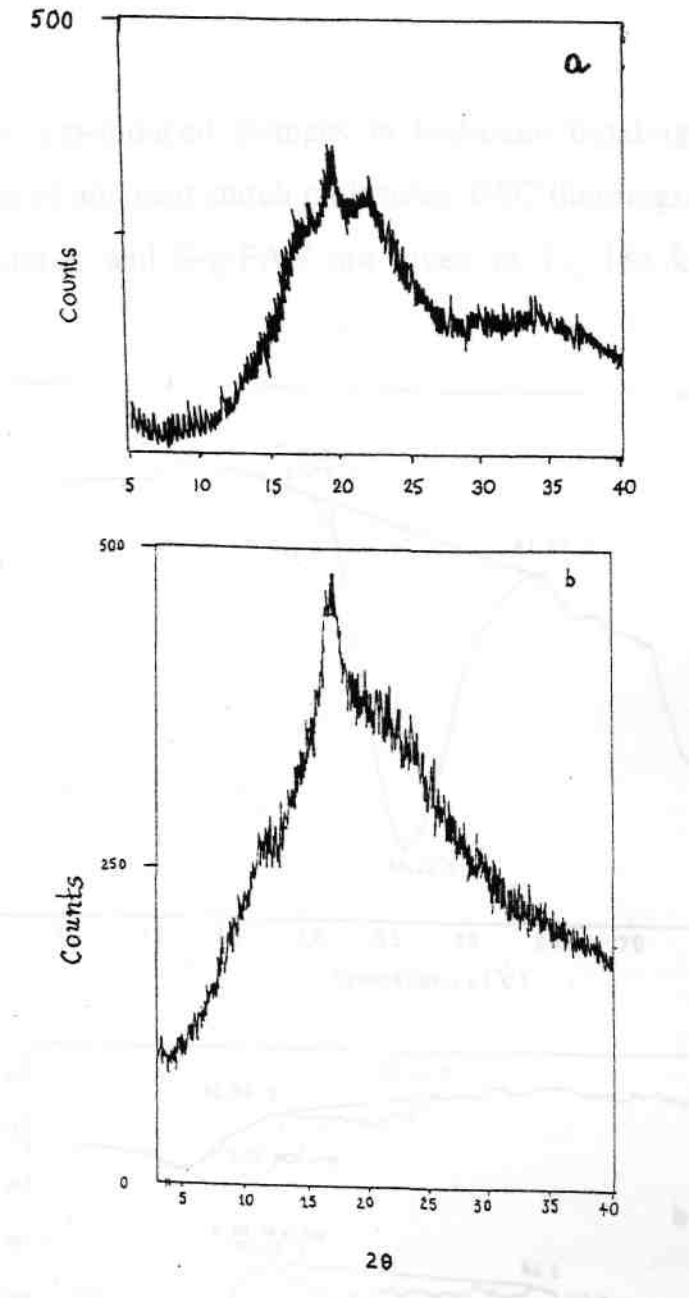


Fig. 17. X-ray diffractograms of native potato starch (a) and S-g-PAN (b)

3.3.4 Differential scanning calorimetry

Structural changes that occur in starch granules during graft-copolymerisation has been studied using differential scanning calorimetry. DSC helps in identifying certain well recognised crystallites in starch and

the consequent heat-induced changes in hydrogen bonding between the hydroxyl groups of adjacent starch molecules. DSC thermograms of defatted native potato starch and S-g-PAN are given in (Fig. 18a & b). A single

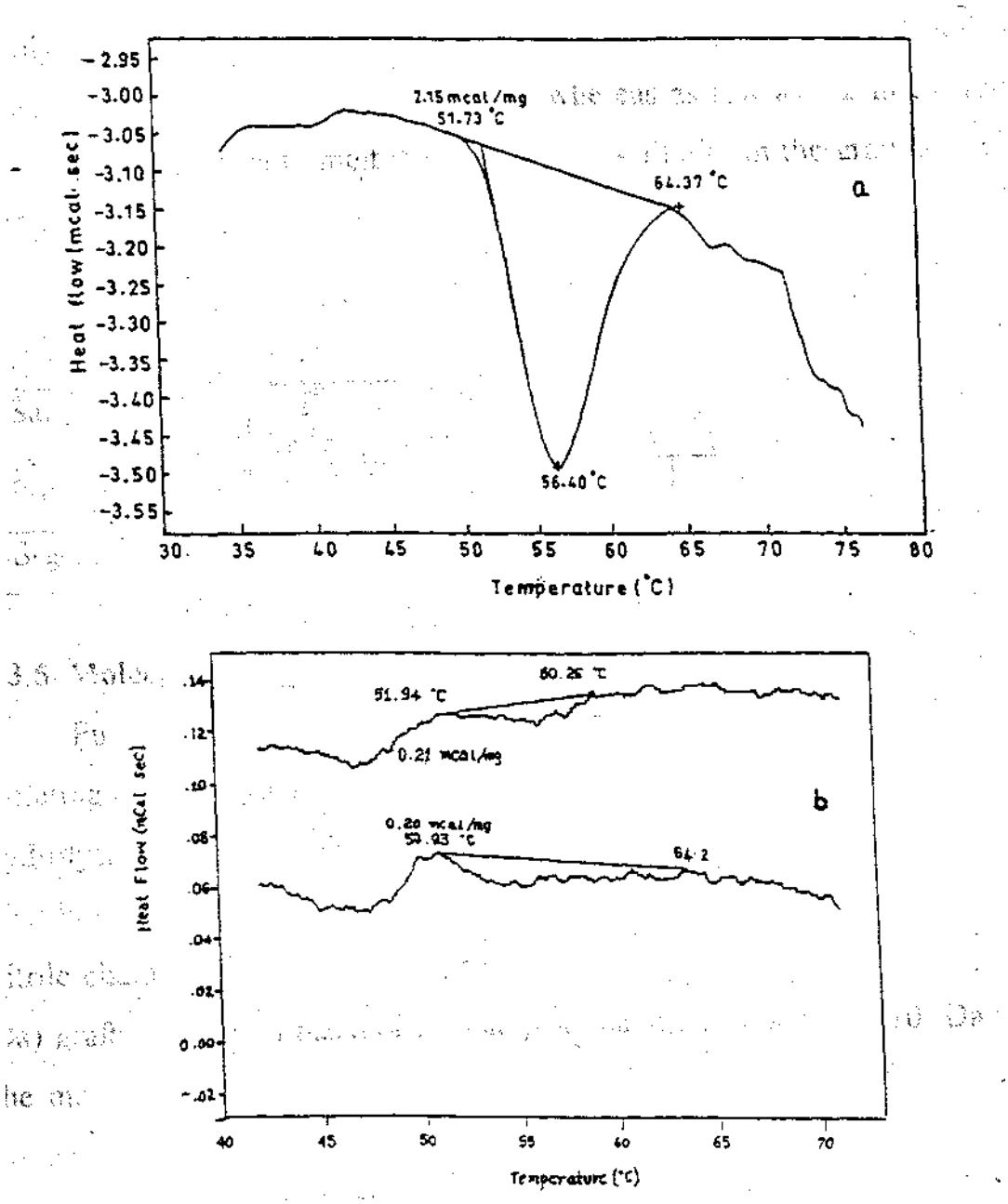


Fig. 18. DSC thermograms of defatted native potato starch (a) and S-g-PAN (b)

endotherm at around 55-58 C corresponding to starch gelatinisation temperature was observed at 50% moisture. The thermogram of S-g-PAN showed neither gelatinisation nor melting peaks, indicating that the original crystalline structure of the starch component has been lost during graft-copolymerisation. As seen in Table 9, an enthalpy of 2.15 m cal mg^{-1} is required to melt the crystallite of starch, whereas as low as 0.2 m cal mg^{-1} was sufficient enough to melt the left over crystallinity in the grafted starch (135).

Table 9. DSC characteristics of potato starch and S-g-PAN

Sample	T^0 onset	T^0 Peak	T^0 conclusion	ΔT (Tc-To)	ΔH m cal mg^{-1}
Potato starch	51.7	56.4	64.4	12.7	2.15
S-g-PAN	51.9	60.3	62.2	10.3	0.20

3.3.5 Molecular weight distribution

Further characterisation of graft-copolymers was carried out by isolating the polyacrylonitrile branches from S-g-PAN through acid hydrolysis. Gel permeation chromatography was used to determine the MW distribution of the hydrolysed products. Fig. 19 shows a range of polyacrylonitrile chains of varying MW (6.3×10^7, 1.5×10^6, 2.5×10^3 and 5.01×10^2 Da) grafted to starch backbone. The peak having a MW 2.5×10^3 Da was the major fraction, it had a retention time of 28.67 mm. The results were comparable to those seen in grafted chain of polyacrylonitrile onto bamboo (128). A major fraction of MW 5.4×10^3 and a minor fraction of 1.4×10^5 Da

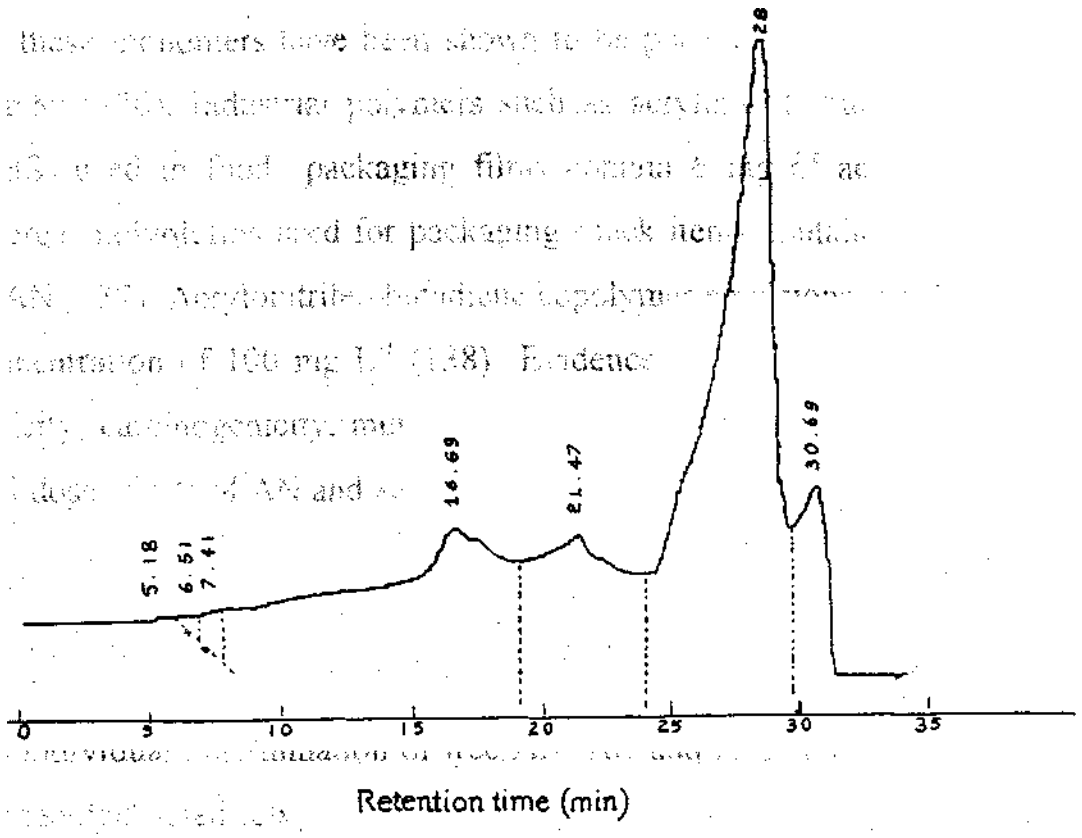


Fig. 19. Molecular weight distribution of polyacrylonitrile chains of S-g-PAN

was observed in the GPC of grafted bamboo. The heterogeneity in molecular weight distribution and the broadness in peak areas indicated the complexity of the grafting reaction, and also revealed the possible existence of multiple grafting sites on starch backbone (128).

3.4 DETERMINATION OF RESIDUAL MONOMERS

During the process of graft-copolymerisation of starch with AN at low pH, partial degradation and hydrolysis of AN occurs resulting in the formation of acrylamide (AM) and acrylic acid (AC). There could also be traces of unutilized AN left over in the process of graft-copolymerisation.

All these monomers have been shown to be potent cumulative neurotoxins to man (136). Industrial polymers such as acrylonitrile-butadiene- styrene (ABS) used in food packaging films contain 6 mg of acrylonitrile kg^{-1}, whereas polyolefins used for packaging snack items contain 0.36-0.72 ppm of AN (137). Acrylonitrile -butadiene copolymer emulsions contain AN at a concentration of 100 mg L^{-1} (138) Evidence has been presented for their toxicity, carcinogenicity, mutagenicity and teratogenicity (139). The LD_{50} oral dose in rats of AN and AC is 93 mg and 2.5 g kg^{-1}, respectively (140).

A wide range of analytical methods viz., spectrophotometric (141), polarographic (142), titrimetric (143) and chromatographic are available for the individual determination of free AN, AC and AM. The vast majority of the methods used rely on gas chromatography (144). A rapid reverse-phase HPLC determination of AN in aqueous samples to detection limits of 5 ppb is reported (145). Determination of AM involves derivatisation to 2,3-dibromopropionamide and extraction of the latter into ethyl acetate followed byGC(146). Determination of AC by GC suffers from interference and a HPLC method and quantitation by UV detection is reported (147). A GC method to determine only the AN content of styrene-acrylonitrile copolymers has also been reported (148).

Earlier GC methods required prewashing and equilibration steps lasting for over 20 min between successive sample injections. The prior derivatisation step suffered from the disadvantages of either partial derivatisation (34-66%) or interference with other compounds and losses due

to evaporation-decomposition. Also the subsequent clean-up steps were found to be laborious. To date, no single method offers the simultaneous determination of AN, AM and AC in a mixture. Hence, attempts were made to develop a rapid and sensitive HPLC method for the simultaneous determination and quantitation of the residual monomers in S-g-PAN. In this method, sample dilution was necessary without which the solvent (acetonitrile) was found to pull AN off the column, thus precluding quantitation. The developed reverse-phase HPLC method involved isocratic separation using 0.05 M KH_2P0_4, pH 5.5. It is evident from Fig.20 that all

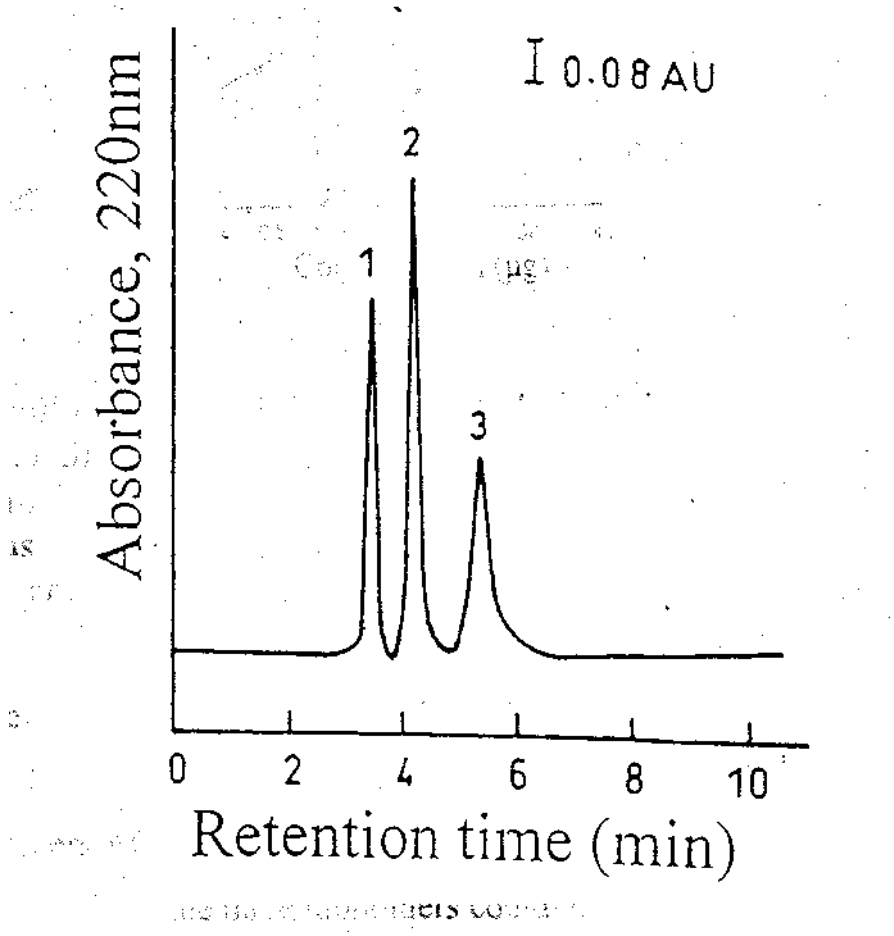


Fig.20. RpHPLC profile of standard mixture of AC (1), AM (2) and AN (3) on Maxil Cis **column**

the three monomers were well resolved from one another in just 5 min with a significant resolution factor (R = 2.3 for AC and AN). The linearity of the peak response to varying concentration of the monomers, examined individually (Fig.21) showed that the limits of detection were at microgram level. AM was detected at very low concentration (0.1-0.5 µg) compared to AN (10-40 µg) and AC (0.5-3 µg).

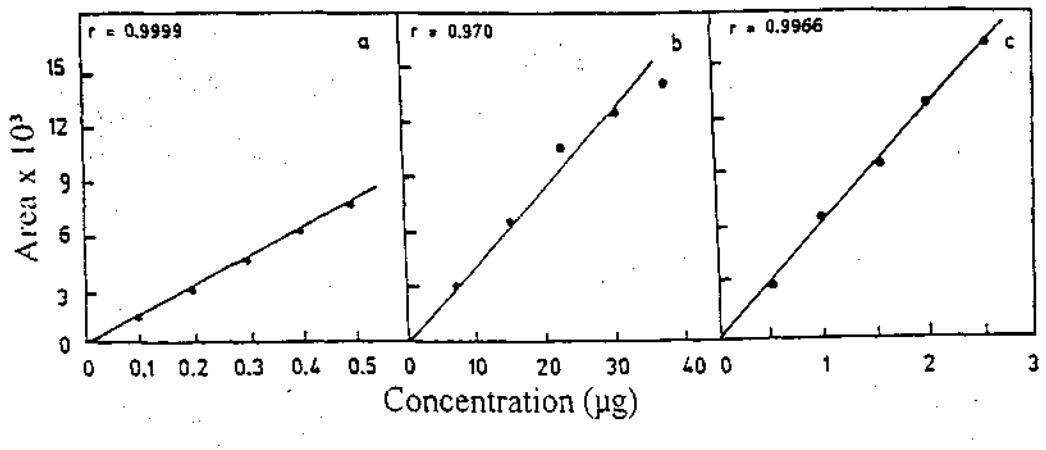


Fig.21. Calibration curves for HPLC analysis of AM (a) AN (b) and AC (c), linear response with good precision obtained for 0.1-0.5 µg, 10-50 µg, and 0.5-3 µg, respectively. Straight lines obtained using linear regression analysis and coefficients of correlation were r=0.9999, r=0.972 and r=0.9966 for a,b and c, respectively

The AC content of S-g-PAN prepared in the laboratory was determined by this method (Fig.22). Using the GC method (Chopra), only two monomers AC and AN could be resolved (Fig.23) whereas in the HPLC method (Fig.20) all the three monomers could be resolved. The AC content

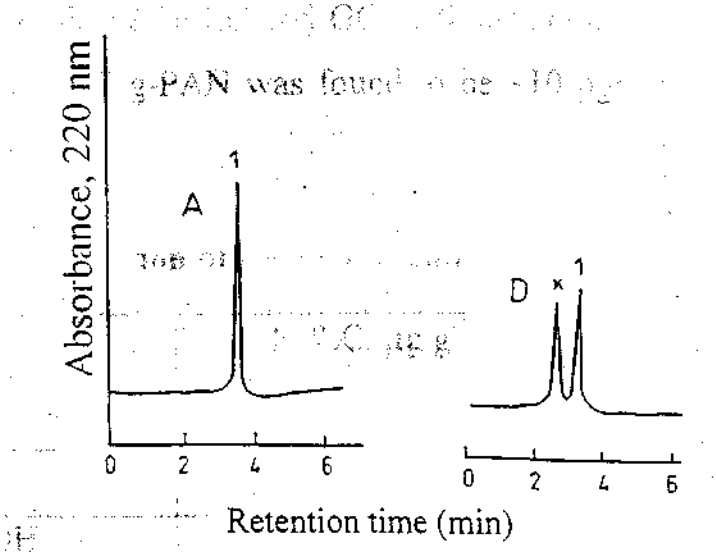


Fig.22. Reverse phase HPLC profile showing AC (1) in S-g-PAN (A) and EtOH washing of S-g-PAN (D).

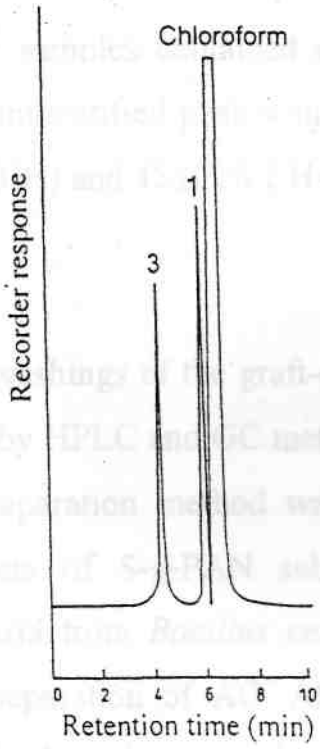


Fig.23. Gas chromatogram of AC (1) and AN (3)

determined by both the HPLC and GC methods are compared in Table 10. The AC content of S-g-PAN was found to be -10 ppb in close agreement with the GC value.

Table 10. Determination of residual monomers in starch-graft polymers

Sample	HPLC, μg g^{-1}			GC, μg g^{-1}		
	AC	AN	AM	AC	AN	AM
S-g-PAN	10.5	ND	ND	11.0	ND	ND
S-g-PAN, EtOH washing	21.5	ND	ND	22.5	ND	ND

ND-Not detected

The grafted polymer samples contained no measurable amounts of AM and AN. GC-MS of unidentified peak x in Fig.22D showed fragment ion peaks at m/z 31 ($CH_2=0^+H$) and 45 (CH_3 $CH=0^+H$) indicating it to be an alcohol-type of compound.

In addition, ethanol washings of the graft-copolymer were also tested for monomer content both by HPLC and GC methods (Table 10) and results were comparable. This separation method was very helpful to identify simultaneously the products of S-g-PAN subjected to degradation by acrylonitrile utilising bacteria from *Bacillus cereus*. The results (Fig.24) indicated a well defined separation of AC, AM and AN, AC being the predominant monomer in the degradation products.

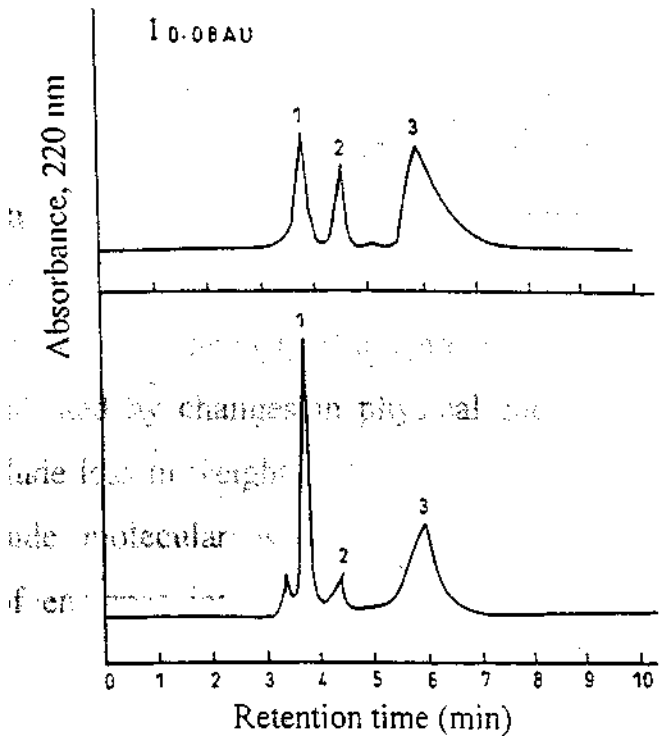


Fig.24. RpHPLC profile of degradation products AC (1), AM (2) and AN (3) in culture broth of *Bacillus cereus* grown on S-g-PAN

The water extracts of industrial copolymers SAN, ABS and ABS (recycled) were tested for their residual monomer content by this method. The results (Table 11) indicated the presence of only AC, as the degradation product of AN.

Table 11. Determination of residual monomers in industrial polymers

Sample	HPLC, µg g •'		
Styrene acrylonitrile (SA)	27.10	ND	ND
Acrylonitrile-butadiene-styrene (AB S)	6.70	ND	ND
Acrylonitrile-butadiene-styrene (ABS) recycled	1.96	ND	ND

3.5 BIODEGRADATION OF S-g-PAN

Pure cultures of *Phanerochaete* and *Streptomyces* sps. have been shown to attack both lignocellulosic wastes and starch-containing plastics such as starch-g-polyethylene (10). Biodegradability of grafted starch films is generally evaluated by changes in physical and biochemical properties. The former include loss in weight and tensile strength whereas biochemical properties include molecular weight distribution, degradation products, identification of enzymes involved and elucidation of the mechanism of enzymatic reaction.

The degradation of nitriles which are the monomers used in starch grafting is quite common among microorganisms. In nature, three different groups of enzymes are involved in the microbial hydrolysis of nitriles. Nitrilases hydrolyze nitriles to the corresponding carboxylic acids, forming ammonia, and nitrile hydratases form amides from nitriles which can be subsequently hydrolysed by amidases. The actinomycete *R.rhodochrous* has been reported to produce both nitrilase and nitrile hydratase/amidase system depending on the specific inducers used (149). There is a single report on the enzymatic hydrolysis of polyacrylonitrile fibres by nitrile-degrading enzymes of *R .rhodochrous* NCIMB 11216 (150).

3.6 Screening and identification of bacteria

Numerous microorganisms that utilize AN have been reported. *Arthrobacter sp.* was reported to use AN as the source of carbon and nitrogen (151). Strains of *Rhodococcus sp.* (152), *Brevibacterium sp.* (153)

and *Klebsilla pneumonia* (154) are also known to degrade AN. Biodegradation of cyanide by *Bacillus cereus var. Myloides* is reported (155).

To identify the best possible strain in terms of high nitrile hydratase and peroxidase activities, of the 10 isolates from soil screened, only one culture showed 80% nitrile hydrolysis. Even in the absence of glucose, this culture showed about 62% hydrolysis when other cultures showed only 20-30% hydrolysis. The soil isolate was gram positive, endospore-producing rod and varying in size (1.0 to 1.2 μm by 3.0 to 5.0 μm) (Fig. 25).

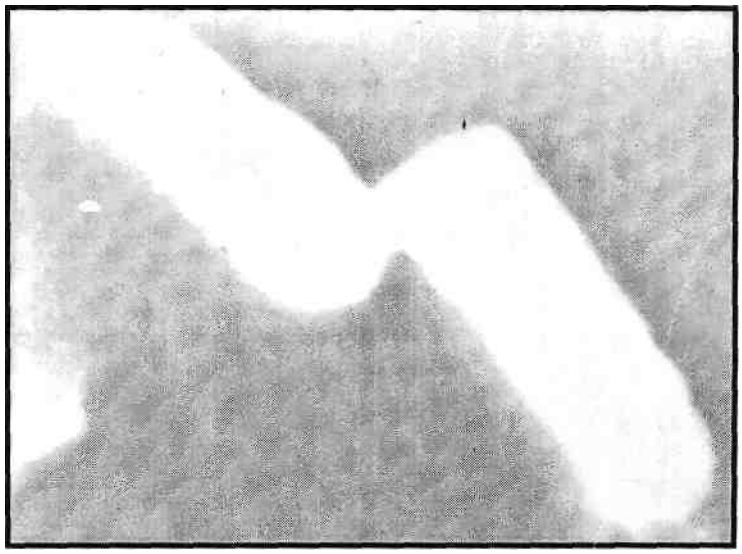


Fig.25. SEM picture of *Bacillus cereus*

The isolate streaked on selective *Bacillus cereus* agar media containing bromothymol blue and egg yolk produced blue colonies surrounded by precipitate of egg yolk which confirmed the isolate to be *Bacillus cereus* (Fig.26) and a pure culture of *B.cereus* is shown in Fig.27.

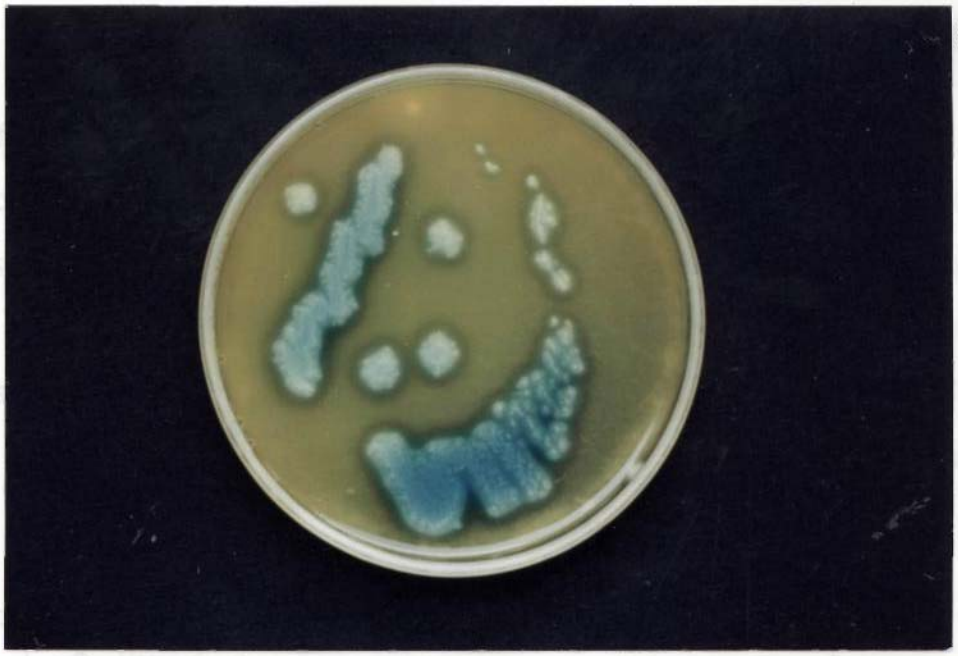


Fig.26. Agar plate showing the blue colonies of *Bacillus cereus* surrounded by precipitate of egg yolk

Fig.27. Pure culture of *Bacillus cereus* in a nutrient agar slant

3.6.1 Optimization

B. cereus cells harvested at 48 h of incubation performed maximum hydrolysis of AN. It has been reported that the induction and growth are optimum when the cells are in log phase of growth (152). Maximum hydrolysis of AN was obtained at 48 h and pH 7.0-7.5 (Fig.28). At 0.4% substrate concentration, *B.cereus* expressed maximal nitrile hydratase activity.

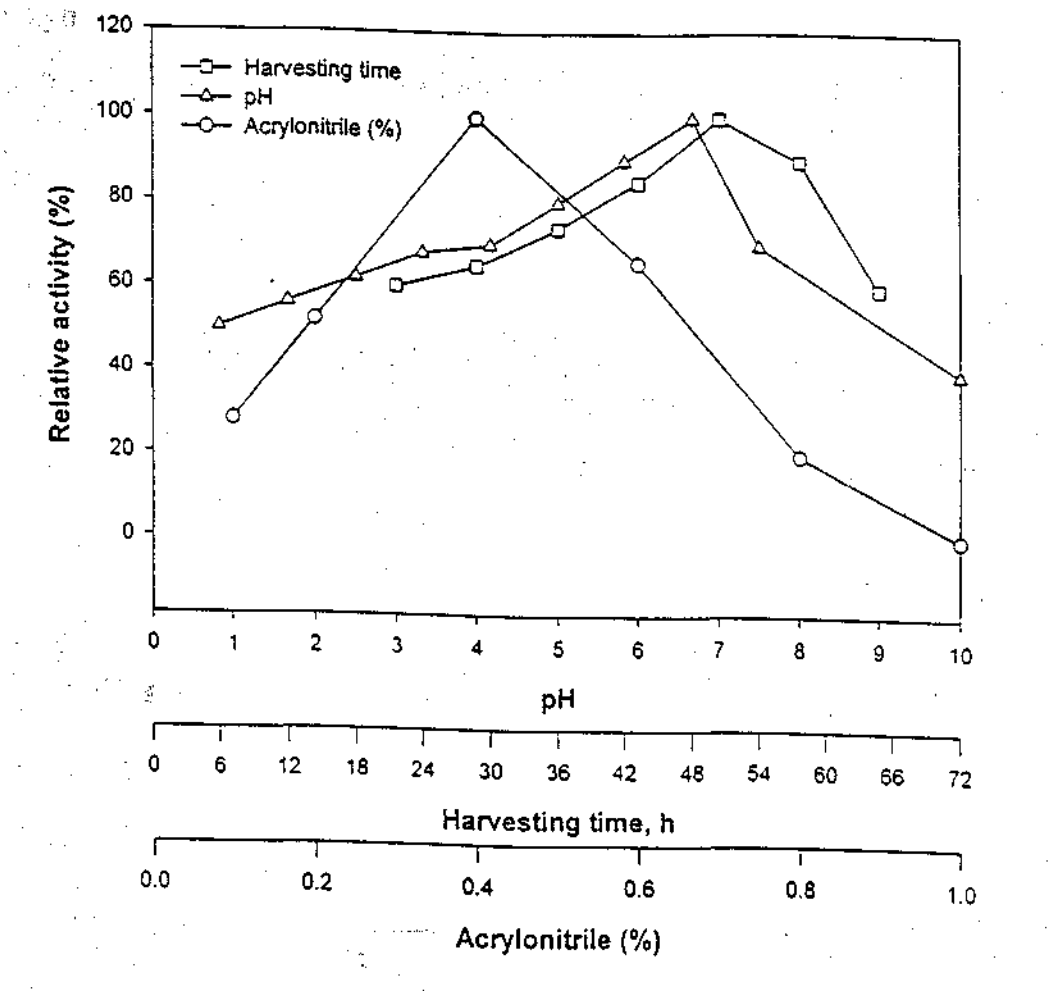
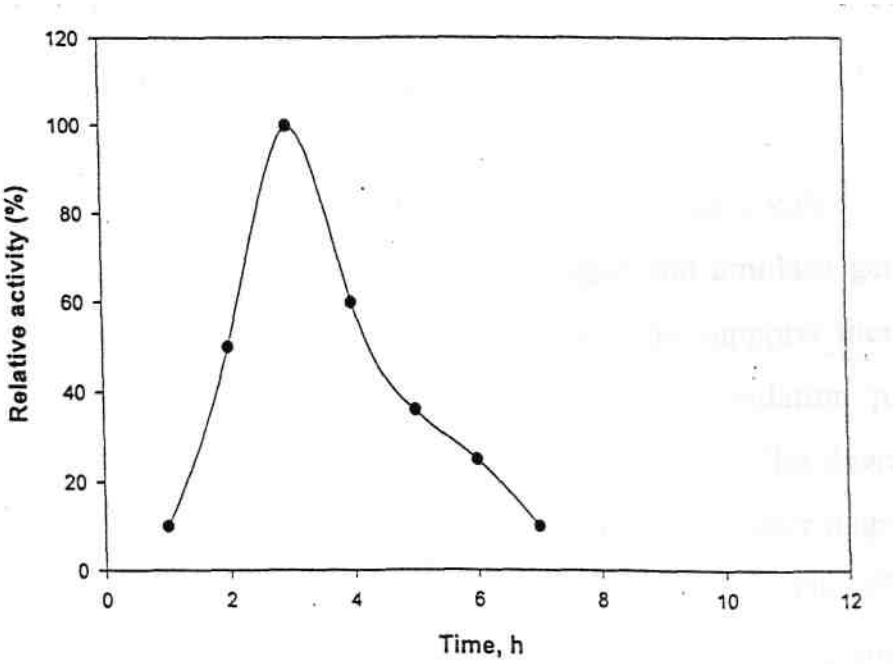


Fig.28. Effect of pH, harvesting time and substrate concentration on nitrile hydratase activity of *B.cereus*

3.6.2 Depolymerisation of S-g-PAN

The appearance of peroxidase activity of *B.cereus* (Fig.29) in the culture filtrate indicated depolymerisation of polyAN. Peroxidases are involved in H_20_2 - dependent cleavage of carbon- carbon bonds of the substrate by free radicals (156). In general microbial cells or hyphae cannot penetrate the interstitial spaces in the macromolecular structure of the polymer such as PAN and there is no evidence for the cells to possess the enzymes needed to break C-C bond endogeneously. Hence aliphatic molecules with average chain length of less than MW.1000 Da are generally susceptible to microbial degradation only by hydroquinone driven Fenton reactions producing H_20_2 (69). Such a reaction is also exhibited by a few microorganisms such as brown rot *Gloeophyllum trabeum* (58).



Fb.29. Peroxidase activity as a function of time at ~ 27°C in the culture filtrate of *B.cereus*

In the depolymerisation of PAN, the extracellular peroxidase reacted with H_2O_2 which is produced by the reduction of an extracellular metabolite such as 2,5-dimethoxy -1,4-benzoquinone (DMBQ) and oxidation of Fe^{3+} present in the culture broth. This reaction generated an oxidized intermediate of enzyme, which in turn oxidized PAN to yield a free radical. The latter underwent C_α-Cβ cleavage (59).

3.6.3 HPLC of degradation products of S-g-PAN

The free monomer (see Fig.30 for HPLC profile) released after depolymerisation of polyAN reached a maximum concentration after 4 h (Fig 30 B). The RpHPLC profile (Fig.30) shows AN, AM and AC, the latter predominating after 36 h. Similarly, during the metabolism of AN by *Arthrobacter sp.* H-1 it was the AC peak which was detected after 48 h (157). Amidase activity appeared to be the rate limiting step during this bioconversion as there was a transient build up of AM in the medium during growth, which was finally converted to AC. The formation of AM and AC indicated that nitrile degradation was by a nitrile hydratase pathway rather than by a nitrilase pathway. Both nitrile hydratase and amidase genes are located in the same region/orientation, which strongly supports their close relationship in the two step reaction of the nitrile degradation pathway (158). The percentage conversion of AN was 70 at 12 h. The degradation pattern of S-g-PAN was slightly different from that of monomer degradation and there was a concomitant increase in pH to 8.2. (Table 12) The efficiency of these conversions was different and dependent upon the nature of the substrate, AN in free form was better degraded than in the polymerised form.

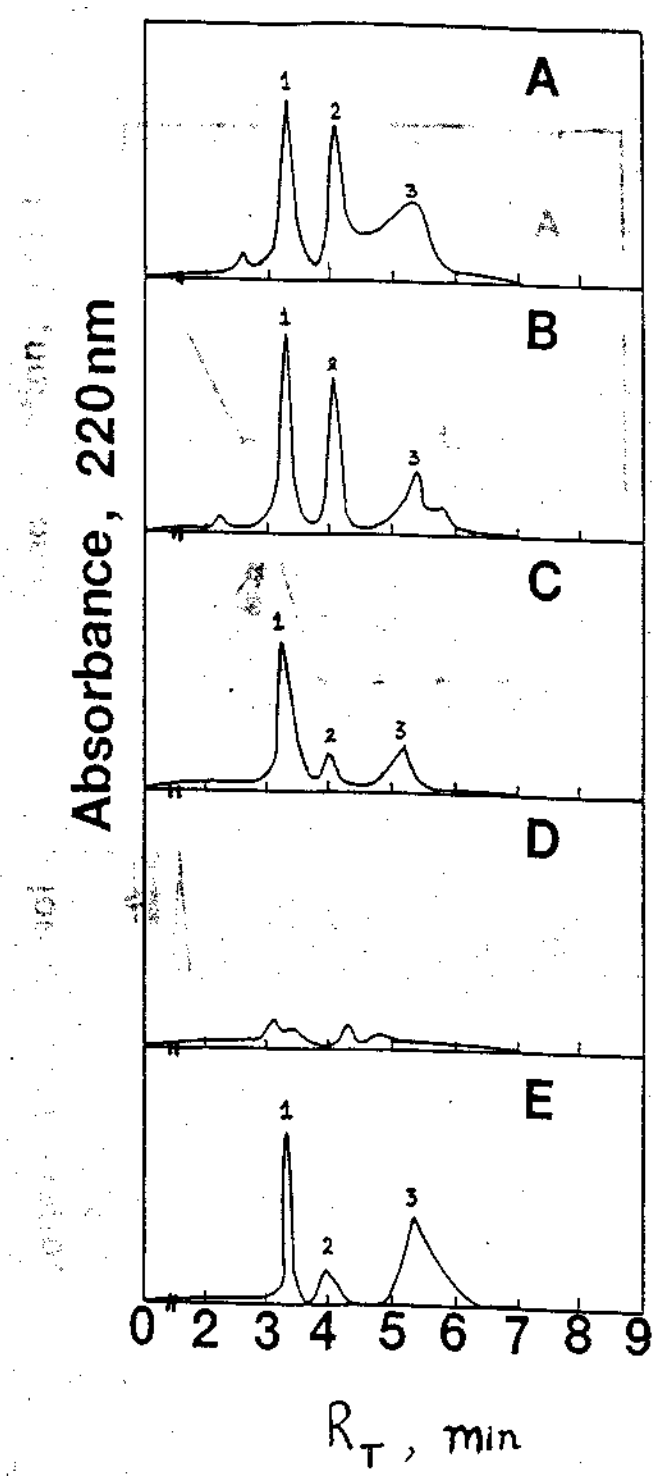


Fig.30. Reverse phase HPLC profile of the hydrolysed products in the culture broth of *B.cereus* grown on S-g-PAN for 6 h (A), 12 h (B), 24 h (C) and 48 h. Standards (E) are AC (1), AM (2) and AN (3)

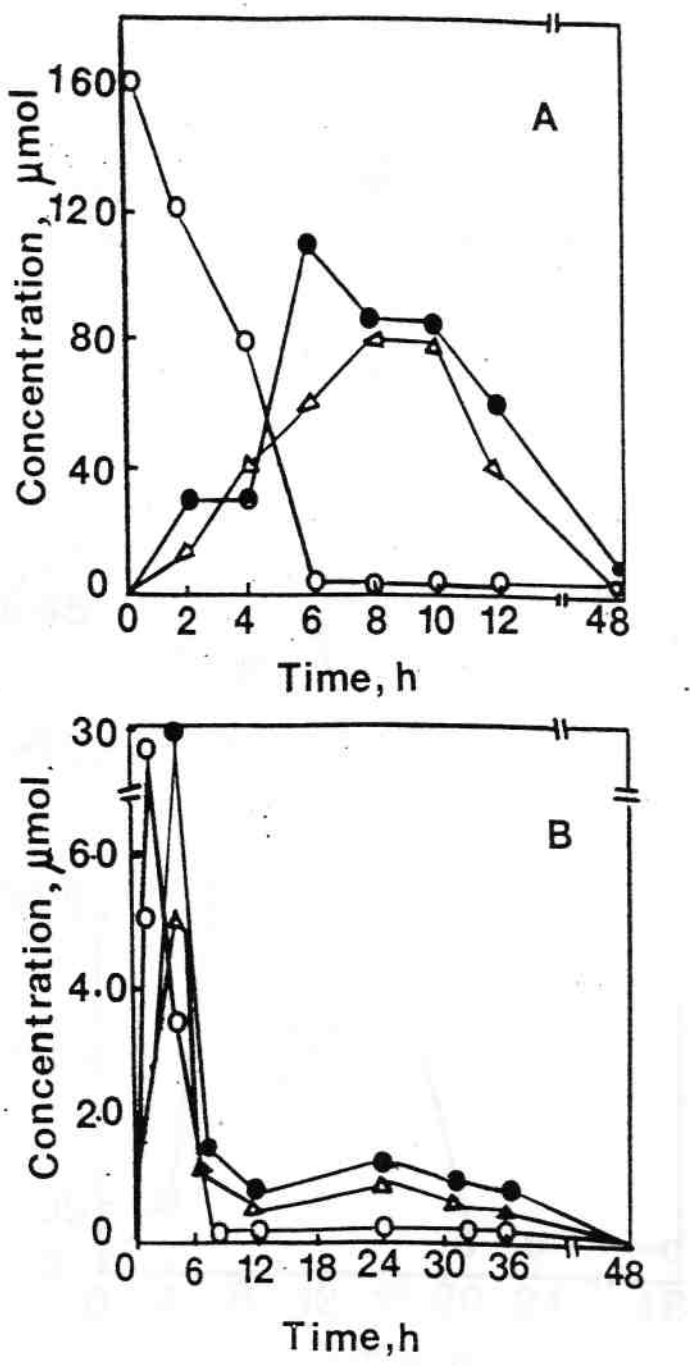


Fig.31. Time course of degradation of free AN (A) and S-g-PAN (B) by *Bacillus cereus* at ~27°C. AN (o), AC (•) and AM (Δ)

The culture filtrate had a maximum α-amylase specific activity of 0.43 units mg-[1] protein at 12 h of cultivation (Fig.32). Further increase in the pH of the culture medium reduced the activity of the enzymes as shown in Table 12. For continuous depolymerisation of PAN, the pH had to be maintained at 7.0, which ensured a stable enzymic activity of bacterial cells. Immobilization of AN transforming cells and recycling of the products formed would seem to prevent the inactivation of enzymes (138).

Fig. 32. α-Amylase activity as a function of time at ~ 27°C in the culture filtrate of *B. cereus*

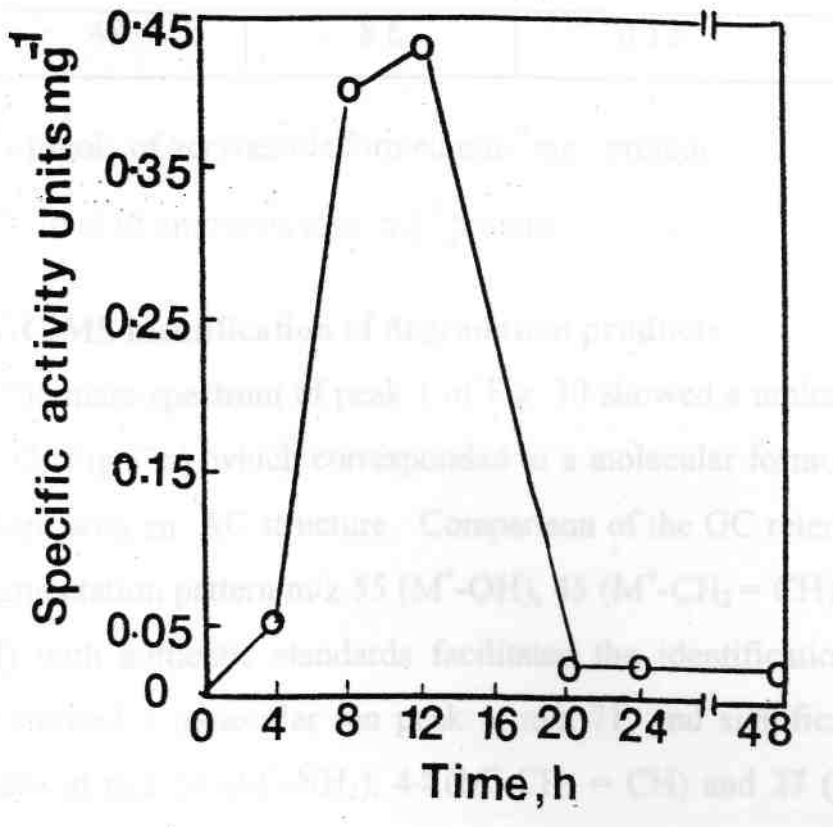


Table 12. Nitrile hydratase and amidase activities in cell-free extracts of *B. cereus* grown on S-g-PAN at pH 7

Time of growth (h)	pH of the growth medium	Nitrile hydratase activity[a]	Amidase activity[b]
2	7.0	4.0	0.02
4	7.0	5.2	0.10
6	7.2	6.0	0.26
12	8.0	3.0	0.13
32	8.2	1.8	0.08
48	8.6	0.12	0.04

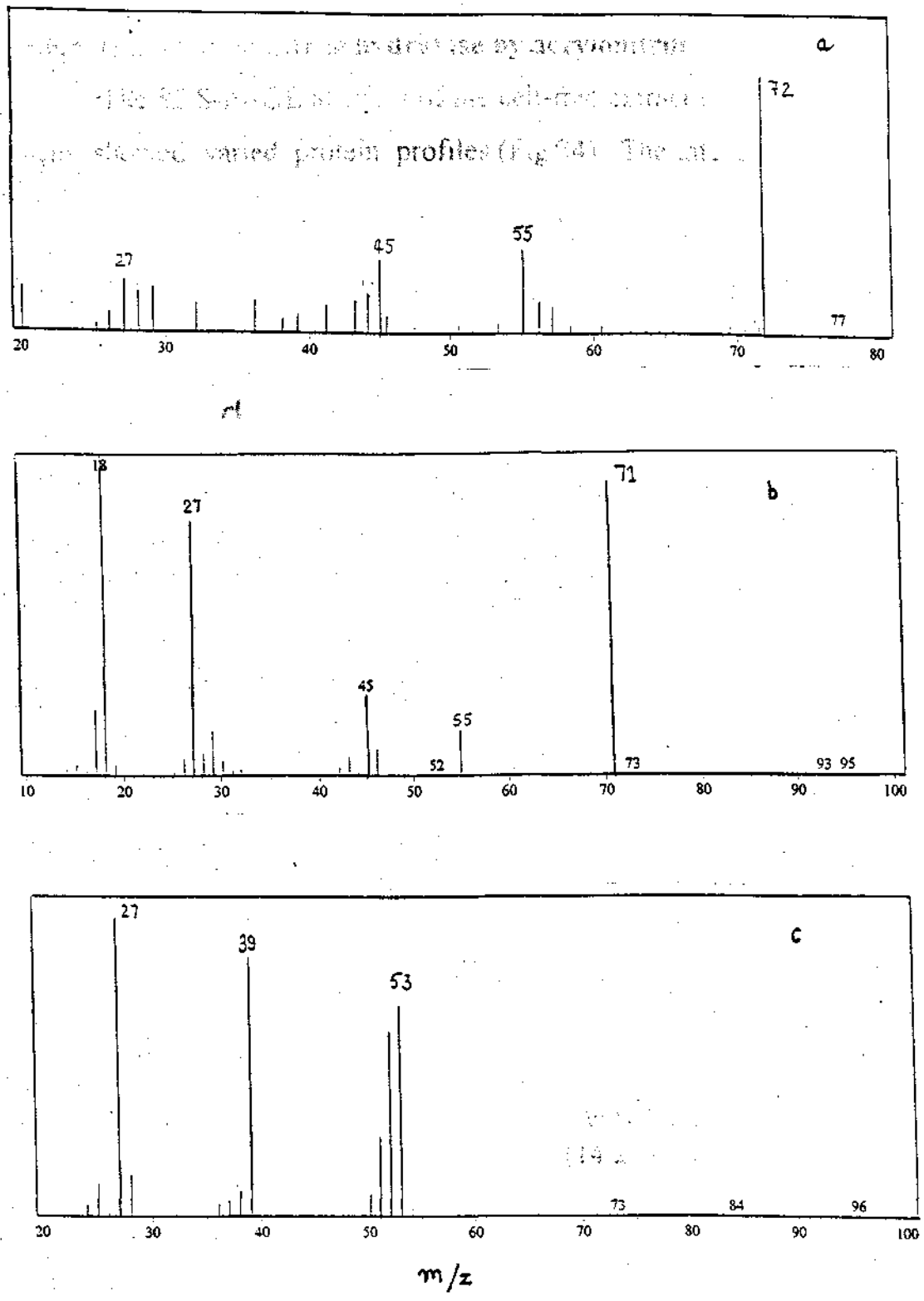
[a] - µmol of acrylamide formed $min^{-1}mg^{-1}$ protein

[b] - µmol of ammonia $min^{-1}mg^{-1}$ protein

3.6.4 GC-MS identification of degradation products

The mass spectrum of peak 1 of Fig. 30 showed a molecular ion peak at m/z 72 (Fig.33a), which corresponded to a molecular formula C3H4O2, in agreement with an AC structure. Comparison of the GC retention time and the fragmentation pattern m/z 55 (M+-OH), 45 (M+-CH_2=CH) and 27 (M+-COOH) with authentic standards facilitated the identification. Peak 2 of Fig.30 showed a molecular ion peak at m/z 71, and significant secondary fragments at m/z 55 (M+-NH_2), 4.5 M+-CH_2=CH) and 27 (M+-CO-NH_2) indicating the compound to be AM (Fig. 33b). Similarly, peak 3 showed a molecular ion peak at m/z 53 and significant fragments at m/z 27 (M+-CN) and 39 (M+-CH_2) indicating the compound to be AN (Fig. 33c).

Fig.33. GC-MS spectra of AC (a), AM (b) and AN (c) 93



3.6.5 Induction of nitrile hydratase by acrylonitrile

The SDS-PAGE analysis of the cell-free extract of differently induced cells showed varied protein profiles (Fig.34). The intensity of the protein

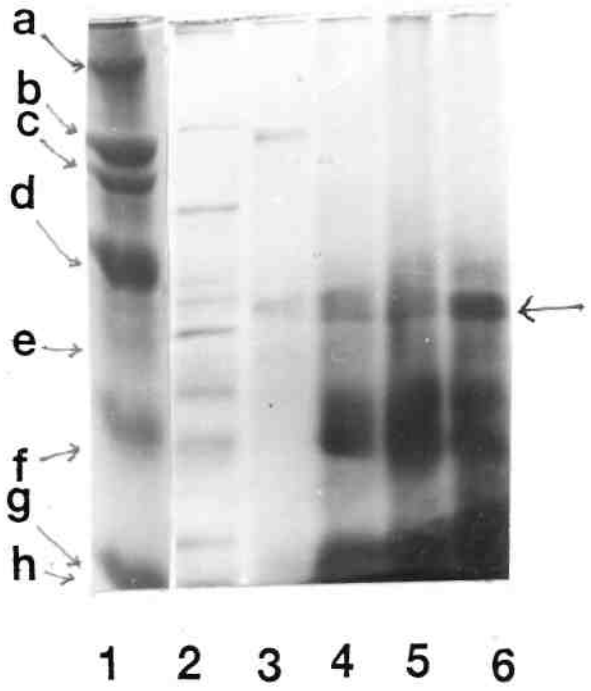


Fig.34. SDS-PAGE protein-profiles of AN-induced *B.cereus* cells cultured under the optimum conditions for the production of nitrile hydratase

Lane 1 had the following molecular mass standards, a, myosin (205 kDa); b, -galactosidase (116 kDa); c, phosphorylase (97.4 kDa); d, albumin (66 kDa); e, ovalbumin (45 kDa); f, carbonic anhydrase (29 kDa); g, trypsin inhibitor (20.1 kDa); h, α-lactalbumin (14.2 kDa); 60 µg protein of uninduced cell-free extract (Lane 2); 60 µg protein of 0.4% and 0.6% (v/v) AN induced cell-free extracts (Lanes 3 and 4); 60 µg protein of 0.4% (v/v) AN + $FeS0_4.7H_20$ (0.001%) induced cell-free extract (Lane 5); 60 µg protein of 0.4% (v/v) AN + $CoCl_2$ (0.001%) induced cell-free extract (Lane 6)

band increased with increase in induction indicating an increase in the production of enzyme. The optimum nitrile hydratase activity and cell growth were seen at 0.4% induction, especially in the presence of $FeS0_4.7H_20$. The specific activity of the enzyme increased 30 times following the addition of $FeS0_4$. $7H_20$ into the culture medium unlike $CoCl_2$, which supports the bacterium to be of Fe-type (Table 13), whereas the protein profiles of the cell free extract of $FeS0_4$ and $CoCl_2$ induced cells remained similar. This is in close agreement with the nitrile hydratase (NH) from *Rhodococcus sp.* N-771 which when compared with *E.coli* in Co-supplemented medium without coexpression of the NH activator, incorporated Co ion onto the catalytic center and produced Co - substituted enzyme, which exhibited rather a weak NH activity (159).

Table 13. Biodegradation of AN by induced *B.cereus* cells

Inducer concentration (%)	Activity[a]
Acrylonitrile, 0.4	0.224
Acrylonitrile, 0.5	0.316
Acrylonitrile, 0.6	0.400
Acrylonitrile, 0.8	0.110
Acrylonitrile, 1.0	—
Acrylonitrile, 0.4 + $FeS0_4.7H_20$, 0.001	6.000
Acrylonitrile, 0.4 + $CoCl_2$, 0.001	0.221

a. μmol acrylamide formed min^{-1} mg^{-1} protein

3.7 *In vitro* amylolytic degradation of S-g-PAN

Studies with amylolytic *Arthrobacter* sp. revealed that bioplasticized starch films are degraded progressively over 56 days (55). Microbial surface interaction between *Lactobacillus amylovorus* and granular starch of starch-g-polyethylene films indicated that colonization by these bacteria on starch granules was important for its degradation (160). Starch-g-PMA film showed an excellent susceptibility to fungal attack in a moist environment (57). An enzymatic method to measure the biodegradability of starch-graft-copolymers is easier and faster than a microbiological study and also it acts as a guide for the choice of microorganisms subsequently to be used to evaluate the bioassimilation of enzymatic biofragmentation products (161).

The effect of time on the hydrolysis of PS, CS, PS-g-PAN and CS-g-PAN by starch degrading enzymes is shown in Fig.35. It can be seen that in the case of native PS and CS the amylolysis was maximum at 4 h with around 80 and 70% hydrolysis, respectively. Further increase in the duration of hydrolysis did not result in any significant improvement in amylolysis. On the other hand the hydrolysis pattern of grafted starches was much different, in that they were maximally hydrolysed at 8 h with about 55 and 50% conversion, respectively for PS-g-PAN and CS-g-PAN.

Glucoamylase showed a better hydrolysis on both native and grafted starches. There was no major change in the hydrolysis pattern between grafted and native starches with pullulanase enzyme. The sequential action

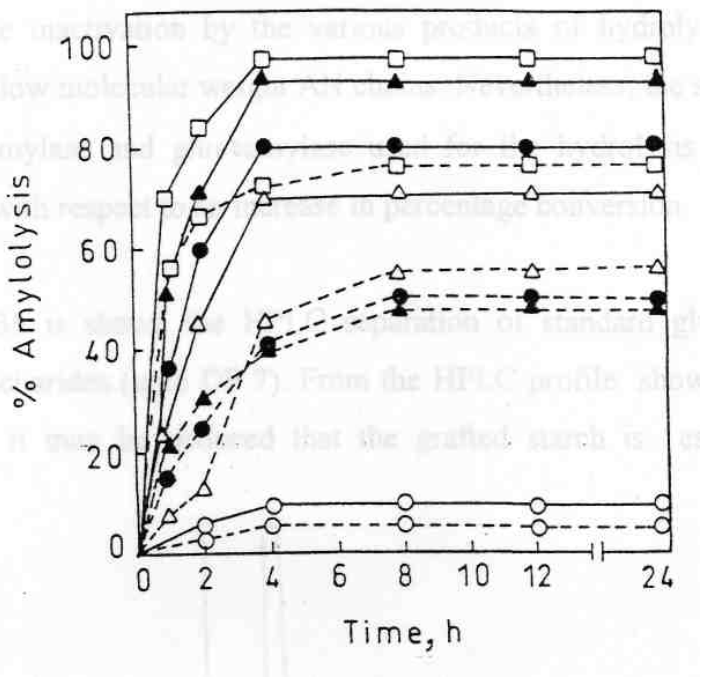


Fig.35. Amylolysis of PS (-----) and PS-g-PAN (-------) as a function of time; (—•—,—•—) α-amylase; (—Δ—,—Δ—) β-amylase; (—Δ—,—Δ—) glucoamylase; (—o—, —o—) pullulanase; (—□—,—D—) sequential action of α-amylase and glucoamylase

of α- amylase followed by glucoamylase produced better results with grafted starches, as the percentage conversion increased to 70% and was in agreement with results obtained earlier (162).

The reduced percentage hydrolysis and conversion of grafted starches by the individual enzymes could be due to the restricted availability of glycosidic linkages of starch backbone to the enzyme, as there are bulky polyacrylonitrile chains sterically hindering for the formation of substrate-

enzyme complex for the hydrolysis to occur. Possibly, there could be **a** partial enzyme inactivation by the various products of hydrolysis (163), especially the low molecular weight AN chains. Nevertheless, the sequential action of α-amylase and glucoamylase used for the hydrolysis produced better results with respect to an increase in percentage conversion.

In Fig.36 is shown the HPLC separation of standard glucose and maltooligosaccharides (upto DP 7). From the HPLC profile shown in Figs. 37a and 37b it may be deduced that the grafted starch is essentially

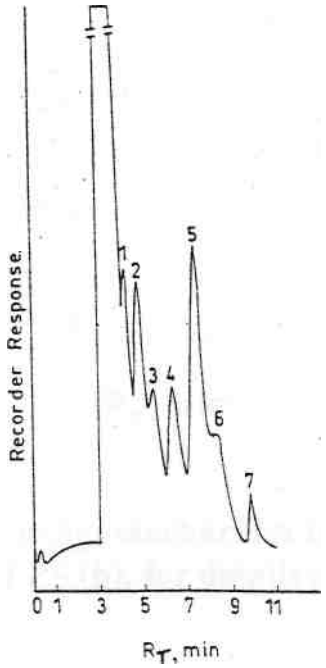


Fig.36. Separation of standard glucose and maltooligosaccharides. Column, Maxil 5 NH_2 (250 x 4.6 mm i.d); mobile phase, acetonitrile - water (75:25); flow rate, 1 ml min^{-1}; column temperature, 30°C. Glucose (1), maltose (2), maltotriose (3), maltotetraose (4), maltopentaose (5), maltohexaose (6), and maltoheptaose (7)

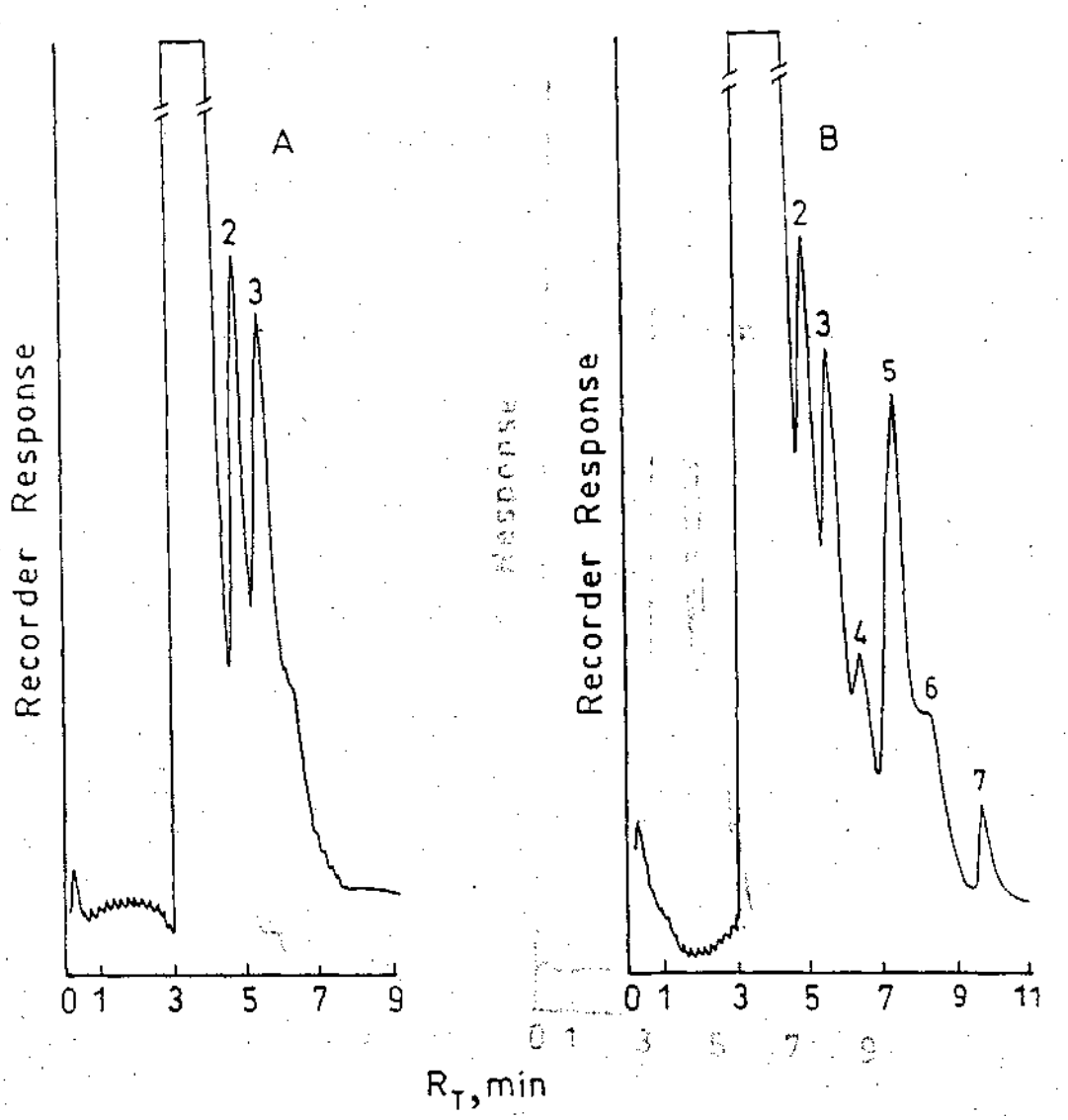


Fig. 37. Separation of maltooligosaccharides in the α-amylase digests of PS-g- PAN (a) and PS (b), for details see Fig.36

hydrolysed by α-amylase to oligosaccharides upto DP 3 in comparison to native starches which showed oligosaccharides up to DP 7 , with DP 4 as the major oligomer. Being an- exoenzyme cleaving sequentially from the non-reducing ends, β - amylase gave mainly maltose (DP 2) in both native and grafted starches (Figs. 38a and 38b).

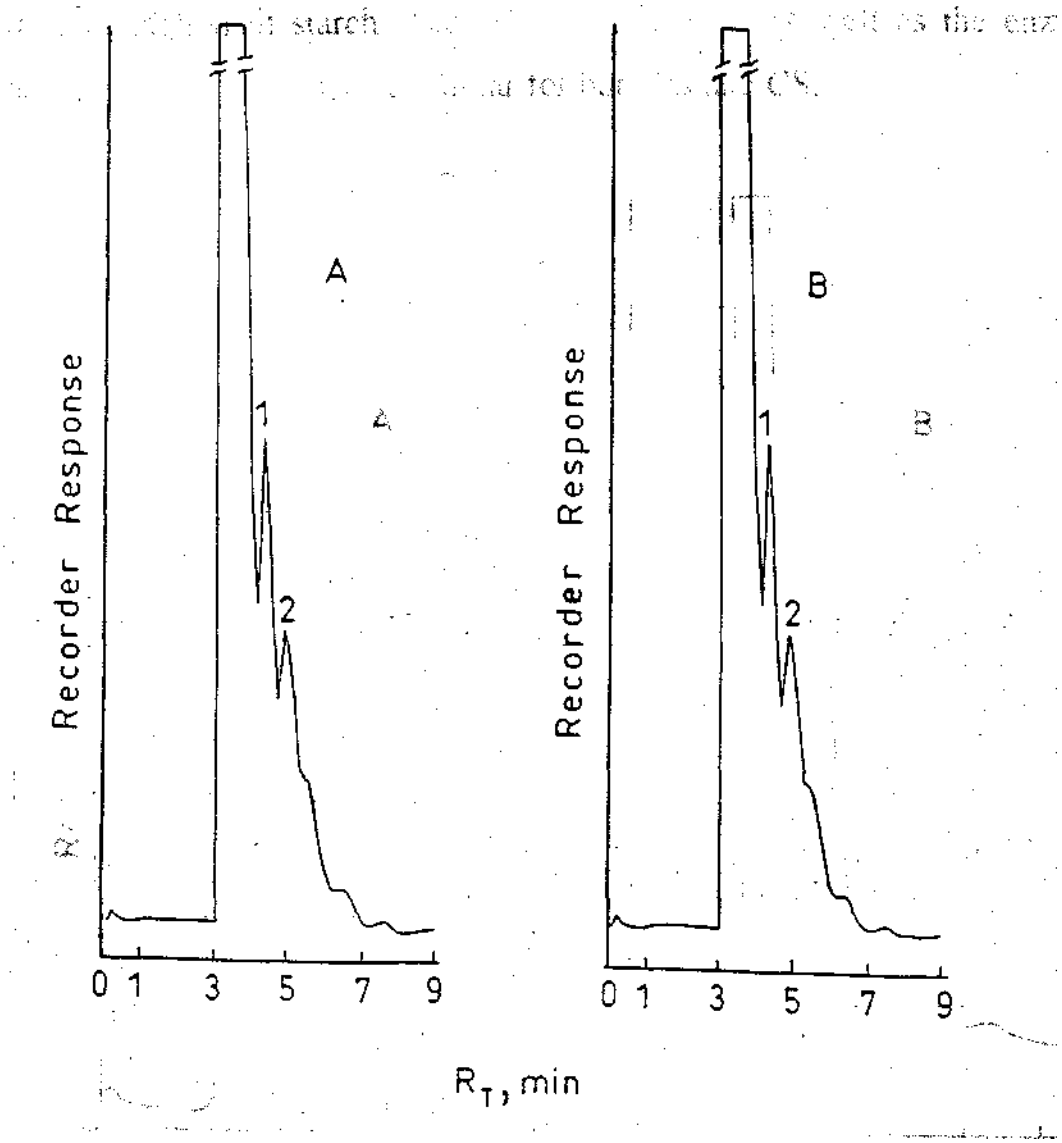


Fig. 38. Separation of maltooligosaccharides in the β-amylase digests of PS-g- PAN (A) and PS (B), for details see Fig.36

Similarly, glucoamylase digestion (Figs.39a and 39b) essentially gave glucose, with very little maltooligomers. The appearance of only lower DP (2-3) maltooligosaccharides in the grafted starch argues in favour of graft copolymerisation of AN on every fourth glucose residue of the linear

amylose fraction of starch. The percent hydrolysis as well as the enzyme degradation profile remained similar for both PS and CS.

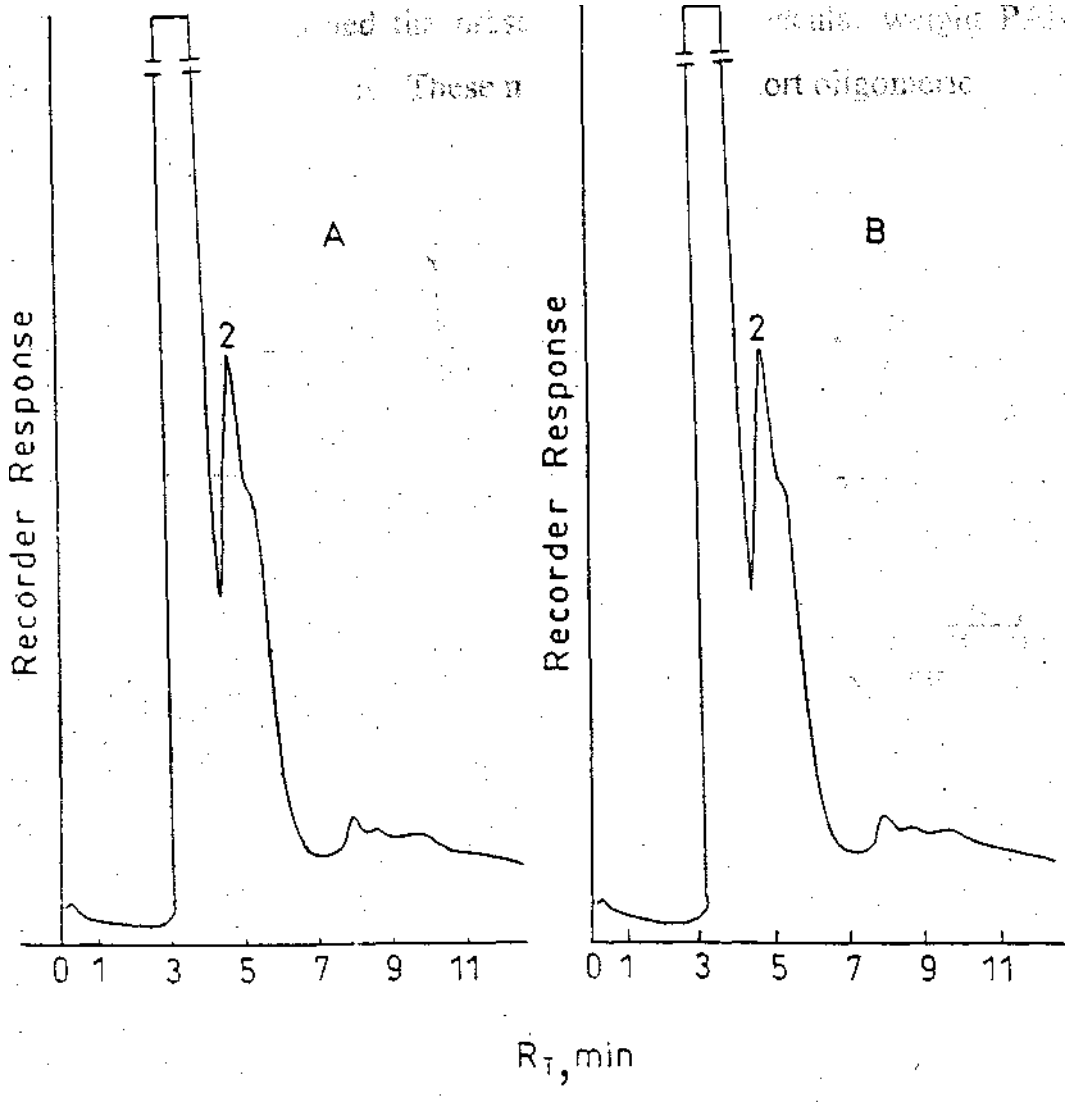


Fig. 39. Separation of maltooligosaccharides in the glucoamylase digest of PS-g-PAN (A) and PS (B), for details see Fig.36

The hydrolysates obtained after amylolytic enzyme treatments of S-g-PAN were analysed for the presence of low molecular weight polyAN chains using a bacterial suspension of *Bacillus cereus* cells (isolated from

soil) (164) andRpHPLC of enzyme digests. The presence of free monomer AN (peak 3) and its hydrolysed product AM (peak 2) and AC (peak X) catalysed by intracellularly present nitrile hydratase and amidase enzymes (Fig. 40 B,C,D,E) confirmed the presence of low molecular weight PAN chains in the grafted materials. These monomers and short oligomeric

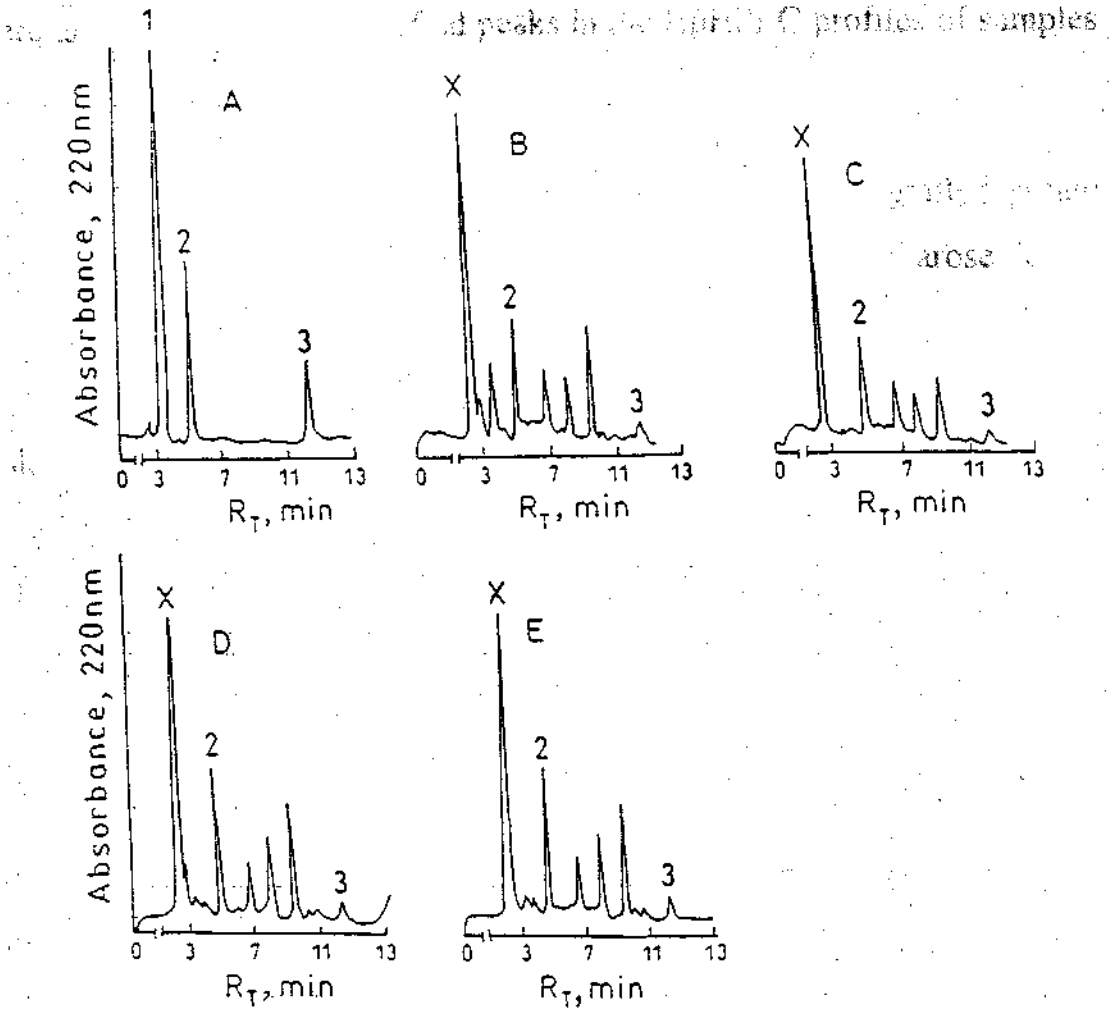


Fig. 40. RpHPLC of *Bacillus cereus* degraded products in the enzyme digests with α-amylase (B), β-amylase (C), glucoamylase (D) and sequential action of α-amylase and glucoamylase (E). Standard mixture (A) of AC (1), AM (2) and AN (3)

chains were rendered water soluble by the presence of hydrophilic oligosaccharide residues attached to them. The peak X on GC-MS analysis showed fragmentation ion peaks at m/z 45 (M+-CH_2=CH) and 31 *(M*+--CH_3) expected by the cleavage of C_α and C_β adjacent to the OH group (165). It could be an alcohol type of compound obtained from AC degradation. There are a few more unidentified peaks in the RpHPLC profiles of samples (Fig.40).

Fig.41 shows the SEC elution pattern of native and grafted potato starches. The former was separated into two fractions on Sepharose CL - 2B, one larger gel-excluded amylopectin fraction and a smaller gel-included amylose fraction (166). In the case of grafted starche only the amylopectin peak and a highly reduced amylose peak were seen. The results showed that it is likely that the amylose component of starch is preferentially undergoing graft copolymerisation with the polyacrylonitrile chains (105).

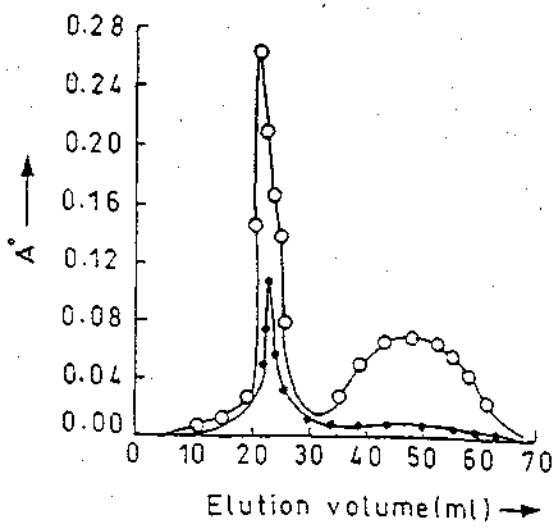


Fig. 41. SEC elution profile of grafted PS-g-PAN (—•—) and PS (—o—)

3.8 COLOURIMETRIC ASSAY OF NITRILE HYDRATASE

Nitriles are highly toxic, organic compounds containing a carbon-nitrogen triple bond (167). Nitriles such as acetonitrile, adiponitrile and acrylonitrile are widely manufactured by the chemical industry and are used as precursors in the manufacture of packaging films, dyes, emulsifiers, cosmetics and antiseptics. All these lead to substantial amount of nitriles in the effluent (168). Apart from this, nitriles enter the environment via their agricultural use as herbicides such as bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and dichlobenil (2,6-dichlorobenzonitrile) (169). Although nitriles are not acted by biological agents, a few of the microorganisms can utilize nitriles as their carbon and /or nitrogen source (170). The microbial degradation of nitriles proceeds through two distinct pathways. Nitrilase catalyzes the hydrolysis of nitriles to corresponding acids (171) and nitrile hydratase (NH) hydrolyses nitrile to an amide, which is latter converted to the corresponding acid by an amidase (172).

A spectrophotometric method for the assay of NH of *Rhodococcus sp.,* based on the difference in extinction coefficient between the substrate acrylonitrile and the product, acrylamide at 235 nm has been described (83). However, acrylic acid the product of amidase also absorbs at 235 nm, resulting in an anomalous absorbance when assayed for NH. Nagasawa et al., reported a GC method for assaying NH of *Brevibacterium* RB12, which converts propionitrile to propionamide (107). Jallageas et al. reported a ^{1}H-NMR method for assaying NH (173). Although these methods are sensitive,

their sample preparation steps are combersome and also they require sophisticated instrumentation. Hence, the need for a simple and sensitive method for assay of NH, which can be utilized for assessing the purity of such enzymes was felt necessary. A simple and quantitative colourimetric assay for NH from *B.cereus,* based on the modified hydroxamate method for the determination of formamide (82) was developed. The colourimetric method involves the determination of acrylamide formed by the action of nitrile hydratase on acrylonitrile and the results are compared with the reported spectrophotometric (83) and HPLC (174) methods.

Formamide reacts with hydroxylamine hydrochloride to give hydroxamic acid which reacts with $FeCl_3$ to give a coloured complex read at 540 nm. Similarly acrylamide, the higher analog in the series undergoes a similar reaction leading to the formation of a coloured complex. The reaction conditions are mild and very specific to an amide group. A linear curve with a regression coefficient of 0.992 was obtained for concentrations ranging from 20-100 μg (Fig.42). Acrylic acid, the product of amide hydrolysis does not interfere in the colour formation (Fig. 42), indicating this reaction to be an amide specific. Hence, this colour reaction can be used to assay NH in the presence of an amidase.

The specific activity of NH in the crude extracts of induced *B.cereus* was 5.49 ± 0.05 units mg^{-1} protein. A few properties of NH were studied using this assay procedure. The initial velocity of the reaction was linear

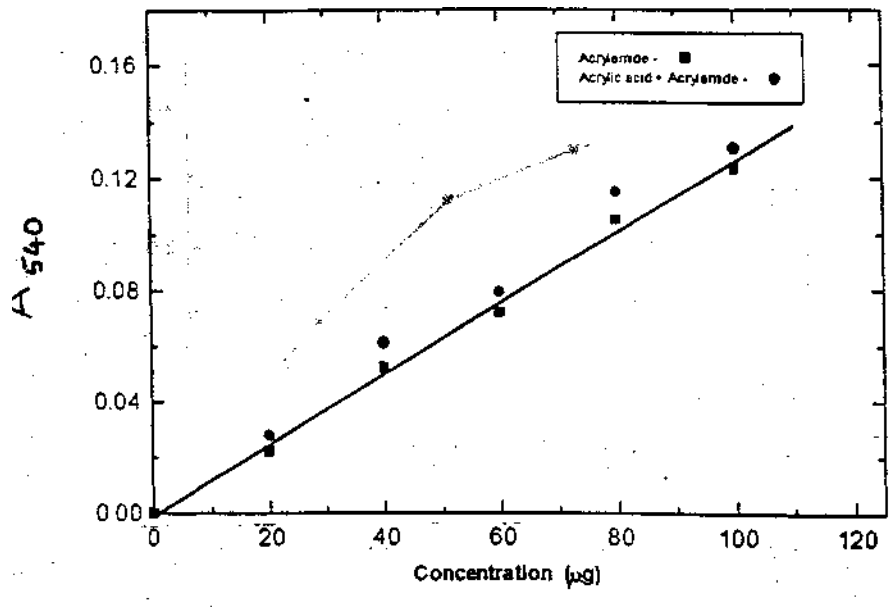


Fig.42. Comparison of the standard curves for acrylamide and acrylamide + acrylic acid

with the added enzyme into the reaction mixture (Fig.43). Thus the initial velocity is directly proportional to $[E_t]$ and therefore can be used to quantitate the enzyme concentration in any preparation, at any stage of purification. The rate of product formation was constant over the entire time interval of the assay. An assay period of 10 min was chosen to ensure that only a small fraction of the substrate acrylonitrile (<10%) was utilised.

3. 8.1 Effect of pH on NH activity

The effect of pH on NH activity was determined using various buffers, viz., 0.05 M sodium acetate (pH 2- 4.8), phosphate (pH 6.0- 7.0) and Tris-HCl (pH 8.0 - 10) at 4°C. The pH activity profile (Fig.44) revealed a pH optimum of 7.0. A cyanide hydratase of *Fusarium solani* (169) and NH of *Brevibacterium* RBI2 (175) were shown to have a similar pH optimum.

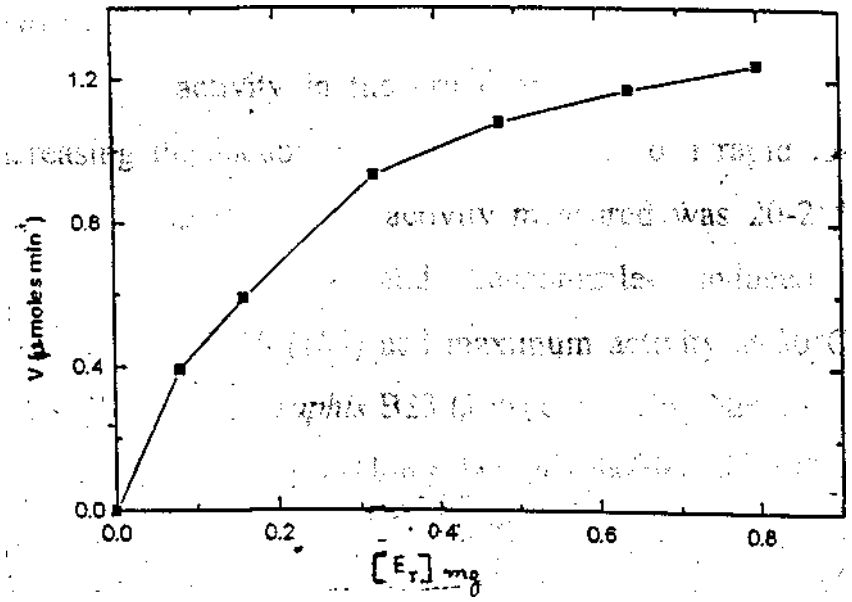


Fig.43. Effect of enzyme concentration on the velocity of the reaction. The enzyme activity was assayed colourimetrically as described under materials and methods

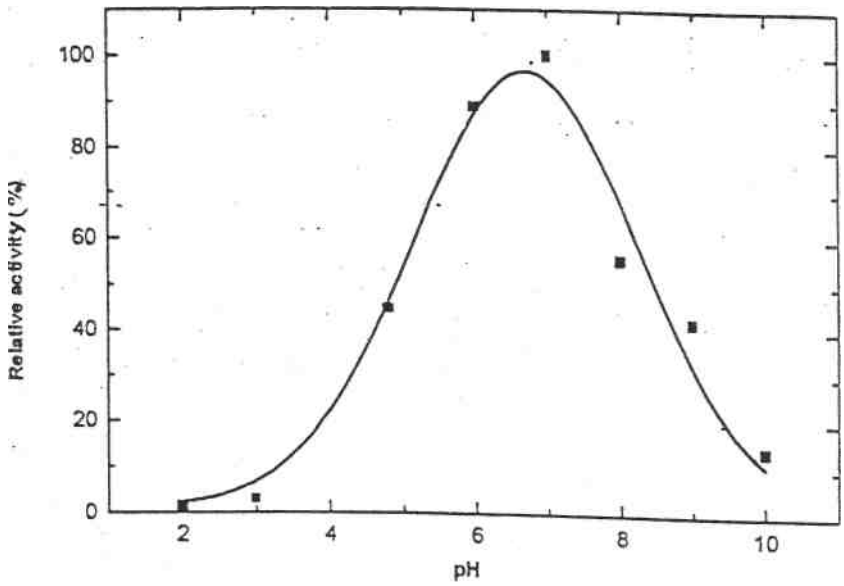


Fig. 44. Effect of pH on the specific activity of nitrile hydratase

3.8.2 Effect of temperature on NH activity

Maximum NH activity in the crude extracts was obtained at 4°C (Fig.45). Increasing the incubation temperature led to a rapid loss in the activity. At room temperature, the activity measured was 20-25% of the maximum. . Both propionitrile- and benzonitrile- induced NH of *R.rhodochrous* NCMB11216 (169) had maximum activity at 30°C and pH 8.0. Resting cells of *P.chlororaphis* B23 (.176) containing NH could convert more than 99% of the substrate acrylonitrile to acrylamide at 10°C.

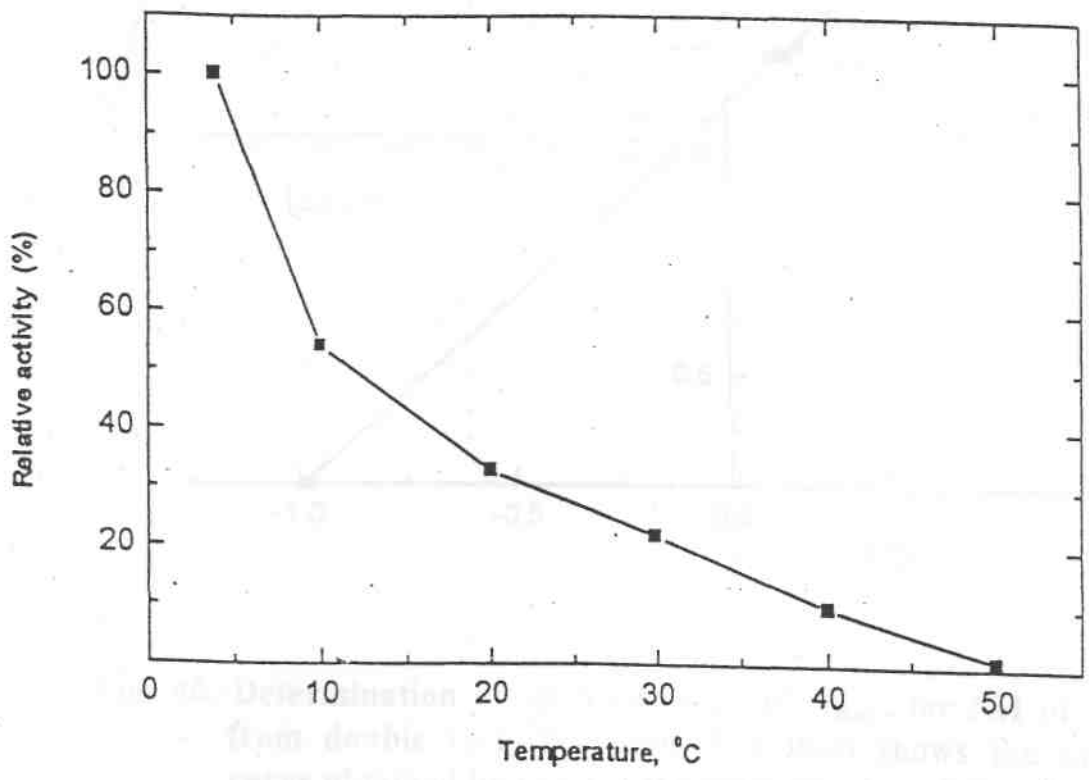


Fig.45. Effect of temperature on the specific activity of nitrile hydratase

3.8.3 Enzyme kinetics

The effect of acrylonitrile concentration ranging from 3.5 mM to 120 mM was investigated. The saturation kinetic data (Fig. 46, inset) show a regular hyperbola indicating that the enzyme NH follows Michaelis-Menten kinetics. The K_m and V_{max} values for NH, determined from the Line weaver - Burk plot (Fig.46), were 1.06 mM and 5.8 µmols min^{-1} mg^{-1} protein, respectively. Under these assay conditions no substrate or product inhibition

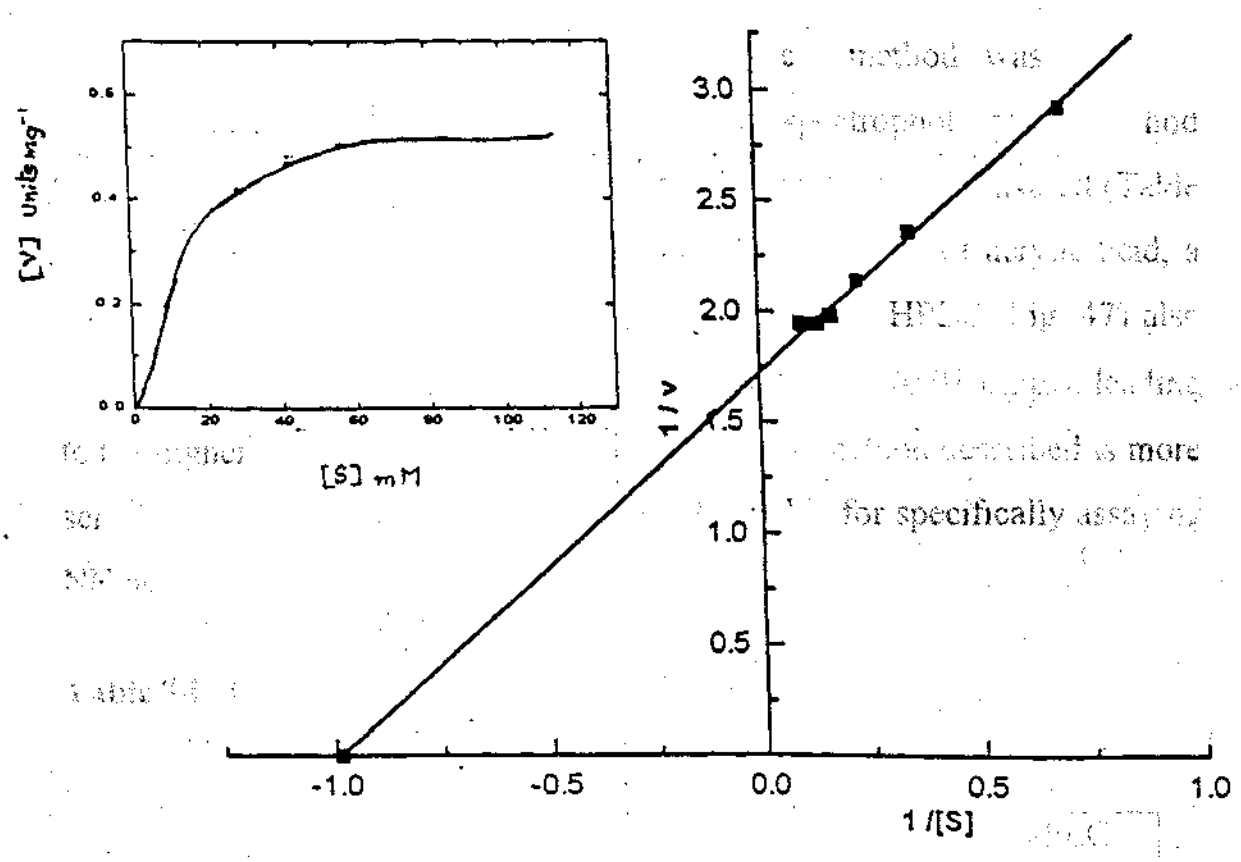


Fig. 46. Determination of apparent K_m and V_{max} for NH of *Bxereus* from double reciprocal plot. The inset shows the saturation curve obtained by varying the concentration of the acrylonitrile

was observed. The apparent affinity for acrylonitrile was 1.06 mM and that reported for benzonitrile for the NH of *R.rhodochrous* (Hoyle) was 2.10 mM.

3.8.4 Comparison of NH activity by different methods

Acrylamide formed by the action of NH on acrylonitrile was quantitated by the reported spectrophotometric (83) and HPLC (174) methods and compared with the described colourimetric method (Table 14). The activity determined by the colourimetric method was in close agreement with that by HPLC, whereas the spectrophotometric method showed slightly higher values at all enzyme concentrations measured (Table 14). This is probably due to the coabsorbance at 235 nm of acrylic acid, a product of amidase. The reaction mixture analysed by HPLC (Fig. 47) also indicated the presence of small amounts of acrylic acid (0.01 µg/µl), leading to the higher absorbance values. The colourimetric method described is more sensitive and specific for acrylamide, and is suitable for specifically assaying NH activity of microorganisms which also produce amidase.

Table 14. Comparison of NH activity by different methods

Enzyme (mg protein)	Enzyme activity[8]		
	Colourimetric method	Spectrophotometric method	HPLC method
0.08	0.39	0.49	0.37
0.32	0.93	0.98	0.95
0.48	1.08	1.13	1.09
0.80	1.24	1.28	1.23

a µmols of acrylamide min^{-1} mg^{-1} protein

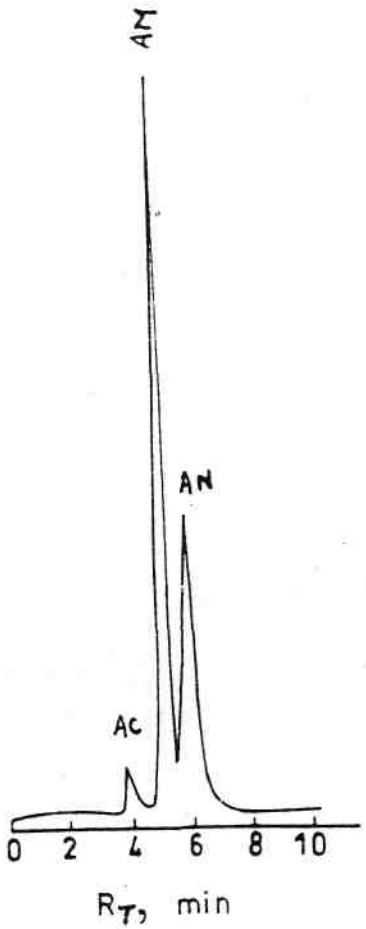


Fig. 47. HPLC profile of assay mixture at 5 min time interval

3.8.5 Induction of NH activity by different nitriles

Amides such as benzamide and acetamide in the concentration range Of 20-100 µg (Fig. 48) could also be determined by using the same colour reaction. Benzonitrile and acetonitrile were used as substrates for the acrylonitrile- induced NH, but the enzyme could not degrade the above substrates. Different nitrile inducers have been reported to induce distinct nitrile - hydrolase activities. *B.cereus* was induced with either benzonitrile

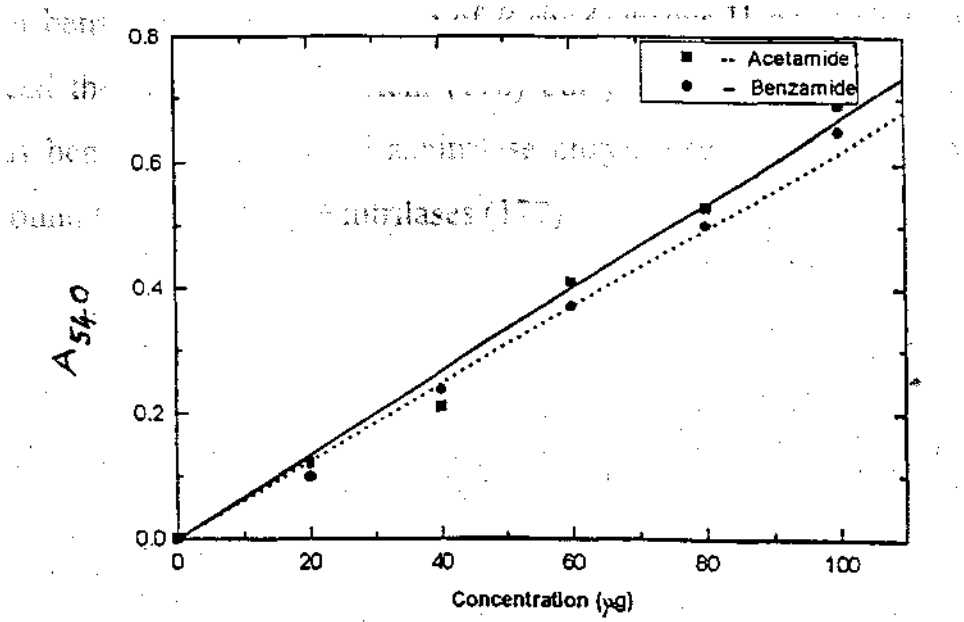


Fig.48. Standard curves for acetamide and benzamide determined by the colourimetric method

or acetonitrile and the NH activity was assayed using benzonitrile, acetonitrile and acrylonitrile as substrates and their respective products assayed by the colour reaction as described. Both benzonitrile- and acetonitrile- induced *B.cereus* NH did not show any activity towards the hydrolysis of the respective inducers. In contrast, both the cell-free extracts could only use acrylonitrile as the substrate. The specific activity measured was 2.39 ± 0.05 and 2.78 ± 0.05 units mg^{-1} for benzonitrile- and acetonitrile-induced NH, respectively. Therefore it can be concluded that a single NH of *B.cereus* although inducible by other nitriles, is very specific to acrylonitrile degradation. In contrast, *R..rhodochrous* produces two similar, but distinct types of nitrilase enzymes in response to induction by propionitrile and benzonitrile (169). The propionitrile-induced enzyme had a K_m of 20.7 mM

for benzonitrile, whereas the benzonitrile induced nitrilase had a K_m of 8.83 mM for benzonitrile (169). Growth of *R..rhodochrous* Jl on aliphatic nitriles produced the NH/amidase system (176) but growth on an aromatic nitrile such as benzonitrile produced a nitrilase enzyme system. Plants have also been found to have multiple nitrilases (177).

SUMMARY AND CONCLUSIONS

In brief, the following are the salient features deduced from this investigation:

> *Chemically modified derivatives of starch and cellulose were prepared and some of their properties evaluated. In comparison with LDPE, HDPE and chitosan films, crosslinked CMC + HPC and crosslinked CMC + HPS films had high WVTR and GTR values, but low tensile strength values. These films, however, could not be used for shelf life extension of mango and capsicum.*

> *The composite coating formulations S_1 and S_2 prolonged the shelf-life of coated banana and mango fruits by displaying retarded colour development, lower acidity, greater firmness, reduced CO_2 evolution and PLW when compared to control and Waxol-coated fruits, but they showed no such effect in the shelf-life extension of capsicum.*

> *The percentage grafting of acrylonitrile onto starch was maximum and there was no difference between grafted starches of potato and cassava.*

> *FT-IR and ^{13}C-NMR techniques confirmed the grafting of acrylonitrile onto starch.*

> *X-Ray and DSC studies revealed that the native starch had lost all its crystallinity during grafting.*

> *Molecular weight distribution by GPC showed a range of polyacrylonitrile chains of varying MW grafted to starch backbone. The heterogeneity in MW and the broadness in peak areas indicated complexity of the grafting reaction and possible existence of multiple grafting sites on starch backbone.*

> *The newly developed RpHPLC method could simultaneously determine and quantitate AN, AM and AC. The method was simple and fast, highly sensitive and reproducible with very good resolution.*

> *The AC content of S-g-PAN, 10.5 µg g^{-1} was significantly low compared to its LD50 oral dose in rats (2.5 g kg^{-1} body weight).*

> *Bacillus cereus, an organism isolated from soil, aerobically degraded S-g-PAN. The extracellular peroxidase activity, reaching a maximum at ~3 h probably initiated the depolymerisation of PAN chains to free monomer (AN).*

> *An inducible intracellular nitrile hydratase/amidase system was involved in the hydrolysis of AN to AM and then to AC.*

> *The extracellular a-amylase degraded the starch moiety to low molecular weight dextrin-type products.*

> *The production of nitrile hydratase increased with increased induction by AN, as evidenced by SDS-PAGE.*

> *Addition of $FeSO_4.7H_2O$ to the medium increased the specific activity of nitrile hydratase, thus supporting the bacterial isolate to be of Fe-type.*

> *In vitro a- and β-amylolysis of the starch moiety of grafted starch showed low values compared to native starches. However, no distinction could be made between the two starches.*

> *The grafted starch hydrolysates on treating with B.cereus cells showed the presence of very low moelcular weight PAN chains, probably grafted onto maltooligosaccharides.*

> *SE-HPLC analysis of grafted starch indicated the amylose component to undergo a preferential graft copolymerization reaction.*

> *A colourimetric assay based on the determination of AM formed by the action of nitrile hydratase on AN is reported.*

> *The initial velocity of this reaction is directly proportional to total enzyme concentration (E(). The pH and temperature optima were 7.0 and 4°C, respectively.*

> *The saturation kinetics data showed K_m 1.06 mM and V_{max} 5.8 µmoles min-1 mg-1[1] protein, and with no substrate or product inhibition.*

> *Benzamide and acetamide could also be determined by this method.*

REFERENCES

1. SmithJP.S., "Food starches and their uses, " in Gum , starch and technology 18th Annual Symposium, Institute of Food Science (1984) 34-42

2. Wolff ,I.A., Davis, H.A., CluskeyJ.E., Gundmm ,L.J. and Rist, C.E. (1951) Ind. Chem. Eng.,21,915-919

3. Roth,W.B. and Mehltretter.C.L. (1967) Food Technol., 21,72-74

4. Jokay, L., Nelson ,G.E. and PowelLE.C. (1967) Food Technol, 21, 21 - 14

5. Stemmler,H. and KnettelV. (1981) Chem. Abstr., 94, 123448a

6. Koike,K., Oshima,T. and Hayakasawa, K. (1979) Chem. Abstr., 90, 105155r

7. Schreiber,K. (1975) Chem.Abstr., 83, 73473e

8. KnechtelA.H. (1963) Amer.Perfum.,78,95-102

9. Roper,H. and Koch,H. (1990) Staerke ,42, 123-130

10. Lee,B.Y., Pometto, A.L, Fratze,A. and BaileyJ.B. (1991) Appl. Environ. Microbiol, 57, 678-685

11. Steinbuchel, A. (1991)Polyhydroxyalkanoic acids. In: Byrom,D. (Ed.), Biomaterials: Novel materials from biological sources, Stockton, New York 124-213

12. Doi,Y. (1990) Microbial Polyesters ,VCH, New York, 126-214

13. ByromJD. (1994) Polyhydroxyalkanoates, In D.P.Mobley (ed.), Plastics from microbes: microbial synthesis of polymers and polymer precursors. Hanser, Munich, 5-33

14. HolmesJP.A. (1988) Biologically produced PHA polymers and copolymers, In BassetζD.C. (ed.), Developments in crystalline polymers, Vol.2, Elseveir,London ,1-65

15. Doi,Y., Mukai,K., Kasuya,K., Yamada,K. (1994) Biodegradation of biosynthetic and chemosynthetic polyhydroxyalkanoates In: Doi,Y. and Fukuda,K. (eds.), Biodegradable plastics and polymers, Elseveir, Amsterdam 39-51

16. Barham,P.J., Keller,A, Otun,E.L., Holmes, P.A,(1984) J. Mat. Sci., 19, 2781-2794

17. Park,H.J. (1991) "Edible coatings for fruits and vegetables " Ph..D. dissertation, University of Georgia, U.S.A.

18. Krochta,J.M., HudsonJ.S. and Avena-Bustillos,R.J. (1990) "Casem-Acetylated Monoglyceride coating for sliced apple products "presented at the Annual meeting of the Institute of Food Technologists, June 16-20, Anaheim, CA

19. Andres,C. (1984) Food Process., 45, 48-49

20. Durst, J.R.. (1967) U. S. Patent 3,323,922

21. GennadiosA, Brandenburg,C.L., Weller,C.L. and Testin,R.F. (1993) J. Agric.Food Chem.,41, 1835-1839

22. McDermott,E.E., Stevens,D.J. and Pace, J. (1969) J. Sci. Food Agric, 20,213-217

23. Wall,J.S. and Beckwıth,A.C. (1969) Cereal Sci. Today, 14, 20-21

24. GennadiosA, McHughJ.H., Weller,C.L and KrochtaJ.M. "Edible coatings and films based on Proteins". In: KrochtaJ.M. and Baldwin (eds), Edible coating and films to improve food quality (1994) 201 Technomic Publishing Company, Inc., Pennsylvania, U.S.A.

25. Levinsky, R.J. (1982) "Allergy aspects of food proteins" In Food proteins FoxJP.F and Condon,J.J., (eds.) , Elsevier Science Publishing Co.,NewYork, 133-143

26. SkerittJ.H., DeveryJ.M. and Hill A S. (1990) Cereal Foods World , 36, 638-639

27. KesterJ.J. and Fennema,O.R.. (1986) Food Technol., 40 ,47-52

28. Dalai, V.B., Eipeson,W.E and Singh,N.S. (1971) Ind. Food Packer, 25, 9-14

29. Durund,V.J., Orian,L., Yanko,U., Zauberman,G. and Fuchs,N.(1984) Hort.Sci., 19,421-424

30. Lakshminarayana,S., Sarmiento,L. and Oritz, J.I. (1974) Proc. Florida State Hort. Sci., 87,325-328

31. Walters,G.G. and Brekke, J.E. (1960) Food Technol., 14,236-238

32. Alvin ,A.S., Arshad, M. and Afzal,M. (1979) Pakistan J. Sci. Ind. Res., 22, 341-345

33. KesterJ.J. and Fennema,O.R. (1989) J. Am. Oil Chem. Soc, 66,1147-1152

34. Whistler,R.L. (1991) Introduction to industrial gums AACC short course on Gum Chemistry and Technology, 1991, Chicago, U.S.A.

35. Krummel,K.L. and Lindsay,T.A. (1976) Food Technol, 30, 36-43

36. Ganz, A.J. (1969) Food Prod. Dev., 3, 65-69

37. Kamper,S.L. and Fennema,O.R. (1985) J. Food Sci., 50, 382-384

38. Nisperos-Carriedo, M.O., Baldwin,E.A .and Shaw,P.E. (1982) Proc. Florida State Hort.Soc, 104, 122-125

39. Mitan,F.J. and Jokay,L. (1969) U.S. Patent 3,427,951

40. Swenson,H.A., MiersJ.C, SchultzJ.H. and Owens,H.S.(1953) Food Technol, 232-235

41. Torres,J.A., Bouzas,J.O. and Karel,M. (1985) J. Food Proc. Preserv., 9, 93-98

42. ByranJD.S. (1972) U.S. Patent 3,707,383

43. Ari,L., Kinumakı,Y. and Fujita, F. (1968) Bull. Tokai Reg. Fish Res. Lab, 56, 89-92

44. ElGhaought,A., Arul, J. Ponnampalan, R. and Boulet, M. (1991) J. Food Sci., 56, 1618-1622

45. Krochta,J.M., Baldwin,E.A. and Nisperos-Carriedo ,M.O.(eds.), (1994) Edible coatings and Films based on polysaccharides. In Edible coatings and films to improve food quality ,Technomic Publishing Co., Inc., Pennsylvania, U.S.A., p. 329

46. Meheriuk,M. and Lau, O.L. (1988) J. Amer. Soc. Hort. Sci., 113, 222-226

47. Griffm,G.J.L. (1974) Adv. Chem. Ser. 134, 159-170

48. Otey,F.H., Westhoff,R.P. and Russell, Ch.R. (1977) Ind. Eng. Chem. Prod. Res. Dev., 16,305-308

49. Otey,F.H., Mark,A.M., Mehltretter,Ch.L and.Russell,Ch.R. (1974) Chem. Prod. Res. Dev., 13, 90-92

50. Rowell,R.M, Schultz,T.P. and Narayan,R. (Eds.) American Chemical Society (1992), Biomass ACS Symp., Ser. No. 476, p. 1987

51. ArthurJ.C, Hinojosa,0. and Bains,M.S. (1968). J. Appl. Polymer Sci., 12, 1411-1421

52. Anvy,Y., Yom-Tov,B. and Zilkha,A.(1965) J.Appl.Polmer Sci., 9,817-821

53. Patil,D.R., Crookston,M.N. and Fanta,G.F. (1995) Staerke, 47, 110-115

54. Abdel-RazikJE.A (1996) Carbohydr. Polymers, 31, 23-27

55. Imam,S.H. and Gould,M.T. (1990) Appl. Environ. Microbiol., 56, 872-876

56. Swanson,C.L., Shogren, R.L., Fanta, G.F. and Imam, S.H.(1991) Starch Plastic Materials: Proceedings of International Symposium on Environmentally Degradable Polymers, Lowell, MA, 241

57. Dennenberg,R.J., Bothast, R.J. and. Abbott, T.P. (1978) J.Appl.Polymer Sci., 22, 459-465

58. Kerem,Z., Jensen, K.A. and Hammel, K.E. (1999) FEBS Lett., 446, 49-54

59. Kerem,Z., Bao,W. and Hammel,K.E. (1998) Proc. Natl. Acad. Sci., 95, 10373-10377

60. Chandhoke,V., Goodell,B., JellisonJ. and Fekete,F.A. (1992) FEMS Microbiol., 90, 263-266

61. Hyde, S.M. and Wood, P. (1997) Microbiol., 143, 259-266

62. Hirano,T., Tanaka,H. and Enoki,A.(1986) Mokuzai Gakkaishi, 41, 334-341

63. Hirano,T., Tanaka,H. and Enoki,A. (1997) Holzforschung, 51, 389-395

64. Goodell,B., JellisonJ., Liu,J., Daniel,G., Paszezynski,A., Fekete,F., Krishnamurthy,S., Jun ,L. and Xu,G. (1997) J. Biotechnol., 53, 133-162

65. Tien, M., Kirk,T., Bull,C., and Fee,J.C. (1986) J. Biol. Chem., 261, 1687

80. Kuniak,L.and Marchesault,R.H. (1972) Staerke, 24,45-49

81. Fanta,G.F., Bagley,B.E., Burr,C.R.and Doane,W.M. (1982) Staerke, 34, 95-102

82. Fry,W.E. and Millar,R.L. (1992) Arch.Bıochem.Biophys.,151 468-474

83. Hjort, C.M.,Godfredsen,E. and Emborg, C. (1990) J.Chem. Technol. Biotechnol., 48, 217-226

84. Fawcett,J.K.and ScottJ.E. (1960) J. Clin. Pathol, 13, 156-159

85. Bernfeld,P. (1951) Adv. Enzymol, 12, 385-386

86. Luck,H. (1965) Peroxidase, In Methods of enzymatic analysis, Bergmeyer,H.O., (ed.) Academic Press, New York, p.895

87. Whistler,R.L. and BeMiller, J.N.Q963) Methods Carbohydr. Chem., 3, . 325-326

88. Johnson,D.P. (1969) Anal. Chem., 41, 859-860

89. ASTM (1980) In Annual book of ASTM Standards, part 20 , p.760-767

90. Rockland,L.B., (1960) Anal. Chem.,32, 1375-1376

91. Donhowe,I.G. and Fennema,0. (1993) J. Food Proc. Preserv., 17, 231-246

92. ASTM (1983) In Annual Book of ASTM Standards, 08.01: 632-647

93. ASTM (1988) In Annual Book of ASTM Standards, 15.09: 324-326

94. McClaveJ.T. and Dietrich,F.H. (1985) In McClave, J.T. Dietrich, F.H. (eds.) Statistics, Analysis of variance comparing more than two means. Dellen, San Francisco, 1-407

95. Miller,L.G. (1959) Anal. Chem., 31, 426-428

96. Rao,P. and Pattibiraman,T.N. (1989) Anal. Biochem., 181, 18-21

97. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem., 193, 265-270

98. Gilbert,G.A. and Spragy,S.P. (1964) Methods Carbohydr.Chem., 4, 168-170

99. Brown, G.A. and Volenec, J.J. (1989) Staerke 41, 247 -251

100. Russell, P.L. and Juliano,B.O. (1983) Staerke, 35, 382-386

101. Zobel,H.F. (1964) Methods Carbohydr. Chem., 6, 22-27

102. Gidely,M.J. (1992) in Developments in carbohdyrate chemistry, Alexander,R.J. and Zobel,H,F. (eds), AACC, St. Paul, Minnesota, p. 163-170

103. McDongall,L.A., Holzaptel,W.A., Schillinger,V., Feely,D.E. and Roignow,J.H. (1994) Int. J. Food Microbiol., 24, 295-308

104. Laemmli,U.K, (1970) Nature, 227, 680-685

105. Kavitha,R. and BeMiller.J.N. (1998) Carbohydr. Polymers, 37, 115-121

106. Lin,K.C, Wu,Y.S., Tan,T.H. and TaiJ.L. (1982) Graft copolymerization of Ligno-cellulosic Fibers, Lin,K.C. (ed.), American Chemical Society,ACS Symp. 187, 236

107. Nagasawa,T., Ryuno,K., and Yamada,H. (1986) Biochem. Biophys. Res. Commun., 139, 1305-1312

108. Greener,I.K. and Fennema,0. (1989) J. Food Sci., 54, 1400-1406

109. Ghaouth,A.El., Arul,J. and Ponnampalan,R. (1991) J. Food Proc. Preserv., 15, 359-368

110. SchultzJ.H., MiersJ.C, Owens,H.S. and Maclay,W.D. (1949) J. Phys. Colloid Chem., 53, 1320-1330

lll.Kittur,F.S., Kumar,K.R. and Tharanathan,R.N. (1998) Z. Lebensm. Unters. Forsch.A., 206, 44-47

112. Smith,S.A. (1986) The Wiley Encyclopedia of Packaging Technology, M.Bakker (ed.), John Wiley & Sons, New York, pp. 514-523

113. DonhoweJ.G. and Fennema,0. (1993) J. Food Proc. Preserv., 17, 231-246

114. Allen,L., Nelson,AX- and Steinberg,M.P. (1963) Food Technol., 17, 1437-1442

115. Hagenmaier, R.D. and Shaw, P.E. (1990) J.Agric. Food Chem., 38, 1799-1803

116. Nisperos-Carriedo, M.O., Baldwin, E.A. and Shaw, P.E. (1992) Proc. Florida State Hort. Soc, 104, 122-125

117. LerdthanangkuLS. andKrochta,J.M.(1996) J.Food Sci.,61,176-180

118. Banks,N.H. (1984) J. Exp. Bot, 35, 127-137

119. Banks,N.H. (1984), Sci. Hort., 24, 279-286

120. Banks,N.H. (1985) Sci. Hort, 26, 149-157

121. ErbiLH.Y. and Mnftngil, N. (1986) J. Food Proc. Preserv., 10, 269-279

122. Dhalla,R. and Hanson,S.W. (1988) Int. J. Food Sci. Technol., 23, 107-112

123. Hirano,S. andNagao,N. (1989) Agric. Biol. Chem., 31,3065-3066

124. Mmo,G. and Kaizerman,S. (1958) J. Polymer Sci., 31, 242-243

125. Ghosh,P. and Paul,S.K. (1986) Ind.J. Technol., 24, 111-113

126. Hebeısh,A., Bayazeed,A., Alfy,El.E. and KhalilJ.M. (1988) Staerke, 40, 223-229

127. Athawale,V.D. and Lele,V. (2000) Carbohydr. Polymers, 41, 407-416

128. Lm,K.C, Wu,Y.S., TanJ.H. and TaiJ.L. (1982) Graft copolymerisation of lignocellulosic fibres, Lin,K.C.(ed.), American Chemical Society, ACS Symp. Series, 187, 243-247

129. Hebeesh,A. and MehtaJP.C. (1968) J. Appl. Polymer Sci., 12, 1625-1632

130. Liang,C.Y. and Krimm,S.J. (1958) J. Polymer Sci., 31, 531-534

131. Yamadera,R.J. (1961) J. Polymer Sci., 50, 54-60

132. Gidley,M.J. (1989)Macromolecules, 22, 351-357

133. Zobel,H.F.(1988) Staerke, 40,1-5

134. Chinnaswamy,R., Hanna,M.A. and Zobel,H.F. (1989) Cereal Foods World, 34, 415-421

135. Shogren,R.L. (1992) Carbohydr. Polymers, 19, 83-87

136. Steıchen,R.J. (1976) Anal. Chem., 48, 1398-1409

137. Gilbert, J. and Starm, R.J. (1992) Food Chem., 9,243-250

138. Battistel, E. Bernardi, A. and Maestri, P. (1992) Biotechnol. Lett., 19, 131-137

139. Schaar,C.J. and Sackett, H.P. (1983) J. Chromatogr., 267, 232-237

140. Merck Index (1960), 7th Edition, p. 17

141. Hall,E.M. and Stivens,W.J. (1977) Anal. Chem., 49, 2277-2282

142. BirdJL.W. and Hall,H.C. (1952). Anal. Chem., 24, 586-591

143. Taubinger,P.R. (1969). Analyst, 94, 28-32

144. Deur-Siftar,D., Svob,V. (1977) Chem. Abs. 87, 58072n

145. Marabo,S.R., Levine,P.S. and Harvey,M.T. (1978) Anal. Chem., 50, 1948-1953

146. Cutie,S.S. andKallos,G.S. (1986) Anal. Chem., 58, 2425-2429

147. Brown,L. (1979) Analyst, 104, 1165-1172

148. Chopra,K.S. and Gupta,L.P. (1978) Indus. Res., 23, 26-32

149. Yamada,H. and Kobayashi,M. (1996) Biosci. Biotechnol. Biochem., 60, 1391-1400

150. Tauber,M.M., Cavaco-Paulo,A., Robra,K.H. and Gubitz,G.M. (2000) Appl. Environ. Microbiol., 66, 1634-1638

151. Yamada,H., Aano,Y. and Tani,Y. (1979) J. Ferment. Technol, 57, 8-14

152. WatanabeJ., Satoh,Y. and Enomoto,K. (1987) Agric. Biol. Chem., 51, 3193-3199

153. Bui,K., Fradet,H., Arnaud,A. and Galzy,P. (1984) J.Microbiol 130, 89-93

154. McBride,K.E., KeimyJ.W. and Stallu,D.M. (1986) Appl. Environ. Microbiol., 53, 325-330

155. Kishore,M.E. (1999). Asian J. Microbiol. Biotechnol. Environ. Sci., 1, 37-46

156. Hammel,K.E., Tıen,M., Kalyanaraman,B. and KirkJ.K. (1985) J. Biol. Chem., 260, 8348-8353

157. Hamde,S. and Ardhale,M.S. (1999) Asian J. Microbiol. Biotechnol. Environ. Sci., 1, 33-36

158. Ikehata,0., Nıshiyama,M., Horinonchı,S. and Beppu,T. (1989) Eur. J. Biochem., 181,563-570

159. Nojiri,M., Nakayama,H., Odaka,M., Yohda,M., Takio,K. and Endo,I. (2000) FEBS Lett., 465, 173-177

160. Doone,W.M. (1992) Staerke, 44, 293-995

161. Coma,V., Couturier,Y., Pascat,B., Bureau,G., Cuq,J.L. and Guilbert,S. (1995) Enz. Microbiol. Technol., 17, 524-527

162. Abraham,E.T., Krishnaswamy,C. and Ramakrishna,S.V. (1987) Staerke, 39, 237-240

163. Fan,L.T., Lee,Y.H. and Beardmore,D.H. (1980) Biotechnol.Bioengg., 22, 177-199

164. Saroja, N., Shamala, T.R. and Tharanathan, R.N. (2000) Process Biochem. (In press)

165. McLafferty,F.W. (1980) Gas chromatography and mass spectrometry interpretations, 3rd Edn, University Science Books, U.S.A. . 190-191

166. Saroja.N. and Tharanathan,R.N. (2000) Eur. Food Res. Technol. (In press)

167. Conn,E.E. (1981) Vennesland,B., Conn,E.E., Knowles,C.J., WetleyJ. and Wissing,F. (eds). Cyanide in Biology, Academic Press, London, 183-196

168. KobayashLM., Goda,M. and Shimizu,S. (1998) Biochem. Biophys. Res. Commun., 253, 662-666

169. Hoyle,A.J., Bunch,A.W. and Knowles,G.J. (1998) Enz. Microbiol. Technol., 23, 475-482

170. Kobayashı,M., Yanaka,N., Nagasawa,T. and Yamada,H. (1992) Biochem., 31,9000-9007.

'55

171.Kobayashi,M. and Shımizu,S. (1994) FEMS Microbiol. Lett., 120, 217-224

172. Kobayashi,M. and Shimizu, S. (1998) Nature Biotechnol, 16, 773-736

173. JallageasJ.G., Arnuad,A. and Galzy,P. (1979) Anal. Biochem., 95, - 436-443

174. Saroja,N., Gowda,L.R. and Tharanathan,R.N. (2000) Chromatographia, 51, 345-348

175. Yamada,H. and Kobayashi,M. (1996) Biosci. Biotech. Biochem., 60, 1391-1400

176. Nagasawa,T., Takeuchi,K., Nardidu,V., Mihara,Y. and Yamada,H. (1991) Appl. Microbiol. Biotechnol, 34, 783-788

177. BarteLB. and Fink,G.R. (1994) Proc. Natl. Acad. Sci., 91, 6649-6653

- Taken from cross-reference, original article not seen