APPLICATION OF MICROBIAL POLYMER HYDROGEL FOR DEVELOPMENT OF BIO-RECEPTOR BASED BIOSENSOR.

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CERTIFICATE

This is to certify that the thesis entitled "***Application of microbial polymer hydrogel for development of bio-receptor based biosensor***" being submitted by **Mr. Narinder Singh (Roll No-601604005)** in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision and conception of Dr. Moushumi Ghosh and that no part-of this thesis has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled "***Application of microbial polymer hydrogel for development of bio-receptor based biosensor***" in partial fulfillment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology Thapar University, Patiala is my own laboratory work during the period of July 2017 to July 2018, under the conception and supervision of **Dr. Moushumi Ghosh,** Professor, Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge

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It is not the right way of expressing by writing few words on a piece of paper for all those who have helped me in the completion of this project, because there is no mode of communication for all those words coming in my heart.

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Place: 05 sept 2018

Dedication

Every challenging work needs self-efforts as well as guidance of elders especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father & Mother,

Whose affection, love, encouragement and prays of day and night make me able to get such success and honour,

Along with all hard working and respected
Teachers

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ABBREVIATIONS

S.No.	Abbreviation	Full form
1.	AChE	Acetylcholinesterase
2.	ATR	Attenuated total reflection
3.	BATH	Bacterial adhesion to hydrocarbon
4.	BPP media	Biopolymer producing media
5.	$CaCl_2.2H_2O$	Calcium chloride
6.	CH	Chitosan
7.	CPC	Cetyl pyridinium chloride
8.	ECH	Epichlorohydrin
9.	EDS	Energy dispersive X-ray spectroscopy
10.	ELISA	Enzyme linked immune-sorbent assay
11.	FAO	Food and agricultural organization
12.	FTIR	Fourier transform infrared spectroscopy
13.	GA	Glutaraldehyde
14.	GC	Gas chromatography
15.	GO	Glyoxal
16.	HPLC	High performance liquid chromatography
17.	K_2HPO_4	Potassium phosphate dibasic
18.	LA	Luria agar
19.	LB	Luria broth
20.	$MgSO_4.7H_2O$	Magnesium sulphate
21.	NaCl	Sodium chloride
22.	OD	Optical density
23.	OP	organophosphorus
24.	PBS	Phosphate buffer saline
25.	PEG	Polyethylene glycol

26.	pHEMA	Polyhydroxyethylmethacrylate
27.	PLA	Polylactic acid
28.	PVA	Polyvinyl alcohol
29.	RGB	Red Green Blue
30.	SEM	Scanning electron microscope
31.	XRD	X-ray diffraction

LIST OF SYMBOLS

S.No.	Symbols	Full form
1.	^{o}C	Degree (s) Celsius
2.	g	Gram
3.	mm	Millimetre
4.	ml	Microliter
5.	nm	Nanometre
6.	%	Percentage
7.	Min	Minute
8.	Sec	Seconds
9.	Cm	Centimetre
10.	kV	Kilo volt
11.	mA	Milli ampere
12.	wt/wt	Weight by weight
13.	w/v	Weight by volume
14.	M	Molarity
15.	Mg	Milligram
16.	U/ml	Units per millilitre
17.	Ppm	Parts per million
18.	m.t.	Metric tons

ABSTRACT

Pesticide residues present in food crops continue to pose a serious concern to human health. Though rapid detection methods for these are crucial for timely intervention, of the several that has been developed, economical and simple methods are presently much sought after. Biomaterials such as biopolymers have recently been investigated as reliable options for biosensors and may provide economical and sustainable options. In an effort to develop a biopolymer-based biosensor for organophosphorus pesticides, microbial polymers were screened with amphiphilic properties and one with good amphiphilicity was selected for analyzing its hydrogelling function. The polymer characteristics were further determined by Scanning electron micrograph (SEM), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). The selected polymer was used for synthesizing the hydrogel and compared visually with poly-vinyl alcohol (PVA) and PVA-Chitosan hydrogels, for their efficacy (Swelling kinetics). The microbial polymer possessed excellent characteristics and swelling property in comparison to the others.

The activity, inhibition kinetics were optimized for acetylcholinesterase (AChE) enzyme with indoxyl acetate as substrate and dichlorvos (organophosphorus pesticide) as its inhibitor. For fabrication of the biosensor, acetylcholinesterase (as bioreceptor) was encapsulated in microbial polymer hydrogel. The AChE based biosensor was then evaluated by analysing dichlorvos residues from commonly consumed food items: cabbage, cauliflower, rice, brinjal, milk and water spiked with various concentrations of pesticide. The results indicated a visual colour change representing response with increasing concentrations. A concentration of dichlorvos ranging above 5 ppm to 100 ppm could be interpreted based documented semi-quantitatively using ImageJ software for image analysis.

The results of this study imply further possibility of refining this method for developing a cost effective and simple kit.

Keywords:- *Microbial polymer, Hydrogel, Acetylcholinesterase (AChE), Indoxyl acetate, Dichlorvos, Organophosphorus, Biosensor.*

1. INTRODUCTION

As abundantly available natural resource, biopolymers are promising materials which are synthesized and refined by evolution to function effectively. Their broad range of functional properties like increasing specificity and structural plasticity has led to their increasing applications in environment, industry and pharmaceutical domains.

Occurrence of pesticide residues invegetables, fruits and water has been an emerging safety issue in many developing countries. For intervention, rapid detection is necessary and pesticide detectors or sensors for on-site detection are important. Amongst pesticides of concern organophosphates are most widely used as insecticides, nerve agents and medication to pests. Commonly used organophosphorus pesticides by farmers on vegetables and crops are dichlorvos, parathion, diazinon etc. In 2008, U.S Department of agriculture reported the traces of organophosphorus compound present in vegetables and crops. The pesticide residue present leads to many severe diseases like cancer, Parkinson, Alzheimer, pregnancy defects etc. which are mostly due to the inhibition of acetylcholinesterase enzyme present in human body. Many analytical techniques have been developed for the detection of organophosphates (Huang et al, 2008). Important examples being Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Enzyme Linked Immuno-Sorbent Assay (ELISA), capillary electrophoresis and various type of spectroscopy (Huang et al, 2008; Barton et al, 2007). Immunoassay based methods are also commonly used for OP detection. The advantage of immunoassay-based methods such as ELISA, radio immunoassay and immunosensors are used due to their high specificity. However, the need to raise antibodies for each pesticide specifically, long analysis times and extensive sample handling are the

major challenges (Lacorte and Barcelo, 1995). Alternatively, spectrophotometric methods are used for OP detection but they are limited by the matrix interference and long detection times (Huang et al, 2008). Though these methods are very sensitive and specific, they are expensive, have long sample preparation times and require trained persons. These issues therefore limit their practicability in detecting pesticide residues in field where untrained personnel are predominantly the main workforce.

Over the decade, biosensors have been developed for pesticides detection such as, electrochemical based biosensors using the pH variation as an indicator (Liu et al, 2005). Rosa et al, (2009) proposed the use of 4-aminophenylacetate as cholinesterase substrate for electrochemical based biosensors further which has limited use due to non-availability of 4-aminophenylacetate commercially. Various biosensors like tyrosine-based biosensors (Kim et al, 2008), alkaline phosphatase-based biosensors (Ayyagari et al, 1995) and enzyme-based biosensors (Du et al, 2009) had been developed by scientists using different techniques. The working of sensors is based on competitive inhibition enzyme kinetics which can be based on physical, chemical, mechanical, electrochemical, or biochemical interaction between bioreceptor and analyte. The process produces a visual color that can be easily quantified by naked eye. The whole mechanism is carried out by co-ordination of the analyte and the bioreceptor like use of enzyme or microorganism to produce the physical response that helps to determine the pesticide (Verma and Bhardwaj, 2015). Major problems of the current developed sensors are low stability, least accuracy, very costly, non-reproducibility and difficult to synthesize. Hence, to overcome all these problem, polymeric materials can be exploited for developing biosensors. Several microorganisms are known to produce diverse polymeric materials. These polymers are hydrophilic (mono, oligo or polysaccharides, peptides), hydrophobic (saturated, unsaturated and hydroxylated fatty acids), as well as

amphiphilic. These different characteristics of biopolymer open up avenues for numerous applications like emulsification, frothing, producing less viscosity of heavy liquids like petroleum (Gautam and Tiagi, 2006; Franzetti et al, 2010).

Many polymers can form 3-dimenional polymeric matrix known as hydrogel. Hydrogel has an ability to swell in water without changing its structure and shape (Drumheller and Hubbell, 1995). Hydrogel can be classified on the basis of method of preparation, physical properties, bio-degradability, nature of swelling, mechanical properties, rheological properties, origin, nature of cross-linker (Qiu & Park, 2001). Most of the hydrogels are synthesized by using a cross-linker (examples:- glutaraldehyde, ethylene glycol) to obtain their permanent structure. Hydrogels can be designed with controllable responses such as to shrink or expand with change in external environmental conditions (Ahmed, 2015). Due to the some important characteristics of hydrogel it is extensively used for many applications like sensors, pharmaceutical, environment, crops, medical surgery etc.

Hydrogels can be prepared by using the synthetic/natural polymers that can be physically or chemically linked together to form a polymeric matrix. In physically linked hydrogel the polymer matrix is bound with weak Vander wall forces or molecular entanglement due to which it has weak mechanical, rheological and swelling properties (Bai et al, 2011). Chemically linked hydrogel exhibits weak swelling kinetics due to the clusters of cross-linking found dissolving in regions of swelling and low cross-link density. This leads to a difference in swelling capacity which needs to be considered while designing the hydrogel (Calvert et al, 2009).

Microbial polymer can be a very good solution to develop hydrogel due to its important properties like safety, biodegradability, amphiphilic and crosslinking properties. Further, these synthesized hydrogel can be used for numerous applications for example developing sensors for different purposes. To date, applications of microbial hydrogels with amphilicity have not been investigated for developing organophosphorus biosensors and may offer a viable alternative to the currently existing biosensors.

The present study therefore attempted to investigate the applicability of extracellular amphiphilic microbial polymer for developing a biosensor for detection of dichlorvos. For this, a hydrogel was synthesized followed by encapsulation of AChE to fabricate the biosensor. The biosensor was tested against pesticide residues in consumable items like vegetables, fruits and water.

SCOPE OF STUDY

Current analytical methods used for detection of pesticides are time consuming, costly and often require skilled personal. For rapid detection of organophosphorus pesticides, AChE biosensors, has been suggested. However, simple, ready to use detection methods are still lacking and assumes criticality for timely detection and subsequent intervention.

The present study addresses these lacunae by utilizing AChE enzyme encapsulated in microbial polymer hydrogel for the fabrication of a simple biosensor. The biosensor was analyzed for its efficacy to detect pesticide residues in commonly consumed food items and water.

OBJECTIVES

The following objectives were framed for addressing the aforementioned gaps in research:

1) Screening of microbial polymers for amphiphilicity and selection of amphiphilic microbial polymer.

2) Production and purification of selected polymer from bacterial strain, determination of chemical and structural properties and synthesis of the hydrogel.

3) Optimization of enzyme activity and inhibition kinetics studies with dichlorvos.

4) Encapsulation of AChE in microbial polymer hydrogels and evaluation of biosensor performance.

2. REVIEW OF LITERATURE

2.1) Microbial polymers

Polymers, either synthetic or natural macro molecule of repeating units (monomers) usually arranged in form of chain. Synthetic polymers are synthesized in large number approximately 140 million tons around the world every year due to their stability in the environment (Premraj et al, 2005) eventually face environmental concerns of biodegradability. Biopolymers are produced naturally by biological systems such as micro-organism, plants, animals, human & are biodegradable and eco-friendly (Armentano et al, 2013). In microorganisms biopolymers are produced under natural conditions, either inside (intracellular) or outside (extracellular) the cell by complex metabolic process. These polymers are composed of tandem repeating units of nucleic acids, amino acids or saccharides (Chassenieux et al, 2013). Several biopolymers have unique characteristics like microencapsulation, act as barrier, smart responsiveness to environmental factors (pH, temperature, light, stress etc.).Chitosan, dextran, starch, proteins, deoxyribonucleic acid (DNA), are examples of biopolymers that can be blended (Rao et al, 2014).

In 2015, Clarinval and Jhalleux classified biopolymer on the basis of source and production; (1) polymers derived from microorganisms such as polyhydroxyalkanoates, bacterial cellulose, (2) polymers isolated from biomass as polysaccharides for example chitosan and proteins, (3) polymers synthesized by bio-derived monomers such as polylactic acid (PLA), (4) polymers produced from natural source like polysaccharides, proteins, (5) polymers isolated from mineral origins as aliphatic polyesters, polyvinyl alcohols, (6) polymers created from modified polyolefins for example polypropylene, polyethylene. Further, Majeed et al, (2015) classified

biopolymers in different categories shown below in fig.2.1 and described as chitosan and polyvinyl alcohol are most abundantly used biopolymer by researcher in the current era for blending hydrogel.

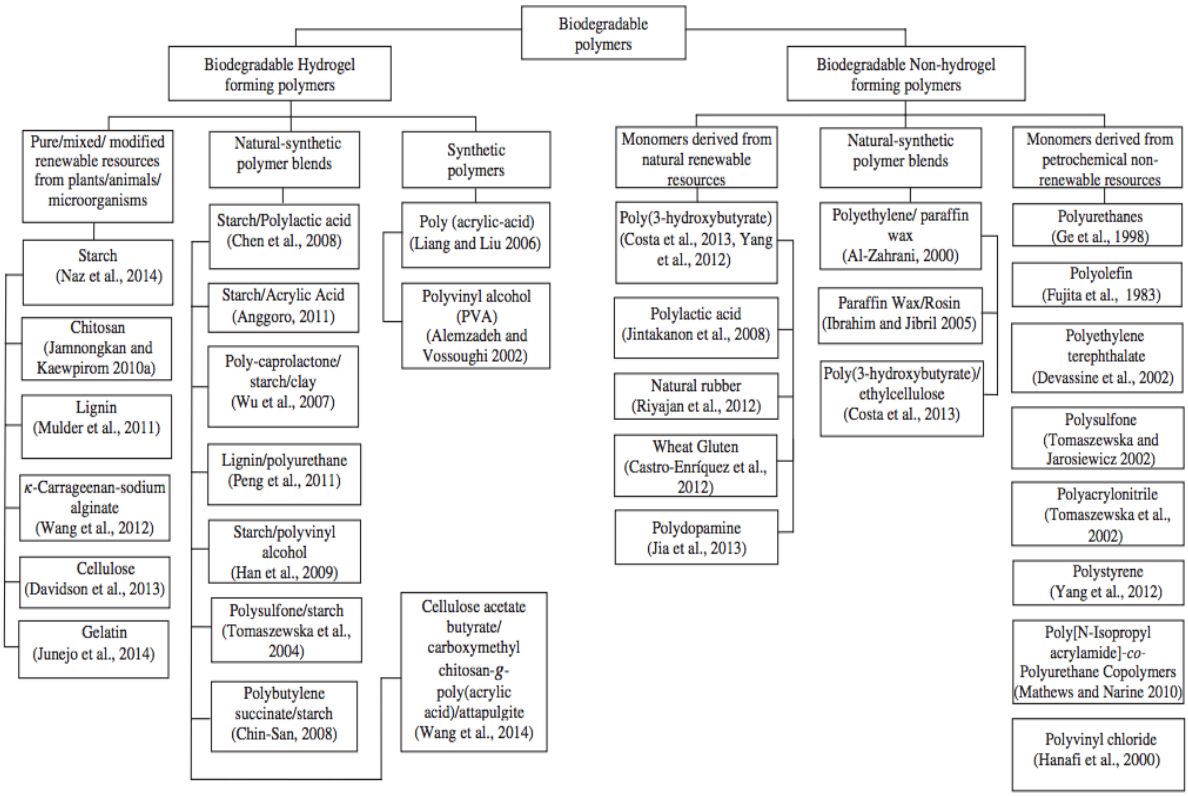


Fig. 2.1: Classification of biopolymers (Majeed et al, 2015)

2.2) Polyvinyl alcohol (PVA)

Most widely used polymer for blending hydrogel is polyvinyl alcohol because of its unique features like non-toxic, water soluble, biodegradability, stability, great physical and chemical properties. $\mathbf{[CH_2CH(OH)]_n}$ is idealized formula of PVA shown in fig.2.2.

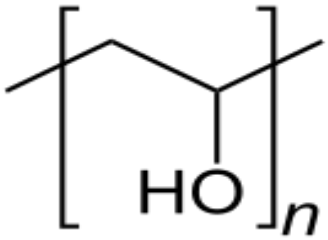


Fig. 2.2 : Formula of PVA (Wikipedia, 2018).
(source:https://en.wikipedia.org/wiki/Polyvinyl_alcohol#/media/File:Polyvinyl_Alcohol_Structural_Formula_V1.svg)

In 1924, Hermann and Haehnel synthesized polyvinyl alcohol (PVA) by hydrolysing polyvinyl acetate in ethanol with potassium hydroxide. In presence of aqueous sodium hydroxide or anhydrous sodium methylate the acetate groups of polyvinyl acetate are hydrolysed by ester and it is commercially produced in a continuous process. Partially hydrolysed and fully hydrolysed are the two classes in which PVA was classified. PVA is synthesized by polymerization of vinyl acetate and followed by partial hydrolysis. Polyvinyl alcohol have magnificent characteristics of blending films or hydrogels, biodegradability, biomechanical properties, hydrophilic properties and adherent properties. Due to versatility of PVA it is used for large number of applications as blending hydrogels, developing sensors, etc. Pereira et al, (2015) developed the time temperature indicators sensors for food packaging with the help of PVA/Chitosan blends. McGovern et al, (2005) used PVA blend for developing humidity measuring sensor.

Biopolymers have key property as “Amphiphilic nature”, due to their unique properties, are promising material for applications in developing sensitive sensors. Properties of biopolymers can be modified by combining them with other polymers that show alteration in mechanical,

swelling, rheological, adsorption, biocompatibility, stability which can be usable for further numerous applications (korotcenkov, 2013).

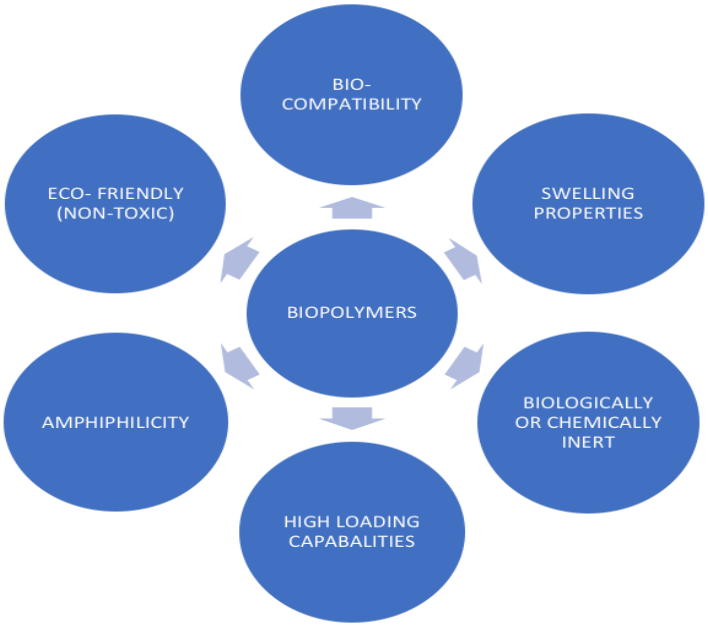


Fig. 2.3: Functional properties of biopolymers (Korotcenkov, 2013).

2.3) Chitosan /chitin

Chitin a β 1-4-linked homopolymer of *N*-acetylglucosamine residues (fig.2.4), is an essential component of the cell wall of fungi, comprising approximately 10% of the cell wall components. Chitin is present in many more other microorganisms and used for many various applications as industrial, environmental, food packaging and biosensors. Chitin is insoluble in its native form, but when deacetylated, it become water soluble (Ohno, 2007). Chitin (fig.2.4) and Chitosan (fig.2.5) have similar type of structure

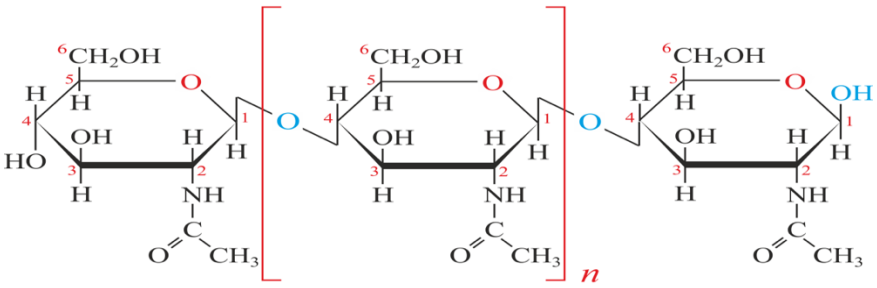


Fig. 2.4: Formula of chitin (Generalic, 2018)

(Generalic, Eni. "Chitosan." *Croatian-English Chemistry Dictionary & Glossary*. 29 Aug. 2017. KTF-Split. 18 June 2018. <https://glossary.periodni.com>.)

Chitosan, poly β (1, 4) 2-amino-2-deoxy-D-glucose (fig.2.5) is synthesized by hydrolysing the amino acetyl groups present in chitin treating with alkaline (Krasaekoopt & Bhandari, 2012). Chitosan is hydrophilic polymer and have some of unique properties like biocompatibility, biodegradability, non-toxic nature, adsorption, ability to blend films etc. So, it used for various application like blending films or hydrogels for environment as biosensors, biomedical for surgery, pharmaceutical for drug release etc.

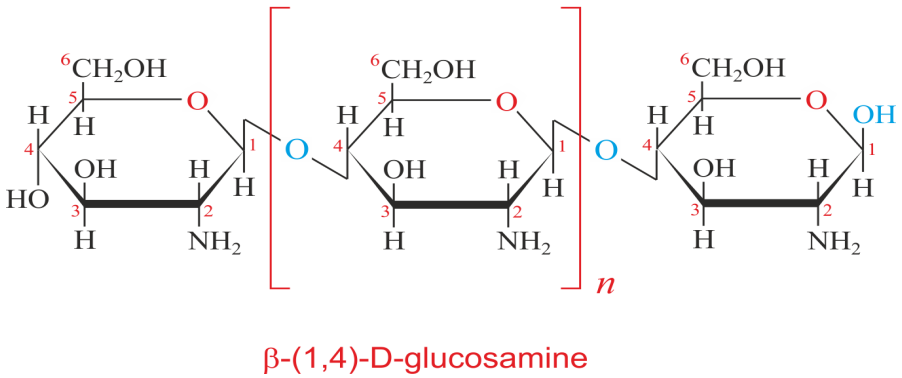


Fig. 2.5: Structure of chitosan (Generalic, 2018).

(Generalic, Eni. "Chitosan." *Croatian-English Chemistry Dictionary & Glossary*. 29 Aug. 2017. KTF-Split. 18 June 2018. <https://glossary.periodni.com>.)

2.4) Amphiphilic polymer

Amphiphilic polymers possess both hydrophilic (polar) and hydrophobic (non-polar) properties. Mostly, amphiphilic polymer is assembly of two monomer. Tribet et al, (1996) successfully developed the first amphipols (class of amphiphilic polymers). Many polymers are produced with the use of micro-organisms which possess both the hydrophilic and hydrophobic properties. Amphiphilic polymer are considered of much importance since they have some of the key functional properties as shown in fig 2.6 (Thanomsub et al, 2004).

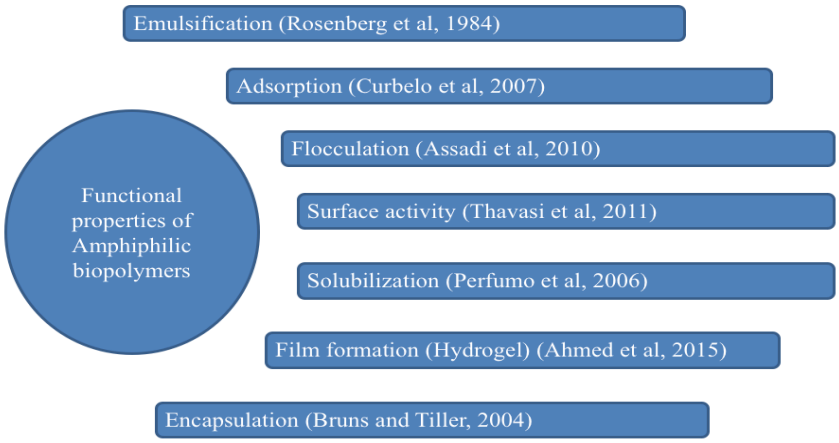


Fig. 2.6: Functional properties of amphiphilic biopolymers

Amphiphilic polymers usually consist of hydrophilic and hydrophobic parts that show emulsification. Emulsification is defined as diffusion of one liquid into another to elicit blends of two immiscible liquids. The property of emulsification (Rosenberg et al, 1986) and de-emulsification (Kosaric et al, 1987) of amphiphilic polymer has been reported by Rosenberg et al, (1986).

Curbelo et al, (2007) described the adsorption property of amphiphilic biopolymers. According to them in adsorption process biopolymers atoms adsorbed on hydrophobic substrates. The

important characteristics of the adsorbent materials are a high porosity along with irregular geometries to deliver a large surface area. Hydrogels or biofilms are blended by the amphiphilic polymers as they obtain the key properties of polymerization and building chains of polymers to from a 3-dimensional structure (Ahmed et al, 2015). Encapsulation with respect to amphiphilic polymer is the action of enclosing something (cells, enzyme etc.). Amphiphilic polymer possesses the unique feature for encapsulation and can be used medical for cell encapsulation (Nicodemus and Bryant, 2008), as sensors (Bruns and Tiller, 2005) and many more vast applications. Amphiphilic polymer has various advantages such biocompatibility, bio-inertness, biodegradability which add on plus point for the safety of environmental.

As mentioned above, some important key properties of amphiphilic polymers can be utilised for synthesizing the hydrogel, attractive for further applications.

2.5) Hydrogels

Hydrogels are described as three-dimensional (3D) polymeric matrix obtained from a class of synthetic and /or natural polymer capable of absorbing and retaining significant amount of water without changing its shape and structure (Rosiak and Yoshii, 1999). Polymers are cross linked by physical or chemical interactions for synthesizing the hydrogel. The 3-dimensional structure and shape of hydrogel allow it to absorb large amount of water as compared to its original volume. Hydrogel possess some idiosyncratic feature as 3-dimensional shape and structure, degree of flexibility, biocompatibility, high adsorption of water or fluid, biodegradability, stimuli responsive to environmental factors, encapsulation, etc. make its use for various application as in medical, pharmaceuticals, plants, environment, food, sensors etc. (Akhtar et al, 2016). Stimuli responsive hydrogel are responsive to environmental condition

as pH, temperature, light, stress etc. Smart hydrogel has been prepared by crosslinking smart polymers which have the capabilities to self-control by responding to environment factors.

Hydrogels are usually classified as natural, synthetic or hybrid, depending on the source of the constituting polymers. They can be crosslinked by chemical, physical interaction or by combination of both. Different interaction like hydration, capillary and osmotic forces which are responsible for water sorption and counterbalancing by exerting the resisting forces by physically or chemically cross-linked polymer chains (Roorda et al, 1986). The swelling kinetics depends on the magnitudes of these opposing effects, and which can be determined to large extent. Some important properties of hydrogel, like absorption of water/biological fluid and diffusion property, and mechanical strength. Many of these properties are depended on swelling kinetics but not totally governed by it. As, some of the properties are influenced directly by physical or chemical nature of polymer and network morphology of hydrogel. Due to the hydrogels property of absorbing and holding high water content, shows resemblance to biological tissues, resulting in an excellent biocompatibility (Mark & Kroschwitz, 2003).

Wichterle and Lim, (1960) successfully synthesized hydrogel and successfully applied as contact lenses. Later, “Smart” hydrogels, or Stimuli-sensitive hydrogels, are discovered which are very different from inert hydrogels in that they can “sense” changes in environmental factors and respond by swelling/shrinking. The swelling behaviour of hydrogels opens a wide range of application as in environment areas like sensors application as environmental monitoring can be trigged by environmental changes (Kushwaha, et al 2012). Smart hydrogels can undergo reversible volume phase transitions upon minute change in environmental conditions such as temperature, humidity, pH, wavelength, intensity of light, electric or magnetic field and responses in different way, swelling/shrinking, becoming conductive, water

permeability changes, etc. (Hoffman, 2013) that allow the use of smart hydrogel in environmental areas for different applications.

Around, 1894 the term 'hydrogel' first appeared in scientific literature when it was used to describe a colloidal gel of inorganic salts (Bemmelen, 1894). Over the two decades following this discovery, hydrogel research remained essentially focused on relatively simple, chemically crosslinked networks of synthetic polymers with applications mainly in ophthalmic, drug delivery, medical, environment, etc. The straight forward network structure was also well-suited for fundamental characterization and modelling of various physio-chemical hydrogel properties such as solute diffusivity and crosslink density. Hydrogels were mainly prepared either by polymerization of water-soluble monomers in the presence of a multifunctional cross linker or by crosslinking of hydrophilic polymers.

In the 1960s, PAM hydrogels were also used for the physical entrapment of cells (Freeman & Aharonowitz, 1981) and enzymes (Hicks & Updike, 1966), as well as for the covalent attachment of proteins. Hydrogels have found widespread environmental application as biosensors for monitoring and detecting many environmental conditions (D'souza, 2011). Inspired by the work of Katchalsky in the 1950s (Katchalsky, et al, 1950) and 1960s on the possibility of transferring chemical energy into mechanical work, in the beginning of the 1970s the hydrogel research focus shifted from relatively simple to stimuli responsive hydrogels or smart hydrogels that can respond to change in environmental conditions such as pH, temperature or concentration of biomolecules (Kopecek, 2007). These environmental triggers can be used to evoke specific events, such as swelling and shrinking of hydrogel.

In the mid-1990s, other physical interactions were recognized and exploited as crosslinking methods that offered the possibility to enhance and finely tune the mechanical, thermal and degradation properties of hydrogels. Many of these interactions also allowed for in situ hydrogel formation. With the increasing knowledge in organic chemistry, a variety of chemically crosslinked hydrogels has been developed. 'Smart hydrogel' is a relatively recent concept that focus on the examination and development of stereo complexed materials (e.g. PEG-PLA interaction) (Buwalda et al, 2014; Yom-Tov, 2014) cross linked by physical interactions (e.g. cyclodextrines).

It is important to note that the "Smart hydrogels", polymeric networks offer a wide range of tuneable properties and trigger stimuli. The theme has been hypothetically described as boundless and the conceivable applications for example, sensors (Chung et al, 2008).

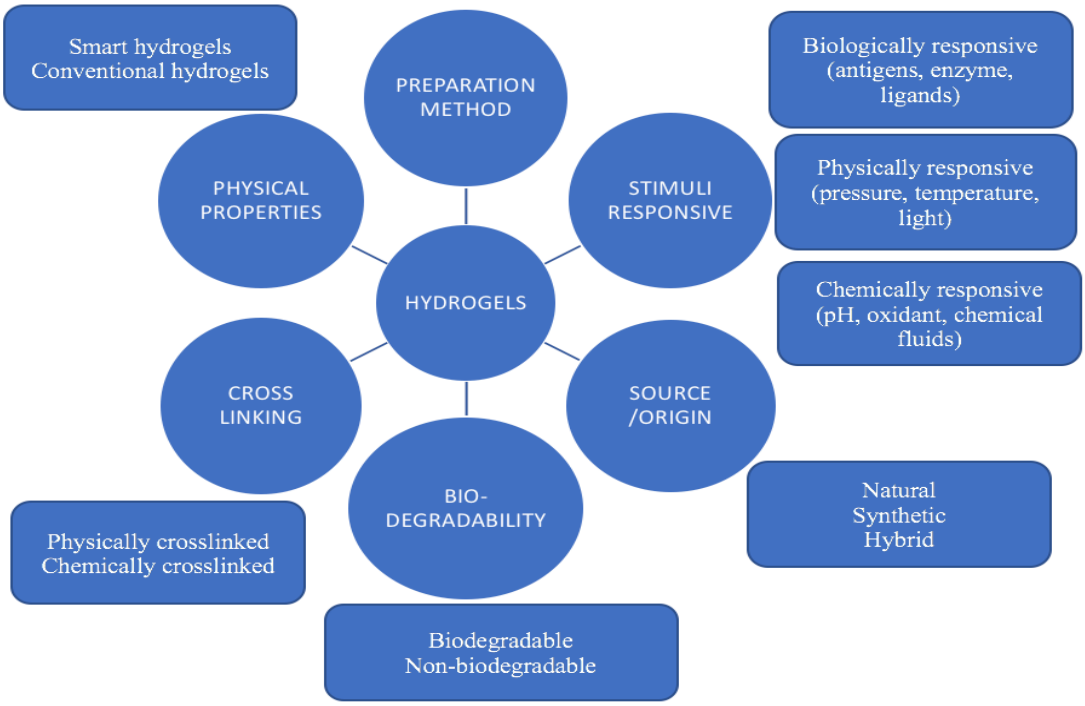


Fig. 2.7: Classification of hydrogel (Ullah et al, 2015)

As discussed above, hydrogels are usually synthesized by physical and chemical methods. Physically cross-linked hydrogel are synthesized in the absence of cross-linkers. Major drawback of physically cross linked hydrogel is the mechanical strength because of weak interactions like hydrogen bonding, Van-der-Wall forces, ionic charge, protein interaction, stereo complex formations etc. some of the example are shown in Table 2.1.

Table 2.1: Examples of physically cross linked hydrogels.

S.no.	Polymers	Method for hydrogel synthesizing	References
1	Polyacrylamide and polyethylene glycol	Hydrogen bonding	Eagland et al, 1994
2	PBT and PEG	Melt polycondensation of PBT and PEG	Bezemer et al, 2000
3	Alginate	Ionic interaction	Gacesa, 1998
4	Polyvinyl alcohol	Freeze thaw method	Yokoyama et al, 1986

Chemically cross linked hydrogels are synthesized by using cross linkers or by chemical method. Due to the good stability and good mechanical strength chemically cross linked hydrogel are widely used for various applications. Chemically cross linked are synthesized by many method like (a) crosslinking by free radical polymerization, (b) crosslinking by high radiation, (c) crosslinking utilizing proteins and (d) crosslinking by complementary groups by reactions like; crosslinking with aldehyde, by addition reactions, by condensation reactions (Akhtar et al, 2016). Some examples of chemically cross linked hydrogel are shown in Table 2.2.

Table 2.2: Examples of chemically cross linked hydrogels

S.no.	Polymers	Method for hydrogel synthesizing	Reference
1	Poly vinyl alcohol	Using cross linker glutaraldehyde	Zu et al, 2012
2	Polysaccharides	Using 1,6-hexamethylenediisocyanate	Brondsted et al, 1995
3	Polyesters and polyamides	Condensation reaction among OH groups with COOH or derivatives	Ray et al, 2010
4	Chitosan and PVA	Cross linking by Aldehyde	Zu et al, 2012

2.6) Effect of cross linker on hydrogel

Various different cross linking chemical are used for synthesizing of hydrogel such as glyoxal (GO), glutaraldehyde (GA), epichlorohydrin (ECH), 1-ethyl-3-(3-dimethylaminopropyl) etc. Cross linking agents have many effect on hydrogel like improved mechanical property, stability, pore size of hydrogel, responsive characteristics swelling and shrinking. An example of hydrophilic polymers having –OH groups is polyvinyl alcohol (PVA), the latter may be cross-linked through glutaraldehyde (Zu et al, 2012).

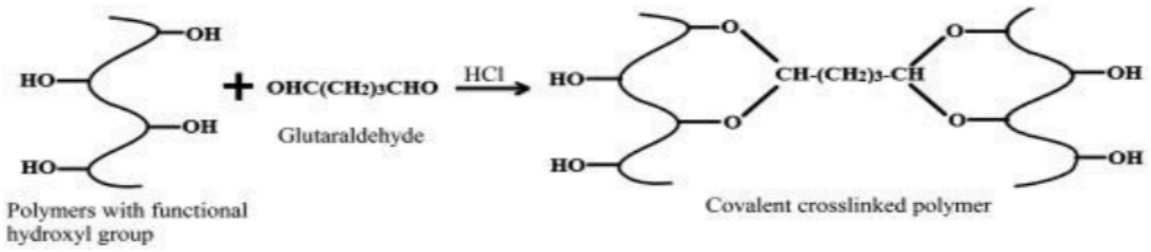


Fig. 2.8: Hydrogel synthesis by cross linking with glutaraldehyde (Zu et al, 2012)

Hydrogel possess various functional properties as swelling and shrinking kinetics (little change in ecological conditions cause change in hydrogel), mechanical property (strength of hydrogel how much weight it can retain), elasticity (elastic nature of gel), biocompatible (compatibility with host), stimuli responsive (change in environmental condition effect the hydrogel).

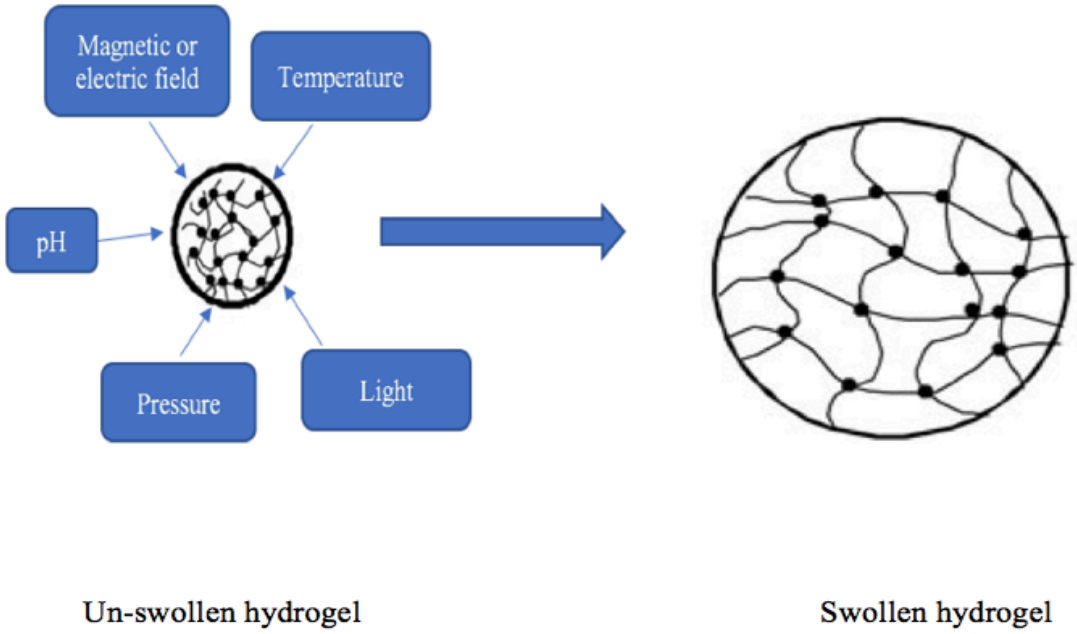


Fig. 2.9: Stimuli responsive hydrogel (Ahmed, 2015)

In light of the above properties, hydrogels have currently several 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 (pesticide detection, phenolic compound detection). One of the vital application which was excessively utilized as sensor to various application as monitoring of the environment pollution, water pollutants, etc. (Banna et al, 2014).

Fig. 2.10: Applications of hydrogels (Lim et al, 2014).

2.7) Hydrogels as biosensors

To ensure quick and accurate analysis, a rapid physical response of the hydrogel is critical for the success of stimuli-responsive hydrogel-based biosensors. Various smart polymers are developed such as chitosan, polyacrylic acid, pluronic, poly(NIPAAM), etc. which are utilized for developing smart hydrogels. Hydrogels can be designed to respond to environmental stimuli further, which can be used as biosensors. Biosensors is an analytical device that have a biological receptor which detect the analyte and produce a signal which is measured by detector (Turner et al, 1987). Different type of biosensors is available for various applications such as glucose sensor (Yoo and Lee, 2010), acetic acid sensors (Mizutani et al, 2001), methane sensor (Damgaard and Revsbech, 1997), alcohol sensors (Kuswandi and Ahmed, 2014) and many more. Biosensors consist of three main parts: (1) the bioreceptor or biological element that recognize the specific molecule from some various molecules (2) the transducer which converts

the biorecognition to a signal (3) a detector which detect the signal and convert it in a readable form. Bioreceptor is the crucial part of the biosensor.

Various biomolecule that can be used for bioreceptor as enzyme, antibodies, aptamers. Hydrogel biosensors are usually synthesized by incorporating the biomolecule in gel forming polymers, they can act as cross-linking agents and make hydrogel network dense. Therefore, when target analyte interacts with the hydrogel produces the response that can be physical, chemical, fluorescence, electro-chemical signal that can be measured by detector. Biosensors can be differentiated based on the signal (fluorescence, electrochemical) produced and bioreceptors (enzyme-based biosensors, antigen- antibody biosensors, ligand binding molecules). The working of biosensors is dependent on the bioreceptor and analyte interactions as analyte is recognized by the bioreceptor and the transducer convert the signal into readable form that can be measured by the detector.

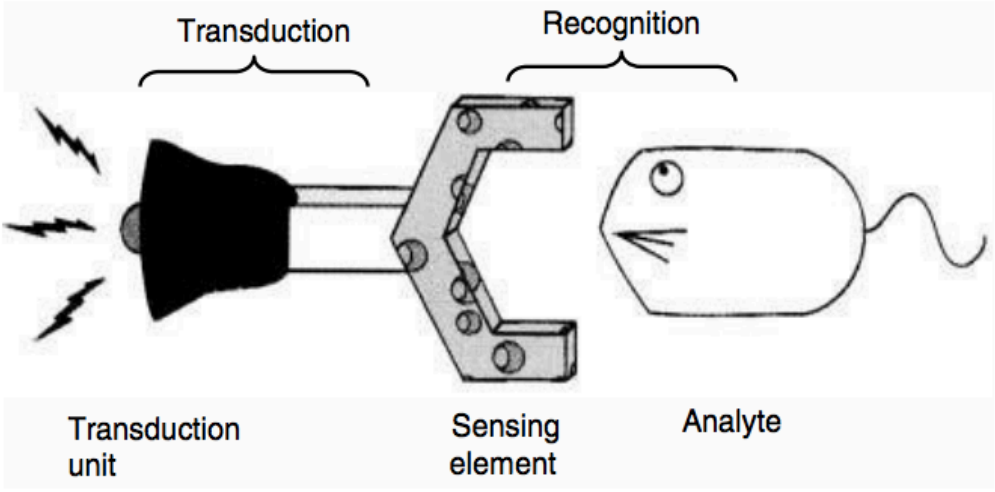


Fig. 2.11: Schematic figure depicting working of biosensor (Fabbrizzi et al, 1995)

Some of the general requirement for the biosensors are listed by Rogers and Gerlach (1996) as shown in Table 2.3.

Table 2.3: General requirement for biosensors (Rogers and Gerlach, 1996)

Requirement	Specification range
Cost	50-5000 per analysis
Portability	Easy to carry by single person, no external power or labour required
Time for assay	1-60 min
Format	Reversible, in situ, continuous
Sensitivity	Parts per million to parts per billion
Range	At least have 2 order magnitude
Specificity	Enzyme/nucleic acids/receptors: specific to one or more groups of the related compound Antibiotics: specific to only one compound

A large number of biosensors has been developed but suffer from major drawbacks like poor sensitivity, low specificity and stability of bioreceptor. So, to overcome this problem hydrogel is used due to its unique property that can maintain enzyme and ligands in its innate structure and give better stability.

2.8) Enzyme based biosensors

Enzymes are proteins that are specifically structured to bind and act on a substrate (reactant molecule) to convert it by catalytic mechanism, that is, by lowering the activation energy of the reaction and accelerates the rate of reaction without being used at the end of the reaction.

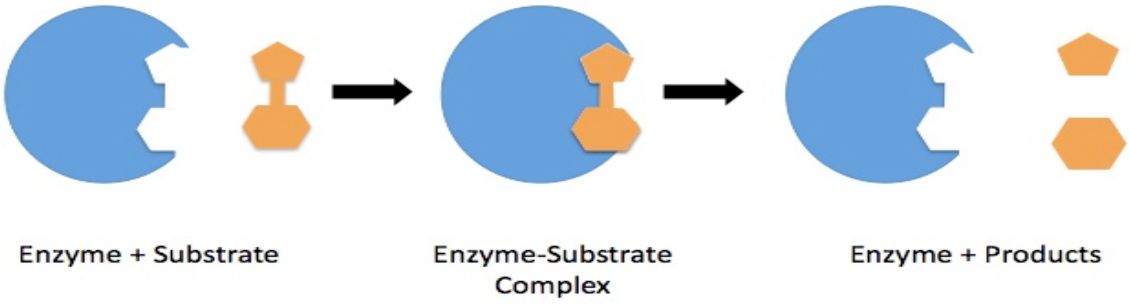


Fig. 2.12: Mechanism of enzyme acting on substrate (Biotechnology, 2013)

Enzyme based biosensors are developed on the basis of functionality as different classes of enzyme carry out different reactions for example glucose oxidase converts glucose to gluco-lactone & hydrogen peroxide, acetylcholinesterase for indoxyl acetate to indole etc.

Enzymes can be classified into six major classes as follow: -

1. Oxidoreductase (Electron transfer reaction)
2. Transferases (Transfer of atom or group)
3. Hydrolases (Hydrolysis)
4. Lyases (Nonhydrolytic bond cleavage)
5. Isomerases (Isomerization reactions)
6. Ligases (Bond formation reactions)

Different biosensors are developed with the help of enzyme that have important applications in different aspects like food industry (determination of glucose, ascorbic acid), environment (contaminant in water, air pollution). In enzyme-based biosensors enzyme act as the receptor that can recognize analyte and produce signal that is converted by transducer and further which is measured by the detector.

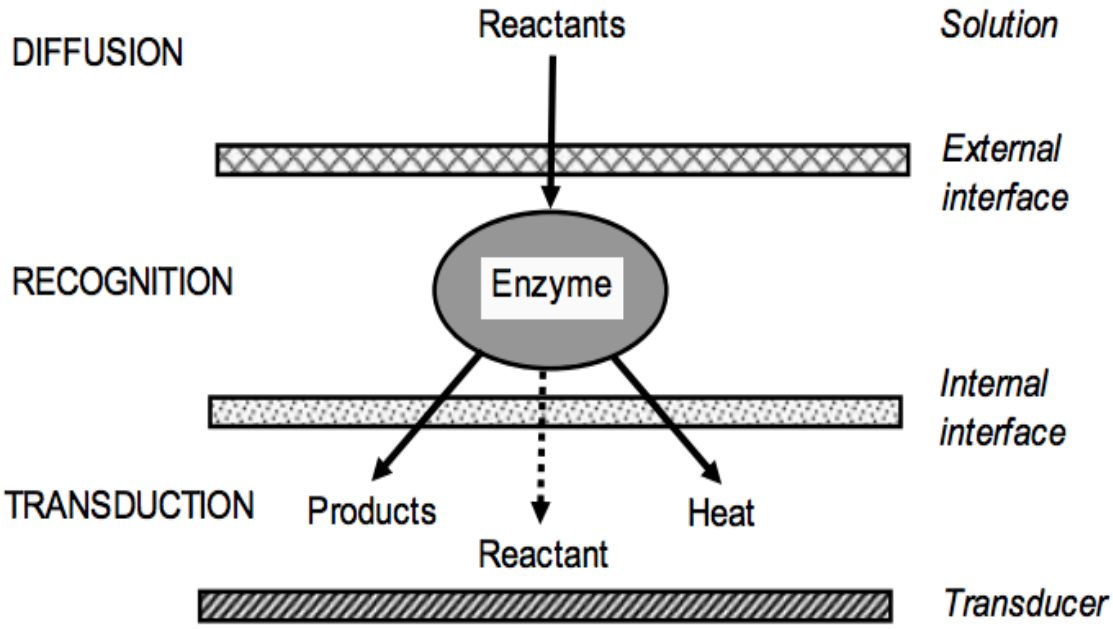


Fig. 2.13: Enzyme based biosensors (Banica, 2012)

In enzyme-based biosensors, the enzyme acts as bioreceptor, which binds to specific substrate and produces response (Guilbault et al, 2004). Two different tactics can be used for analysis the analyte by using enzyme-based biosensor: (1) if enzyme converts the analyte into product then the product formation is measured for determining the analyte, and (2) if analyte inhibits the enzyme from conversion then decrease in product formation can be measured and correlated to analyte concentration (Arduini et al, 2009).

Biosensors based on the principle of enzyme inhibition have been extensively applied for detecting organophosphate insecticides (OP), organochloride insecticides, heavy metals and glycoalkaloids. The choice of enzyme/analyte system is based on the fact that these toxic analytes inhibit the normal enzyme function (Amine et al, 2006).

2.9) Acetylcholinesterase enzyme

Acetylcholinesterase (AChE) is a type-B carboxylesterase enzyme located primarily in the synaptic cleft with a smaller concentration in the extra junctional area (Van den berg, 2016). Acetylcholine is the most abundant neurotransmitter in the body, playing important roles in both the peripheral and central nervous systems.

Acetylcholine is released into the synaptic cleft of motor neurons in order to activate muscles, and can also be used as a neuromodulator within the brain in order to regulate groups of neurons at a time. After acetylcholine has served its purpose within the synaptic cleft, it is rapidly degraded into choline and acetyl CoA through hydrolysis by the enzyme acetylcholinesterase (AChE), and reabsorbed by the neuron to be recycled and used again (Barnard, 1974).

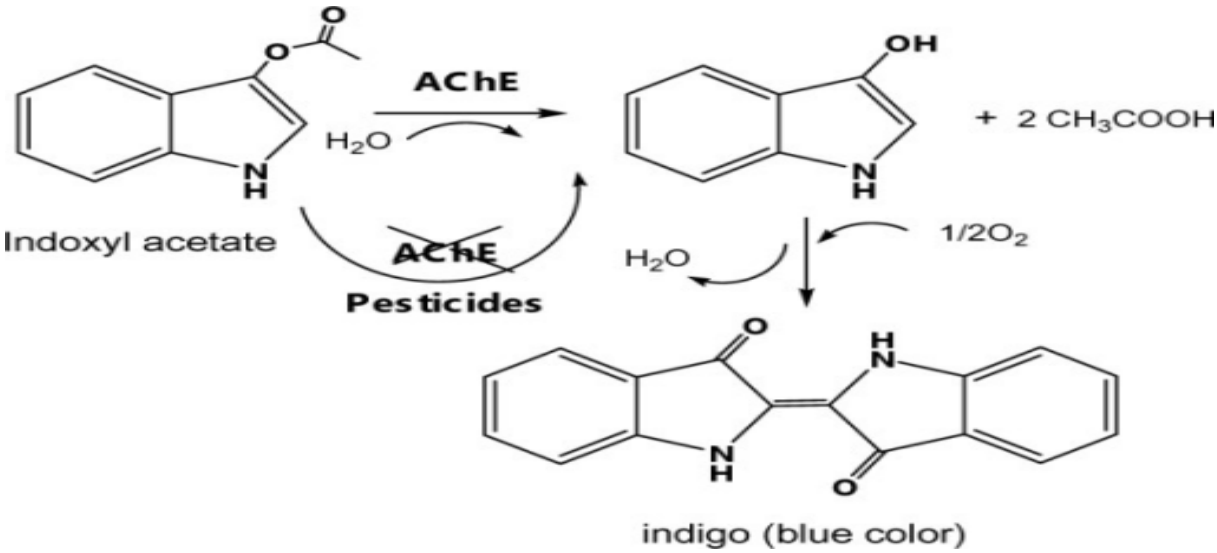


Fig. 2.14: Mechanism of AChE enzyme (Apilux et al, 2015)

When AChE is interfered by enzyme inhibitors, acetylcholine is not degraded and reabsorbed and its action is prolonged. This can then lead to incessant stimulation of muscles, glands, and

the central nervous system, and depending on the dose, can be fatal (Devic et al, 2002). Acetylcholinesterase (AChE) acts primarily as a regulatory enzyme of cholinergic neurotransmission by hydrolysing Acetylcholine to choline and acetate.

Pesticides inhibits acetylcholinesterase enzyme from hydrolysing acetylcholine to choline and acetate. The increase in the quantity of acetylcholine in the body leads to many fatal problems like stimulation of muscles, Alzheimer disease, Cancer, Parkinson disease etc.

2.10) Pesticides

Pesticides are the toxic group of chemical that are deliberately applied to protect the agriculture and industrial products by supressing plant and animals pests. However, the majority of pesticides are not specifically targeting the pest only and during their application they also affect animals, non-target plants and indirectly humans (Amine et al, 2006). A large number of pesticides are used on crops for protecting the crop from pests as pesticides are not easily degradable, so they remain in crops as pesticide residue and some quantity of pesticide persist in soil and leach to groundwater and surface water and contaminate the environment. Depending on the chemical properties as pesticides possess they can enter the food chain and influence human health. Pesticides affect the human health as it enter the human body by different routes through crops or vegetables that have residual pesticide. It can also enter through ground and surface water which is in contact of pesticides thus, entering in the food chain. The different type of pesticides available and most widely used are carbamates, organochlorines, organophosphorus etc. (Jeyaratnam, 1990). Organophosphorus compounds are organic esters of phosphoric, phosphonic or phosphinic acids. These compounds are powerful acetylcholinesterase inhibitor and used as insecticides and anthelmintic.

Organophosphorus are classified into 13 different type : phosphates, phosphonates, phosphinates, phosphorothioates, phosphonothioates, phosphorothioates (S-substituted), phosphonothioates (S-substituted), phosphorodithioates, phosphorotrithioates, phosphoramidates, phosphoramidothioates, phosphorofluoridates and phosphonofluoridates. These different type can be used under different pesticide names like dichlorvos, diazinon, malathion, di-isopropyl etc. A study reported by Reddy and Colman, (2017) showed three different incident of poisoning by organophosphates compounds out of which in India they observed 8 case videos and concluded that the people suffering from organophosphorus pesticide show 59% mild, 18% moderate and 23% severe symptoms. Though detection of organophosphorus pesticide many analytical techniques such as gas chromatography, high performance liquid chromatography, enzyme linked immunosorbent assay etc are operational, these are cost intensive, require skilled labour and time consuming, rendering them inapplicable especially in resource poor settings.

2.11) Acetylcholinesterase biosensor

Over the decades, acetylcholinesterase-based biosensors have been favored as a sensitive and rapid technique for pesticide analysis in food, quality control and environmental monitoring. The choice of AChE enzyme as bioreceptor enables the concurrent detection of many toxic pesticides. For analysis of pesticides residue in food generally requires large number of samples and there is need of rapid, sensitive and low-cost methods. The main purpose for fabrication of acetylcholinesterase biosensors is to provide reliable alternative to classical methods which are currently used in analytical laboratories for pesticide analysis, like Gas Chromatography (GC) or High-Performance Liquid Chromatography (HPLC) coupled with mass selective detectors.

These techniques are highly expensive, need skilled staff and time-consuming (Andreescu and Marty, 2006).

The AChE biosensor activity depends on quantitative estimation of the product formation when introduced to target analyte. The inhibition kinetics of AChE enzyme depends on the interaction with inhibitor which is quantitative based on inhibitor concentration and incubation time (Guerrieri et al, 2002; Ivanov et al, 2003). As a result, the AChE enzyme activity is inversely proportional to inhibitor concentration (Amine et al, 2006).

Substrates and inhibitors bind to three distinct binding sites on the AChE: (1) the choline binding site binds specifically to the substrate's choline group and is blocked by tricyclic inhibitors (acridine, phenothiazine and their derivatives); (2) the size of the acyl pocket regulates substrate specificity and facilitates sensitivity towards the transition-state analogous inhibitors (organophosphorus and carbamates) of different size; (3) the peripheral anionic site is located near the entrance of the gorge and is blocked by charged mono and bi quaternary inhibitors (propidium, decamethonium) (Radic et al, 1993).

Acetylcholinesterase biosensors have been shown to be functional, and many biosensors using cholinesterase as the bioreceptor in combination with Amperometric (Mionetto et al, 1994; Palleschi et al, 1992; Bachmann and Schmid, 1999; Schulze et al, 2002a), Potentiometric (Ghindilis et al, 1996; Evtugyn et al. 1996; Lee et al, 2001), Optical (Choi et al, 2001; Danet et al, 2000) and Piezoelectric (Abad et al, 1998; Makower et al, 2003) transducers have been developed. The biosensors working and fabrication is totally based on substrate specificity, enzyme concentration and system (mono or multiple enzymes) and finally on the applications of the device.

Nevertheless, the application of acetylcholinesterase biosensors is limited for toxic analysis in food and environment. AChE-based biosensors suffer from major drawbacks: the stability of the enzyme, poor sensitivity and reversibility of the enzyme. Moreover, a large number of AChE biosensors reported in literature have been analyzed on standard sample solutions but, not on real sample solutions. This makes it difficult to fully validate their practicality in real sample analysis.

A recent trend in AChE biosensor manufacturing is to use materials and composites which can provide large surface area and an increased sensitivity. Because of their large surface area, these materials are especially attractive for optical biosensors. As large surface allow the maximum interaction with the enzyme and substrate and increasing its sensitivity towards the sample (Andreescu et al, 2005). In present study, microbial hydrogel was used for developing the biosensor which has the advantages of providing a large surface area thus allowing proper interaction of analyte and bioreceptor which will increase its accuracy and sensitivity. Hydrogel provide better protection and stability to the acetylcholinesterase enzyme for the long term usage of biosensor, apart from being non-toxic, robust and easily available.

3. MATERIALS AND METHODOLOGY

3.1 Reagents and Chemicals

All the reagents and chemicals used were purchased from HI Media (Mumbai, India), Sigma Aldrich (Bengaluru, India) and Lobachemie (Mumbai, India). Experiments were carried out using Luria broth, Luria agar and BPP (Biopolymer producing) media. The composition of BPP 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. Enzymatic assay performed for acetylcholinesterase and indoxyl acetate were procured from Sigma Aldrich (Bengaluru, India). Dichlorvos was brought from local retailer.

3.2 Sample collection

Amphiphilic biopolymers producing microbial strains were obtained from a collection of pre-existing collection from the repository of Dr. Moushumi Ghosh, Department of biotechnology. A total of 10 microbial strains were revived in Luria broth (LB) media. Purity of microbial strains was checked by streaking on Luria agar (LA) plate. The glycerol stocks of microbial strains were made and stored at 4^{o}C for further analysis and gram staining.

3.3 Screening of biopolymer producing microbial strains

Overnight grown revived microbial cultures were used as inoculum for BPP media at 37^{o}C for 48 hours with shaking. The culture broth of BPP media was used for 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 BPP media (Siegmund, and Wagner, 1991). Wells were made in the agar plates and 20 µl of the inoculum was introduced. Then the incubation for 48 hour at 37^{o}C was given and later stored at 4^{o}C for 24 hour. Next day, 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, (1986) 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 and suspended in a buffer solution (K_2HPO_4-16.9 gL^{-1} and KH_2PO_4-7.3 gL^{-1}). The pellets were further diluted to obtain an optical density (OD) of ~ 0.5 at 610 nm. 100 µl of diesel oil were added to 2ml cell suspension in test tube 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

Thavasi et al, (2011) described the E_{24} test for checking the emulsification of biopolymer. The test was performed by using 5 different solvents (Petrol and Diesel (HP petroleum pump, Patiala), Kerosene (Depo, Patiala), mustard oil (Saffola, India) and Xylene (HI media, Mumbai, India). Equal amount of cell supernatant was added to 2ml oil test tube and vortexed for 2min 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).

$$E_{24} = \frac{Height\ of\ emulsified\ layer}{Total\ height} \times 100$$

d) Drop collapse test

Drop collapse test (Shahaliyan et al, 2015) was performed by adding 2 µl of diesel to the 96 well micro titre plates. The plates were equilibrated at 37^{o}C for 1 hour. After that 5 µl 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 sulphate) 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 petri 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 37^{o}C. All the petri dishes were visually examined for a clear zone around the colony (Rodrigues et al, 2010).

3.4 Extraction of extracellular Biopolymer

Extraction of extracellular amphiphilic biopolymer was carried out by growing cultures in BPP media. Microbial culture in Luria broth was incubated overnight at 37°C with shaking. 1% culture was used for inoculating BPP Media at 37°C for 48 hours with shaking. The overnight grown culture was harvested by centrifugation, supernatant was collected and concentrated to $1/10^{th}$ of its original volume by lyophilizing. The biopolymer was precipitated by using equal amount of ethanol for 24 hours at 4°C. The crude biopolymer sample was purified by treating with CPC (Cetyl Pyridinium Chloride) followed by dialysis. The biopolymer obtained was stored in powdered form after lyophilization (Ghosh et al, 2009).

3.5 Characterization of biopolymer

3.5.1 Scanning electron microscopy (SEM)

SEM was performed to understand the surface topography and composition of biopolymer. Sample (G_2) was analysed on SEM system (JSM541-V, JOEL, Japan) using an acceleration voltage of 10 kV and magnification ranging from 100 to 1000 folds (Mao et al, 2010).

3.5.2 X-ray diffraction (XRD)

Biopolymer properties regarding crystallinity, inter chain separation and inter planar spacing was obtained using X-ray diffraction. XRD patterns were recorded on Diffractometer system (XPERT-PRO, Japan) for G_2 sample. The voltage of 40 kV and 30 mA current was applied with CuKα radiation. The G_2 was scanned from 1.4° to 50°at 2θ (da Silv eta et al, 2010).

3.5.3 Fourier transform infrared spectroscopy (FTIR)

The functional groups present in the sample (biopolymer) was analysed by using FTIR. Purified amphiphilic biopolymer (1 mg) was mixed with KBr (potassium bromide (100mg)) which was used for background reference (Jeong et al, 2007) and pressed with 7500 kg for 30 secs 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).

3.6 Preparation of hydrogel

Three different hydrogel (PVA, chitosan and biopolymer (G_2)) 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°C (solution A). pH of cooled PVA solution was maintained at 2.00 ± 0.005 with 1.0 M HCl. Powdered 2.5 g chitosan was dissolved in 250.0 ml of Milli-Q water with 2% acetic acid, under constant stirring for 48-72 hours (solution B). Biopolymer solution was prepared by dissolving 1.25g of G_2 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 mixed and pH was adjusted to 4.00 ± 0.05 with 1.0 M NaOH solution. The mixture was kept on magnetic stirrer for 5-10 min and cross-linker reagent glutaraldehyde (GA) was added slowly. The final concentration of GA in hydrogel 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).

3.7 Characterization of hydrogel

3.7.1 Scanning electron microscopy (SEM)

The structure, surface topology and cross linking interaction of the hydrogel was analysed through SEM. Hydrogel samples were prepared by cutting them into small pieces and fixed with 1% cold GA solution at 4°C for 2 hours. Further, hydrogels was frozen and lyophilized for analysing on SEM (JSM541-V, JOEL, Japan). The microscope was coupled to an energy dispersive spectrometer (EDS). The samples were metallized to make them conductive and analysed at 15 kV voltages (Mathews et al, 2008).

3.7.2 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to characterize the presence of specific functional groups in the hydrogel. Hydrogel blends cross-linked with GA (PVA), (PVA:CH::1:1), (G_2:PVA::1:1) were obtained as fine powdered and analysed through FTIR using attenuated total reflection (ATR) modes. Spectra of FTIR were obtained in the transmission wavelength range of 4,000 to 400 cm^{-1} during 64 scans, with 1 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).

3.7.3 X- ray diffraction (XRD)

Wide-edge X-beam powder diffraction profiles were recorded for hydrogels 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).

3.7.4 Swelling behaviour

Swelling behaviour of hydrogel was quantified by weighing the dried hydrogel and soaking it 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 each time.

The swelling percentage was calculated by:

$$\text{Swelling } \% = \frac{W_S - W_d}{W_d} \times 100$$

Where, W_s is the swollen weight of hydrogel and $W_d$is the dry weight of hydrogel (Gulrez et al, 2011).

3.8 Enzyme kinetics

Factors affecting enzyme kinetics were studied by varying concentration of enzyme, substrate, incubation time. The pesticide concentration was varied for studying the enzyme inhibition kinetics.

3.8.1 Solutions preparation

Acetylcholinesterase (AChE) enzyme stock solution of 1000 units/ml was prepared in phosphate buffer saline (PBS) of 7.4 pH and kept at -20°C .The substrate indoxyl acetate stock solution of 10 mg/ml was prepared in methanol and kept at -20°C (Apilux et al, 2015). Stock solution of dichlorvos pesticide was prepared in water. Blocking solution was prepared by mixing 50mM boric acid with 0.5% casein. Washing solution was prepared by 50mM PBS buffer with 0.1% SDS.

3.8.2 Enzyme inhibition assay:

The enzyme assay was performed in 96 well plate using acetylcholinesterase enzyme and indoxyl acetate as substrate. AChE enzyme undergoes catalytic hydrolysis to produce a blue coloured chromophore (fig. 3.1). Assay was performed by taking 5 µl of acetylcholinesterase concentration ranging from 100-1000 units/ml along with control lacking enzyme. For inhibition assay, each enzyme concentration was made to react with 5 µl of dichlorvos pesticide as inhibitor ranging from 500-20000 ppm. After 10 min of incubation 5 µl of indoxyl acetate concentration ranging from 1-10 mg/ml was added. The reaction mixture was again incubated for 50 min at 45°C (Assis et al, 2010) before measuring the final absorbance at 670 nm.

Inhibition assay was carried out by two set of controls which was devoid of inhibitor and substrate respectively (Pohanka et al, 2011).

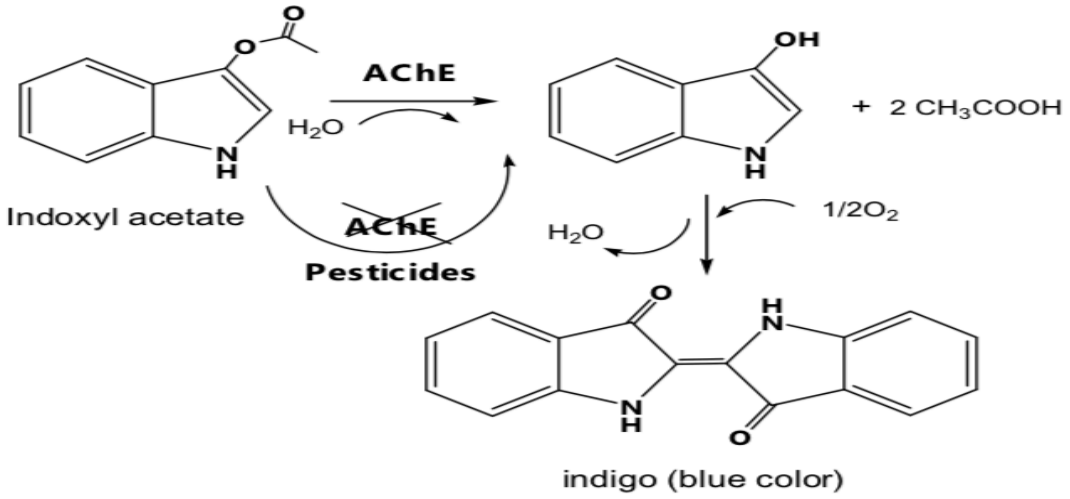


Fig. 3.1: Mechanism of AChE enzyme with indoxyl acetate (Apilux et al, 2015)

3.8.3 In vitro assay for acetylcholinesterase enzyme

Enzyme assay was performed with indoxyl acetate substrate and AChE enzyme. Different concentration of AChE enzyme were prepared ranging from 100U/ml to 1000U/ml in PBS buffer of 7.4 pH (Apilux et al, 2015). The concentration of substrate and other factors were kept constant. Enzyme activity was optimized in 96 well plate at 45°C for 50 min and absorbance was measured at 670 nm (Pohanka et al, 2011).

3.8.4 Determination of substrate concentration

Different concentration of indoxyl substrate ranging from 1mg/ml to 10mg/ml were prepared and assay was performed in 96 well plate at 670 nm with optimized 800 units/ml concentration of enzyme in previous assay (Apilux et al, 2015).

3.8.5 Optimization of pH and Temperature

The pH of enzymatic assay was optimized by varying the pH of indoxyl acetate substrate from 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. Three different buffers were prepared namely citrate Tris HCL (9 to 7.2), citrate phosphate (7.5 to 4) and HCl (4.5 to 2.5). These were utilized for setting up the required pH for substrate. Individual controls were set up for each varied substrate pH which was devoid of enzyme. Any sought of substrate hydrolysis due to pH variation was corrected by subtracting control values from activity shown by enzyme (Assis et al, 2010).

Assay was also performed for optimizing temperature by carrying out enzymatic activity at different temperature ranging from 4^{o}C, 20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 37^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C and 65^{o}C (Assis et al, 2010).

3.8.6 Rate of reaction

Velocity of the reaction was calculated by executing the AChE enzyme assay with indoxyl acetate substrate by incubating the reaction mixture for different time incubation. Rate of

reaction will give us the velocity at which one unit of enzyme catalyses substrate into product concentration (Pohanka et al, 2011).

3.8.7 Inhibition kinetics

Inhibition assay was performed by 800U/ml AChE enzyme and substrate 5mg/ml where pesticide (dichlorvos) used as inhibitor to the reaction. Different dilutions of pesticides ranging from 20000 ppm to 500 ppm were used for performing inhibition assay. The AChE enzyme and pesticides were incubated for 15 min at 45°C followed by addition of substrate (indoxyl acetate) to see the inhibitory effect. Inhibition kinetics was obtained by the difference between the AChE enzyme and its incubation with pesticide (Pohanka et al, 2011).

3.9 Fabrication of AChE based biosensor

AChE based biosensor strips was prepared from Whatman filter paper of size (1*5cm). The strips were sterilized at 121° C for 15 min before use. The AChE enzyme was immobilized onto strip and allowed to dry for 1 hour at room temperature. This was followed by immersing the strip into blocking solution to prevent non-specific binding and was allowed to dry at room temperature for 2 hours. The blocked enzyme strip was then washed by washing solution to remove excess enzyme and kept for drying at room temperature for 2 hours. The prepared enzyme strips were directly immersed into hydrogel solution (G_2 and PVA) for encapsulation.

3.10 Design and working of the biosensor

The design of developed biosensor was a strip type that is encapsulated in hydrogel so to provide enhanced absorbing property. All samples for pesticide residue were tested by washing the samples with water/PBS prior to analysis. The residual pesticide if any present in sample was removed in washed solution. The solution was then used for determining dichlorvos.

The working of the biosensor was determined by immersing the biosensor in the PBS buffer for 5 min. Later, it was dipped in pesticide solution for 5 min and kept on incubation for 15 min. At last, the final result are obtained by drenching the biosensor in substrate solution for 5 min and kept for incubation for 15 min. if any pesticide present in sample, then the colour intensity produced by biosensor was observed.

3.11 Pesticide detection using AChE based biosensor

The performance of AChE based hydrogel biosensor was tested for pesticide detection in cabbage, cauliflower, brinjal, rice, water and milk samples. The optimized concentrations of pesticide was spiked to different samples and were allowed to equilibrate for 24 hrs at room temperature. Next day the vegetable samples were chopped into small pieces and immersed in PBS buffer for washing. The washing of sample allow the draining of pesticide residue in it. The supernatant was subjected to pesticide analysis by AChE based biosensor. After incubating for 15 min substrate was added and colour production was noticed by naked eye.

3.12 Data processing and Statistical analysis

All experiments were run in triplicates and data presented are mean of the observations. The color signal generated by the reaction of AChE with the indoxyl acetate substrate can be determined by naked eyes for a qualitative analysis. The images were captured using a digital camera and analyzed by ImageJ software (National Institutes of Health, USA).

The color signal at the test zone image was analyzed by measuring the mean intensity in the RGB channel. The mean intensity value of each test zone was obtained by subtracting the intensity from that of the background (the area above the test zone). Next, the background-subtracted intensity values were used to obtain a calibration curve.

The dichlorvos residue in sample solutions from cabbage, cauliflower, brinjal, rice, water and milk were analysed with the help of AChE based biosensor. It was determined with the colour signal produced by reaction of AChE enzyme with indoxyl acetate. The chromophore signal enable quantification of pesticide residue in samples.

4. RESULTS AND DISCUSSION

4.1 Screening of amphiphilic biopolymer producing microbial strains

Amphiphilic biopolymer production ability of 10 different microbial 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 10 microbial strains (G_1, G_2, G_3, G_4, G_5, G_6, G_7, G_8, G_9 and G_{10}) were examined in which 2 microbial strains (G_2, G_5) were positive and rest of the microbial 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 plate, indicates negative results. Sodium dodecyl sulphate (SDS) and water were used as positive and negative control for this test respectively. Since this test is specific for some anionic amphiphilic biopolymers, another screening method was opted for better result (i.e. hemolytic test).

Hemolytic assay is generally performed to screen amphiphilic biopolymer producing microorganisms. This assay yields superior results in many cases (Banat, 1993). Result of the hemolytic test indicated that out of 10 microbial strains, 2 strains (G_2 and G_5) showed positive results and other microbial strains were found partially haemolytic or non- haemolytic (Table 4.1). Mulligan et al, (1984) suggested that blood agar lysis as a preparatory screening strategy for amphiphilic biopolymer generation.

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 G_2 and

G_5 microbial isolate. Clear zone around the colonies on blood agar plate introduced these microbial isolates as an amphiphilic biogenic polymer producer, while less defined zone around the colonies demonstrated lower hemolysis activity in G_6 and G_9 microbial isolates. In other words, G_2 and G_5 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 pores constraints in extracellular amphiphilic biopolymer production particularly when microorganisms are studied directly on blood agar plate. To avoid anomalies, the G_2 and G_1 microbial 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 microbial cell surface (Al-Tahhan et al, 2000).

Drop collapse test had been reported by 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 microbial strains, four strains (G_2, G_5, G_9 and G_{10}) were positive and showed flat drop after 1 min. With strains G_4, G_9, G_7 and G_1 lesser flat drop while G_6 and G_3 strains showed least flat drop as compared to negative control (distilled H_2O). 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 G_1, G_5, G_9 and G_{10} microbial isolates received a score of (+++). Amphiphilic biopolymers are found to form a layer on the oil surface causing the suspensions of G_2, G_5, G_9 and G_{10} microbial isolates to degrade immediately. It is generally accepted that drops of culture which gets flat rapidly and formed circle, indicate high

concentration of amphiphilic biopolymer. Isolate G_6 and G_3 did not form flat drop immediately so these microbial 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 G_6 and G_3 cannot be designated as non-produced (Shahaliyan et al, 2015).

Table 4.1 Screening of amphiphilic polymer producing microbial strains

S No.	Bacterial Strain	CTAB Assay	Haemolysis Assay	Drop collapse Assay	BATH Assay
1	G_1	-	-	++	-
2	G_2	++	++	+++	+++
3	G_3	-	-	+	++
4	G_4	-	-	++	+++
5	G_5	++	++	+++	++
6	G_6	-	+	+	-
7	G_7	-	-	++	+
8	G_8	-	-	++	-
9	G_9	-	+	+++	+
10	G_{10}	-	-	+++	++
11	SDS (Positive control)	+++		+++	
12	Water (Negative 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 negative control
BATH assay: '+++' cell adhesion > 45%, '++' cell adhesion > 15%, '+' cell adhesion > 0.6%

BATH test was conducted in this study with 10 microbial strains out of which G_5 showed highest adhesion to diesel i.e. 45% and G_2 showed 40% adhesion to hydrocarbon. Other strains

showed very less or no adhesion to diesel oil. Results of all the 10 microbial 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 microbes and did not leave the water phase. This could be possibly affecting the adhesion of the microbial cells.Rosenberg et al, (1986) 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 G_5.

By inoculating the microbial strains into BPP medium was used to check the maximum production of 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 tests were demonstrated that out of 10 microbial strains, only 4 microbial strains showed positive result against four hydrocarbons (petrol, diesel, kerosene and xylene), and the response of 6 microbial strains were not found significant. However, the microbial strains with mustard oil showed no emulsification stability (Table 4.2). Emulsification index of G_2 with petrol, diesel, kerosene and xylene was 61.5, 60, 25 and 30 respectively; whereas emulsification index of G_5 and G_9 was 35, 40, 25, 30 and 50, 60 respectively. As G_2, G_5 and G_9 shows emulsification index but the microbial strain G_2 show better result as comparative to G_5 and G_9. So, G_2 strain was selected for biopolymer production. The emulsification of petrol, diesel, kerosene and xylene by microbial culture was determined through visual observation. It was mentioned that microbial 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 microbial strains showed no variation with

mustard oil. When the hydrophobic compounds (petrol, diesel, kerosene and xylene) mixed with microbial culture broth, stable and static emulsion layer was formed that indicated the presence of amphiphilic biopolymer. The polymer creates emulsion between two immiscible liquids as reported by Makkar and Cameotra (2002).

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

S No.	Bacterial Strain	Petrol	Diesel	Mustard oil	Kerosene	xylene
1	G_1	-	-	-	-	-
2	G_2	+++ (61.5)	+++ (60)	-	++ (25)	++ (30)
3	G_3	+ (4.5)	+ (11)	-	-	+ (10)
4	G_4	-	-	-	-	+ (5)
5	G_5	++ (35)	++ (40)	-	++ (25)	++ (30)
6	G_6	-	++ (25)	-	+ (10)	-
7	G_7	-	-	-	+ (5)	+ (10)
8	G_8	-	-	-	+ (5)	-
9	G_9	+++ (50)	+++ (50)	-	-	-
10	G_{10}	+ (15)	+ (15)	-	-	-
11	SDS (Positive control)	+++	+++	+++	+++	+++
12	Water (Negative control)	-	-	-	-	-

Values are in percentage

The present study suggested that microbial isolate G_2 had higher capability for the formation of emulsion layer with petrol, diesel, kerosene and xylene which suggested that microbial isolate G_2 could produce extracellular amphiphilic biopolymer. Release of extracellular

polymer decreases the surface tension and the microbial culture absorbed and metabolised the hydrocarbons used in present experiment and formed emulsification (Chukwudi, 2010).

Therefore, on the basis of above mentioned screening methods, one potential microbial strain was selected for further studies.

4.2 Amphiphilic biopolymer production:

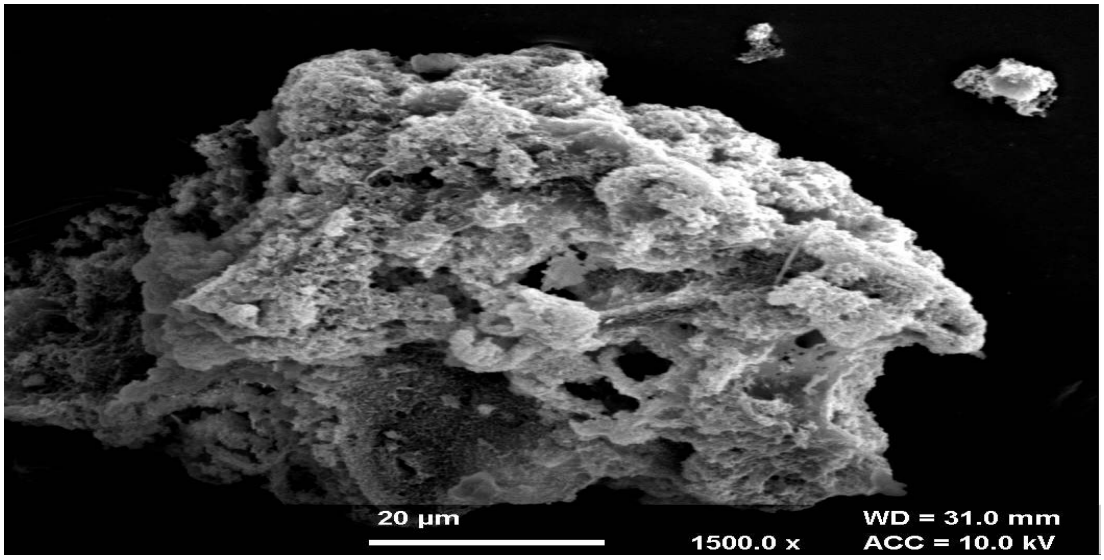
Amongst the biopolymers chosen from repository, only one from strain G_2 possessed high amphiphilicity as determined from the initial tests. Therefore, this strain was chosen for further experiments. The microbial strain G_2 was cultured in the BPP media for 48 hours and biopolymer obtained from supernatant. The yield of biopolymer was 0.258 gram/L and was used for further experiments. The BPP media has been suggested by earlier workers as an optimal media for obtaining biopolymers of extracellular nature, therefore this media was the one for choice to obtain the biopolymer (Kaur et al, 2013).

4.3) Characterization of biopolymer

4.3.1) Scanning electron microscopy (SEM)

SEM micrographs are used to study the composite and morphology of synthesized materials. The purified biopolymer G_2 was observed to be white to off white fluffy granular powder. The biopolymer was examined under scanning electron microscope and observed to be fibrous and porous in nature as shown in Fig. 4.1. The fine granules of biopolymer are irregular in shape with size <20 um in the longest dimension. Granular shapes were observed as aggregates within

the range <1500 um in the largest dimension. Grooves and ridges were distinguishable on its surface in range 600 um indicating a large surface area of biopolymer (Tushar et al, 2014).



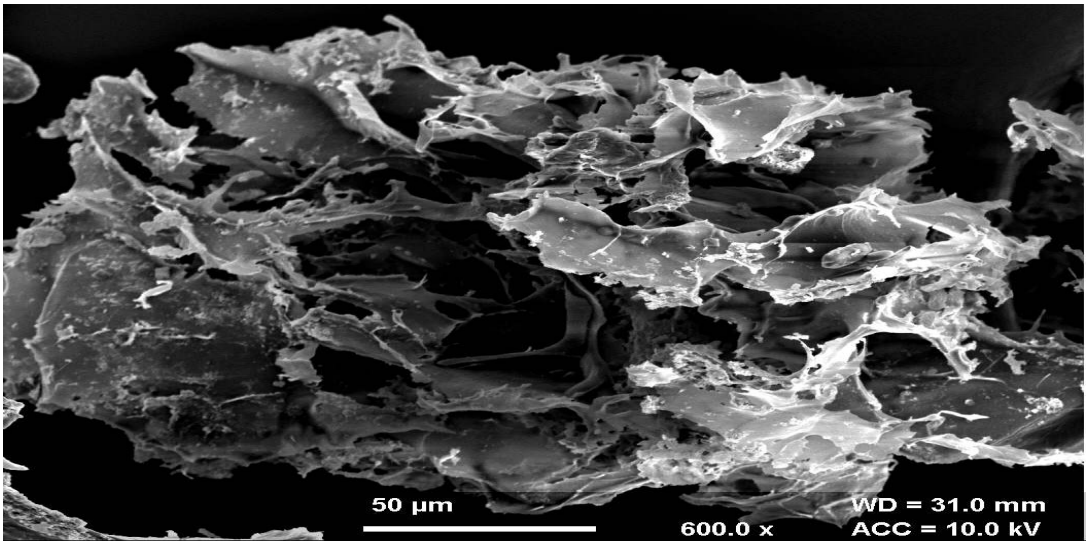


Fig. 4.1: Biopolymer G_2 as observed under scanning electron microscope with different magnifications (a) Magnification 1500 X, Bar size 20 µm, (b) Magnification 600 X, Bar size 50 µm.

4.3.2) Fourier transformed infrared spectroscopy of biopolymer G_2

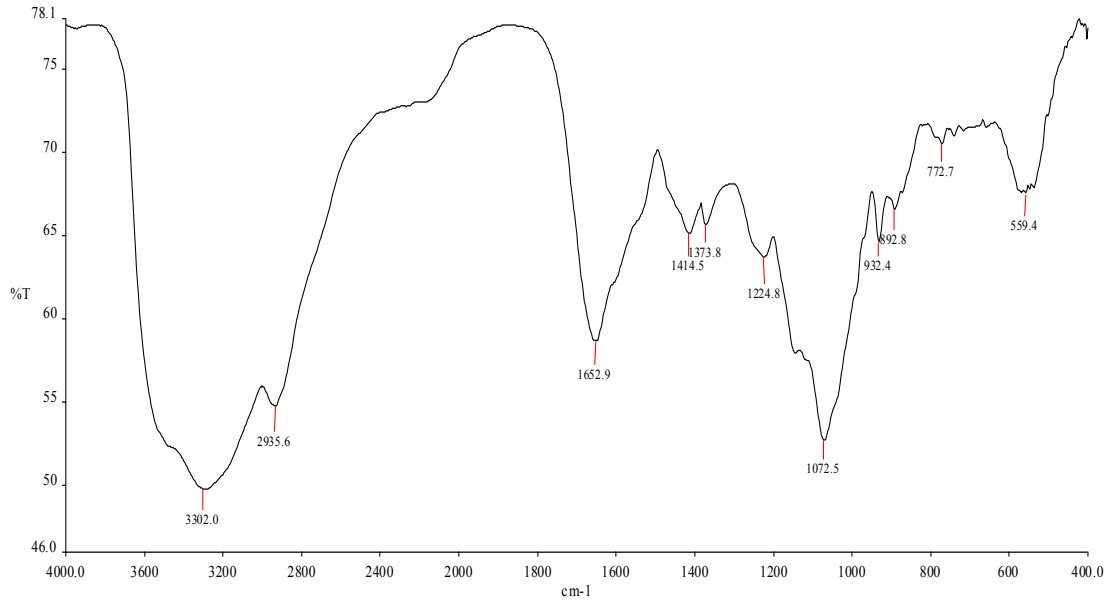


Fig. 4.2: Fourier transformed infrared spectroscopy spectra of biopolymer (G_2)

FTIR spectroscopy was used to analyze the functional groups present in biopolymer G_2. The FTIR spectra obtained was between the wavelength (cm^{-1}) and the transmission percentage. The stretching vibration is due to presence of alkyl group (C-X) at 559 cm^{-1}. The vibration peak of alkene (C-H) was seen on spectra at 772 cm^{-1} and 892 cm^{-1}. The peak at 932 cm^{-1} represent hydroxyl group (O-H). The long stretching peak was observed at 1072 cm^{-1} due to aliphatic amines (C-N). The peak at 1224 cm^{-1}, 1373 cm^{-1} and 1414 cm^{-1} represents the presence of haloalkane (C-H), phenol (O-H) and sulphate (S=O) respectively. The deep stretching of peak at 1652 cm^{-1} show the presence of alkene (C=C). methylene and amine group show a strong vibration weak at 2935 cm^{-1} and 3301 cm^{-1} respectively. The results indicated FTIR spectra of a typical polysaccharide.

4.3.3) X-ray diffraction

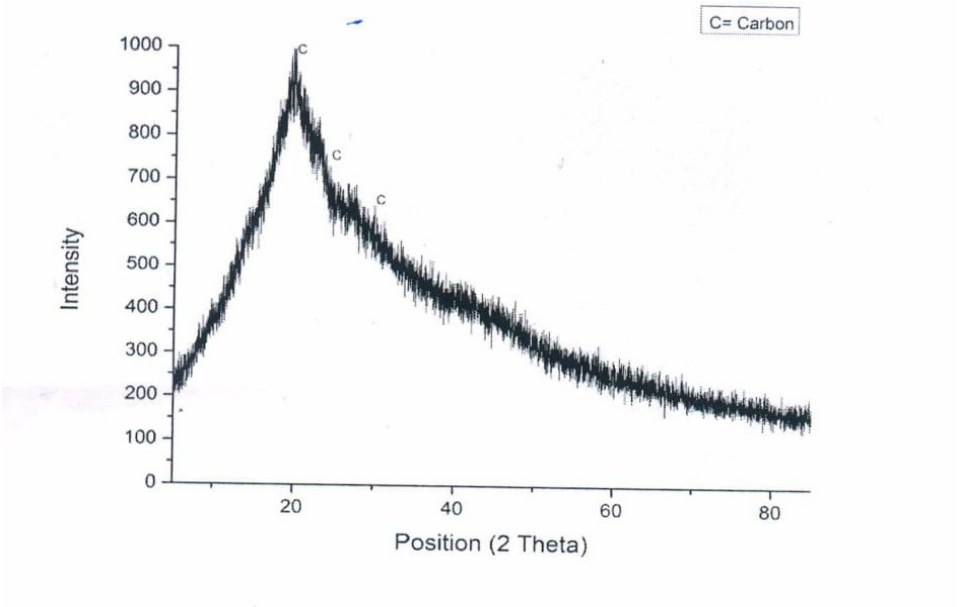


Fig. 4.3: X- ray diffraction spectra of microbial polymer (G_2)

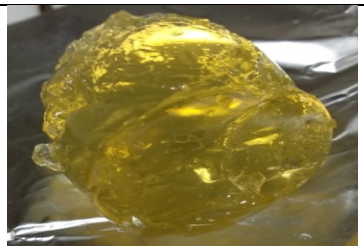
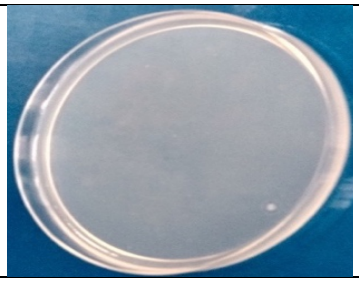
XRD was performed for G_2 to analyze the structural properties such as degree of crystallinity, amorphous etc. It was observed from XRD spectra that G_2 possessed an amorphous in nature. Only carbon were present in G_2 in range of 20^o to 35^o (Fig. 4.3). Pure diffraction peak of sample G_2 was at $2\theta=20^o$ (Tripathi et al, 2009).

4.4) Preparation of hydrogels

Three different hydrogels were prepared successfully as shown in table 4.3. The first hydrogel comprised of PVA alone but was fragile with transparent appearance. Next hydrogel prepared comprised of PVA: Chitosan (1:1) possessed good elasticity and yellow transparent color due to chitosan. The third hydrogel was synthesized by replacing chitosan with G_2 with same ratio as above. The hydrogel prepared was found to be transparent, show high elasticity among the

three hydrogels. All hydrogels were observed to be homogenous in nature during solubility and phage segregation.

Table 4.3: Different type of hydrogel and their appearance.

S. no.	Sample	Different Hydrogel Concentration	Visual Appearance of Hydrogels
1	PVA	1:0	
2	PVA: Chitosan	1:1	
3	PVA: Biopolymer(G_2)	1:1	

Mathews et al, (2008) prepared different hydrogel with varying concentration PVA: Chitosan. On similar lines it was possible to successfully prepare the three different hydrogels with concentration of PVA: Chitosan (1:1), PVA (alone), PVA: Biopolymer (1:1). The microbial hydrogel possessed good visual clarity in comparison to the other hydrogels. It is important for hydrogels to have sufficient clarity as this enables coloration patterns if the hydrogels are to be uses as visual indication in determining presence or absence of any compound by qualitative means.

4.5) Characterization of hydrogels

4.5.1) Scanning electron microscopy (SEM) and EDS

SEM has been described as an invaluable tool for understanding the architectural pattern of polymers. In order to gain insights of the structure of the hydrogels developed, SEM along with EDS was carried out. EDS aids in determination of the principal chemical components.

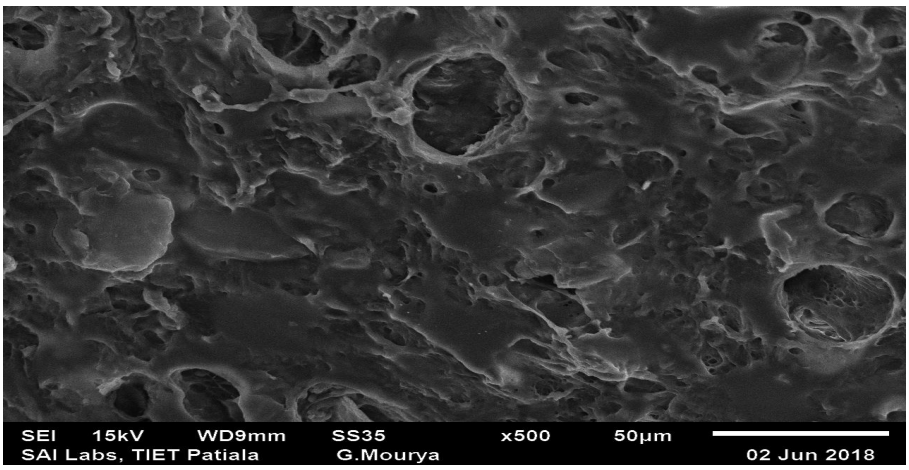


Fig. 4.4: Scanning electron micrograph of PVA: Chitosan with 500X magnification & bar size of 50 µm

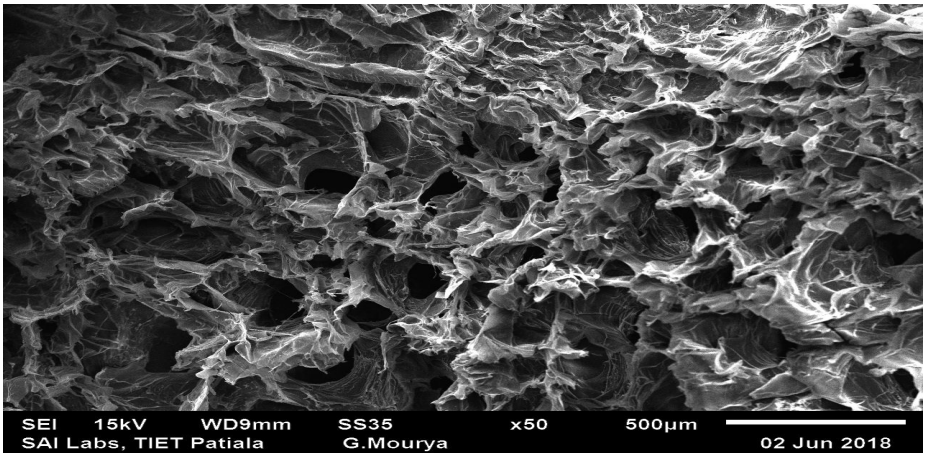


Fig. 4.5: Scanning electron micrograph of PVA: Biopolymer (G_2) with 50X magnification & bar size of 500 µm

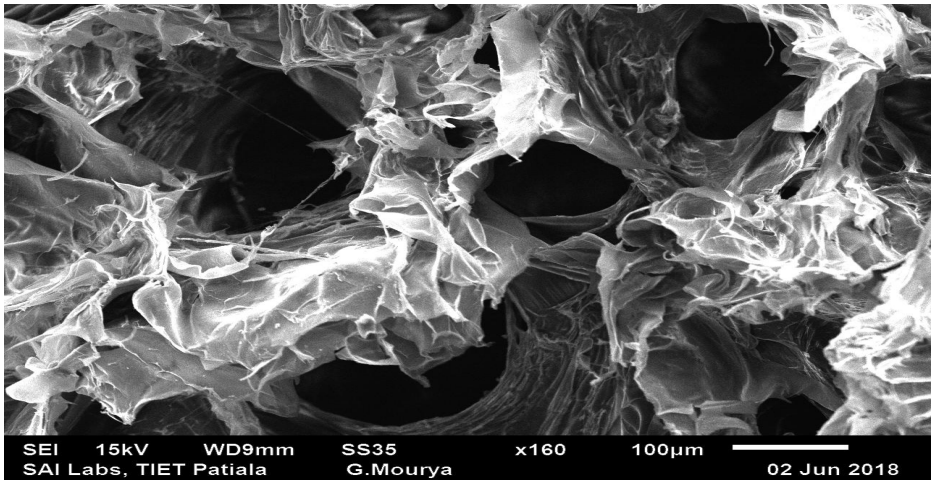


Fig. 4.6: Scanning electron micrograph of PVA with 160X magnification & bar size of 100 µm

Hydrogels synthesized by varying the polymer and its concentration were examined under Scanning electron microscope to observe the surface topography. PVA, PVA: Chitosan, PVA: Biopolymer (G_2) hydrogels were analyzed by subjecting the sample to scanning electron microscopy for comparing the pore size and surface properties. The microporous hydrogel blends synthesized with PVA: Chitosan and PVA: biopolymer was investigated and compared with PVA hydrogel. The effect on pore size and surface morphology of hydrogel using chitosan or biopolymer was reported by Mathews et al, (2008). The SEM image gives high magnification graph of all three different hydrogel. The lyophilized hydrogels were examined and observed the porous nature of the hydrogel. The hydrogel composed of PVA only was observed under SEM having a homogenous porous structure. But when biopolymer (G_2) or Chitosan was added to PVA a significant change in porosity and surface morphology was observed in Fig. 4.5 and Fig. 4.4 respectively.

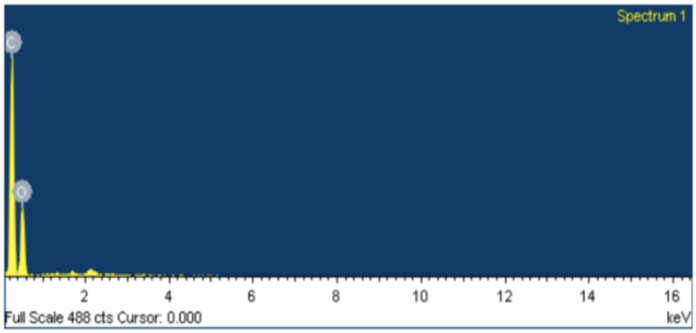


Fig. 4.7: EDS spectra of PVA

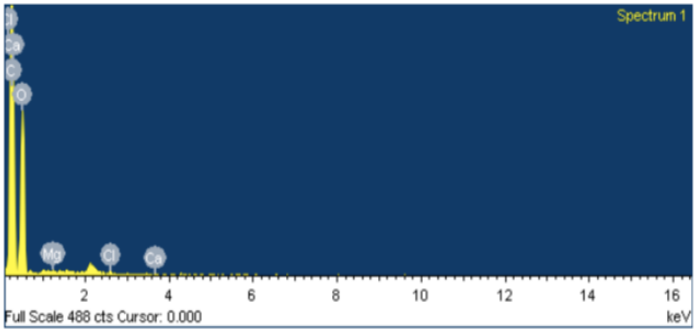


Fig. 4.8: EDS spectra of PVA: Chitosan

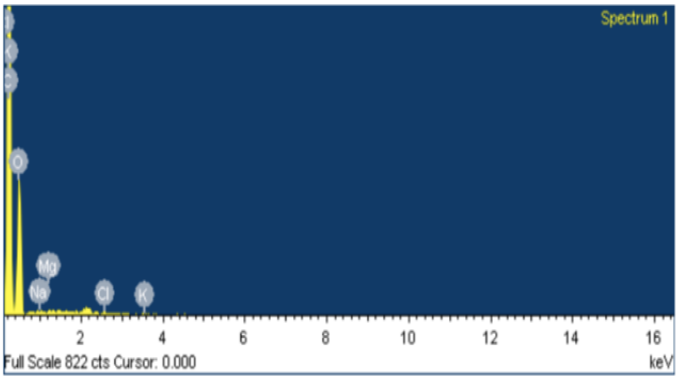


Fig. 4.9: EDS spectra of PVA: Biopolymer

The chemical composition of hydrogels was determined by EDS along with SEM. The carbon and oxygen were found in abundant amount in PVA hydrogel (Fig. 4.7). In PVA: Chitosan hydrogel the peaks are obtained which determines the presence of carbon and oxygen in large quantity and traces of magnesium, calcium and chlorides were found as shown in Fig. 4.8. In PVA: biopolymer (G_2) spectra was obtained by EDS which result in the presence of carbon

and oxygen in large amount and traces of sodium, magnesium, chloride and potassium was analyzed as shown in Fig. 4.9.

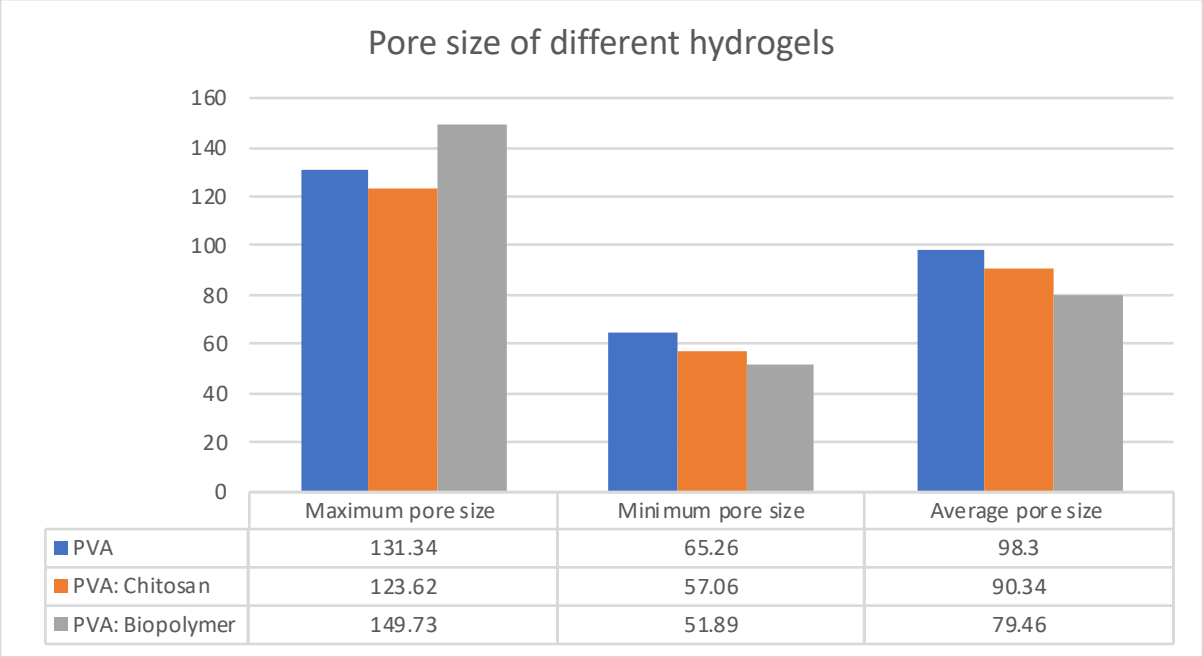


Fig. 4.10: Comparison of Pore size of different hydrogels.

The pore size of different hydrogels was examined the SEM image by using the Image J software. A significant change in pore size was observed in hydrogel blends as average pore size of PVA, PVA: Chitosan, PVA: Biopolymer (G_2) was recorded 98.3, 90.34 and 79.46 um respectively as shown in Fig 4.10. A decrease in pore size was noticed as chitosan was used for hydrogel blend and further chitosan was replaced by biopolymer for hydrogel synthesized and it was noted that a significant decrease in pore size of PVA: biopolymer hydrogel. The small pore size implies a strong possibility of encapsulating AChE enzyme in the microbial hydrogel (Pierre, 2004). Overall, results of SEM demonstrated that chitosan and biopolymer have a significant effect on the morphological and porous nature of hydrogel. The SEM images clearly depicted the filamentous surface topology of hydrogel indicating the opposing behavior of additives during transportation through surface membrane.

Mathews et al, (2008) reported the pore formation in membrane of PVA: chitosan hydrogel. Whereas, in present study PVA: Chitosan and PVA: biopolymer hydrogels were synthesized which showed the filamentous membrane structure indicates the formation of pore in hydrogel. Moreover, a significant change in pore size of hydrogel was noticed in PVA, PVA: Chitosan and PVA: Biopolymer (G_2).

4.5.2) Fourier transform infrared spectroscopy

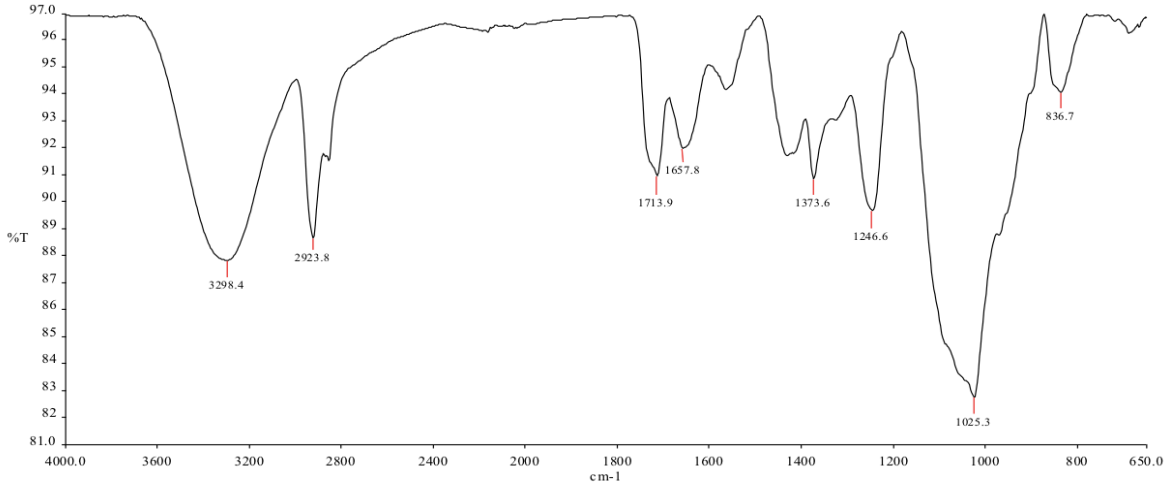


Fig. 4.11: Fourier transformed infrared spectroscopy spectra of PVA: Chitosan.

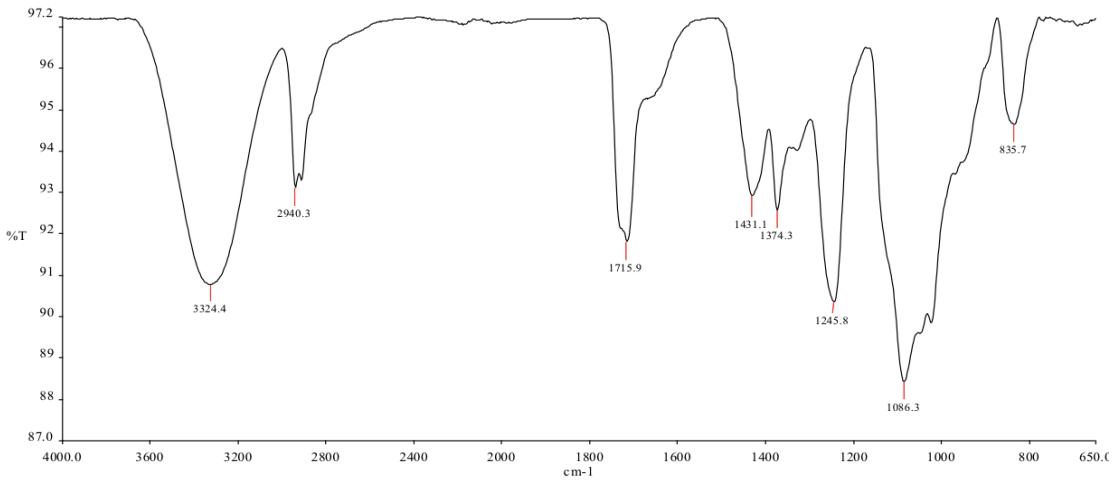


Fig. 4.12: Fourier transformed infrared spectroscopy spectra of PVA.

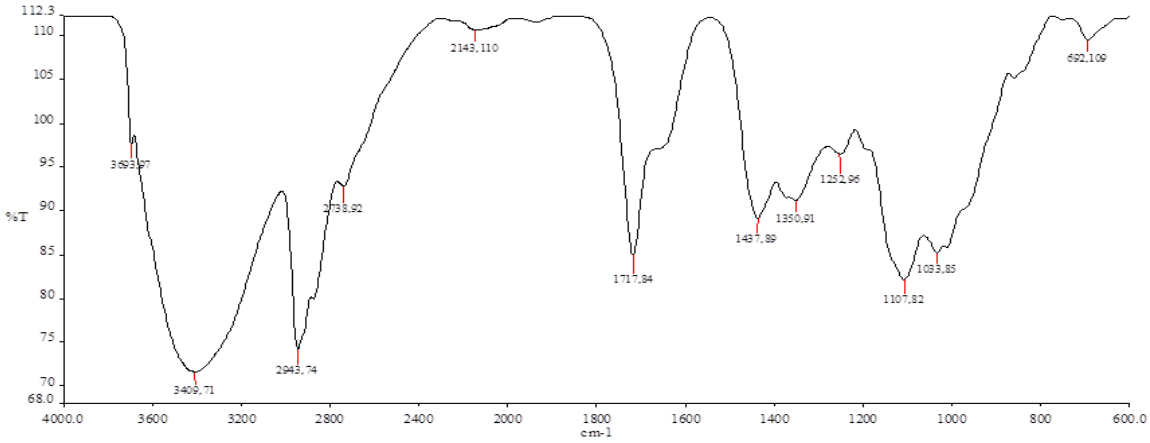


Fig. 4.13: Fourier transformed infrared spectroscopy spectra of PVA: Biopolymer.

FTIR analysis yielded important observations regarding the functional groups present in hydrogel. The FTIR spectra of PVA hydrogel (Fig. 4.12) exhibited medium narrow vibrational peak at 835 cm^{-1} indicating the presence of alkyl group (C-X). The strong narrow peak obtained at 1086 cm^{-1} was due to the presence of amine group (C-N). The presence of alcohol group (C-O) shows a narrow strong peak at 1245 cm^{-1}. the vibrational peak obtained at 1374 cm^{-1} and 1431 cm^{-1} is due to the presence of alkanes and aromatics amine groups respectively. The peak obtained for double bond region is narrow and strong due to presence of carbonyl group (C=O). Alkane groups shows the peak at 2940 cm^{-1}. The broad strong peak was seen for presence of alcohols and phenol group at 3324 cm^{-1}.

The FTIR spectra of PVA Chitosan (Fig.4.11) hydrogel revealed a peak at 836 cm^{-1}, which represents the presence of alkyl groups (C-X). The deep narrow peak obtained at 1025 cm^{-1} attributes to alcoholic group (C-O). The peak at 1246 cm^{-1} and 1373 cm^{-1} was medium narrow represents stretching of haloalkanes and alkanes (C-H) respectively. Amine group (N-H) was present they contributed by showing peak at 1657 cm^{-1}. 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). 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).The stretching of carboxylic group was at 1713 cm^{-1} which was formed by the presence of acetic acid, utilized for chitosan dissolving (Jegal and Lee, 1999). The narrow medium vibrational due to alkane (C-H) at 2923 cm^{-1}. Alcohols and phenols shows a broad medium peak at 3298 cm^{-1}.

The FTIR spectra of PVA Biopolymer (Fig. 4.13) indicated a peak at 692 cm^{-1} attributed to the presence of bending of alkyl bromide group (C-X) (Parida et al, 2011). The narrow strong vibrational peak obtained at 1033 cm^{-1} and 1107 cm^{-1} was due to stretching of aliphatic amine groups (C-N). The medium narrow peaks are obtained at 1252 cm^{-1}, 1350 cm^{-1} and 1437 cm^{-1} was due to stretching of haloalkanes (C-H), alkanes (C-H) and aromatic amine groups (C-C) respectively. The presence of alkyl chloride group (C=O) show peak in double bond region due to stretching of group at 1717 cm^{-1}. The peak at 2143 cm^{-1} and 2738 cm^{-1} represents the presence of alkyne group (C=C) and alkane (C=H) respectively. At 2943 cm^{-1} the narrow stretched peak was observed due to presence of carboxylic acid (O-H). The broader medium peak represents the presence of alcohols and phenols at 3409 cm^{-1} and 3693 cm^{-1}.

4.5.3) X-ray diffraction

The XRD is performed for all three different hydrogels and spectra is obtained as shown in Fig. 4.14. The diffraction peak of PVA hydrogel was obtained in range of 19.4^{o} and 40.3^{o}. the strong diffraction peak indicates the high crystallinity of PVA hydrogel. Whereas, the diffraction peak of PVA: Chitosan hydrogel was at 19.30^{o} and for PVA: Biopolymer (G_2) was at 19.38^{o}. PVA hydrogel intensity to form crystalline in blends was decreased by addition of Chitosan and Biopolymer (G_2). A decrease was noticed after the addition of chitosan and biopolymer at 1870, 1665 and 1549.

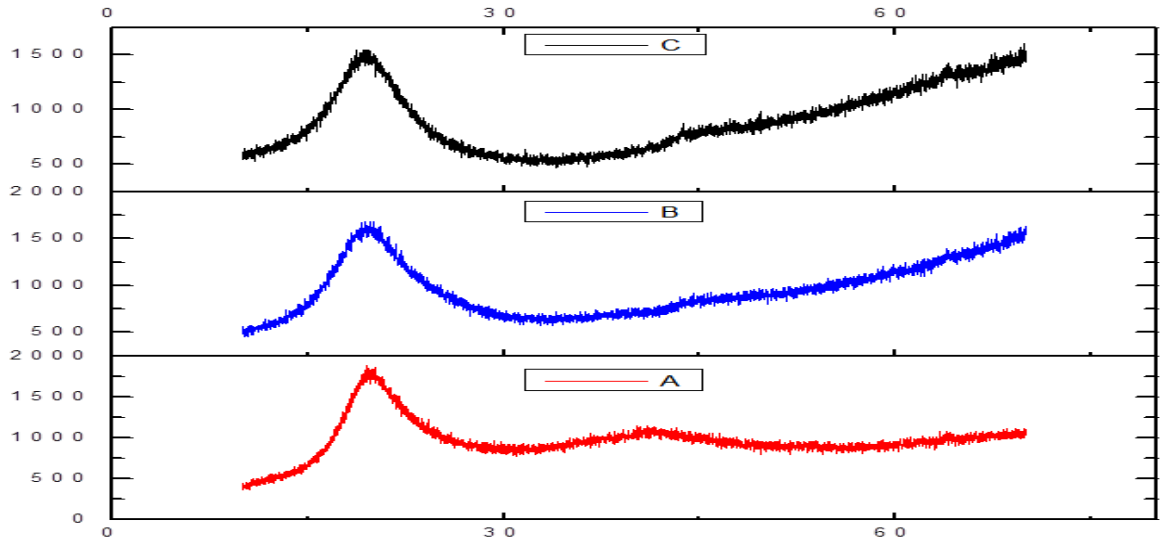


Fig. 4.14: X-ray diffraction spectra of : A) PVA, B)PVA: Chitosan, C) PVA: Biopolymer (G_2)

From above obtained spectra it implies the crystallinity of all three different hydrogels. The crystallinity capability of PVA was opposed by chitosan and biopolymer. Similar reduction in crystallinity of hydrogel was observed by Pal et al, (2007).

4.5.4) Swelling kinetics

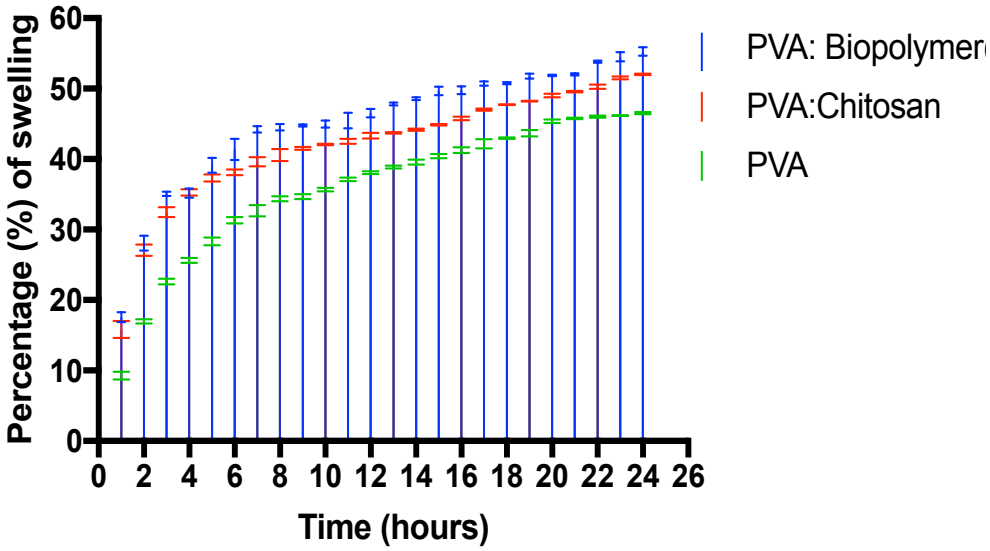


Fig. 4.15: Swelling behavior of different hydrogels

Water retention by hydrogel was mostly due hydrogen bonding between O-H group and water. A steady rise in water retention was observed till 24 hours marking the equilibrium point. The rigidity of hydrogel matrix was based on the concentration of GA used. The higher or lower concentration of cross linker contributes to the high or low swelling property of hydrogel (Parida et al, 2011).Since the cross linker binds the matrix restriction of the movement of macromolecule is observed during swelling over time. The swelling values at equilibrium for PVA, PVA: Chitosan, PVA: biopolymer (G_2) were calculated as: 460%, 520%, 550% respectively. Accordingly PVA: biopolymer combination showed maximum swelling kinetics.

A study carried out by Bajpai and Singh, (2006) on swelling kinetics of different hydrogels, the equilibrium obtained by water intake was found to be 1150%, 652%, 551% respectively. But, in the present study the swelling values at equilibrium for hydrogels were lower than these authors.

4.6) Determination of Enzyme concentration

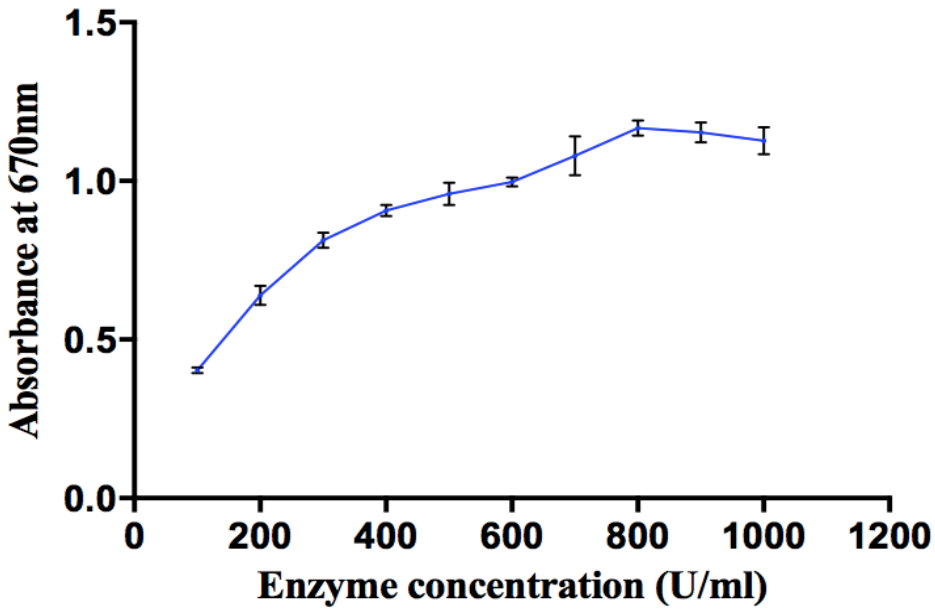


Fig. 4.16: Determination of optimal acetylcholinesterase enzyme concentration.

The optimum concentration of enzyme was determined by the enzyme activity with 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 units/ml of enzyme concentration. Fig. 4.16 revealed that with an increase in enzyme concentration, the absorbance increases for AChE linearly for up to 800 units/ml of AChE. A decline in absorbance values follows thereafter. Absorbance measured is directly proportional to the colorimetric product formed by AChE enzyme and indoxyl acetate substrate. The highest product formation was recorded at 800 units/ml of enzyme concentration. The V_{max} and K_m for acetylcholinesterase enzyme was calculated as 0.621 U/mg and 3.754 respectively. Similar results have been noted by Apilux et al, (2015).

4.7) Determination of optimal Substrate concentration

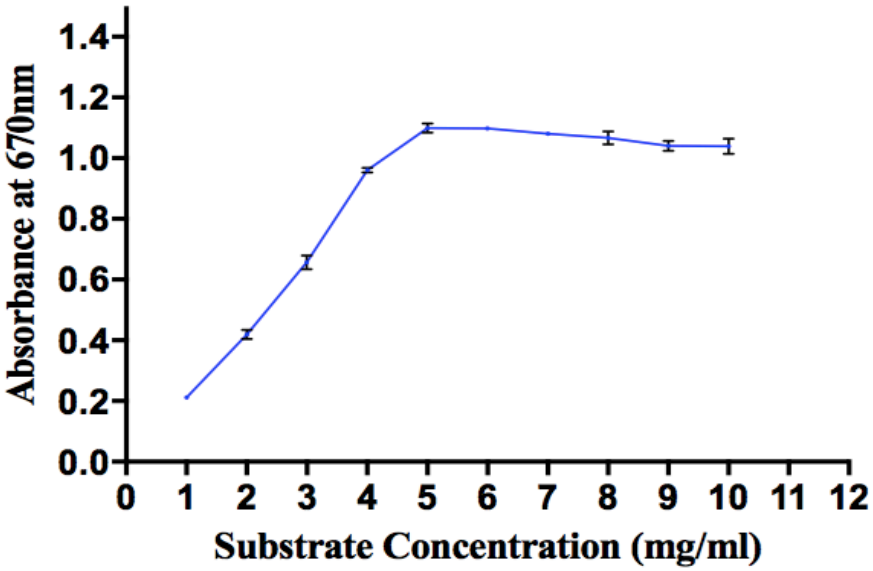


Fig. 4.17: Determination of optimal concentration of indoxyl acetate substrate.

The optimal value of substrate was determined by using different concentrations of substrate indoxyl acetate ranging from 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/ml as shown in fig. 4.17. It was observed that the highest activity of enzyme was observed at 5mg/ml of substrate followed by

negligible change in absorbance. The results were similar to those obtained by Apilux et al, (2015) with indoxyl acetate at 5mg/ml concentration.

4.8) Optimization of pH and Temperature

Assay was performed varying pH and the highest enzyme activity was found to be in the range of 7 to 8 pH. It was observed that the decrease or increase in pH than optimum a decline in enzyme activity was seen in fig. 4.18. Assis et al, 2010 have recorded the similar pH range for enzyme activity.

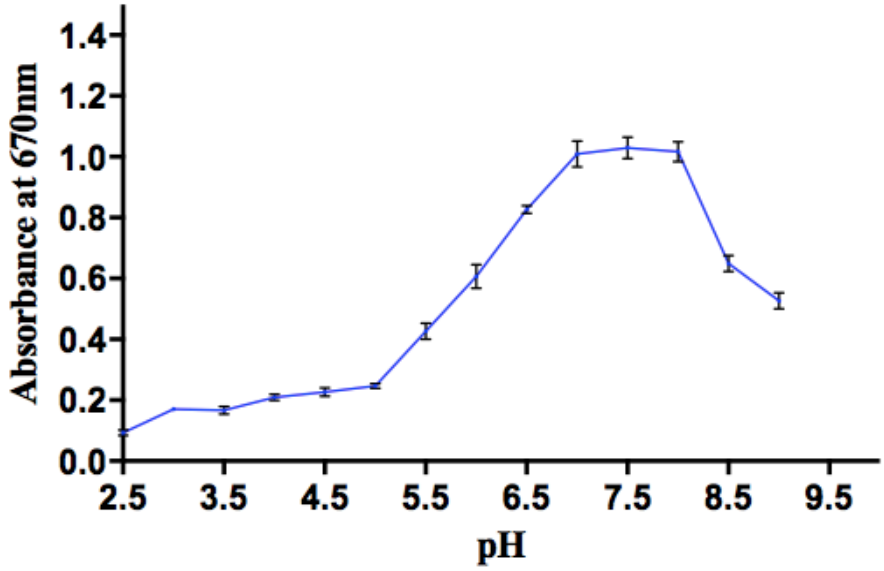


Fig. 4.18: Optimization of enzyme activity on different pH

The temperature was also optimized for enzyme so to observe the effect of temperature on its activity. From fig 4.19, highest enzyme activity was observed at 45^{o}C temperature. At low temperature below optimum, a decrease in activity was due to enzyme substrate loss binding or due to enzyme efficiency. At high temperature above optimum the denaturation of enzyme

starts which leads to decrease in enzyme activity. Assis et al, 2010 optimized the temperature for enzyme and observed that at 45°C enzyme show maximum activity.

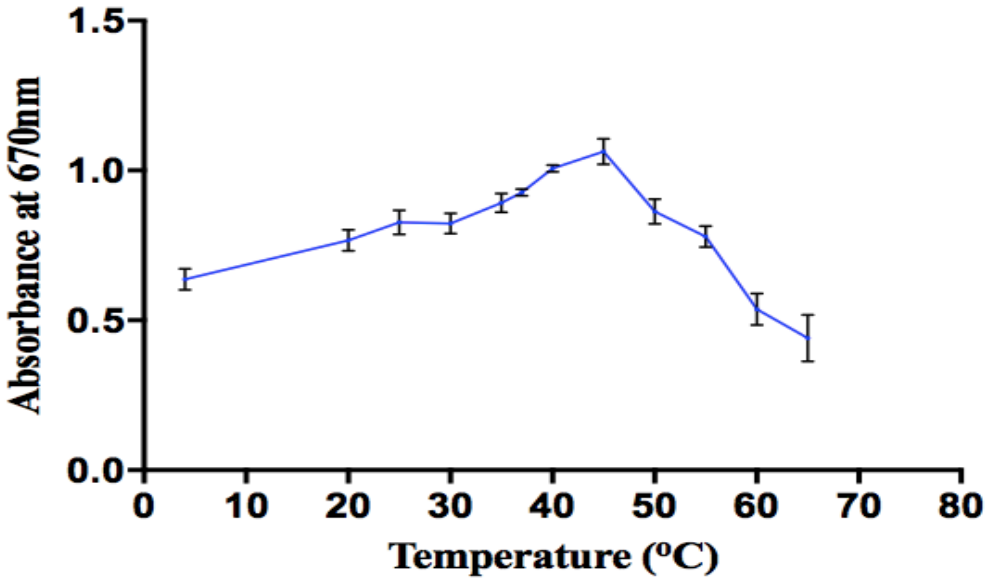


Fig.4.19: Optimization of enzyme activity at different temperature.

4.7) Rate of reaction

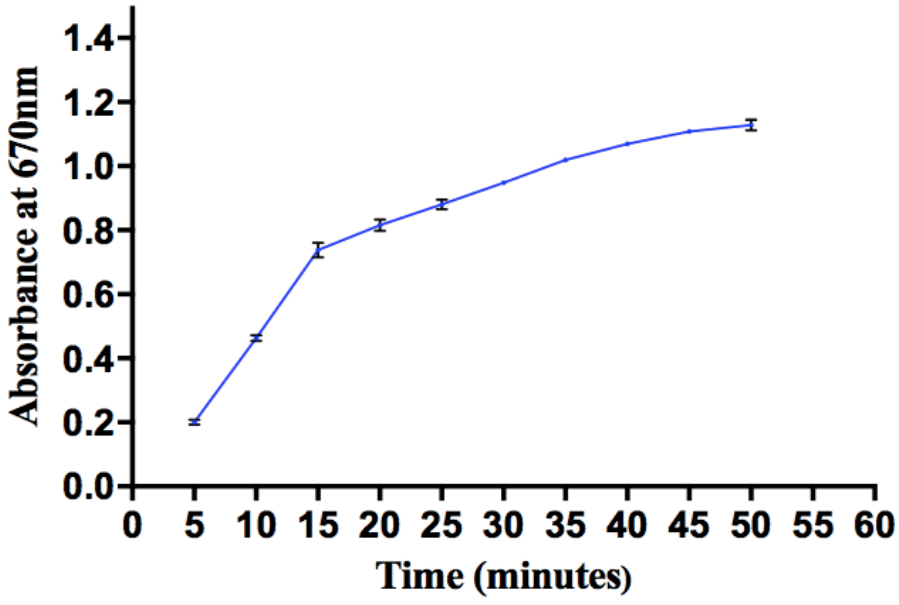


Fig. 4.20: Rate of reaction by AChE enzyme and indoxyl acetate with different incubation times.

Enzyme assay for different incubation time was carried out for determining the enzyme activity. Enzyme activity is expressed in units as one unit of enzyme that catalysis the conversion of 1.25 mg/ml of substrate per minute under conditions optimized. Enzyme activity (v) is calculated by initial reaction velocity when there is least effect.

$$v = \frac{Product\ Formation\ or\ Substrate\ conversion}{Time}$$

4.9) Inhibition assay

Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate is an organophosphate, widely used as an insecticide to control household pests, in public health and protecting stored product from insects. Its prevalence in water, toxicity and presence of residues in commonly consumed food materials has reached alarming proportions in several countries. Several reports in India have indicated the presence of this in a variety of consumable items.

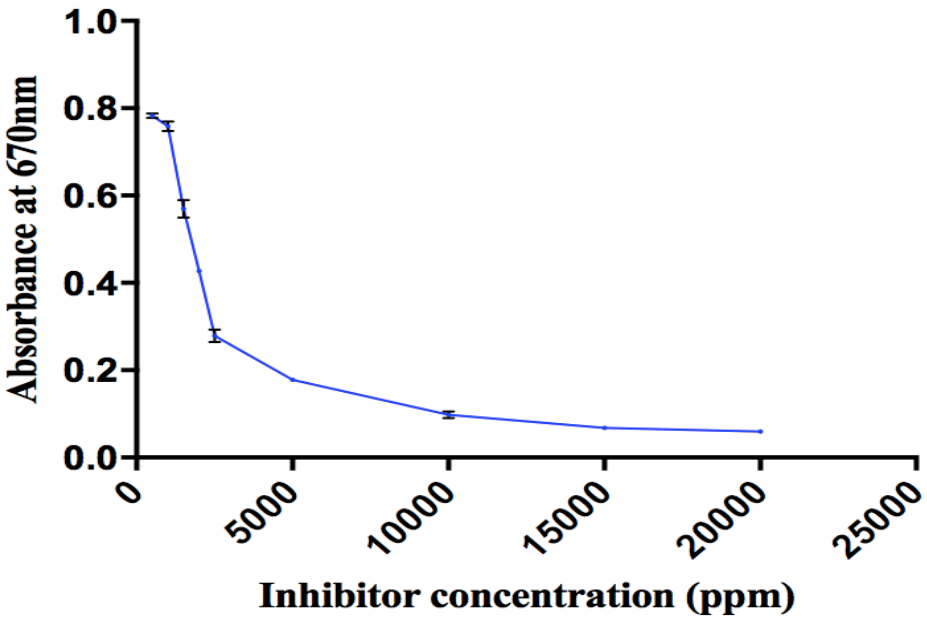


Fig. 4.21: Enzyme (AChE) inhibition kinetics by dichlorvos.

Therefore, this organophosphate pesticide was chosen as a model inhibitor for acetylcholinesterase activity. The concentration of dichlorvos in each well was kept as 500, 1000, 1500, 2000, 2500, 5000, 10000, 15000 and 20000 parts per million. AChE enzyme was completely inhibited by dichlorvos at 10,000 ppm. The inhibition (%) calculated was respectively 21.4%, 22.7%, 40.6%, 57.5%, 70.46%, 82.2%, 93.55% for up to 10,000 ppm. At higher concentrations, little or no variation in enzyme activity was observed due to total inhibition carried out by dichlorvos. Linhares , (2013) performed inhibition kinetics with AChE enzyme and dichlorvos in RBC_s and observed the sensitivity and specifity of enzyme towards dichlorvos was 83.3%, in line with the observations, however the sensitivity and specificity of enzyme calculated from inhibition kinetics graph was 93.5% in the present study.

4.10) Analysis of organophosphorus pesticides using biosensor

Dichlorvos residues present in consumable items was quantified using the biosensor. The biosensor reacts with pesticide solution and produces a colorimetric response, the bioreceptor and analyte interactions leads to the decrease in the colorimetric signal.

In the current study, enzyme-based biosensor with the use of microbial hydrogel provided the advantage of absorbing large amount of water or biological fluid which helps to get accurate result for the samples by allowing complete contact with the enzyme. Besides, Hydrogel encapsulation retained the enzyme, provided stability to the enzyme for the long-term/reuse of biosensor. For assessing storage stability, the biosensor was stored (PBS solution) at 4^oC, 28^oC and 40^oC for 15 days. The biosensor retained 93% activity whereas at 28^oC activity was retained. Loss of activity was notable at 40^oC, these results were in line with observations with

those earlier indicating loss of AChE enzyme activity and inability of encapsulation to protect the enzyme.

In order to judge whether the biosensor could discriminate varying quantities and could be used for a range of products: - milk, water, cabbage, cauliflower, brinjal and rice were chosen. Production of blue indigo color (15 minutes) resulted as a response with indoxyl acetate. The inhibition in coloration was clearly discernable by naked eye and can be judged by the intensity of the color.

However, at very high concentrations of dichlorvos, only confirmation of the presence and absence was possible. For example, at a concentration of 5000 ppm dichlorvos, the biosensor remains colorless showing total inhibition of AChE enzyme. At concentrations below 1000 ppm, biosensor indicates a dark indigo blue color, while concentrations lying within a range of 1000-2500 ppm displayed tint indigo blue color. In range from 2500-5000 ppm a very light indigo blue color with very low intensity is observed. However, the image analysis results were too erratic and the values of pesticides could not be distinguished.

Therefore, a lower concentration (100ppm) of dichlorvos was spiked in the vegetable, rice, water and milk samples. The intensity of color in all cases were measured by ImageJ software for semiquantitative data. The results of dichlorvos spiking of food items at lower concentrations indicated consistency in the semi-quantitative values from image analysis. For rice sample, dichlorvos was observed around 10-20 ppm concentration. In water and milk samples levels of dichlorvos pesticide lay in the range 50-60 ppm. Amongst the vegetable samples like brinjal, cabbage and cauliflower were used for pesticide residue testing. In cabbage, the color intensity corresponded to 30-40 ppm of dichlorvos. Brinjal sample solution

indicated a concentration of 20-30ppm. Highest concentration of dichlorvos (60-70ppm) was detected in Cauliflower, followed by cabbage and brinjal respectively.

Table 4.4: Coloration profile of the AChE biosensor with high initial spiking of dichlorvos in food items:

Coloration observed within 15 minutes	Concentration of dichlorvos	Remarks
Colorless	≥5000ppm	Complete inhibition of AChE
Very light blue-indigo	2500-5000ppm	Near complete inhibition of AChE (Concentration not distinguishable)
Tint of blue indigo	1000-2500ppm	Low–moderate inhibition (Concentration not distinguishable)
Dark blue indigo	500- 1000ppm	Low inhibition (Concentration not distinguishable)
Dark blue indigo with low intensity	<500ppm	Lowest inhibition

However as specified by Apilux et al, (2015) color intensity could not be analyzed below 5ppm concentrations of dichlorvos, which remained the limit of detection (LOD).

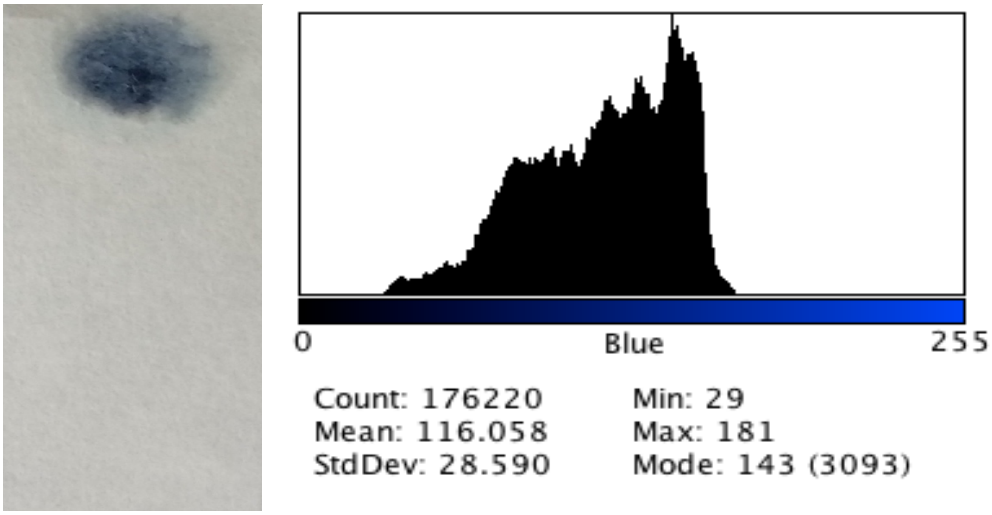


Fig. 4.22: Efficacy of biosensor determined in control wash solution & the colour intensity was recorded with ImageJ software.

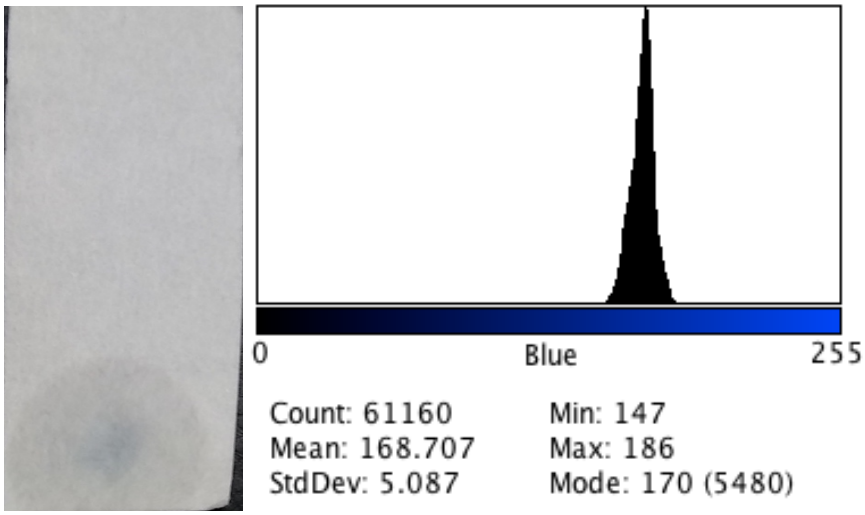


Fig. 4.23: Efficacy of biosensor determined in cauliflower wash solution & the colour intensity was recorded with ImageJ software.

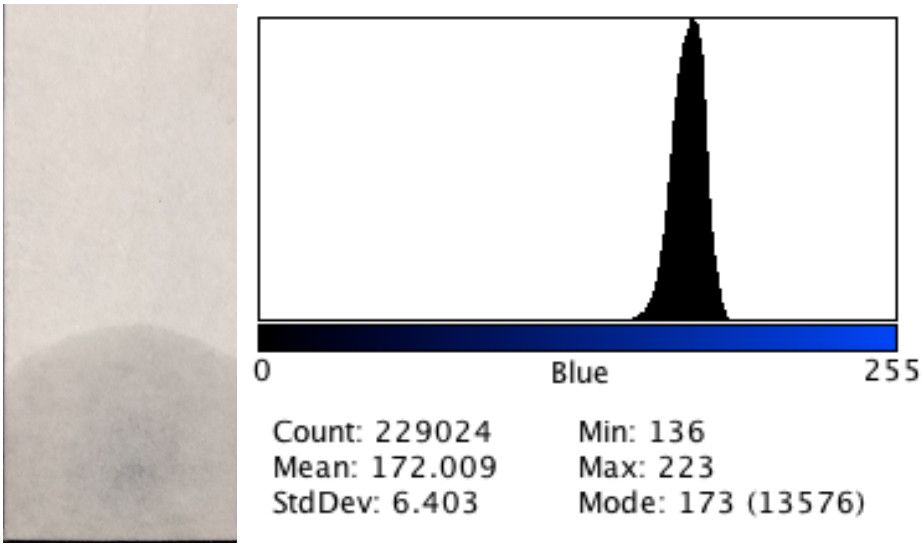


Fig.4.24: Efficacy of biosensor determined in cabbage wash solution & the colour intensity was recorded with ImageJ software.

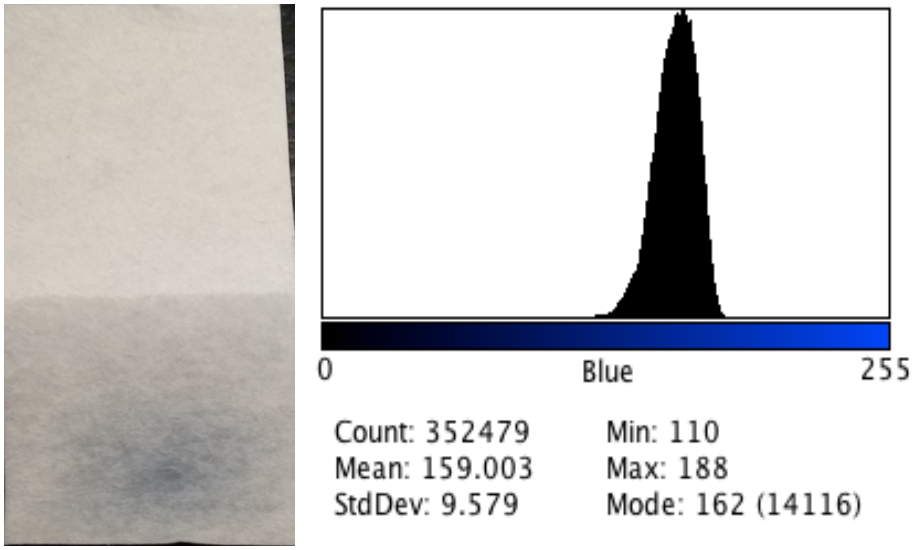


Fig. 4.25: Efficacy of biosensor determined in rice wash solution & the colour intensity was recorded with ImageJ software.

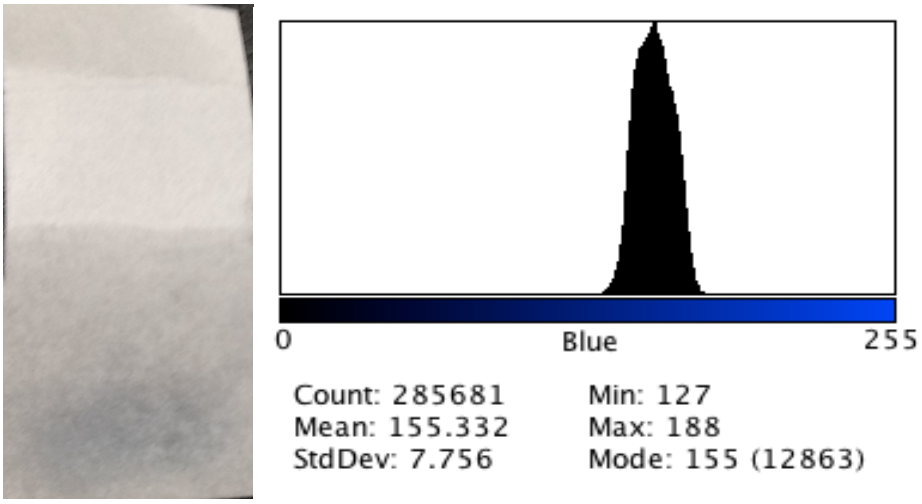


Fig. 4.26: Efficacy of biosensor determined in brinjal wash solution & the colour intensity was recorded with ImageJ software.

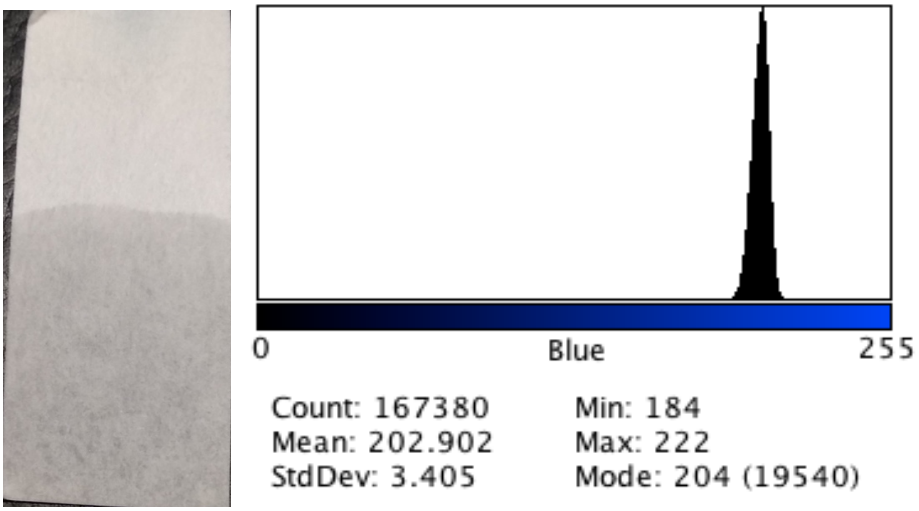


Fig. 4.27: Efficacy of biosensor determined in water solution containing pesticide & the colour intensity was recorded with ImageJ software.

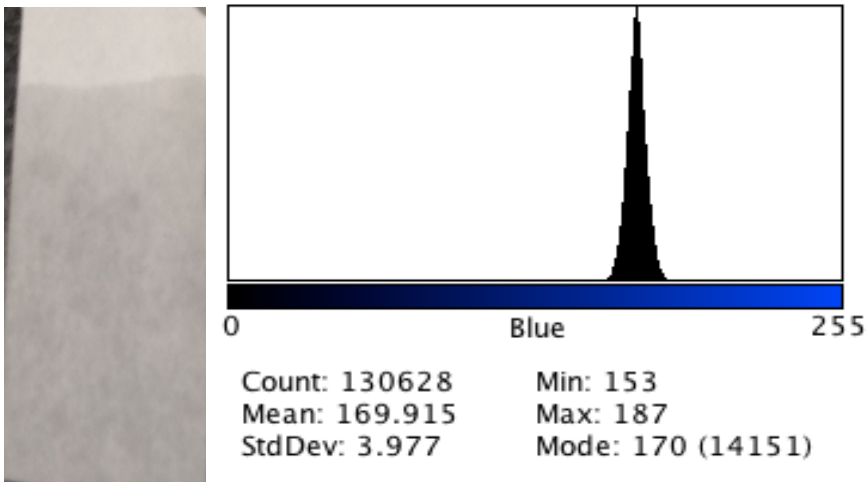


Fig. 4.28: Efficacy of biosensor determined in milk solution contacting pesticide & the colour intensity was recorded with ImageJ software.

Table 4.5: Range of dichlorvos concentration analyzed based on color intensity measurements from food items spiked with dichlorvos. *Color intensity is depicted as a.u

Food items used	Color intensity (a.u)*	Approximate concentration of dichlorvos
Water	3.6	50-60ppm
Rice	4.7	10-20ppm
Milk	3.5	50-60ppm
Cabbage	4.0	30-40ppm
Cauliflower	2.4	60-70ppm
Brinjal	4.3	20-30ppm

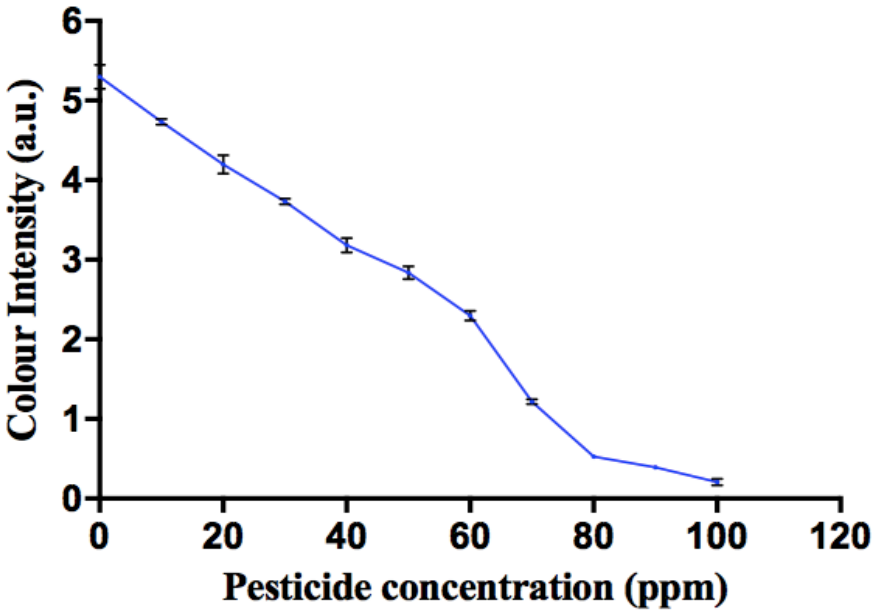


Fig 2.29: Colour intensity of different pesticide concentration measured by ImageJ software.

International regulations permit dichlorvos presence of 0.1-1 ppm (mg/Kg)in vegetables. In India, the Food Safety and Standards (Contaminants, Toxins and Residues) regulations, 2011 deals with compliance of various contaminants, toxins and residue standards prescribed in foods. These regulations provides maximum limits of various metal contaminants, crop contaminants, residues of insecticides/pesticides, antibiotic and pharmacologically active substances in different foods. To date, several studies have been conducted as part of the central scheme 'Monitoring of Pesticide Residues at National Level', are operational since 2005 in India. A summary of these reports indicate that a total of 16,790 food samples have been analysed and out of these 509 samples were found to harbour pesticide residues several folds above MRLs (Maximum Residue Limit) as prescribed under FSSAI and CODEX. Dichlorvos in these lists occurs at concentrations far above the set MRL. It is important therefore, that simple yet robust processes or methods are in place for enabling greater and effective detection based approaches in the supply chain of food products so that segregation can be made early. Such measures will be crucial for safeguarding the health of consumers and also

facilitate quantitative analysis for pesticides further using sophisticated methods. Based on the results obtained in this study, further work to explore the applicability will be worthwhile. This can be done through rigorous validation in more diverse food items, application of the QuEchRS extraction methods and concurrent use of GC-MS for validation of both concentrations and establishing a correlation with semi-quantitative data.

5. CONCLUSION

The salient features of the study are listed below as:

- An extracellular microbial polymer (designated as G_2) was selected for its high amphilicity, from amongst a repository of 10 polymers produced by bacterial strains.
- FTIR of the microbial polymer indicated presence of hydroxyl, carboxylic functional groups as predominant structural constituents. X-ray diffraction (XRD) indicated the amorphous nature of G_2.
- To further investigate whether the bacterial polymer could form hydrogels, three different hydrogels were prepared as- PVA, PVA: Chitosan, PVA: Biopolymer (G_2) using glutaraldehyde. The three hydrogels visually inspected were transparent, highly uniform yellow transparent and uniform white transparent hydrogel respectively.
- Characterization of the hydrogels were carried out: Swelling kinetics indicated the high swelling ratio of microbial hydrogel than others with small pore size in comparison to the others. These features were important in utilizing the microbial hydrogel as biosensor for encapsulating AChE.
- Acetylcholinesterase activity (kinetics), substrate concentration and inhibition kinetics by dichlorvos was optimized.
- Microbial polymer hydrogel was used to encapsulate the acetylcholinesterase-based biosensor and tested with different concentrations of AChE.
- The biosensor could discriminate dichlorvos present at different concentrations within a period of 15 minutes.
- The designed biosensor possessed stability for up to 15 days in 4^oC followed by 28^oC during storage as judged by the activity.

- The performance of the biosensor was assessed in wash solutions of food items like cauliflower, cabbage, water, milk, brinjal and rice spiked with dichlorvos. The color produced by the biosensor was discernable visually and the quantitation of dichlorvos in high, medium or low ranges was possible.

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