NOVEL AMPHIPHILIC BIOPOLYMER-HYDROGEL
AS
TIME-TEMPERATURE SENSOR

A Thesis
Submitted in partial fulfilment of requirement
for award the degree of
Masters in
Biotechnology

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled as **"NOVEL AMPHIPHILIC BIOPOLYMER-HYDROGEL AS TIME-TEMPERATURE SENSOR''** in partial fulfilment of the requirement of award of degree of Masters in science of biotechnology (DBT) Thapar University, Patiala, India is an authentic record of my dissertation work during six months from January 2017 to July 2017 under the guidance of Dr. Moushumi Ghosh, Professor and head of department (HOD) of biotechnology (DBT) Thapar University, Patiala. I had not submitted the matter embodied in this thesis for award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled **"NOVEL AMPHIPHILIC BIOPOLYMER-HYDROGEL AS TIME-TEMPERATURE SENSOR"** submitted by Saruchi in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is record of student's own work carried out by her. The report has not been submitted for the award of any other University or Institute.

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ABSTRACT

Temperature is an important factor affects the quality and safety of various food products. Thus, it is very crucial to control the temperature during both distribution and storage of food products. An intelligent and biodegradable time/temperature indicator (TTIs) was developed which was based on microbial extracellular amphiphilic biopolymer-hydrogel (EAB). TTIs constitute an important part of responsive packaging; they are cost-effective and user-friendly devices to monitor, record, and translate the overall effect of temperature history on food quality in the chill chain down to a product unit level.

In the present work, ten bacterial strains were screened for the production ability of extracellular amphiphilic biopolymer (EAB) based on variety of tests like; CTAB (cetyltrimethylammonium bromide) agar plate method, BATH (bacterial adhesion to hydrocarbon), Emulsification stability (E_{24}) test, Drop collapse test and Hemolytic test. Four bacterial strains with higher E_{24} activity were selected but only one bacterial strain with 61.5% emulsification activity was finally used for the EAB production. Higher EAB production of 0.248 g/l was achieved in EAB media. EAB was further characterized by Scanning electron micrograph (SEM); Fourier transforms infrared spectroscopy (FTIR) and X-ray diffraction (XRD). Furthermore, hydrogel (polyvinyl alcohol (PVA), PVA/chitosan and PVA/EAB) was synthesed using glutaraldehyde (GA) as a cross- linker. Then characterized using SEM, XRD, FTIR and swelling behaviour. Then the TTI (time-temperature indicator) sensor was prepared with all the three different hydrogel and was activated with lactic acid by keeping at 37^{o}C and 4^{o}C. The results of TTI conducted at 4^{0}C and 37^{0}C for 24 hours indicated the change in the colour of PVA, PVA/chitosan and PVA/EAB-SA1 as yellow to red, yellow to dark brown and yellow to light red respectively. The result of the study this EAB/PVA-hydrogel has possible application in the food industry.

Keywords: Amphiphilic biopolymer, hydrogel, time-temperature indicator, emulsification, hydrocarbons, hydrophilic, hydrophobic, biopolymers.

LIST OF ABBERVATIONS

TTIs	Time-temperature indicators
EAB	Extracellular amphiphilic biopolymer
PVA	Polyvinyl alcohol
SAC	Surface active compound
PLA	Poly lactic acid
SFT	Surface tension
IFT	Interfacial tension
CTAB	Cetyl trimethyl ammonium bromide
BATH	Bacterial adhesion to hydrocarbons
pHEMA	Polyhydroxyethylmethacrylate
PEG	Polyethylene glycol
PBT	Polybutylene terephthalate
GA	Glutaraldehyde
CH	Chitosan
ATH	Anthocyanin
LMB	Leuco methylene blue
MB	Methylene blue
TVB-N	Total volatile basic-nitrogen
LA	Luria agar
LB	Luria broth
OD	Optical density
SDS	Sodium dodecyl sulphate
MR-VP	Methyl red- vogesproskauer
SIM	Sulphide indole motility
CPC	Cetyl pyridinium chloride
FTIR	Fourier transforms infrared spectroscopy
SEM	Scanning electron microscope
XRD	X-ray diffraction

LIST OF SYMBOLS

ºC	Degree (s) Celsius
g	Gram
ml	Millimeter
µl	Microliter
g/l	Gram per litre
nm	Nanometer
%	Percentage
mm	Millimeter
min	Minute
v/v	Volume by volume
sec	Seconds
kg	Kilogram
mg	Milligram
cm	Centimeter
kV	Kilo volt
mA	Milli ampere
wt/wt	Weight by weight
w/v	Weight by volume
M	Molarity

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Chapter 1

INTRODUCTION

A gamut of perishable products intended for human use must resort to strict quality and safety. This increasing range is exemplified by several lifesaving drugs/pharmaceutical products, regular use products, food, horticulture products (Koutsoumains et al, 2005).

The two fundamental factors that add to the loss of hygienic, nutritional, functional and tactile nature of perishable items are time and temperature (Taoukis and Labuza, 2003). Time-Temperature Integrators (TTIs) or sensor consolidates both time and temperature making an interpretation into a single noticeable outcome. In this manner, it demonstrates the total time-temperature history of the related items. TTIs should essentially be straightforward, economical device, have high precision, reproducibility and should attach to the products (Fu et al, 1991). Application of TTIs can substantially control the damage over the supply chain and help economic losses and safeguard both quality and safety (Taouksi, 2001).

The basic guideline of integrators is a temperature-subordinate process, which can be mechanical, physical, chemical, biochemical, or electrochemical. The process brings about an irreversible, quantifiable and regularly visual change, for instance a change in colour. The process utilized can be chosen to coordinate the distinctive time-temperature through reenactment of the chemical, biochemical (ex: development of microorganisms) and physical responses occurring during dealing with and distribution, and the signal delivered shows the aggregate impact of time and temperature at any given moment (Carrillo et al, 2003).

Over decade various time-temperature indicators has been developed for example, a TTI strip based on enzyme reaction Rani and Abraham (2006), Galagan and Su (2008) developed fadable ink colorimetric TTI. TTI system based on Lactic acid bacteria growth and metabolic activity for monitoring the quality food products was described by Vaikousiet al, (2008). However few such sensors have found commercial use. While several technological factors have been associated for limited applicability of currently available time temp sensors, polymeric materials have emerged as the most favorable material option for sensor development; this is due to the possibility of modifications apart from mechanical integrity (Congress et al, 1993). In the current past, efforts have concentrated on applications of natural polymers or biopolymers since they are biodegradable, non-toxic and possess no untoward reaction towards most of the components in use. Depending on the applications of TTIs, two important characteristics of biopolymers have been sought (a) amphilicity and (b) ability to form hydrogels.

The microbial compounds containing hydrophilic (mono, oligo or polysaccharides, peptides) as well as hydrophobic (saturated, unsaturated and hydroxylated fatty acids) components are known as amphiphilic biopolymers. The dual functional property of amphiphilic biopolymers have evoked their applications in various areas such as emulsification, frothing, diminish the viscosity of heavy liquids, such as unrefined petroleum etc. (Gautam and Tiagi, 2006; Franzetti et al, 2010). Hydrogels on the other hand is hydrophilic polymer network that can absorb large quantities of water without dissolving. Their unique swelling ability arises from the fact that polymer backbone contains hydrophilic functional groups, which allow the hydrogel to absorb water. The presence of crosslinks between network chains prevents the hydrogel from dissolution and swelling capacity of hydrogel is determined by the nature of the polymer chains and the crosslinking density (Hubbell, 1996). Hydrogel can be classified on the basis of source, polymer composition, configuration, or the most useful one is in the terms of applications and type of crosslinking. Physically or chemically produced hydrogels consists of covalently cross-linked networks and make use of a cross linker (e.g. glutaraldehyde, ethylene glycol) to obtain their permanent network structure. As compared to physically produced hydrogels, chemically produced hydrogels are stronger because of their covalent linkage and due to their different type of crosslinking. Physical hydrogels also have a limitation of possessing reversible nature by default (Ahmed, 2015).

Both physically and chemically produced hydrogels can exhibit defects in their network structure, influencing the elastic properties and swelling behaviour of gel. In physically cross-linked hydrogel clusters of molecular entanglements, creates homogeneities and fail to contribute the elastic nature of networks (Bai et al, 2011). On the other hand chemical hydrogels also exhibit reduced elasticity as a result of free chain ends. Furthermore, due to aggregation (hydrophobic) of crosslinking agents, chemically linked hydrogels contains the regions which show low water swelling and the clusters of crosslinking found to be dissolving in regions of swelling and low crosslink density. This leads to a difference in swelling capacity which needs to be considered while designing the hydrogels (Calvert et al, 2009).

It is imperative from the above deliberations that a combination of both properties is rare amongst most biopolymers but would be highly desirable. Few studies, however to date have attempted to characterize microbial biopolymers in terms of their amphilicity and hydro gelling property simultaneously restricting the choice for developing biosensor integrators based on Time –Temperature regime.

There present study therefore focuses to screen amphiphilic biopolymers from amongst a library of microbial biopolymers, explore the hydrogel formation in combination with PVA and finally apply their functionality in Time Temperature Integrator biosensors for determining the product stability. The target analyte chosen was lactic acid, commonly generated in most perishable products due to growth of Lactic acid bacteria.

SCOPE OF THE STUDY

Microbial polymers have unique functional diversity, adequate up-scalability, besides being nontoxic and biodegradable, their exploitation for a suitable TTI sensor assumes significance as opposed to synthetic polymers. Therefore this study attempted to characterize microbial polymers with amphiphilic property and develop PVA (Poly Vinyl alcohol) hydrogen composites in order to fabricate a TTI biosensor. Chitosan was used as standard reference for this purpose.

OBJECTIVES

The following objectives were framed in order to address the gaps in research:

- Screening, production and characterization of amphiphilic biopolymers producing bacterial strain(s).
- Development of hydrogel from amphiphilic biopolymers as PVA composites.
- Application of amphiphilic biopolymer-hydrogel composite as TTI biosensor.

Chapter 2

REVIEW of LITERATURE

2.1 Biopolymers

Approximately 140 million tons of synthetic polymers are created everywhere around the world. Due to their great stability problem, their disintegration cycles in biosphere are inexhaustible (Premraj et al, 2005). So, the materials produced from these synthetic polymers are non-biodegradable and not get decomposed properly and cause environmental issues (Chanprateep et al, 2010). Because of this expanding awareness, there is a need to use polymers from natural sources to create biopolymers. This production of biopolymers is an alternative to secure the environment by decreasing toxic discharge (Godoi et al, 2011; Hassan et al, 2013; Mousaviou et al, 2010). Polymers produced naturally by biological systems such as microorganisms, animals, plants, insects, humans are known as "biopolymers" (Armentano, et al, 2013). These naturally produced polymers are formed inside the cells of living organisms by complex metabolic process. Their backbones are made up of repeating units of saccharides, nucleic acids or amino acids and different extra chemical side chains (Chassenieux et al, 2013). So, they create a great significance in current research to develop newer biodegradable polymers. A various number of biodegradable polymers have been blended in nature during the development cycles of all life forms (Rao et al, 2014). These polymers include starch, proteins, deoxyribonucleic acid (DNA), Chitosan and dextran etc.

Pawar and Purwar (2013) studied that there had been a great interest of using biopolymer in packaging which helped the food to protect and preserved from the external contamination. These protections retard the deterioration, extend the life of product and maintained its safety and quality of food which are packaged. So, these biopolymers are the one which satisfies all of the functions without making any harm to nature. The conviction of these biopolymer materials will reduce the requirement of synthetic polymer.

Rastogi et al (2015) partitioned biopolymers into three fundamental classification in light of their source and generation; (1) Polymers straight forwardly separated or expelled from biomass, for example, polysaccharides such as chitosan and proteins. (2) Polymers created by traditional substance from bio-derived monomers, for example PLA. (3) Polymers delivered directly by microorganisms for example, polyhydroxyalkanoates, bacterial cellulose and so forth. (Mensitieri et al, 2011). Further, Othman (2014) classified the biopolymers as shown in Figure 2. Among all the biopolymers chitosan and polyvinyl alcohol (PVA) is most widely used in the current era of research as described by Congress et al, 1993.

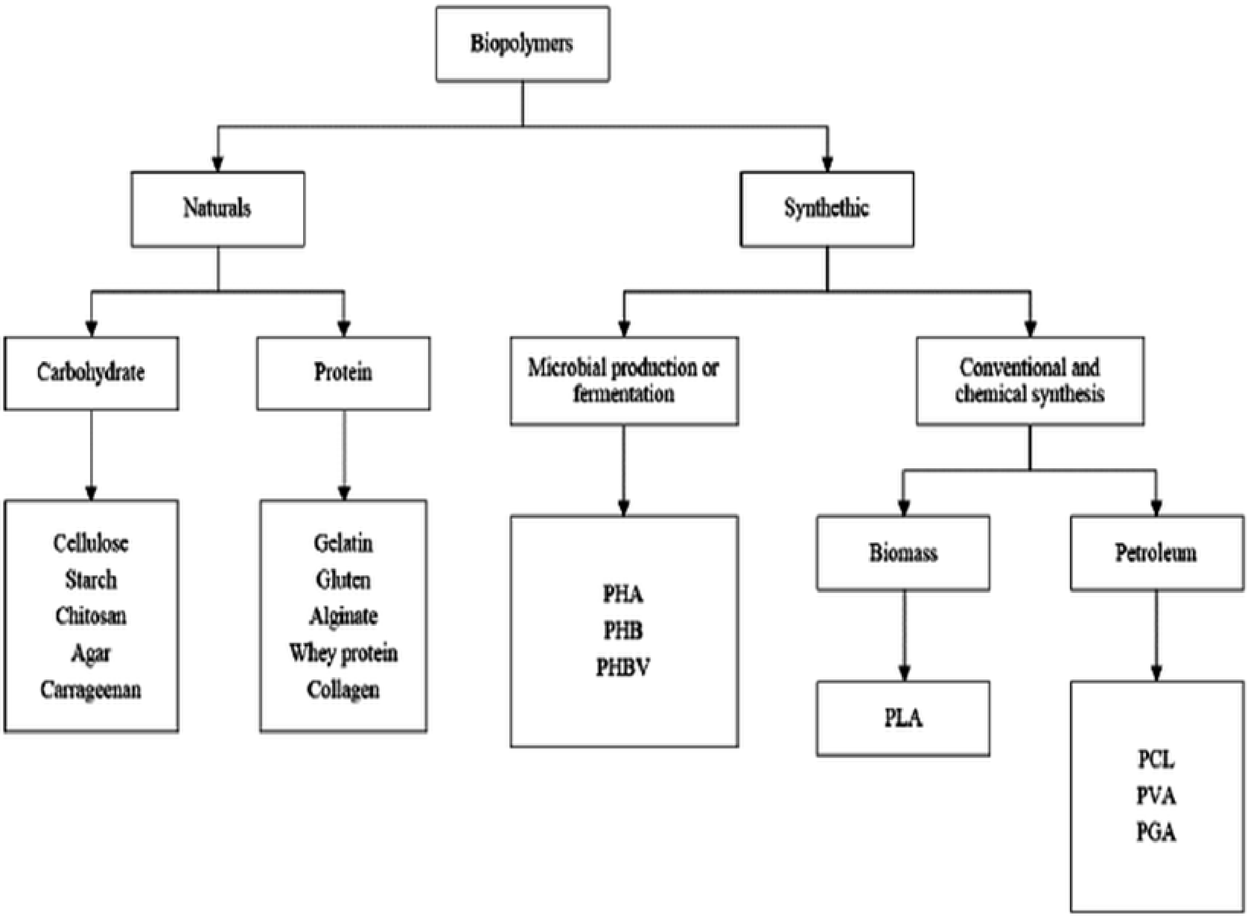


Figure 2.1 Categories of the biopolymers (Othman, 2014)

Chitin and Chitosan

Chitin is a skeletal polysaccharide made up of fundamental shell constituent of crabs, shrimps and creepy crawlies. Chitin is insoluble in its local form, but when slightly deacetylated, it becomes water solvent. Chitosan has various magnificent properties like biocompatibility, biodegradability, adsorption and capacity to frame films (Brugnerotto et al, 2001). Due to their various properties they are generally utilized as a part of food industry, because of their sensing application to protect the food from damage and which in turn protects the quality and safety of food products (Vanderroost et al, 2014). Chitin and chitosan have similar structure as shown below (Figure 3).

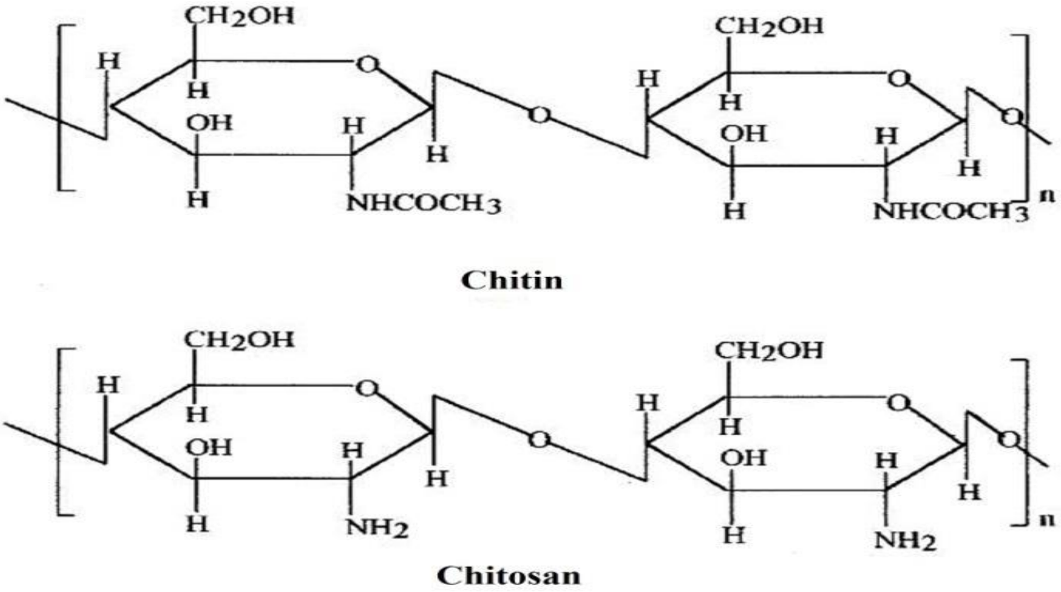


Figure 2.2 Chemical structures of chitin and chitosan (Rao et al, 2014)

Polyvinyl alcohol (PVA)

Polyvinyl Alcohol (PVA) is nontoxic, water soluble synthetic polymer, which is broadly used for the development of polymer blends due to its great physical and chemical properties.

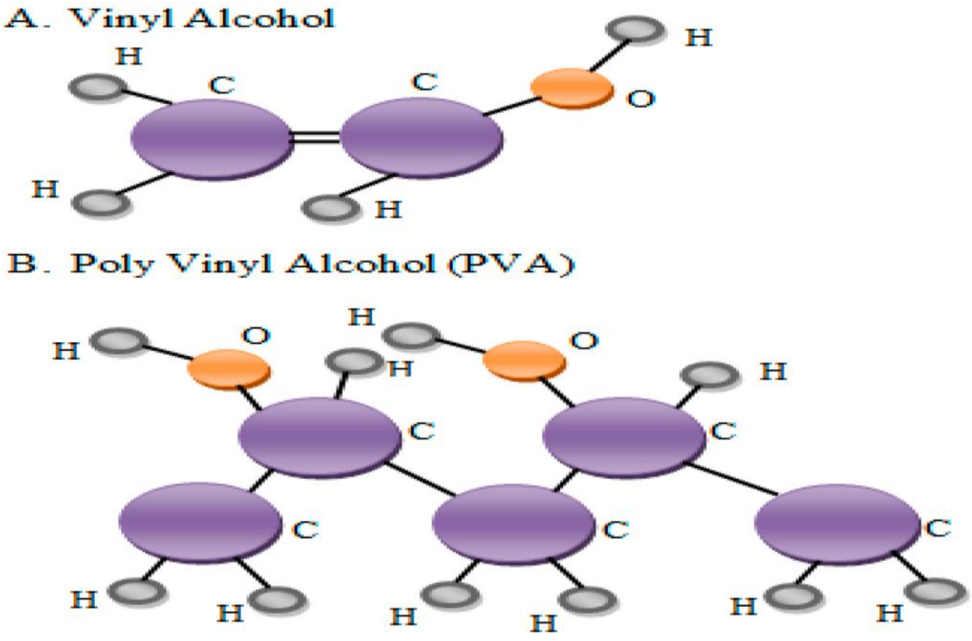


Figure 2.3 Structure of (a) vinyl alcohol and (b) PVA (polyvinyl alcohol) (Grote, 2008)

PVA has excellent film shaping qualities, bio-inertness, emulsifying capacity and adhesive properties. Due to these various properties, they play an important role in various applications, for example membrane preparations, reusing of polymer and packaging etc. (Fromageau et al, 2007). De Souza et al (2009) made a PVA blends with chitosan and used for food packaging system.

Grote (2008) describes numerous properties of biopolymers like, being inexpensive, biodegradable, high loading capacity and biocompatibility and providing an appropriate microenvironment. But main focused they gave on the microbially produced biopolymers. These biopolymers have very important and unique property such as “Amphiphilic nature”. Due to this nature they shows two valuable properties; (1) they reduce the pressure of surface when consume at particular intermix (i.e air-water or oil-water), (2) they can self-assemble (Mousaviou et al, 2010).

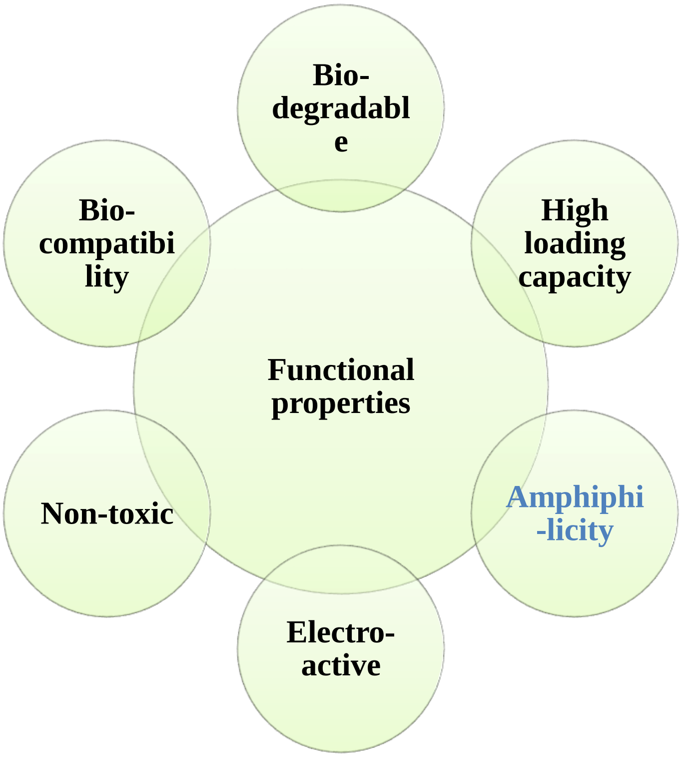


Figure 2.4 Functional properties of Biopolymers (Grote and James, 2008)

This unique functional property makes microbial produced biopolymers most preferable in various applications like; in food industry (Congress et al, 1993).

2.2 Amphiphilic Biopolymers

According to Thavasi et al (2011) amphiphilic biopolymers are made up of “hydrophilic” (water loving) and “hydrophobic” (water hating) parts. Many microorganisms can create variety of amphiphilic compounds with both hydrophilic and hydrophobic elements. These elements are present within a molecule and enable them to display surface activities at interfaces. Banant (1993) partitioned that these biopolymers play a very important role in industries due to their advantages over synthetic biopolymers. The advantages like; they are biodegradable, non-toxic, and highly selective, performs a particular action at extraordinary temperature and pH.

Amphiphilic biopolymers have pulled into much consideration due to their various unique functional properties as shown in Figure 6 (Thanomsub et al, 2004).

Figure 2.5 Functional properties of Amphiphilic biopolymers

- **Surface activity (Thavasi et al, 2011)**
- **Emulsification (Rosenberg et al, 1986)**
- **De- emulsification (Nadarajah et al, 2002)**
- **Flocculation (Assadi et al, 2010).**
- **Adsorption (Curbelo et al, 2007)**
- **Solubilization (Perfumo et al, 2006)**
- **Film formation (Hydrogel) (Amalraj et al, 2017)**

Surface activity of these amphiphilic biopolymers defines that strong adsorption of materials at surfaces or interfaces in the form of monolayer. So, surface tension (SFT) and interfacial tension (IFT) are one of a vital property of these biopolymers. So, SFT of solid intermolecular attract the power to build the tension on the surface (Thavasi et al, 2011). Another important property is emulsification and de-emulsification which was reported by Rosenberg et al (1986) and Kosaric et al (1987) respectively. Emulsification is the diffusion of one liquid into another to prompts the blend of two immiscible liquids, process used to form this immiscible liquid is known as emulsion. Emulsifier structure has two characters in the molecules, one is adaptable to water (hydrophilic group) and other is not adaptable to water (hydrophobic group). It shows a cellular solubilisation and the resultant solubilized particles are considerably greater. On the other hand, procedure of de-emulsification breaks emulsion by disturbance of stable surface among the mass and the household stage, which is accomplished by adjusting the states of thermodynamic at the interface. Nadarajah et al (2002) describes that this process had the ability to blend bacterial culture which were normally tried by using Kerosene-water and oil emulsion framework, where up to 96% of de-emulsification is accomplished. Blended culture items displayed high de-emulsifying action and contrasted the best effective culture.

Akiyoshi et al (2000) defined that flocculation process as emulsion beads comes very close to each other to form a bunch. These bunch could be separated by the mechanical activity and re-establishing the emulsion. Whereas Curbelo et al (2007) talked about the adsorption property of amphiphilic biopolymers. According to them in adsorption process biopolymers atoms adsorbed on hydrophobic substrates. Solubilisation was one of the important properties of microbial produced biopolymers which enhanced the solubilisation of insoluble material. At high concentration of these biopolymers, micelle structures developed. These micelle structures represented the insoluble molecules and brought into solution at heavy amount. Perfumo et al (2006) described that this property develops the substances which are water-insoluble in aqueous solutions or in natural solvents. Amalraj et al (2017) outline one of the most important property of amphiphilic biopolymers i.e. they had the capability to form film such as hydrogel, which had its vital role in checking food quality etc.

Gautam and Tiagi (2006) illustrated that depending upon amphiphilic biopolymers various functional properties; they can be screened by various screening methods. Siegmund and Wagner (1991) developed CTAB or blue agar plate method which was used for the recognition of the anionic surfactants. On the other hand Saravanan and Vijayakumar (2012) developed another prominent screening test for microbially produced biopolymers i.e. emulsification index. This test was sensible for a first screening biosurfact conveying life forms. Then there was a method to check the surface hydrophobicity i.e. BATH (Bacterial Adhesion to Hydrocarbons) is described by Busscher et al (1984). There was many more screening test for these biopolymers namely; Drop collapse method (Jain et al, 1991), Hemolytic activity test (Satpute et al, 2010) etc.

Siracusa et al (2008) portrayed that shift in the research of packaging promoted the use of biopolymers produced by microbes, which were used in decreasing waste disposal. So, the edible coating material was used as coating in food packaging material microbial produced biopolymer was coated with (Azeredo et al, 2012). In food industry these amphiphilic biopolymers played an important role (Cutter, 2006) and growing in food packaging systems to check quality and safety of food products (Mensitieri et al, 2011), to enlarge their shelf-life. Various advantages of these biopolymers alone and used in the food industries are; (1) extracted from replenishable resources (2) had biocompatibility (3) show biodegradability which helps in maintaining ecological safety (Prashanth and Tharanathan, 2007). The properties like; (thermal stability, flexibility, good barrier to gases and water, resistance to chemicals, biocompatibility and biodegradability) depends upon the monomer used in biopolymer preparation (George et al, 2006).

All the functional properties mentioned above made the amphiphilic biopolymers to be used for the formation of matrix i.e. hydrogel matrix (Amalraj et al, 2017).

2.3 Hydrogel

Hydrogel is three dimensional lattice polymer matrixes and have hydrophilic groups due to which these matrixes can swell up to many times of their original volume in liquid medium. They consist of various functional properties which made them appropriate for many applications like; in food industries.

Further, the one of the important functional property of hydrogel is to show the changes in their structure (e.g. deformation, swelling, dissociation of functional groups) due to alterations in the external environment (physical/chemical/mechanical stimuli). This important property of the hydrogel made scientists to use this material for the preparation of "intelligent" materials which had the capacity to self-control from the external environment (Amalraj et al, 2017).

As hydrogel is the polymeric material. So, they can be divided into three classes (natural polymer hydrogels, synthetic polymer hydrogels and combinations of the two). Based on the cross-linking they can be classified as physical hydrogel (networks holds together by physical forces like; ionic, H-bonding or hydrophobic) and chemical cross-linked hydrogel (covlently cross-linked) (Amalraj et al, 2017).

Anyway, the material which was described by Lee et al (2013) was not described as hydrogel it was just a colloidal gel structure with some inorganic salts. It is interesting to know the history of the term itself (Balint et al, 2014). Anyhow, the first cross-linked hydrogel material described its water affinity properties. Hydrogel (polyhydroxyethylmethacrylate (pHEMA)) developed in 1960, with the goal of using them in permanent contact lenses with human tissues, so, it is the first material to be used inside the patient (Kopecek, 2007).

Buwazlda et al (2014) and Yom-Tov et al (2014) described the history of hydrogel and divided it into three generations. In first generation hydrogel was composed of crosslinking procedures by chemical modifications of a polymer with an initiator. The main goal was to develop the hydrogel with high swelling, good mechanical properties and relatively simple procedure.

Then, in the seventies starting, hydrogel different concept grew and named as second generation hydrogel. This generation material was responsible for environment stimulus (variation in temperature, pH or concentration of specific molecules in solution) (Wichterle et al, 1960). Finally, a third generation of hydrogel main focused on the examination and development of stereo complexed materials (e.g. PEG-PLA interaction) (Buwalda et al and Yom-Tov, 2014) cross linked by physical interactions (e.g. cyclodextrines) (Abebe et al, 2012). This advance in hydrogel's science was rapidly prompts increment the enthusiasm for the development of the"smart hydrogels", polymeric networks with a wide range of tuneable properties and trigger stimuli. The theme was hypothetically boundless and the conceivable applications for example, sensors (Chung et al, 2008).

Since the spearheading work of Wichterle and Lim in 1960 on cross-connected hydrogels, and in light of their hydrophilic character and potential to be biocompatible, hydrogels had been of extraordinary enthusiasm to biomaterial researchers for a long time (Kirakci et al, 2014) (Kono and Teshirogi, 2015). The imperative and compelling work of Lim and Sun in 1980 exhibited the effective utilization of calcium alginate microcapsules for cell epitome. Later in the 1980s, Yannas and collaborators consolidated normal polymers, for example, collagen and shark ligament into hydrogels for use as wound dressings. Hydrogels in light of both regular and manufactured polymers had kept on being of enthusiasm for embodiment of cells (Yannas et al, 1989) and most as of late such hydrogels had turned out to be particularly alluring to the new field of "tissue building" as networks for repairing and recovering a wide assortment of tissues and organs (Sefton, 2000).

In today’s era of research hydrogel is in much consideration to be used as intelligent packaging in food industries to check the quality of the food products and increasing the self-life of those food products (Maciel et al, 2012). In today’s era of research hydrogel can be synthesised by two different methods; physical and chemical. Physically cross-connected hydrogel is because of the absence of cross-linkers which was used for synthesis process. One main drawback of physical cross-linked hydrogel is that its mechanical strength is not so good (Ahmed, 2015). Table 1 gave some examples of physically cross-linked hydrogels polymer; method type and loaded drug are given in each case.

Table 2.1 Physically cross-linked hydrogels examples:

S No.	Polymer	Method type	Loaded drug	Reference
1.	PEG and PBT	Melt polycondensation of PEG and PBT	Lysozyme	Bezemer et al, 2000
2.	Pullulan	Hydrogel nanoparticles	Adriamycin	Akiyoshi et al, 1996
3.	Polyacrylamide	Antigen-Antibody Interaction	IgG	Miyata et al, 1999

The different methods to synthesize physically cross-linked hydrogels; (a) by hydrogen bonds, (b) from amphiphilic graft and block polymers (polymer of -PLA and PEG or PBT and PEG, hydrophobized polysaccharides), (c) crosslinking by crystallization in homopolymer systems and by stereo complex formation, (d) crosslinking by ionic interactions (e) crosslinking by protein interactions like; genetically engineered proteins using, and by antigen-antibody interactions (Miyata et al, 1999).

In chemical cross-linked hydrogel cross linkers are used for its synthesis. Chemically cross-linked hydrogel in current era has increased interest due to good mechanical strength. Table 2 gave some examples of chemically cross-linked hydrogels polymer, method type and drugs loaded in each case (Ahmed, 2015).

Table 2.2 Chemically cross-linked hydrogels examples:

S No.	Polymer	Method type	Loaded drug	Reference
1.	Chitosan-PVA	Crosslinking with aldehyde	Nano-insulin	Zu et al, 2012
2.	Chitosan	Crosslinking with aldehyde	Mitoxantrone	Jamella and Jayakrishnan, 1995
3.	PVA	Condensation reaction	Diltiazem hydrochloride	Ray et al, 2010
4.	Gelatin	Crosslinking with aldehyde	Lysozyme	Kuijpers et al, 2000

Following were the different methods to synthesize chemically cross-linked hydrogels. (a) crosslinking by free radical polymerization, (b) crosslinking by high radiation, (c) crosslinking utilizing proteins, (d) crosslinking by complementary groups by reactions like; crosslinking with aldehyde, by addition reactions, by condensation reactions (Akhtar et al, 2016).

Crosslinking with glutaraldehyde

Hydrophilic polymers having –OH groups e.g. polyvinyl alcohol (PVA); may be cross-linked through glutaraldehyde (Zu et al, 2012). To set up cross-connecting, tight conditions are enforced (low pH, methanol included as a quencher, high temperature). On the other hand, polymers having amine groups may get cross-connected by utilizing same cross linker under mild conditions in which Schiff bases are framed**.** It was specially designed for the cross-linked protein synthesis for example, gelatin and albumin (Akhtar et al, 2016).

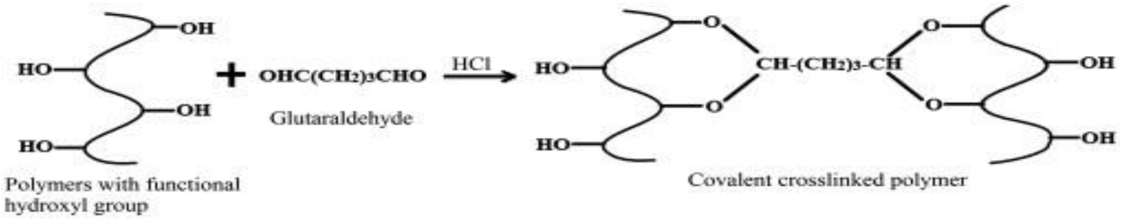


Figure 2.6Synthesis of hydrogel by crosslinking method with Glutaraldehyde (Zu et al, 2012).

According to Amalraj et al (2017) hydrogel had various functional properties namely; swelling (little change in biological and ecological conditions cause’s changes in hydrogel), Mechanical (this property of hydrogel tell about the strength of the hydrogel i.e. how much weight it can retain), biocompatible (this property checks whether the hydrogel is compatible to the host or not; for e.g. in case of controlled drug delivery), and environment responsive.

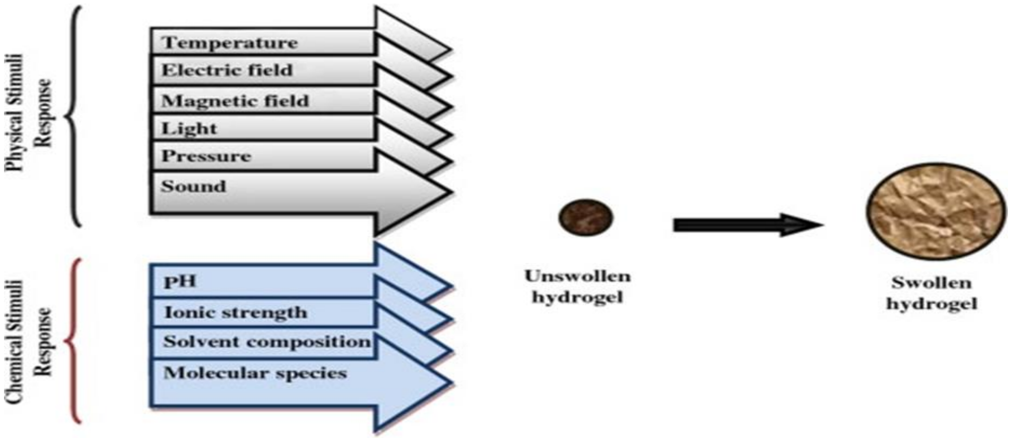


Figure 2.7 Stimuli responsive hydrogel (Ahmed, 2015)

In light of all the above properties, hydrogels had many general applications like in Domestic uses (e.g. Diapers, Watering dabs for plants), Medicine and human services (e.g. Wound dressing, controlled drug delivery) and environmental. One of the vital application which was excessively utilized as sensor to check for temperature and as needs to check the item quality and time span of the item usability can be increased (Li et al, 1996).

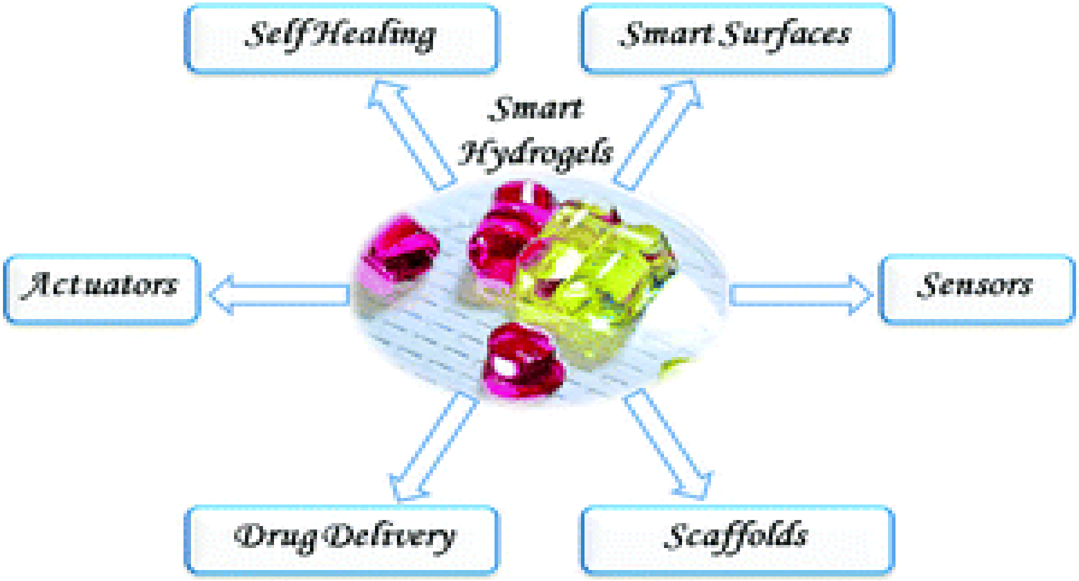


Figure 2.8 Applications of hydrogel (Nho et al, 2014).

2.4 Hydrogel used as sensors

At the point when hydrogel is used as a part of detecting, typically one of the three fundamental attributes is exploited: (a) their semi wet and dormant structure that predetermines hydrogel as host-system, (b) intensification impact of their affectability on sub-atomic level that is converted into microscopic impacts like; change in swelling degree, and (c) capacity to control the dispersion conduct of particles through the polymer framework (El-Sherif et al, 2010).

Kuswandi et al (2011) told that over several decades' chemical sensors and biosensors results in novel and interesting devices which were used in various applications including food technology. They incorporated sensors into food packaging technology and resulted as what today called smart or intelligent packaging. This smart packaging technology used to make variety of sensors or biosensors to monitor the food quality and safety of food from producers to consumers such as freshness, CO_2, leakage, oxygen, pH, pathogens, degradation of food with respect to time or temperature. So, this innovation made a great potential in the development of new sensing systems which gets integrated into the food packaging. Many studies have been done to use hydrogel as sensors. Maciel et al (2012) built up a biodegradable temperature indicator material which was created in light of characteristic and warmth touchy colour (anthocyanin-ATH). They fused ATH into chitosan film that was made on card paper system (CH-Sys). They observed that CH-Sys changed colour from light violet to light yellow when kept at different temperature (40-70^{o}C). They demonstrated that, TTI system had the potential applications in different domains like in food technology to check the quality and safety of food products (Yoshida et al, 2014). So, it is a powerful and choice temperature indicator which was used for packaging material and gained perspective of biodegradable and safe materials (chitosan, card paper and ATH). So, the indicator related with intelligent packaging indicates huge possibility for safe and quality nourishment of products (Maciel et al, 2012).

Whereas Yan et al (2008) portrayed temperature is a very important factor which affect the quality and safety of food items for both distribution and storage. They observed that TTI sensor which gave a visual synopsis of products which accumulated in history of chill-chain and recorded the effects of both temperature and time (Nopwinyuwong et al, 2010). So, they developed a prototype TTI which was based on the process called “diffusion” of lactic acid with temperature. They made four lactic acid based TTI sensor with different concentration of substrate and observed the colour change which was due to the pH reduction (Yan et al, 2008). So, these TTI sensors considered a good candidate to monitor the quality and shelf life of food with different time-temperature exposure (Kerry et al, 2006). Futher, Marek et al (2013) made TTI sensor which was based on oxidation of leuco methylene blue (LMB) to MB. The sensor works as it changes the colour from yellow to green in presence of oxygen and was incorporated into polyvinyl alcohol matrix. Oxygen gets diffused into the polymeric matrix and gets uptake due to oxidation reaction which decides the time observing of the sensor. So, on this observation they created a physical model which accounts for both diffusion and oxidation reaction. For this reason they experimentally determined the reaction kinetics, diffusion coefficient of oxygen and concentration profiles in polymer matrix modelled. In view of these modelling, this TTI sensor could be calibrated exactly and used widely in application such as, for monitoring the freshness of consumer goods.

On the other hand, Wu et al (2013) developed TTI sensor which was based on chemical reaction between urease and carbamide and they explored the discoloration kinetics of this urease based TTI sensor. By using mathematics formula they revealed the relationships of colour changes of TTI sensor with time and temperature and established this sensor. They concede that this type of TTI sensor had the potential to be applied in some time temperature dependent foods. However, Puligundla et al (2012) developed different types of CO_2 sensor (optical sensors, polymer films, polymer hydrogels etc.) in demand for the better quality of food products. They studied that occurrence of CO_2 gas indicated the spoilage of food in packed foods. So, they incorporated this CO_2 sensor into the package of food and monitored the product quality until it reached the customer.

Hsiaoand Chang (2016) developed microbial TTI sensor for vacuum packed fish fillets and test this sensor for change in the biochemical and microbiological reactions in fish fillets. So, under static and dynamic storage conditions they noticed colour change and based on total volatile basic nitrogen (TVB-N) levels they gave three grades to fish freshness (very fresh, fresh and spoiled). This TVB-N made this microbial TTI as a good indicator for the freshness of fish products. This TTI is important when fish microbiological and TVB-N analyses do not match well.

Further, Bento et al (2015) demonstrated that pH indicator in smart packaging had the potential use which allowed the consumer to evaluate quality of food. They developed a film based on PVA, chitosan, gelatin and red cabbage extract to monitor the food quality. Film was characterized and was tested with Ricotta cheese to evaluate the potential use for spoilage of food.

Chapter 3

MATERIALS and METHOD

3.1 Chemicals and Reagents

All the chemicals and reagents used were of highest analytical grade and were ordered from Sigma Aldrich (St. Louis, MO, USA) and Himedia (Mumbai, India). In order to screen the extracellular amphiphilic biopolymer (EAB) producing microbial strains Luria broth (LB), Luria agar (LA) medium and extracellular amphiphilic biopolymer (EAB) medium were used. The composition of EBP media was in grams per litre of distilled water; peptone (5.0), diammonium sulphate (2.0), yeast extract powder (1.0), $CaCl_2.2H_2O$ (0.7), NaCl (0.1), $MgSO_4.7H_2O$ (0.2), K_2HPO_4 (1.0), Dextrose (1.0), pH 7.0 ± 0.2.

3.2 Sample collection

Amphiphilic biopolymers producing microbial strains were screened from pre-existing microbial library comprising of biopolymer producing bacteria (Thapar University, Patalia). A total of 10 isolates were revived in Luria broth (LB) media. Purity of isolates was checked by streaking on the Luria agar (LA) plate and glycerol stocks were made and stored at - 20^{o} C for further analysis and gram straining. All cultures were grown thrice prior to initiation of experiments.

3.3 Screening of EAB producing bacterial strains

Overnight grown cultures of revived strains were used as an inoculum for EAB media. The inoculated media was incubated at 37°C for 24 hour. The culture broth of EAB media was used for the screening of extracellular amphiphilic biopolymers producing microbial strains. Five different screening experiments were performed in duplicates and the mean values were reported.

a) CTAB (Cetyltrimethylammonium bromide) agar or Blue plate method

The CTAB agar plate method is a quantitative assay for the identification of anionic surfactants. Plates were prepared by addition of 0.1g cetyltrimethylammonium bromide

(CTAB), 0.0025g methylene blue (MB), 7.5 g agar and 10 g glycerol as a substrate to 500 ml of EBP media (Siegmund, and Wagner, 1991). Wells were cut in the agar plates and 20 µl of the inoculum was introduced. Then the incubation for 48 hour at 37°C was given and later stored at 4^{o}C for 24 hour. After that the plates were observed for presence of dark blue halos surrounding the colonies (April et al, 1999).

b) BATH (Bacterial adhesion to hydrocarbon) test or cell surface hydrophobicity Test

The method of Rosenberg et al (1984) was used to understand cell hydrophobicity measured by bacterial adherence to hydrocarbons. Cell pellets of overnight grown culture were used for the test. The pellets were washed, suspended in a buffer solution (K_2HPO_4-16.9 gL^{-1} and KH_2PO_4-7.3 gL^{-1}) and were diluted to obtained an optical density (OD) of ~ 0.5 at 610 nm. To 2 ml of cell suspension in test tubes, 100 µl of diesel oil were added and vortexed for 3 min. Then the test tubes were kept undisturbed at room temperature for 2 hour for separation of unrefined petroleum and aqueous phases. The optical density of the aqueous phase was then measured at 610 nm in a spectrophotometer (UV1800, Shimadzu). Using the following formula, rate of cells attached to unrefined petroleum was calculated.

$$\% \text{ of bacterial cell adherence} = (1-(OD_A / OD_B)) \times 100$$

Where: OD_A was the absorbance of the cell suspension after addition of oil

OD_B is the absorbance of cell suspension (set as 0.5)

c) Emulsification stability (E_{24}) test

E_{24} test was performed as described by Thavasi et al, (2011). Test was conducted using five different solvents (Petrol and Diesel (Dhillion oil pump, Bhadson Street, Patiala), Xylene (Himedia, Mumbai, India), Kerosene (Depo, Tripuri, Patiala) and mustard oil (Patanjali, India). To each 2ml oil, equal amount of culture supernatant was added and vortexed for 2 min and allowed to stand at room temperature overnight. The E_{24} index was observed as the height of emulsified layer (mm) by the total height of the liquid column (mm).

$$E_{24} = \frac{\text{height of emulsified layer}}{\text{total height}} \times 100$$

d) Drop collapse test

Drop collapse test (Shahaliyan et al, 2015) was performed by adding two microliter of diesel to the 96 well micro titre plates. The plates were equilibrated at 37 ºC for 1 hour. After that five microliter of culture supernatant were added onto the surface of the diesel oil. The shape of drop on the surface of oil was observed after 1 min for flat and deep shape. SDS (Sodium dodecyl sulfate) was used as positive control.

e) Hemolytic test

A hemolytic test was performed by using human blood (5% v/v) agar petri dish. The bacterial culture was streaked on blood agar perti dish (sheep blood 37 g/l, blood 5-10% (v/v) and pH 7.4±0.2) and incubation was given for 48 hour at 37ºC. All the petri dishes were visually examined for a clear zone around the colony (Rodrigues et al, 2010).

3.4 Characterization of extracellular amphiphilic biopolymer producing bacterial strains

a) Methyl Red Test

Overnight grown cultures were inoculated with MR-VP (methyl red and vogesproskauer) broth under sterile conditions and incubated at 37ºC for 24 hour. After incubation few drops of methyl red indicator were added and presence or absence of red colour was observed (Barry et al, 1970).

b) Voges-Proskauer Test

24 hour grown cultures were inoculated with MR-VP (methyl red and vogesproskauer) broth under sterile conditions and incubated overnight at 37ºC. After incubation few drops of Barrett’s reagent (mixture of alcoholic α-naphthol and 40% potassium hydroxide solution)

were added. Then the presence and absence of a deep rose colour in the culture was observed (Werkman, 1930).

c) Citrate Utilization Test

Cultures were inoculated by streak inoculation on the Simon citrate agar (ammonium di-hydrogen phosphate 0.5 g, di-potassium phosphate 0.5 g, sodium chloride 2.5 g, sodium citrate 1.0g, magnesium sulphate 0.1g, agar 7.5g, bromothymol blue 0.04g and distilled water 500 ml (pH 6.6±0.2)) slants under proper sterile conditions and incubated at 37ºC for 24 hour. The presence and absence of growth on the surface of the slant along with blue colour was observed (Koser, 1924).

d) Indole Production Test

Sulphide indole motility medium (SIM) agar (peptone 15g, beef extract 1.5g, ferrous ammonium sulphate 0.1g sodium thiosulfate 0.0125g, agar 1.5g and distilled water 500 ml pH (7.3±0.2))tubes were inoculated with overnight grown cultures with stab inoculation and incubated at 37ºC for 24 hour. After incubation few drops of Kovac’s reagent (consists of p-dimethylaminobenzaldehyde, butanol and hydrochloric acid) were added. The culture was observed for presence and absence of red layer on surface (Rahman et al, 2002).

e) Starch Hydrolysis Test

Starch agar (peptone 2.5g, beef extract 1.5g, starch 1.0g, agar 7.5g and distilled water 500ml pH (7.2±0.1)) plates were made for inoculation and divided into small sections. By spot inoculation method the plates were inoculated with culture broth and incubated at 37ºC for 24 hour. After that the few drops of Gram’s iodine solution was dropped onto the plates for 30 sec. Then the plates inoculated with cultures were observed for the presence and absence of the blue-black colour colony formation by the growth of each culture (Dhail&Jasuja, 2012).

f) Hydrogen Sulphide Test

Sulphide indole motility medium (SIM) agar tubes were made and inoculated with overnight grown cultures via stab inoculation. Then the tubes were incubated at 37ºC for 24 hour. The strains were observed for presence of black coloration along the line of the stab inoculation. The non-inoculated tube was taken as negative control (Barry et al, 1970).

3.5 Extraction of extracellular amphiphilic Biopolymer

Extraction of extracellular amphiphilic biopolymer was carried out by growing cultures in EBP media. Overnight grown culture was harvested and concentrated to 1/10th of its original volume. Equal amount of ethanol was added to precipitate the amphiphilic biogenic polymer. The pellet obtained was washed using deionized water. CPC (cetylpyridinium chloride) and dialysis treatment for purification were given. The amphiphilic biopolymer obtained was stored in powdered form after lyophilization (Ghosh et al, 2009).

3.6 Characterization of extracellular amphiphilic Biopolymer

a) Fourier transforms infrared spectroscopy (FTIR)

FTIR analysed the presence of functional groups in the extracellular amphiphilic biopolymer. 1 mg purified amphiphilicbiogpolymer was mixed with 100 mg of KBr (potassium bromide) which was used for background reference (Jeong et al, 2007) and pressed with 7500 kg for 30 sec to obtain proper translucent pellets. Infrared ray spectra were recorded with the wave number of 4 and 0.01 cm^{-1} (Spectrum RX-IFTIR, Perkin-EImer).

b) Scanning electron microscopy (SEM)

SEM was utilized to analyse the structure of extracellular amphiphilic biopolymer (EAB) on SEM system (JSM541-V, JOEL, Japan). EAB-SK1 sample was analysed, using an acceleration voltage of 10 kV and magnification ranging from 100 to 1000 fold (Mao et al, 2010).

c) X-ray diffraction (XRD)

XRD patterns was recorded on (Diffractrometer system= XPERT-PRO, Japan) for powdered form of EAB-SA1 sample. The 40 kV voltage and 30 mA current was applied with CuKα radition. The sample was scanned from 1.4 to 50^{o} at 2θ (Mauad et al, 2010).

3.7 Preparation of hydrogel

Three different hydrogel (PVA, chitosan and EABSA-1) solutions were prepared. Solution of PVA was prepared by dissolving 5.0 g of PVA powder in 100 ml of Milli-Q water, under constant stirring at 75 ± 2^{o}C (solution A). pH was maintained at 2.00±0.005 with 1.0 M HCl of cooled solution A. 2.5 g of chitosan was dissolved in 250.0 ml of Milli-Q water with acetic acid (2%), under constant stirring for 3 days (solution B). EAB solution was prepared by dissolving 1.25g of extracellular amphiphilic biopolymer in 250 ml of distilled water for 48 hour (solution C) (De Souza et al, 2009).

Different concentrations of solution A, B and C were added with PVA and pH was adjusted to 4.00 ± 0.05 with 1.0M NaOH solution. The mixture was kept on magnetic stirrer for 5 min and cross-linker reagent glutaraldehyde (GA) was added slowly. The final concentration of GA in hydrogel solution precursors was 1% (wt/wt). Further, the mixture of three different solutions were poured into test tube and dried for 72 hour at room temperature(De Souza et al, 2009).

3.6 Characterization of hydrogel

a) Scanning electron microscopy (SEM)

The structure of hydrogel was analysed by SEM (JSM541-V, JOEL, Japan). The microscope was coupled to an energy dispersive spectrometer (EDS). Before the observation of SEM samples, hydrogel were cut into small pieces and fixed with 1% cold GA solution at 4^{o}C for 2 hour. Then the hydrogel solution was frozen and lyophilized. The hydrogel was placed in a minivice for the examination of cross sectional structure of hydrogel. The samples were metallized to make them conductive and was analysed with 15 kV voltages (Mathews et al, 2008).

b) Fourier transforms infrared spectroscopy (FTIR)

FTIR was used to characterize the presence of specific chemical groups in the hydrogel. Hydrogel blends cross-linked with GA (PVA/Chi/GA), (PVA/GA), (EABSA-1/PVA/GA) were obtained as fine powdered form and analysed through FTIR technique using attenuated total reflection (ATR) modes. Spectra of FTIR were obtained in the wavenumber range of 4,000 to 650 cm^{-1} during 64 scans, with 2 cm^{-1} resolution (Spectrum RX-IFTIR, Perkin-EImer). The FTIR spectra's were obtained and major vibration bands associated with main chemical groups were observed (Mansur et al, 2008).

c) XRD (X- ray diffraction)

Wide-edge X-beam powder diffraction profiles were recorded at room temperature, with a (Diffractrometer system= XPERT-PRO, Japan) CuKα radiation generated at 40 kV and 40 mA; the range of diffraction angle was 10.00 to 70.00 at 2θ (Pal et al, 2007).

3.7 Swelling behaviour

Dried hydrogel were weighed and soaked in distilled water. At regular time interval the swollen hydrogel was removed from the distilled water, dried with the help of filter paper and weighed, then again placed in the same bath. The swelling percentage was calculated by;

$$\text{Swelling \%} = \frac{\mathrm{Ws}-\mathrm{Wd}}{\mathrm{Wd}} \times 100$$

Where, Ws is the swollen weight of hydrogel and W_d is the dry weight of hydrogel (Parida et al, 2011).

3.9 Preparation of lactic-acid based TTI sensor

Two dyes (bromothymol blue and methyl red) were used to set up a colour blend (Nopwinyuwong et al, 2010). This indicator solution was prepared by mixing bromothymol blue (1% v/v) and methyl red (0.1% w/v) in ethanol (50% v/v) respectively. Both solutions were mixed in 2:3 ratios, and tested with a solution of lactic acid as pH indicator which was observed with naked eye (yellowish colour) (Wanihsuksombat, 2010).

After that the dye mixture was mixed with PVA, Chitosan/PVA, and PVA/EAB hydrogel in 1:1 ratios. Then the solutions were kept on magnetic stirrer for 5 min to form homogenous mixture. Approximately 20 ml solution of each mixture was poured into petri plate (90 mm diameters) to form solid gel. To activate TTI sensor 5.0 ml of 1 M lactic acid was dropped on filter paper and placed in the cover glass of petri plate. This cover glass was then immediately placed onto the petri plate and kept at different temperature for different time intervals. The colour change at different temperature with different time was observed (Wanihsuksombat, 2010.

Chapter 4

RESULTS and DISCUSSION

4.1 Screening of extracellular amphiphilic biopolymer (EAB) producing bacterial strains

EAB production ability of given bacterial strains was confirmed by different screening methods namely; CTAB agar plate method, drop collapse test, haemolytic technique, BATH (bacterial adhesion to hydrocarbons) test and emulsification index technique.

CTAB method was used to detect anionic extracellular amphiphilic biopolymers (Siegmund& Wagner, 1991). Results of this method in Table 4.1 depicts that ten bacterial strains (SA1, SA2, SA3, SA4, SA5, SA6, SA7, SA8 and SA9 and SA10) examined two bacterial strains (SA1, SA5) were positive and rest of the bacterial strains showed negative response. The positive result is indicated by blue colonies around the well whereas lack of blue coloration around well made in the agar plat, indicates negative results. Sodium dodecyl sulphate (SDS) was used as positive and water used as negative control for this test respectively. Since this test is specific for some anionic extracellular amphiphilic biopolymers, another screening method was opted for better result (i.e. hemolytic test).

Hemolytic assay is generally performed to screen extracellular amphiphilic biopolymer producing bacteria. This assay yields superior results in many cases (Banat, 1993). Result of the hemolytic test indicated that out of ten bacterial strains, two strains (SA1 and SA5) showed positive results and other bacterial strains were found partially haemolytic or non-haemolytic (Table 4.1). It has been suggested that blood agar lysis as a preparatory screening strategy for extracellular amphiphilic biopolymer generation (Mulligan et al, 1984).

A hemolytic assay was a qualitative assay for the evaluation of the amphiphilic biogenic polymer production on the blood agar plate which indicated the hemolytic activity of SA1 and SA5 bacterial isolate. Clear zone around the colonies on blood agar plate introduced these bacterial isolates as an amphiphilic biogenic polymer producer, while less defined zone around the colonies demonstrated lower hemolysis activity in SA6 and SA9 bacterial isolates. In other words, SA1 and SA5 isolates were capable of lysing red blood cells producing clearance in the agar medium (Satpute et al, 2010). Youssef et al (2004) concluded that extracellular amphiphilic biopolymers and extracellular enzymes had remarkable performance on lysis of blood cells. The blood agar medium is a complex medium and poresconstraints in extracellular amphiphilic biopolymer production particularly when microorganisms are studied directly on blood agar plate. To avoid anomalies, the SA1 and SA2 bacterial isolates were streaked onto blood agar plate to visualize haemolysin. These isolates had the capability to promote emulsification of hydrocarbons with the release of lipopolysaccharides responsible for the hydrophobicity of bacterial cell surface (Al-Tahhan et al, 2000).

Drop collapse test had been reported by several scientists (Youssef et al, 2004) for screening of extracellular amphiphilic biopolymer (low molecular weight). This test was carried out using petrol, diesel, kerosene and xylene. The result of this test indicated that out of ten bacterial strains, four strains (SA1, SA5, SA9 and SA10) were positive and showed flat drop after 1 min. With strains SA4, SA9, SA7 and SA2 lesser flat drop while SA6 and SA3 strains showed least flat drop as compared to negative control (distilled H2O). Table 4.1 showed the results of this test.

In drop collapse test the amount of oil droplets dispersed in the test is reported from (+) very less flat drop to (+++) flat drop in which SA1, SA5, SA9 and SA10 bacterial isolates received a score of (+++). Extracellular amphiphilic biopolymers are found to form a layer on the oil surface causing the suspensions of SA1, SA5, SA9 and SA10 bacterial isolates to degrade immediately. It is generally accepted that drops of culture which gets flat rapidly and formed circle, indicate high concentration of extracellular amphiphilic biopolymer. Isolate SA6 and

SA3 did not form flat drop immediately so these bacterial culture can attribute to hydrophobicity of oil density and droplet gets accumulated. However the test has been shown to be inconclusive in many cases, therefore based on the results obtained SA6 and SA3 cannot be designated as non-produced (Shahaliyan et al, 2015).

BATH test was conducted in this study with 10 bacterial strains out of which SA5 showed highest adhesion to diesel i.e. 45% and SA1 showed 40% adhesion to hydrocarbon. Other strains showed very less or no adhesion to diesel oil. Results of all the 10 bacterial strains are depicted in Table 4.1. In this test cells were removed from aqueous phase which depended on the adhesion of the hydrocarbon phase. So, it is a sensitive method to check the surface area of two liquid phases of diesel droplets in aqueous phase. The formation of droplets gets influenced by the conditions of mixing, type of vessel in which mixing was done etc. Thus a drawback of this method is that small diesel droplets were formed which was stabilized by bacteria and did not leave the water phase. This could be possibly affecting the adhesion of the bacterial cells.

Rosenberg et al (1984) observed the hydrophobicity of *Pseudomonas* sp. with hexadecane to be 60%. In the current study the maximum hydrophobicity was found to be 45% for isolate SA5.

Table 4.1 Screening of EAB producing bacterial strains

S No.	Bacterial Strain	CTAB Assay	Haemolysis Assay	Drop collapse Assay	BATH Assay
1.	**SA1**	++	++	+++	+++
2.	**SA2**	-	-	++	-
3.	**SA3**	-	-	+	++
4.	**SA4**	-	-	++	+++
5.	**SA5**	++	++	+++	++
6.	**SA6**	-	+	+	-
7.	**SA7**	-	-	++	+
8.	**SA8**	-	-	++	-
9.	**SA9**	-	+	+++	+
10.	**SA10**	-	-	+++	++
11.	**SDS (+control)**	+++		+++	
12.	**Water (-control)**	-		-	

CTAB assay: ‘++’ blue zones with > 0.4 cm, ‘+’ blue zones with 0.4 cm
Haemolytic assay: ‘++’ haemolytic, ‘+’ partially haemolytic, ‘_’ non- haemolytic
Drop collapse assay: ‘+++’ flat drop after 1 min, ‘++’ less flat drop after 1 min, ‘+’ little flat drop as compared to –ve control
BATH assay: ‘+++’ cell adhesion > 45%, ‘++’ cell adhesion > 15%, ‘+’ cell adhesion > 0.6%

By inoculating the bacterial strains into EAB medium was used to check the maximum production of extracellular amphiphilic biopolymers by emulsification stability or emulsification index test. This test was performed with different kinds of oils (e.g. petrol, diesel, kerosene, xylene and mustard oil). Result of this test demostrated that out of ten bacterial strains, only four bacterial strains showed positive result against four hydrocarbons (petrol, diesel, kerosene and xylene), and the response of six bacterial strains were not found significant. Howerver the bacterial strains with mustard oil showed no emulsification stability (Table 4.2). Emulsification index of SA1 with petrol, diesel, kerosene and xylene was 61.5, 60, 25 and 30 respectively; whereas emulsification index of SA5 and SA9 was 35, 40, 25, 30 and 50, 60 respectively. The emulsification of petrol, diesel, kerosene and xylene by bacterial culture was determined through visual observation. It was mentioned that bacterial culture produced bubbles and emulsified all the crude oils in aqueous phase of medium. After that tiny droplet of crude oils were emulsified after overnight incubation. All the bacterial strains showed no variation with mustard oil. When the hydrophobic compounds (petrol, diesel, kerosene and xylene) mixed with bacterial culture broth, stable and static emulsion layer was formed that indicated the presence of extracellular amphiphilic biopolymer. The polymer creates emulsion between two immiscible liquids as reported by Makkar and Cameotra (2002).

The present study suggested that bacterial isolate SA1 had higher capability for the formation of emulsion layer with petrol, diesel, kerosene and xylene which suggested that bacterial isolate SA1 could produce extracellular amphiphilic biopolymer. Release of extracellular polymer decreases the surface tension and the bacterial culture absorbed and metabolised the hydrocarbons used in present experiment and formed emulsification (Chukwudi, 2010).

Table 4.2 Emulsification Index of extracellular amphiphilic biopolymer producing bacterial strains towards different hydrocarbons.

S No.	**Bacterial Strains**	**Petrol**	**Diesel**	**Xylene**	**Kerosene**	**Mustard Oil**
1.	**SA1**	+++(61.5)	+++(60)	++(30)	++(25)	-
2.	**SA2**	-	-	-	-	-
3.	**SA3**	+(4.5)	+(11)	+(10)	-	-
4.	**SA4**	-	-	+(5)	-	-
5.	**SA5**	++(35)	++(40)	++(30)	++(25)	-
6.	**SA6**	-	++(25)	-	+(10)	-
7.	**SA7**	-	-	+(10)	+(5)	-
8.	**SA8**	-	-	-	+(5)	-
9.	**SA9**	+++(50)	+++(60)	-	-	-
10.	**SA10**	+(15)	+(4.5)	-	-	-
11.	**SDS (+ control)**	+++	+++	+++	+++	+++
12.	**Water (- control)**	-	-	-	-	-

Values are in percentage

Therefore, on the basis of above mentioned screening methods, four potential bacterial strains were selected for maximum extracellular amphiphilic biopolymer production ability and were further characterized.

4.2 Morphological and biochemical characterization of extracellular amphiphilic biopolymers producing bacterial strains

Morphological and biochemical characterization of four bacterial strains were analysed using Bergey's manual of bacteriology. These biochemical characterization tests includes seven tests (Grams strain test, methyl red test, indole production test, Voges-Proskauer (VP) test, citrate utilization test, starch hydrolysis test and hydrogen sulphide test). Out of four bacterial strains three (SA5, SA6, SA9) were found to be methyl red positive, whereas in case of hydrogen sulphide test only SA1 showed positive result. On the other hand SA1 and SA9 showed positive result for both starch hydrolysis and citrate utilization test and only SA5 showed positive result for VP test. Table 4.3 depicts the result of the entire seven characterization test for selected four bacterial strains.

Table 4.3 Biochemical identification of EAB bacterial strains

Name of tests	SA1	SA5	SA6	SA9
Gram Strain	-	-	-	-
VP Test	-	+	-	-
Methyl Red Test	+	+	-	+
Starch Hydrolysis Test	+	-	-	+
Citrate Utilization Test	+	-	-	+
Indole Production Test	-	+	+	-
Hydrogen Sulphide Test	+	-	-	-

Biochemical identification test demonstrated that bacterial isolates SA5, SA6 and SA9 belonged to *Pseudomonadaceae*family and other SA1 bacterial isolate belongs to

Enterobacteriaceae family. However, the cultures further needs to be subject for 16S sequencing for confirmed result. Due to time constraint the result could not be achieved.

4.3 Extracellular amphiphilic biopolymer production

On the basis of screening test for amphiphilic biogenic polymers, four bacterial strains (SA1, SA5, SA6 and SA9) were selected for further studies. These strains were inoculated in the EAB medium, extracted and purified. The yield of SA1, SA5, SA6 and SA9 was found to be 0.248g, 0.148g, 0.125g and 0.212g respectively. The SA1 sample was used for further studies due to their high yield production of EAB.

4.4 Characterization of extracellular amphiphilic biopolymers (EAB)

4.4.1 Scanning electron microscopy (SEM)

The structure morphology of EAB-SA1 was studied using JSM541-V, JOEL scanning electron microscope (Japan). The scanning electron micrograph of EAB-SA1 (Figure 4.1) indicated of large number of grooves and projections implying large surface area. It was also observed that EAB-SA1 sample had a porous and fibriller structure.

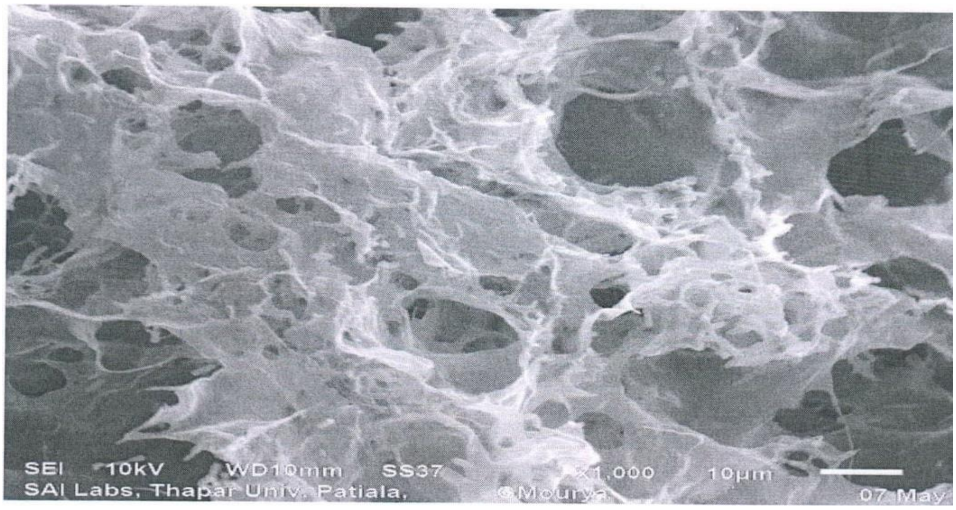


Figure 4.1 Scanning Electron Micrograph of freeze-dried EAB-SA1

4.4.2 XRD (X- ray diffraction)

XRD analysis was used to study the solid state of structural properties like; degree of crystallinity for amorphous, semi-crystalline and composite materials which could be described by the XRD spectra. It was observed from this characterization study that sample EAB-SA1 was amorphous in nature. Only carbon was present in EAB-SA1 in range of 20^o to 35^o (Figure 4.2). Pure diffraction peak of sample EAB-SA1 was at $2\theta=20^o$ (Tripathi et al, 2009).

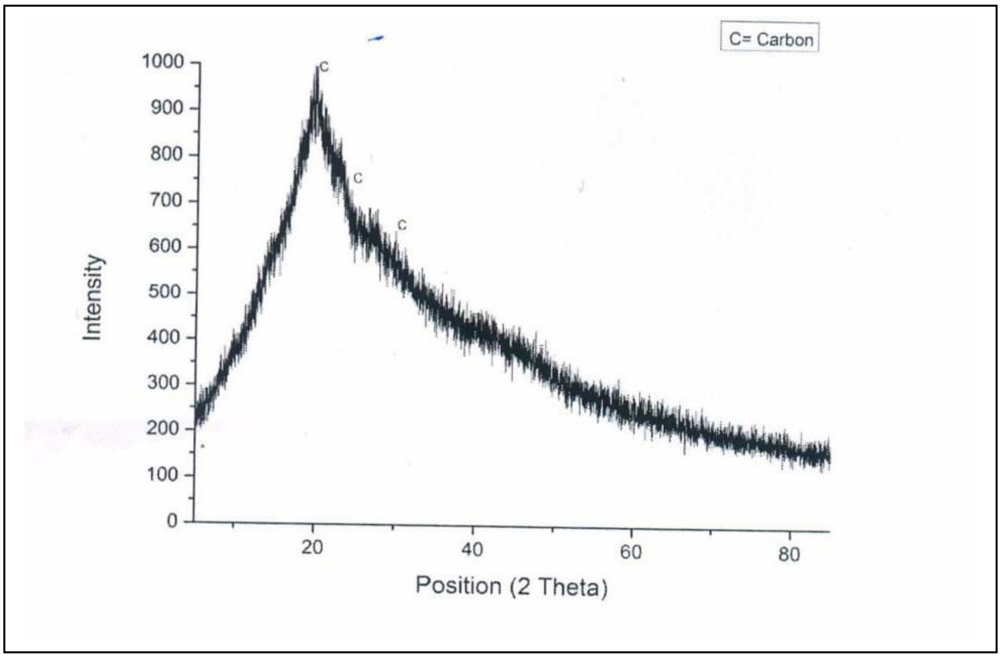


Figure 4.2 XRD spectra of EAB-SA1

4.4.3 FTIR (Fourier transform infrared spectroscopy)

FTIR spectroscopy was used to analyse the functional groups in extracellular amphiphilic biopolymer (EAB). Figure 13 depicts the FTIR spectra of EAB-SA1, which inferred that wave number 3646cm^{-1} indicated the strong and sharp peak which was associated with the O-H stretching from intermolecular and intramolecular hydrogen bonds. A strong C-H stretching of asymmetric vibrations was found at 2981 cm^{-1} and 2888 cm^{-1} which confirmedthe presence of alkanes. Whereas the infrared band at 2359 cm^{-1} was due to C=C stretching, which confirmed the presence of alkynyl. On the other hand the bands at 1715 cm^{-1} and 1071 cm^{-1} correspond to asymmetric stretching vibration of C=O bonds in carboxylic group and stretching vibration of hydroxyl group (O-H) respectively. The doublet band at 1157 cm^{-1} was due the presence of ester sulphate groups. Formation of band at 1379 cm^{-1} and 1250 cm^{-1} showed strong stretching of N-O and C-O functional group respectively. The band at 806 cm^{-1}in the spectrum was due to C-H bending.

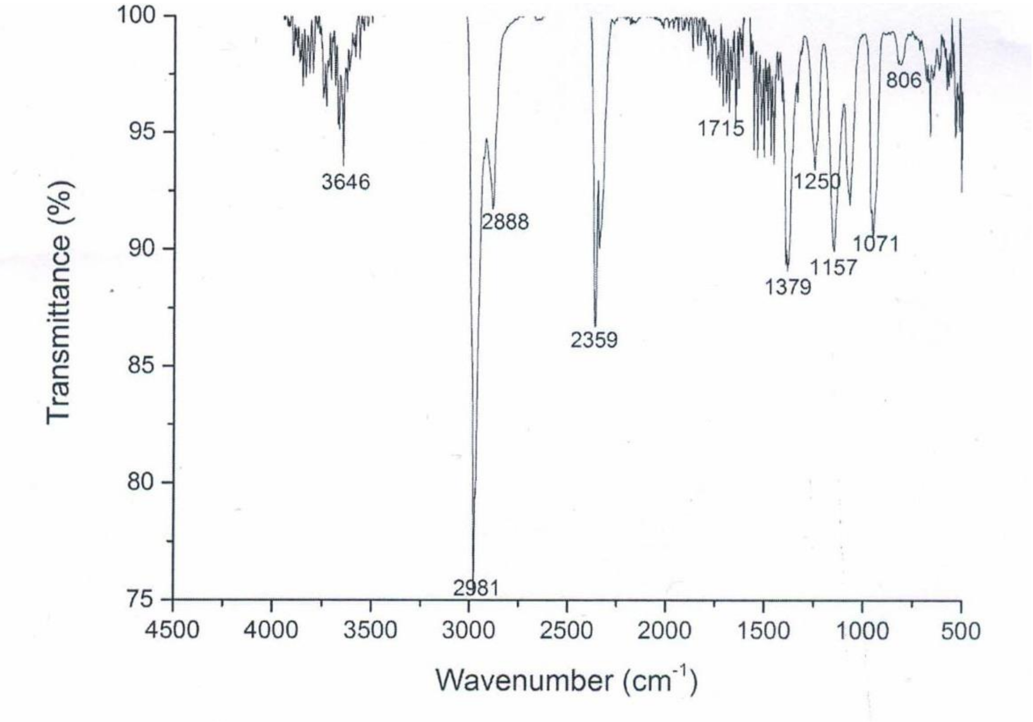
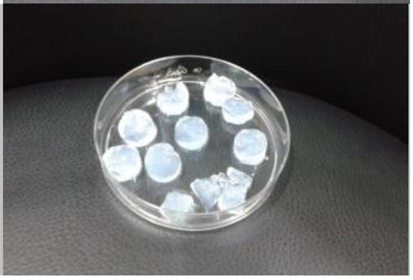


Figure 4.3 FTIR spectra of EAB-SA1

4.5 Preparation of hydrogel

Three different types of hydrogels (PVA; PVA/chitosan and PVA/EAB-SA1) were formulated with different composition and cross-linked with glutaraldehyde (GA), solidified and cut into small pieces as shown in Table 4.4.

Table 4.4 PVA, PVA/Chitosan and PVA/EAB-SA1 hydrogel with different composition

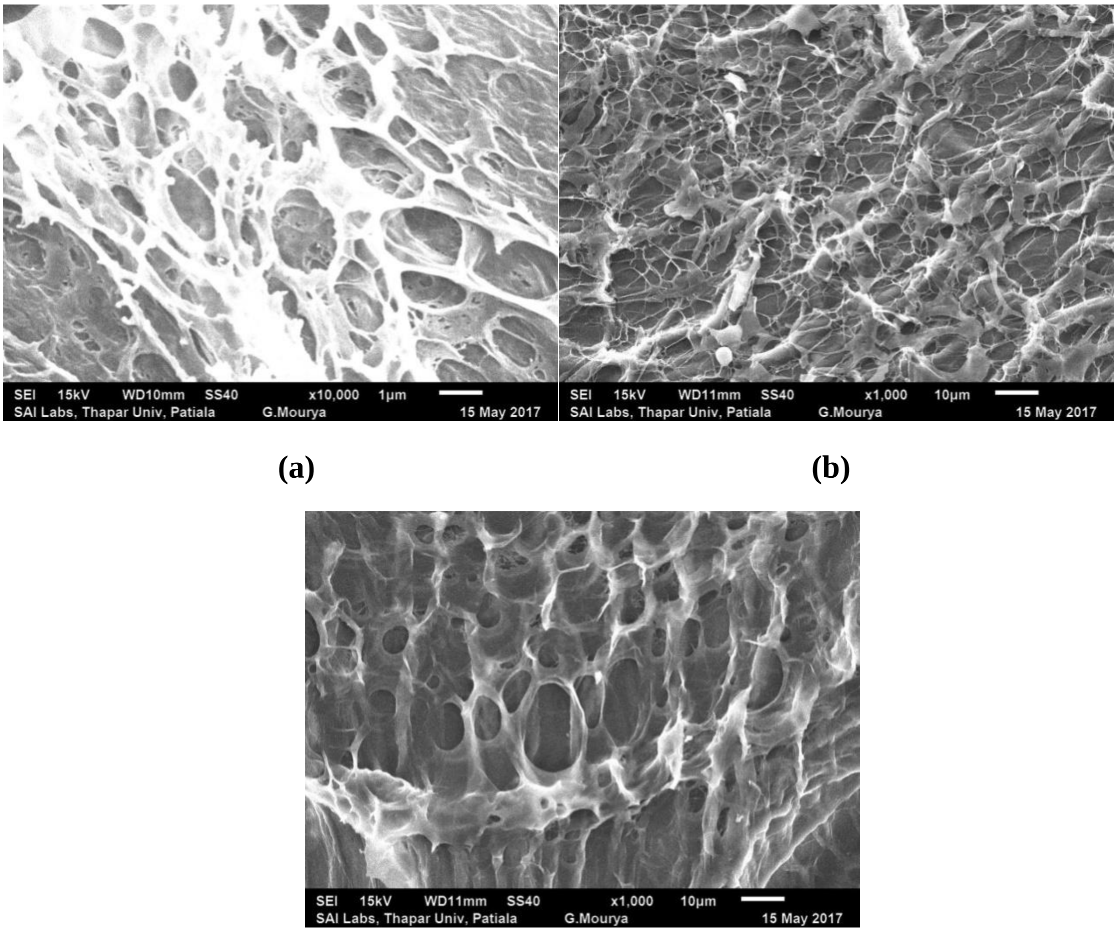
Hydrogel	Ratio	Composites
PVA	1:0	
PVA/chitosan	1:1	
PVA/EAB-SA1	1:1	

The hydrogel film was clear and no heterogeneity was observed regarding to solubility and phase segregation. All the three different hydrogel blends were visually inspected as transparent, highly uniform yellowish transparent and uniform white transparent gel respectively. In the previous study the hydrogel was made with different concentration of PVA/chitosan (Mathews et al, 2008) but in present study the three different types of hydrogel were made with concentration of 1:0, 1:1 and 1:1 respectively.

4.6 Characterization of hydrogel

4.6.1 Scanning electron microscopy (SEM)

The structure morphology of different types of freeze-dried hydrogels was examined by scanning electron microscopy. This was important to compare the difference between the pore sizes of PVA; PVA/chitosan and PVA/EAB-SA1 hydrogel blends with GA. Figure 4.4 described the electron micrograph of different three hydrogels.



(a) **(b)**

(c)

Figure 4.4 Scanning electron micrographs of freeze-dried (a) PVA, (b) PVA/chitosan and (c) PVA/EAB-SA1 hydrogels.

Macro porous hydrogel blends based on PVA with chitosan and EAB-SA1 polymer were investigated with the PVA hydrogel. 5% PVA hydrogel lacking chitosan and EAB-SA1 polymer was also examined. The effect of PVA blending with chitosan and EAB-SA1 polymer on structure and pore distribution has been reported by Mathews et al (2008). The SEM images of PVA, chitosan/PVAand EAB-SA1/PVApolymer provided high-magnification micrograph of three different hydrogels. A hydrogel membrane appeared porous with different composition and the material used for each freeze-dried hydrogel.

Figure 4.4 Showed porous structure morphology of PVA hydrogel and suggested a homogeneous structure. But when chitosan and EBA-SA1 polymer were added to the PVA to form a PVA/chitosan and PVA/EAB-SA1 polymer blends respectively (Fig (b) and Fig (c)), significant change appeared in the porous structure of both hydrogels.

Significant change ($p<0.05$) in the average pore size of PVA, PVA/chitosan and PVA/EAB-SA1 polymer hydrogels 96.1, 89.34 and 82.64 respectively was observed (Fig 4.5), also see Table 4.5. Besides, there was decrease in the average pore size of PVA/chitosan and PVA/EAB-SA1 polymer membrane in comparison to PVA membrane.

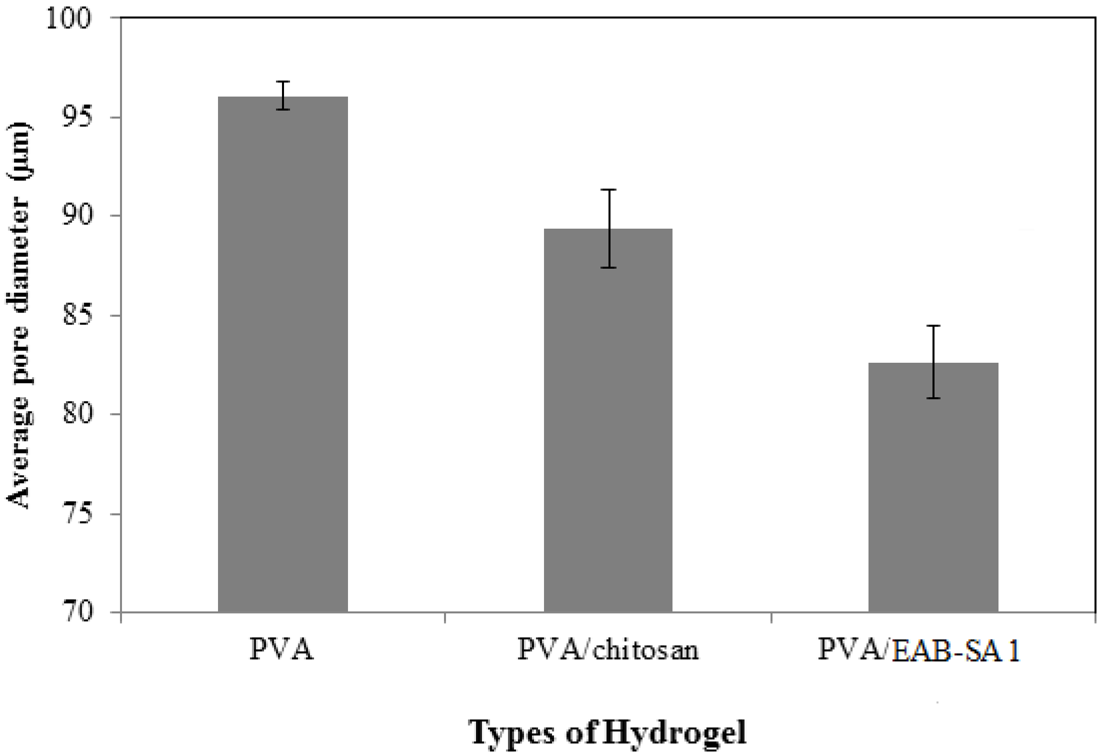


Figure 4.5 Average pore sizes of PVA, chitosan/PVA, and EAB-SA1/PVA polymer (evident from the scanning electron microscope cross sectional images of freeze-dried hydrogel membranes). The error bars indicate standard deviation from mean of triplicate experiments.

Table 4.5 Pore size of different hydrogel from SEM images using imageJ.

Hydrogel composities	Maximum (µm)	Minimum (µm)	Average (µm)
PVA	122.34	69.86	96.1
PVA/chitosan	121.62	65.93	89.34
PVA/EAB-SA1 polymer	159.73	55.63	82.64

Overall result of SEM demonstrated that chitosan and EAB-SA1 polymer possessed dissimilar effect on morphological structure of hydrogel. Scanning electron micrograph of all the three hydrogel showed porous filamentous membrane by which additives could not get transported through the membrane.

Mathews et al, (2008) reported the hydrogel formulated by PVA/chitosan which was soluble in water and PVA/chitosan which was insoluble in water, supporting the vascular cell culture, indicated the formation of pores in hydrogel membrane. Whereas, in present study PVA, PVA/chitosan, PVA/EAB-SA1 polymer hydrogel was prepared and its filamentous membrane structure indicated the formation of pore in hydrogel. Moreover, there was a great difference in average pore size of PVA/chitosan water soluble and PVA/chitosan water insoluble and PVA, PVA/chitosan, PVA/EAB-SA1 polymer hydrogel membranes (see table 4.5).

4.6.2 XRD (X- ray diffraction)

The X- ray diffraction of three different types of hydrogel (PVA, PVA/chitosan and PVA/EAB-SA1) matrix is shown in Figure 4.6. A diffraction peak of pure PVA hydrogel was at the range of 19.4^o and 40.3^o were observed. These diffraction peaks were more strong and intense suggesting high crystalline structure of PVA. Whereas, diffraction peak of PVA/chitosan and PVA/EAB-SA1 hydrogel was at 19.30^o and 19.38^o respectively. Intensities of the above mentioned three hydrogel matrixes were 1870, 1665 and 1549 with decrease in intensities upon addition of chitosan and the EAB-SA1 (Tripathi et al, 2009). This addition suppressed the PVA capacity to crystallize in blends. These observations implied the crystallinity of the three different types of hydrogel membrane was because of chitosan and EAB-SA1 as opposed to PVA. Similar to the reduction in crystallinity of hydrogel membrane observed by Pal et al (2007).

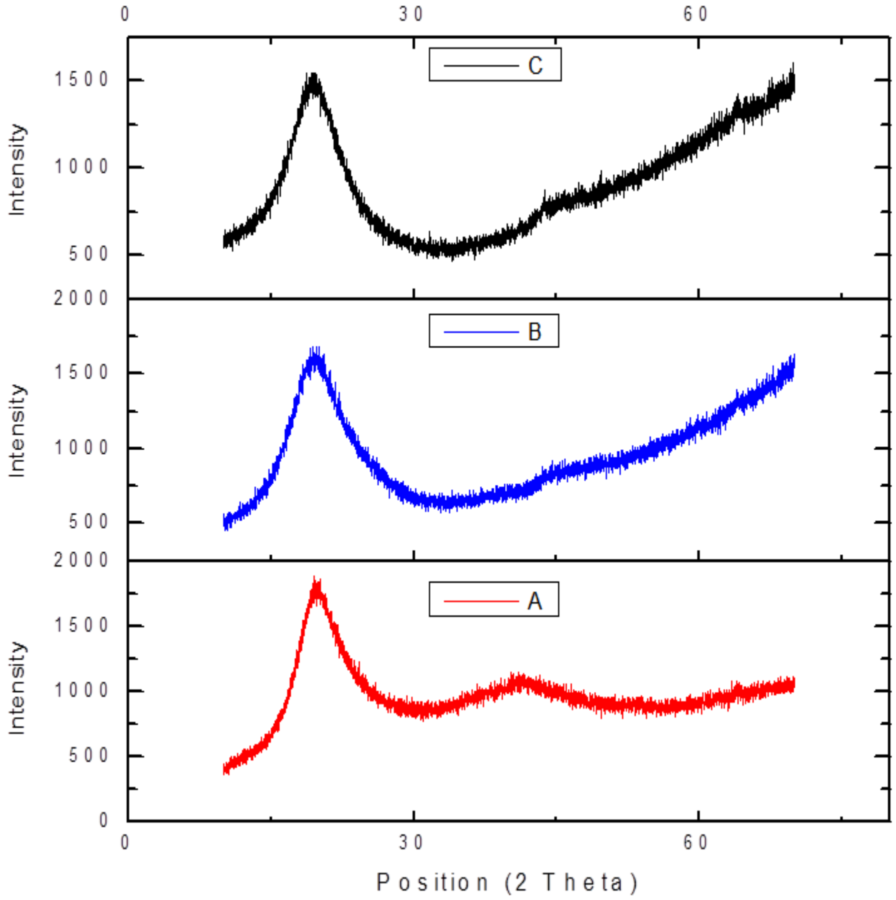
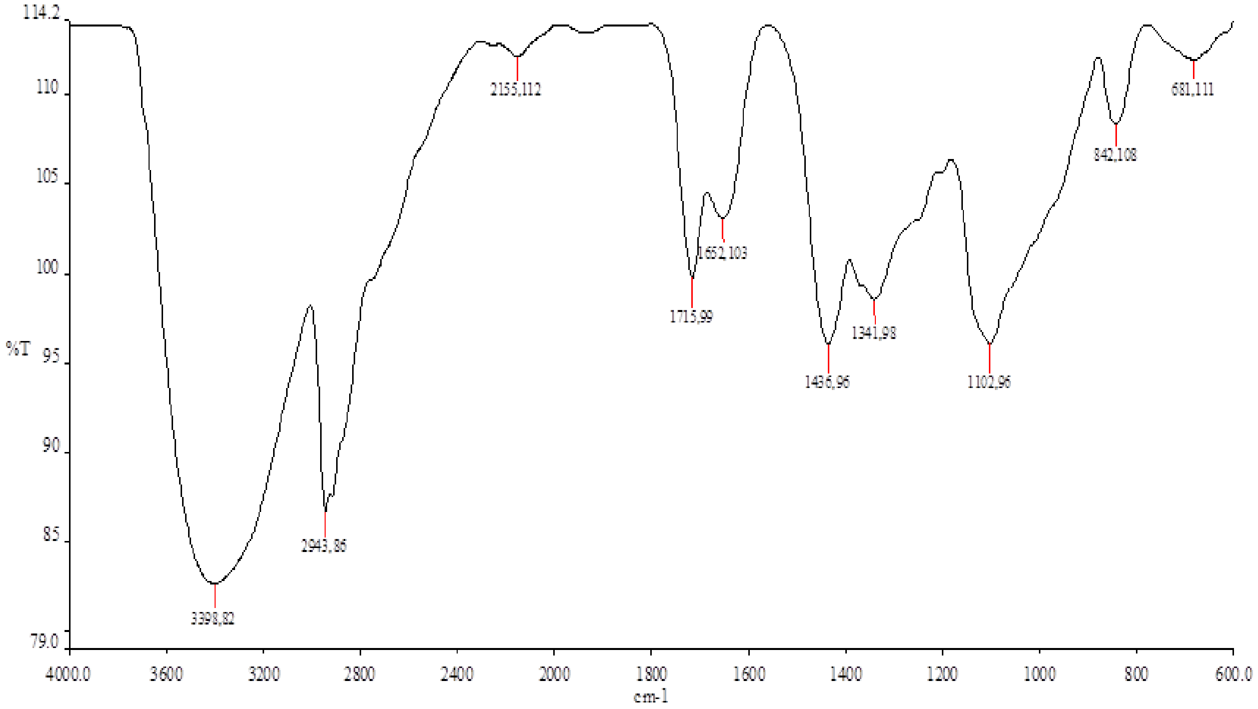
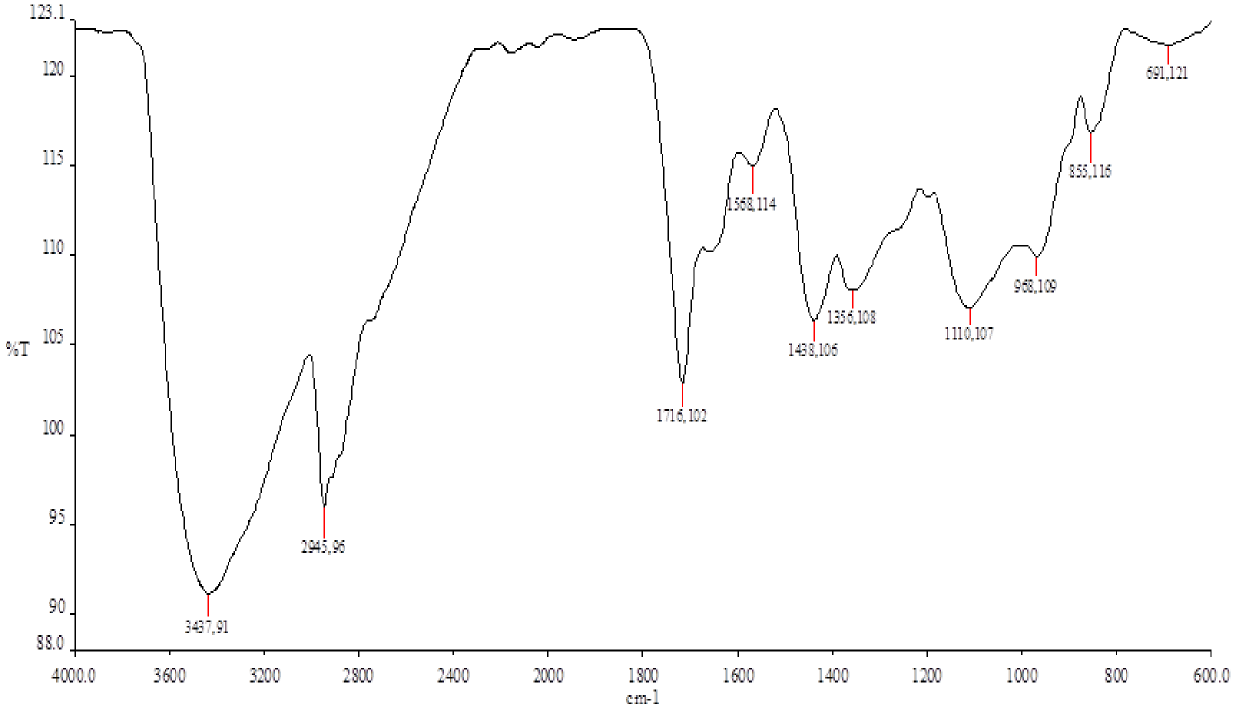


Figure 4.6 XRD spectra of three different hydrogels (A) PVA hydrogel, (B) PVA Chitosan hydrogel and (C) PVA-EAB-SA1

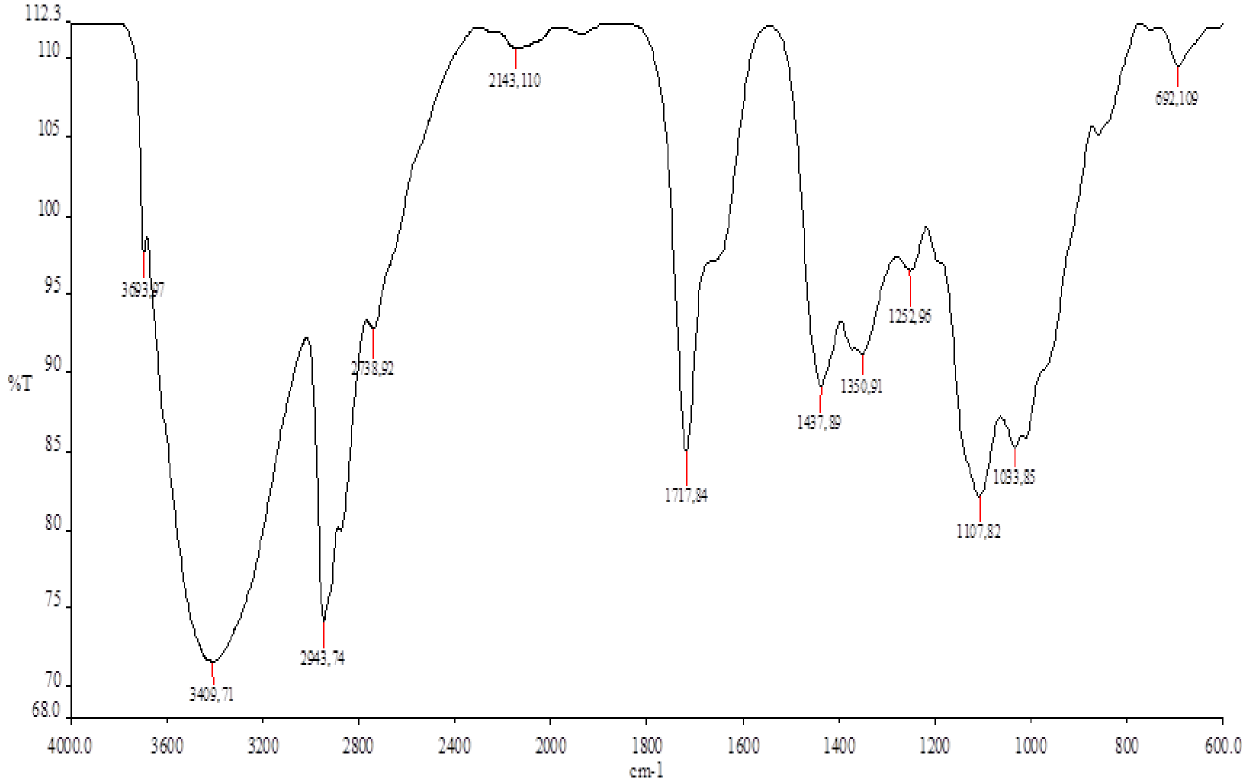
4.6.3 FTIR analysis



(a)



(b)



(c)

Figure 4.7 FTIR spectra of three different hydrogel (a) PVA hydrogel (b) Chitosan/PVA hydrogel and (c) EAB-SA1/PVA hydrogel.

FTIR analysed the functional groups and also helped in the investigation of the three different types of cross-linked network of hydrogel (PVA, PVA/chitosan and PVA/EAB-SA1). The FTIR spectra of PVA hydrogel(Figure 4.7 (a)) demonstrated the main peaks which were similar to O-H and acetate groups. The stretching of O-H groups with the association of polymers and amide from hydrogen bonds (intermolecular and intramolecular) was showed by the broad band at 3398 cm^{-1}. The bands at 2943 cm-1 and 2155 cm-1 was due to C=H and C-H stretching. Whereas C=O and C-O stretching was at 1715 cm^{-1} and 1662 cm^{-1} was due to the presence of acetate group which was left by PVA upon the reaction of saponification of polyvinyl acetate.

Further, C-H bending vibrations were showed at 1436cm^{-1} and 1341 cm-1, also the band at 1100cm^{-1} demonstrated C-N functional group. On the other hand, C-H groups were showed in the region of 915-450 cm^{-1} (Chandy and Sharma, 1992).

The infrared spectra of PVA/chitosan hydrogel presented in figure 4.7 (b). Two distinct bands were showed at 1438 cm^{-1} and 1568 cm^{-1}. Formation of band at 1568 cm^{-1} showed NH_3 deformation due to amino group's ionization. The band at 1438 cm^{-1}indicated carboxylic acid presence in hydrogel. So, the band at 1716 cm^{-1} was due to carboxylic acid which was formed by the presence of acetic acid, utilized for chitosan dissolving (Jegal and Lee, 1999).

The FTIR spectra of PVA/chitosan hydrogel with 1:1 proportion, at 1% of glutaraldehyde (GA) chemical cross-linker showed that the bands at 1110 cm^{-1}, 1438 cm^{-1} and 1356 cm^{-1} were mainly due to PVA. Further the peaks which were present at the above mentioned wavenumbers were similar to carboxylic acid and imines which were formed by amine groups present in the GA (cross-linker) in the formation of chitosan. On the other hand, vibration band at 1438 cm^{-1} was due to higher intensity and alteration in band of CH_2 group. Due to the presence of amine groups in the chitosan helped in the formation of covalent bond in the PVA/chitosan formulated hydrogel (Mathew and Kodama, 1992). This chemical crosslinking of PVA/chitosan hydrogel was explained with the help of Schiff base formation which was indicated by C=N and NH_2 groups present at 1568 cm^{-1} and 1438 cm^{-1} wavenumber respectively (Edlund and Albertsson, 2002).In chitosan derived blends imine group band increases which were formed due to nucleophilic reaction of chitosan's amine group with aldehyde (Parida et al, 2011). Moreover, PVA blend formed by GA (cross-linker) showed significant result in the bands alterations of O-H, which ultimately formed Acetyl Bridge (Jegal and Lee, 1999).

Figure 4.7 (c) showed the FTIR spectra of PVA/EAB-SA1 hydrogel. The bands at 1717 cm^{-1} and 1033 cm^{-1}was due to C=O and O-H stretching respectively. The doublet bands at 1107 cm^{-1} showed the presence of ester sulphate functional groups. Moreover, bands at 3503 cm^{-1} and 3479 cm^{-1} indicated N-H stretch and O-H broad stretching respectively. Wavenumber at 2943 cm^{-1}, 2143 cm^{-1}, 1487 cm^{-1} (C-H bending) and 2738 cm^{-1}showed alkyl C-H stretching which confirmed alkanes. C=O (ketone group) was showed by band at 1717 cm^{-1}. Whereas, alcohol and amine bands at 1350 cm^{-1} and 1252 cm^{-1} showed O-H bends and C-N stretching.1107 cm^{-1}, 1033 cm^{-1} and 692 cm^{-1} band demonstrated C-O, CO-O-CO, C-Br stretching which indicated secondary alcohol, anhydride and halo compounds (Parida et al, 2011).

Overall, the FTIR spectral analysis for all the three different hydrogel showed that these hydrogel blends were the mixtures of carbon, hydrogen, oxygen and nitrogen compounds.

4.6.4 Swelling ratio

In order to characterize the swelling behaviour, three different types of hydrogels were allowed to equilibrate in the distilled water at room temperature for 24 hour. The results of the uptake of dynamic water were showed in Figure 4.8.

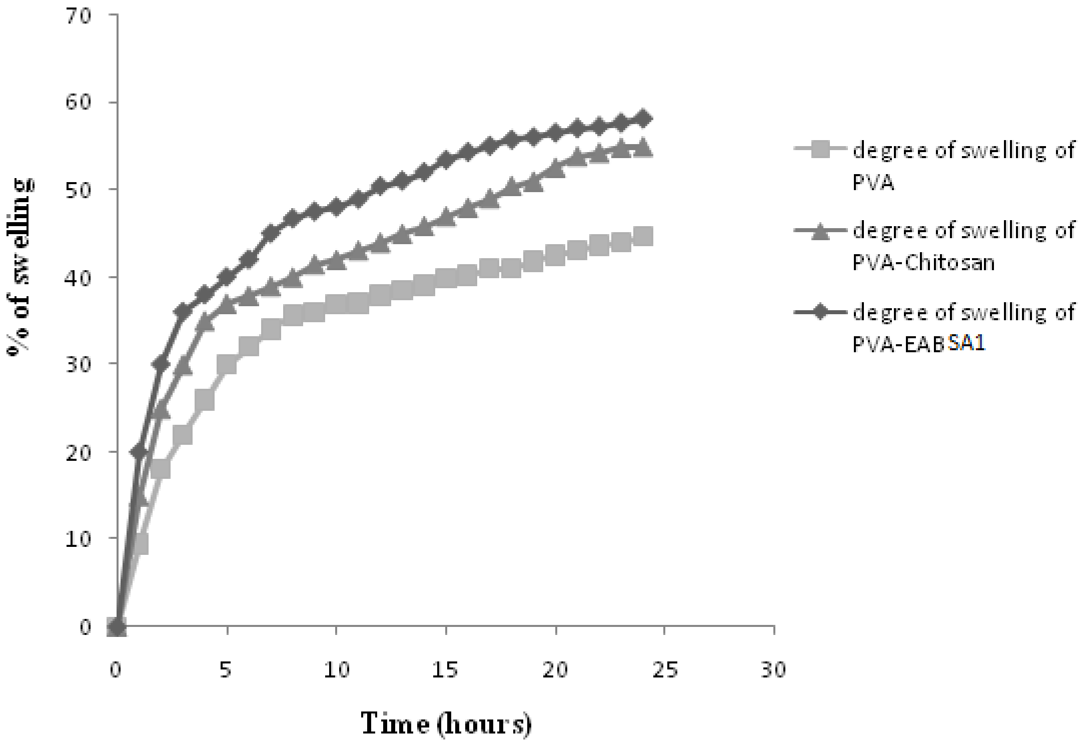


Figure 4.8 Dynamic uptake of water at different time intervals of various hydrogel samples using distilled water

As shown in figure 4.8 the water uptake of all the three different hydrogel increased with increase in time interval till 24 hour, after that the gel attained equilibrium. This increase in the water uptake was attributed to the O-H (hydroxyl) group's presents in the hydrogel. When the cross-linker was used, it would reduce the uptake of water in all the samples. The concentration of GA was based on the utilization of the functional groups present in polymers (hydroxyl and amino) in the hydrogel. Due to this cross-linking reaction of hydrogel showed less capability for hydrogen bonding (Parida et al, 2011). Moreover, when there was increase or decrease in the rigidity of the hydrogel network, which restricted the movement of macromolecular chains in the hydrogel matrix and contributed towards the lower degree of swelling. The equilibrium water uptake values for PVA, PVA-chitosan and PVA-EAB-SA1 polymer hydrogel were found to be nearly 260%, 410% and 560% respectively. The study made by Bajpai and Singh (2006) on the swelling behaviour of different hydrogel, the equilibrium water uptake was found to be nearly 1150%, 652% and 551%. Moreover in the present study it was found to be nearly 260%, 410% and 560% respectively.

4.7 Application of extracellular amphiphilic biopolymer-hydrogel as TTI (Time-Temperature Indicator)

Responsive packaging involves integration of a sensor or sensing interface on the packaging film for real time monitoring. The packaging material reacts to stimuli and thereby provides a response to the consumer. Time/temperature indicators or integrators (TTIs) constitute an important part of responsive packaging; they are cost-effective and user-friendly devices to screen, record, and interpret the general impact of temperature history on sustenance quality in the chill bind to an item unit level (Van den Berghe et al, 2006). Depending on the working principle, TTI systems are classified as chemical, physicochemical, or biological systems, with their integrated time-/temperature-dependent change being manifested as an irreversible visible color development, a movement toward a color change, or a mechanical change in consistency.

In the current study, we investigated the applicability of a microbial hydrogel and compared its performance with lactic acid as stimulant. The irreversible colour advancement that the TTI shows is because of the colour change of a chromatic indicator which happens upon pH diminish which may be presumed as the end product, lactic acid resulting from metabolism of spoilage microorganisms most frequently, Lactic acid bacteria. The fundamental preferred standpoint of the created polymeric hydrogel based TTI compared to other available TTIs, is the non-toxicity and sustainable nature of the polymer, being obtained from microorganisms. Besides its mechanical and intrinsic properties of modification could be important add-on attributes for monitoring other parameters of the product. The Indian curd or yoghurt is a typical food product which was taken as a case for application. It is outstanding that the time span of usability of curd/yogurt is connected to the development and metabolic exercises of the LAB (lactic corrosive microbes) and tangible rejection is watched when the LAB achieve a specific populace level (~ 7 to 8 log CFU) which result in an inadmissible acrid/acidic/mushy smell.We envisaged that temperature being the parameter that predominantly determines the shelf life and final quality of curd/yoghurt, both improper shelf storage or consumer abuse could lead to monitorable changes using the TTI. The results of the experiments conducted at 4 ^{0} C and 37 ^{0} C for 24 hours is depicted in Figure 4.8. Visual observation and comparison with PVA biopolymer hydrogels and Chitosan PVA hydrogels showed almost similar color changes in response to lactic acid challenge over time, however notable intensity alterations were observed after 24 hours. A difference in migration may offer an explanation for this phenomenon; however more experiments may prove the actual reason.

Overall, the results indicated graded changes in color intensity with time in proportion with the lactic acid. A critical comparison can only be made with similar microbial amphiphilic hydrogels rather than chitosan; unfortunately no studies have yet applied microbial biopolymers for TTI application. The approximate amount of lactic acid for reaching the endpoint could be correlated with actual experiments which involved log CFu (colony forming units) Vstitrable acidity of curd samples (results not shown).

In accordance with our perceptions, parallel Arrhenius plots gotten for the TTI endpoint and the μmax of L. sakei (R2 = 0.946) show a similar temperature reliance between microbial development and the TTI reaction. The assessed an incentive for the Eα of L. sakei'sμmax was 103.2 kJ/mol, which is near the Eα of the TTI endpoint (97.7 kJ/mol) (Vaikousi et al, 2008).Though further studies are required to finalize application of the amphiphilic microbial polymer, the current results clearly suggest an interesting prospect for further technological exploitation of this biopolymer for responsive packaging.

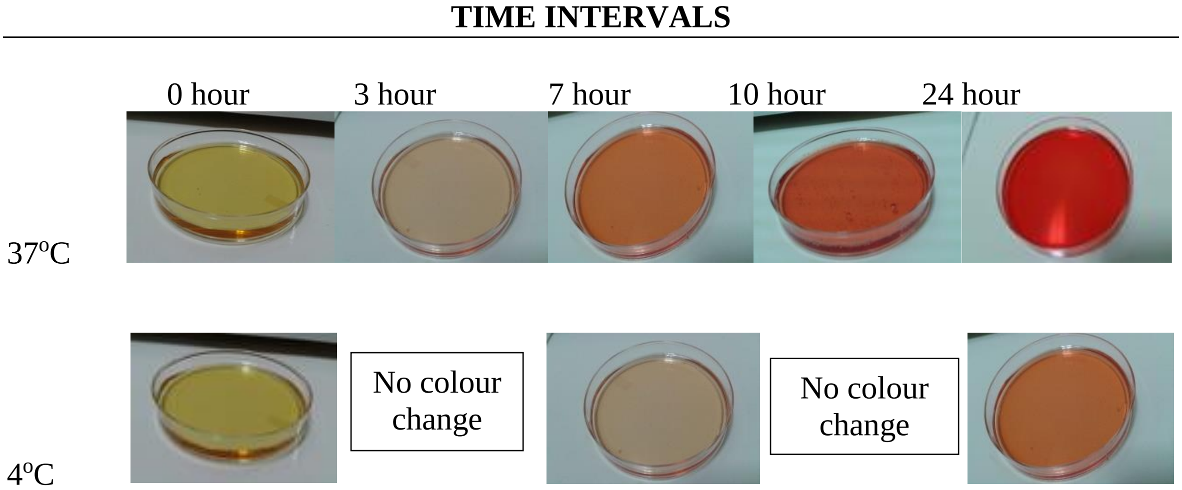


Figure 4.8 (a) Colour change of lactic-acid PVA hydrogel TTI at different temperatures.

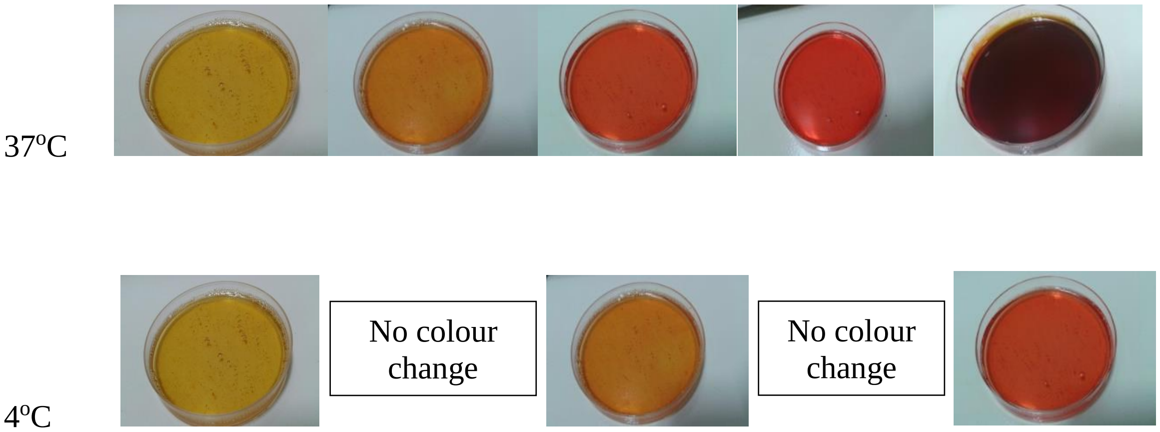


Figure 4.8 (b) Colour change of lactic-acid PVA/chitosan hydrogel TTI at different temperatures.

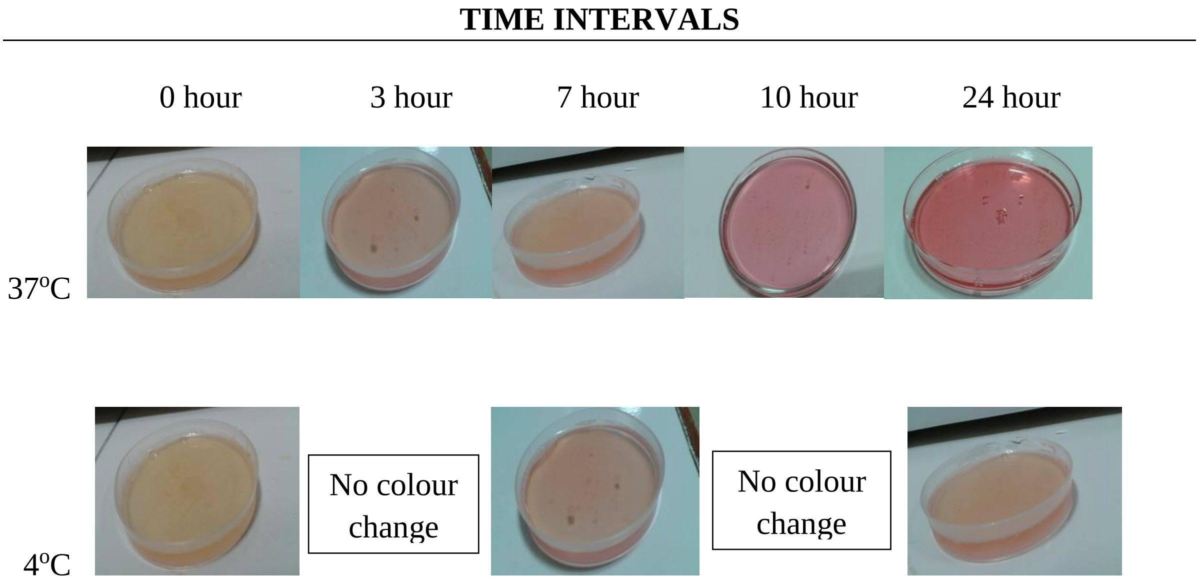


Figure 4.8 (c) Colour change of lactic-acid PVA/amphiphilic biopolymer hydrogel TTI at different temperatures.

CONCLUSION

The salient findings in this study are:

- Bacterial strains from microbial library were screened for their amphiphilic property.
- Four bacterial strains producing EAB with their higher emulsification activity were selected. Studies showed that the highest EAB yield (0.248g/l (SA1), 0.148g/l (SA5), 0.125g/l (SA6), 0.212g/l (SA9)) and emulsification activity (61.5% (SA1), 40% (SA5), 25% (SA6), 60% (SA9) was at 48 hours.
- FTIR (Fourier transform infrared spectroscopy) of EAB-SA1 indicated carboxylic, hydroxyl functional groups as predominant structural constituents whereas SEM (scanning electron micrograph) describes the fibriller structure of EAB-SA1. On the other hand XRD (X-ray diffraction) indicated the amorphous nature of EAB-SA1.
- Further the hydrogel of PVA (polyvinyl alcohol), PVA/chitosan and PVA/EAB-SA1 were synthesized and chemically cross-linked with bi-functional aldehyde. The results of these three different hydrogel were formulated with no heterogeneity and visually inspected as transparent, highly uniform yellowish transparent and uniform white transparent gel.
- However the characterization results of these three different hydrogel indicated the porous structure, semi-crystalline nature and carbon, hydrogen, oxygen and nitrogen functional groups of hydrogel.
- Then the TTI (time-temperature indicator) was prepared with all the three different hydrogel and were activated with lactic acid by keeping at 37^{o}C and 4^{o}C. The results of TTI conducted at 4^{0}C and 37^{0}C for 24 hours indicated the change in the colour of PVA, PVA/chitosan and PVA/EAB-SA1 TTI as yellow to red, yellow to dark brown and yellow to light red respectively.
- Visual observation and comparison with PVA biopolymer hydrogels and Chitosan PVA hydrogels showed almost similar color changes in response to lactic acid

challenge over time, however notable intensity alterations were observed after 24 hours.

- The applicability of a microbial hydrogel and compared its performance with lactic acid as stimulant was investigated. The irreversible colour development that the TTI manifests is due to the colour change of a chemical chromatic indicator which occurs upon pH decrease which may be presumed as the end product, lactic acid resulting from metabolism of spoilage microorganisms most frequently, Lactic acid bacteria.

The current results clearly suggest an interesting prospect for further technological exploitation of this biopolymer for responsive packaging.

PUBLICATION

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