Evaluation of protective biopolymers for cosmeceutical applications

Dissertation

Submitted in the partial fulfilment of the requirement for

The award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

Under the guidance of :

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CANDIDATE S DECLARATION

I, hereby declare that the work presented in this thesis entitled **Evaluation of protective biopolymer for cosmeceutical applications** in partial fulfilment of the requirement for the award of the degree of Masters of Technology in Biotechnology, Department of Biotechnology (DBT), Thapar university, Patiala, is an authentic record of my work during the period of one year from july 2014 to june 2015 under the guidance of **Dr. Moushumi Ghosh,** Associate Professor Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled Evaluation of protective biopolymers for cosmeceutical applications submitted by Prerna Tiwari in partial fulfilment of the requirement for the award of Degree of Masters of Technology in Biotechnology to Thapar University, Patiala, is a record of student s own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other degree or certificate in this or any other University or Institute.

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ACKNOWLEGDEMENT

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. It gives me immense pleasure to acknowledge with attitude. The help and guidance rendered to me by a lot of people.

First of all, I sincerely acknowledge my gratitude to almighty GOD for his compassion made me fell to have and to see the moment whose euphoria is not vanishing from the mind will remain till eternity.

I beat the rays of my illuminant gratitude to my honourable guide, Associate Professor, **Dr. Moushumi Ghosh** for her excellent guidance. Her discipline, principles, simplicity, caring attitude, constructive criticism and provision of fearless work environment will be cherished in all walks of my life. I am very much grateful to her for valuable guidance and everlasting encouragement throughout my course.

It gives me great pleasure to express my deep sense of gratitude to **Dr. Dinesh Goyal,** Professor and Head, Department of Biotechnology (DBT), Thapar University, Patiala, for allowing me to continue my dissertation work by providing necessary laboratory facilities to fulfil of this work.

I also wish to express my thanks to **Mrs. Taranpreet kour**, **Ms. Parul and Ms. Supriya Arora,** research scholars, DBT for their valuable companionship and suggestions. I am thankful to **Ms. Komal Sharma,** Junior research fellow, DBT for her constant encouragement and help.

I am grateful to laboratory staff **Mr. Babban, Mr. Mohinder, Mr. Surinder and Mr. Chandan** for their timely help and assistance while completing this project.

From the core of my effective domain, with an uncountable avalanche of emotions of gratitude & sincerity I would like to express my cost sincere thanks to **Nitika vats, Yashila girdhar, Ankita pandey** and **Sandeep kour saggi** who have been the source of guidance & inspiration for me. I am immensely grateful to them for their all time co-operation, valuable suggestions to complete my project in stipulated time.

Word of reverence, gratification and affection for my beloved **Parents** who brought me to this stage and indebted for their big moral support for me in the completion of my higher studies. It was their confidence, affection and prayer for my success and progress which made it possible for me to complete the task relentlessly in time.

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ABSTRACT

Cosmeceuticals have drug like benefits because of there active ingredients such as vitamins, enzymes, phytochemicals, essential oils, antioxidants and enzymes. Beneficial effect of Cosmeceuticals on human health have attracted increased attention. Antioxidant activity is a very important factor present in cosmeceuticals but mainly artificial antioxidant molecules are used in cosmeceuticals these days. Cosmeceuticals could be more beneficial if natural antioxidant molecules were used instead of artificial antioxidant molecules. In the present study natural biopolymers which were isolated from bacteria and used for the antioxidant activity in Cosmeceuticals. There are diverse structural and functional biopolymers synthesized by bacteria of all taxa and secreted in external environment, bacterial generation in large numbers and biopolymers isolation is a cheaper process. Noble biopolymers were isolated and there antioxidant activities were evaluated *in vitro* by various assays of antioxidant activity such as- determination of total antioxidant activity, scavenging of Scavenging of 1,1 – diphenyl -2- dipcrylhydrazyl radicals, assay of H_2O_2 scavenging activity, determination of reducing power. In all the assays of antioxidant activities antioxidant capabilities of various concentration of biopolymers were compared with standard compound and observed the increase in antioxidant activity as the concentration of the biopolymer increases. The biopolymers and the combinations of different biopolymers which were showing the best results during the antioxidant assays, mixed with virgin coconut oil. The concentration of biopolymers added to the coconut oil was dependent on the concentration of biopolymer showing best antioxidant activity in the antioxidant assays. Acid value, in different time periods were noted to find out the biopolymer having best antioxidant activity when applied with the coconut oil. During the experiments bultylated hydroxyl toulene were acting as positive control since it have a good antioxidant activity. There were combination of two polymers which was protecting the oil from degradation throughout the experiment even more than the butylated hydroxyl toulene. The aim of present study was the identification of the type of biopolymers which was having the highest antioxidant activity. The aim of the study was fulfilled since the biopolymers having maximum antioxidant activity was isolated. From the observed results it can be concluded that the synthetic antioxidants can be substituted with the natural biopolymers, or natural antioxidants can be used as antioxidant molecules in the next generation cosmeceuticals.

List of Abbreviations

ROS	Reactive oxygen species
TAC	Total antioxidant capacity
DPPH	1,1- Diphenyl-2-picryl-hydrazyl
BHA	Butylated hydroxylanisole
BHT	Butylated hydroxytoluene
LA/LB	Luria agar/broth
BPB	Biopolymer producing broth
OD	Absorbance
DNS	3,5- dinitrosalicylic acid
FTIR	Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
SEM	Scanning electron microscopy
XRD	X-Ray diffraction

List of symbols

L	Litre
G	Gram
µl	Microlitre
Mgl^{-1}	Milligram per litre
rpm	Rotation per minute
nm	Nanometre
mM	Milimolar
M	Molar
μgml^{-1}	Microgram per millilitre
$Mgml^{-1}$	Miligram per millilitre
ºC	Degree celsius
Aº	Angstrom
cm^{-1}	Inverse centimetre
mV	Millivolt
µm	Micrometre

1. INTRODUCTION

Cosmeceuticals derived from the words 'cosmetic' and 'pharmaceutical' may be defined as substances with drug like properties besides serving its basic function to protect or enhance the appearance or order of the body. Consumer demand for more healthy cosmetics have revolutionized both research and driven the industry in incorporating bioactive molecules derived from natural sources in cosmetics to create cosmeceuticals. In the current past substantial body of literature have demonstrated that bioactives, apart from those traditionally used from plant sources for amending cosmetics, could be potentially exploited from microorganisms for developing cosmeceuticals. This is because; microorganisms are extremely heterogeneous and produce a unique multitude of diverse bioactive molecules including polymeric materials. Amongst those that are being extensively studied, biopolymers have the potential to be applied as cosmetic, cosmeceuticals, enzymes, pharmaceuticals. Furthermore, utilization of simple to complex substrates with varied chemical properties causes bacteria to produce diverse biopolymers. Bacterial biopolymer was first discovered in mid-19th century in wine, later on that biopolymer known as dextran and the prokaryote responsible for its production was *Leuconostoc mesenteroides* (Rehm *et al*., 2010; Linker *et al*., 1966).Microbial extracellular polymers or EPS are fairly ubiquitous and are synthesized and secreted into the external environment by the bacteria, can be hetropolymeric or homopolymeric in composition and of diverse molecular weight. Biopolymers, mostly extracellular have various applications in pharmacological, neutraceuticals, functional food, cosmeceuticals, herbicides and insecticides (Nwodo *et al*., 2012).One major impediment for the application of biopolymers in industries is their high cost of production relative to their commercial value, however this problem can be overcome by using cheaper substrates, optimizing fermentation conditions, developing high yielding strains by mutagenesis or genetic manipulations and optimizing downstream processing (Rehm *et al*., 2010). Unique properties of biopolymers cause the high value application (Castleton *et al*., 1999). Free radical mediated damage and subsequent loss of functionality is predominant in oil based cosmetics; clearly designing safer yet effective approaches which can quench free radicals can lead to extend the quality of oil and thereby the cosmetics. An obvious choice lies in antioxidant molecules to protect most of the oil and lipid based cosmeceuticals from oxidative damage- currently synthetic molecules such as BHA or BHT are being used extensively.Natural antioxidant molecules have been recorded and are being intensely

researched upon both from plants such as ascorbic acid, tocopherols herbal extracts like sage and rosemary as well as from microorganisms as possible choices. Biopolymers derived from microbial sources have begun to be exploited for multiple functionalities, including antioxidant potential. Therefore exploration of biopolymers with antioxidant properties may be envisaged as an important and interesting option for seeking safe yet novel alternatives to antioxidants currently used for cosmetics. Biopolymers are soluble or insoluble polymers secreted by microorganisms. The diversity of chemical composition of microbial biopolymers results in the variety of properties which cannot be found in the plant biopolymers (Kambourova *et al*., 2009).

Cosmetic formulations usually contain fragrances, natural fats and oils all of which are susceptible to auto-oxidation by exposure to the air causes off-odors and other instabilities. Adding antioxidants to the raw material is a way to preserve its stability. Natural antioxidants such as tocopherols (Vitamin E derivatives) are weaker antioxidants than BHA and BHT but much more friendly in their skin benefits. In the same way biopolymers can also be explored as viable natural antioxidant molecules then the present antioxidants.

Moreover, microbial biopolymers can be used as the carrier for the antioxidant molecules in fact free *radical grafting* technique allows synthesis of antioxidant biomacromolecule without the use of organic solvents. It has been reported that quercetin, catechin and gallic acid were bound to chitosan, starch, insulin and alginate exhibit good antioxidant activities (Gianfranco *et al.*, 2014), free radical grafting can therefore be attempted onto the biopolymer matrix to enhance the functionality.

Scope of study

Antioxidants are added in cosmeceuticals to prevent it from rancidification specially,There are two chemical antioxidants which are most widely used i.e. BHA and BHT, it has been estabilished that the acceptable daily intake of both of theses antioxidants should not be more than 0.25 mg/kg bw/day for BHT and 1.0 mg/kg bw/day for BHA it was noted that the exposure should not be increased from these intakes (Carocho, *et al.,* 2013). NDGA (Nordihydroguaiaretic acid) renal cystic disease in rodents (Evan and Gardner, 1979). From the various studies it was find out that the synthetic antioxidants causes a lot of adverse effects in human health. Therefore to find out a alternative of these antioxidants or to remove the side effects of synthetic antioxidants a natural biomolecule was discoverd as a antioxidant molecule in the present study. These natural biomolecules were the biopolymers isolated from different bacterial sources.

Objectives of the study

i) Screening and characterization of antioxidant-biopolymer from bacterial isolates.
ii) Establishment of the antioxidant potential of biopolymers screened and optimization of culture variables for enhanced production of selected biopolymers
iii) Real time evaluation of biopolymers as cosmeceuticals additive: dosage optimization, oxidation status, functionality, shelf stability.

The study were aimed to fulfil all the objectives.

2. REVIEW OF LITERATURE

Before using biopolymers as antioxidants in cosmeceuticals, the study of the microorganisms or the natural bioactive components used in cosmeceuticals till now was understood by the literature survey. Cosmeceuticals are not the drugs but they have drug like benefits, they protect the body today's well known cosmeceuticals lotions, creams, ointments, theses are known as antioxidants because they contain molecules which protects and nourishes human body (Kim, *et al.,* 2008). The cosmeceuticals contain phytochemicals, essential oils, vitamins etc. Antioxidants are added in cosmeceuticals to prevent it from randification, low cost, owing to their high performance and wide availability.

Presently there are many cosmeceuticals in the market which contain antioxidant molecules like Butylated hydroxyl anisole (BHA), Butylated hydroxyl toluene (BHT), di-tertbutyl-4-hydroxymethylphenol (IONOX-100), Tertiary butyl hydroquinone (TBHQ), Propyl gallate (PG), Octyl gallate (OG), Nordihydroguaiaretic acid (NDGA) and 4-hydroxyresorcinol (Guan *et al.,* 2005 and Guo *et al.,* 2006). Although they are helpfull in protecting the quality but the excess intake of theses antioxidants by body can cause mutagencities, toxicities and thus endanger the health of people (Williams 1993, 1994).

2.1) Cosmeceuticals derived from various organisms

Nowadays there are many researches has been conducted on the physical and chemical properties, biotechnological applications, chemical structure of bioactive substances derived from various marine organisms. This indicates there rich source of chemical products having applications in cosmetic, pharmaceuticals, cosmeceuticals and even food industries (Anderson, et al., 2000 and Faulkner, et al ., 2000)

2.1.1) Extracts from algae

Algae extracts are rich in polsacchrides and bioactive substances.DNA helps in regenerating the damaged or dried skin and prevents moisture loss within the skin. DNA has been isolated from marine organisms and applied in cosmeceuticals for skin protection and moisturizing effects (Fitton, *et al.,* 2007; Karawita, *et al.,* 2007; Yuan, *et al.,* 2006).

Table 2.1 : Different types of algae and their application in cosmeceuticals

Name of algae	Content in algae	Function in cosmeceuticals
Chondrus crispsus (red algae) (Fitton, *et al.,* 2007)	Polysaccrides and minerals (zinc, maganease, calcium etc)	Moisturizing, smoothing, healing, conditioning
Codium tomentosum (green algae) (Fitton, *et al.,* 2007)	Glucouronic acid	Water distribution within the skin
Laminaria saccharina (Fitton, *et al.,* 2007)	Protein, vitamin, minerals and carbohydrates	Sebaceous gland activity, anti-inflammatory and healing
Crithmum maritimum (Majudar, 2007)	Minerals, polyphenol, essential oils, vitamin C, flavonoids	Protein synthesis in connective tissue (collagen, elastin), antseptic, anti-inflammatory
Ascophyllum nodosum (brown algae) and *Asparagopsis armata* (red algae) (Karawita, *et al.,* 2007)	Anti-irritant components	Reduces the level of VEGF (high level can cause sensitive red skin)
Entermorpha compressa (green algae) (Yuan, *et al.,* 2006).	Glycosamine, hydroxyproline, and polysaccharides	Increases blood circulation

2.1.2) Extracts of microalgae

These are small, coloured, unicellular organism. Microalgae can occur in water but mostly they are photoautotropic. Microalgae are a rich source of many chemical products which have various applications in pharmaceutical, nutritional, cosmetic and medicinal industry. Extracts of microalgae is rich in minerals, vitamins and proteins.

There are some active ingredients which were isolated from microalgae and used in cosmeceuticals, which repairs damaged skin and blemishes, inhibits the inflammation process

and maintains moisture in the skin. Extracts from microalgae species *Arthrospira* and *Chlorella* have been used in the commercial products in the skin care market (Stolz, *et al.,* 2005).

Chlorella a unicellular green algae, contain valuable protein used in biotechnology industries, food and aquaculture. Extracts of chlorella contains various biologically active compounds such as wound healing substances, antioxidants, growth factors and anti-inflammatory compounds. *Dunaliella* widely used for the production of valuable biochemicals such as carotenoids, polyunsaturated fatty acids and colouring agents for cosmetic and food industry. Through molecular engineering approaches in *Dunaliella*, the carotenoid content has been increased which makes it more attractive for biotechnological applications (Jin, *et al.,* 2003). Microalgae are mainly used in cosmetic or skin care products. The extracts of *Laminaria digitata* is a skin penetrant (Mungo, *et al.,* 2005).Similarly, extracts of *Chorella vulgaris* stimulates collagen formation in the skin which can be used in the products supporting wrinkle reduction and tissue. Regeneration.(Dermatochlorella, St. Malo, Codif, France) (Spolaore, *et al.,* 2006). According to a new study extracts from microalgae (*Nannochloropsis oculata*) launched by Pentapharm (Switzerland) have excellent tightening effects. Extract from *D. Salina* that is Blue Retinol stimulates proliferation and skin cells growth. Marine algae extracts has been used to prepare Marestil which is a strong electicizing, moisturizing and toning complex.

2.1.3) Carotenoids

Many microorganisms and plants produce a lipid soluble pigment known as carotenoids. They not only act as pigment but also have beneficial effects on human health by working as antioxidant, have role in cancer prevention and enhancing immune responses. Commercially caretenoides used in pharmaceutical, animal feed and neutraceuticals (Armstrong, *et al.,* 1994 and Sandmann, *et al.,* 2001). Carotenoids have vitamin -A precursor, β-Carotene as vitamin -A precursor can be converted into vitamin-A (retinol) enzymatically and applied in cosmeceuticals. Recent research has described the role d cantaxanthin and zeaxanthin in various applications apart from acting as antioxidants.

2.1.4) Polysaccharides

Polysaccharides have various applications in different fields like pharmaceutical, food, healthcare industry and cosmeceuticals. Polysaccharides like D- glucose, D-mannose, D-glucouronic acid and D-galactose isolated from algae have application in cosmetics as excipients because of its high bonding, gelling and viscosity increasing properties. **Fucoidan** which is a sulphated polysaccharide found in various brown algae. It has been suggested from the study that the fucoidan have skin protecting, anti-aging and antioxidant activity. (Kang, *et al.,* 2008 and Lee, *et al*., 2008). Fucoidan have some other functions like anti-coagulant, anti-viral, anti-inflammatory and anti-tumor properties (Usov, *et al.,* 2001). Nowadays cosmetics are generally made up of a base and partially hydrolysed fucoidan; partially hydrolysed fucoidan was isolated from the heated algae (Lee, *et al.,* 2008). Various forms of fucoidans such as sulfated fucogalacton, sulfated fucan and sulfated fucoglucuronomannan isolated from *Kjellmaniella crassifolia* which are used in cosmeceuticals. **Carrageenans,** isolated from red algae of family *Rhodophyceae* which is a linear sulfated polysaachride with molecular weight of 300 and 600 kDa approximately have alternating 1,3-linked β-D-galactopyranose and α-1,4-linked-D-galactopyranose (Mangin *et al.,* 2001 and Lecacheux, *et al.,* 1985). Molecular weight difference is due to the presence of different sulfate and anhydro group. Carrageenans are highly flexible and have helical structure, there most common source is *Gigartina* from Southern Europe (Viebke *et al.,* 1995). Properties of Carrageenans depends on the number and postions of sulfate groups, they provides textures to various cosmetic products such as lotions, creams, sprays and foams (Yermak *et al.,* 1997). Carrageenans also used as thickening agent and viscocity changer in various pharmaceutical preperations, food products and cosmeceuticals (Cabello-Pasini, *et al.,* 2005). **Alginates** containing α-(1-4)-linked L-glucouronic acid (G) residues and β-(1-4)-linked D-mannuronic acid (M) are linear unbranched polymers (Smidsrod, *et al.,* 1996). Bacterial alginates are acetylated, bacterial acetylase can be used to produce acetylate to increase its water binding capacity. Raw material for alginates is isolated from brown algae, alginate is used to stabilize and thicken emulsions at low pH. Stabilizing acidic skin care preparations have Protanal alginates (Smidsrod, *et al.,* 1996). Alginic acid can be isolated from brown algae using mineral acids or alkali. Alginic acid can be converted in to various alginate like sodium alginate. This type of structure depends on the growing conditions of algae and source of

algae. There are alginates which are isolated from different species like *Durvillea* and *Ascophyllum* contains high amount of mannuronic acid so that theses alginates forms softer gel low porosity and high elacticity. *Laminaria hyperborea* stems contain glucouronic acid, so the alginates isolated from them have more rigid gels with more porosity.

Agar consists of alternating D- and L- galactopyranose units isolated from red algae, it is unbranched polysaccharides can be used as thickening agent in cosmetics. *Gracilaria chilensis* (Chile) and *Gracilaria gracilis* (Atlantic) produces more than 50% of agar (Cabello-Pasini, *et al*., 2005), red algae such as *Pterocladia, Gelidium* and *Pterocladiella* produces high quality of agar. Agar is mainly used for the thickening and viscosity-altering in cosmetics, food and pharmaceutical preperations (Prasad, *et al.,* 2007).

2.2) Antioxidant activity

During the process of metabolism of oxygen, reactive oxygen species such as hydroxyl and superoxide anion radicals are produced which are highly reactive. These are the product of biological reactions, some play positive role while some may cause damage to cell membrane and DNA which results in membrane lipid peroxidation and decreased membrane fluidity (Finkel & Holbrook., 2000; Melov *et al*., 2000). Many studies have shown that the ROS is responsible for the Alzheimer's disease, epilepsy, Parkinson's disease, lung injury, inflammation (Raouf *et al*., 2000).

All the organisms have antioxidant defence and repair system, but the damage caused by ROS is unable to be prevented by these system (Ke *et al*., 2009). Synthetic antioxidants, like BHA and BHT are being used extensively, but they can exhibit cytotoxicity. (Grice, 1988; Luo & Fang, 2008; Valentao *et al.,* 2002). Polysaccharide based polymers isolated from microorganisms have been reported to possess antioxidant capability (Krizkova *et al*., 2006; McCue & Shetty, 2002) besides lacking toxicity.Major part of yeast cell wall dry weight is polysaccharides and it defines its stability and morphology. Recent studies suggests that the polysaccharides have antioxidant activity that can act as protective agents such as antioxidant, antimutagens and antigenotoxic agents (Kogani, *et al*., 2008). Since it has been established that the oxidative damage caused by the ROS and free radicals can cause many chronic diseases such as neurodegenerative, cancer and cardiovascular diseases which is the reason of death (Halliwell *et al.,* 1994). The polysaccharides of *Saccharomyces cerevisiae*

and *Candida utilis* has been extensively investigated for the antioxidant related antigenotoxic or antimutagenic and anticancer properties (Bobek *et al*., 1997). According to the study there are protective effects of CMG on lipid peroxidation in liposomes (Klein *et al.,* 1970).

2.3) Exopolysacchrides

Polysaccharides are most abundant in extracellular biopolymers (Dogsa *et al*., 2005). They are found as constituents in teichoic acid and serve as protective and structural process. They can take the form of covalently bound cohesive layer outside the cell, which is known as capsule (Hugenholtz *et al* 2002) or can be released in environment as slime (Cerning *et al*., 1995). Theses capsules mainly serve as adhesive agents of cells to other cells, These can be overproduced if there is need of sugar to become reservoir of carbohydrates for the metabolism (Decho *et al*., 2010 and Flemming *et al*., 2010).

The distinction between unattached or loosely attached extracellular material is depends on the functional and structural relationship with the cell. The non-toxic nature and biocompatibility of bacterial biopolymers has made them suitable for their use in various medical applications like drug delivery, tissue engineering and wound dressing, which makes them more attractive in comparison to the polysaccharides isolated from microalgae and plants (Rhem *et al*., 2010, Sutherland *et al*., 1998 and Otero *et al*., 2003).

Some of the biopolymers are gradually degraded in-*vivo* which makes them suitable for use in controlled drug delivery and in tissue replacement (Rhem *et al*., 2010). Beneficial effects of biopolymers have mainly concentrated on biopolymers elaborated by probiotic microorganisms. Beneficial effect can be physiological, therapeutic and nutritional, mechanism of action of end product of "pro-bio active substances" (Naidu et al., 1998) in many cases identifiable to exocellular polymers. Biopolymers may thus be looked upon as an important class of probioactive molecules.

Table 2.2 : Exopolysacchrides their source and applications

Exopolysaccahrides	Source	Applications
Acetan	*Acetobacter xylinum*	Gelling agent and vicosifier
Alginate	*Pseudomonas aeruginosa* *Azotobacter sp.*	Microencapsulation and immobilization
Cellulose	*Acetobacter xylinum* *Pseudomonas sp.,* *Agrobacterium sp.,* *Rhizobium sp.,*	Acoustic membrane in audiovisual equipment, natural nondigestable fibers and temporary artificial skin.
Mulsan	*Acinetobacter calcoaceticus*	Immobilization and emulsification
Curdlan	*Alcaligenes faecalis var. Myxogenes*	Gelling agent
Dextran	*Lactobacillus hilgardii,* *Leuconostoc dextranicum,* *Leuconostoc mesentroides,* *Streptococcus mutans*	Microcarrier in cell/tissue culture, blood flow improving or blood plasma extender and cholesterol lowering agent
Cyclosophorans	*Rhizobium sp.,* *Agrobacterium sp.,* *Xanthomonas sp.*	Food component and encapsulation of drugs
Gellan	*Sphingomonas paucimobilis,* *Aureomonas elodea,* *Sphingomonas elodea*	Gelling agent/ solidification
Kefiran	*Lactobacillus hilgardii,* *L. rhamnosus, L. kefir,* *L. kefiranofasciens*	Viscoelasticity and gelatination
Hyaluronic acid	*Streptococcus sp.*	Synovial fluid replica and moisturization
Levan	*Bacillus subtilis,* *Alcaligenes viscosus,* *Zymomonas mobilis*	Similar as dextran
Welan	*Alcaligenes species*	Vicosifier and stabilizer

Succinoglycan	*Alcaligenes faecalis var. Myxogenes*	Immobilization and gelling agent
Xanthan	*Xanthomonas campestris*	Gelatination and emulsification

(Sutherland, 1998; Kumar et al., 2007; Vu et al., 2009; Sutherland, 1997; Soetaert 1995; Vu et al., 2009 and Satpute et al., 2010)

Table 2.3: Most common components of bacterial exopolysacchrides

Components	Example
Hexose sugars	D- galactose, D- glucose, D- mannose, L-rhamnose, D- allose, L- fucose
Pentose sugars	D- ribose, D- arabinose, D-xylose
Amino sugars	D- galactosamine, D- glucosamine
Organic substituents	Acetate, succinate, pyruvate, glycerate, hydroxybutanoate
Uronic acid	D- galacturonic acids, D- glucuronic acids, D- mannuronic acids
Inorganic substituents	Phosphate, suphate

(Kenne and Lindberg, 1983)

2.3.1) Properties and applications

The current commercial interest on biopolymers have expanded phenomenally due to their scope of applications in biomedical, food, pharmaceutical, bioleaching and bioremediation industries due to some of their unique properties, rheological, physical and structural diversity.

Table 2.4: Source of EPS with health benefits

Microbial source of exopolyshaccaride polymers	Health benefits/Functionality
Bifidobacterium bifidum	Antiulcer activity (Nagaoka *et al.,* 1994)
Lactobacillus casei	Activated mouse macrophages (Nomoto *et al.,* 1989)
Lactobacillus rhamnosus	Stimulated mouse lymphocytes (Chebot *et al.,*1992)
Bifidobacterium lactis Bb12	Proliferated mouse lymphocytes (Amrouche *et al.,* 2005)
L.delbrueckii ssp. *Bulgaricus*	Stimulated murine splenocytes (Kitazawa *et al.,* 1998)
Bacillus licheniformis	Immunostimulatory and antiviral activities (Arena et al., 2006)
Lactobacillus plantarum	Antimutagenic activity (Tsuda et al., 2007)
Lactobacillus kefiranofaciens	Increased gut mucosal immunity (Vendarola, 2006)
Pantoea agglomerans	Free- radical scavenging activity (Wang et al., 2006)
Bacillus coagulans RK-02	Antihyperglycemic activities (Kodali & Sen, 2008)

2.3.1.1) Dairy Industry

EPSs mainly used for the mouth-feel, texture, taste and stability of dairy product, these are also used for the production of fermented food products in Asia, Eastern Europe, Northern Europe. The bacteria producing EPS has been widely used for the production of fermented milk (Ruas-Madiedo, 2002 & 2003, De vuyst *et al.,*2001).

2.3.1.2) Health benefits

EPSs have shown many health benefits like cholesterol lowering (Welman & Mddox, 2003).

The study on EPS is mainly "*in-vitro*" however clinical studies have demonstrated the functional claims of EPS. For instance, the EPS isolated from kefir grains have shown the property of retarding the tumor growth when administered orally; probably the oral immune enhancement when EPS is administered is due to T-cell not by B-cell (Zubillaga *et al.,* 2001).Further research is necessary for the oral administration of EPSs for the use of EPSs in functional food. It has been proved that the EPS have antidiabetic, antiulcer (Naidu *et al.,* 1999) and cholesterol lowering activity (Nakajima *et al.,* 1992) but the detailed mechanism need to be studied.

The above deliberations indicate that few studies have actually been conducted for evaluating the antioxidant function of microbial biopolymers for oil based cosmetics. In view of the lack of scientific data, this study was designed to systematically investigate potential biopolymers from microbial cultures as antioxidants. The structural properties of these biopolymers were also elucidated.

3. MATERIALS AND METHOD

3.1) Chemicals and media `

The reagents and chemicals used were purchased from Sigma and were of highest analytical grade. BPB medium (biopolymer producing broth) (Annexure I) (Ghosh et al., 2009) was prepared, was used as screening medium for EPS producing microorganisms. Components for the standard media were purchased from Sigma Aldrich (USA) or Fisher Scientific (USA) and Hi-media from Mumbai (India). Media and other chemical reagents were autoclaved at 121º C and 15 psi for 15 min prior to use.

3.2) Screening of biopolymer producing isolates

A preexisting library of biopolymer producing bacterial strains, isolated from diverse sources. and maintained in the laboratory was screened, by inoculating them in biopolymer producing media .

3.3) Preservation of isolated strains

Glycerol stocks were used to preserve the isolated strains. The cultures were streaked on the LA plates, and incubated in LB tubes. For this, 3ml of culture were taken and centrifuged at 8000 rpm for 5 min. Saline (1ml) was added to the pellet and vortexed. The mixture was centrifuged at 8000 rpm for 5 min. Again pellet were mixed with 1ml of saline, vortexed and centrifuged at 8000 rpm for 5 min. 500 µl of LB was added in to the pellet and vortexed; 500 µl of suspension were added into 500 µl 80% glycerol in cryovials and stored at -80^0C.

3.4) Growth profile of the isolates

To optimize culture variables for enhanced production of EPS the selected isolates were grown to mid-log phase and then 1% of the adjusted inoculum (O.D approx. 0.02) was added to 100 ml of LB media. Aliquots were withdrawn initially after every 2 hours till the O.D became constant which means the stationary phase was reached, then the samples were withdrawn at an interval of 12 hours till the polymer production decreases (maximum biopolymer produced during stationary phase only). Yield of the polymer was analyzed based on the amount of polymer produced by bacterium with respect to time to know the incubation period for maximum biopolymer production.

3.5) Biopolymer production by isolates (Ghosh *et al.,* 2009)

Selected strains were grown for 48 hrs at 37^0C and 120 rpm in biopolymer producing broth. After 48 hrs, samples were centrifuged (CF15RX11, Hitachi, Japan) at 1000 rpm and 4ºC for 10 min for the removal of cells. Two volumes of chilled alcohol were added to the supernatant collected for the precipitation of EPS and kept overnight at 4ºC. Pelletize the suspension by centrifugation. Centrifuged again at 12000 rpm for 20 min at 4ºC to isolate the precipitate from the suspension, then the CPC treatment and dialysis were conducted on the precipitate. The precipitates were washed resuspended in distilled water and lyophilised to get the EPS in powdered form.

3.6) Assays of antioxidant activities

i) Determination of total antioxidant capacity (TOC) (Mitsuda, *et al*., 1996)

Pure biopolymer extract was used to determine the total antioxidant capacity reagent A (Annexure I) were mixed and the volume was made upto 250 ml with distilled water, this mixture was labelled as total antioxidant capacity. Different concentrations of 0.1 ml biopolymer extract were dissolved in 1 ml of TOC and after 15 min absorbance was taken at 695 nm. Ascorbic acid was used as standard in this process.

ii) Assay of H_2O_2 scavenging activity(Gulcin, *et al.,* 2004)

Hydrogen peroxide assay was used to determine the free radical scavenging activity using pure biopolymer extract. Phosphate buffer saline (0.1 M pH 7.4) was used to prepare hydrogen peroxide solution (10 mM). 2 ml of hydrogen peroxide solution was mixed with the 1 ml of different concentration of biopolymer extracts. After 10 min of incubation at 37ºC the absorbance was measured against blank (without hydrogen peroxide) at 230 nm using UV spectrophotometer. Percentage scavenging activity of H_2O_2 was calculated using formula,

percentage of scavenging = $(A_0 – A_1) / A_0 * 100$,

A_0 is the absorbance of control and A_1 is the absorbance of sample.

iii) Determination of reducing power (Yenhum, et al., 1997)

2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, W/V), 2.5 ml phosphate buffer saline (pH 6.6, 0.2 M) and 0.5 ml of biopolymer extract were mixed together. 2.5 ml of trichloroacetic acid (10%, W/V) was added to the reaction mixture after incubating the reaction mixture at 50ºC for 20 min. After centrifugation at 1400 rpm for 10 min, collected 0.5 ml of upper layer of solution mixed with 0.4 ml of $FeCl_3$ (0.1%, W/V) and 2.0 ml deionised water. The mixture was incubated at room temperature for 10 min and absorbance was taken at 700 nm by spectrophotometer. Higher absorbance shows the higher reductive potential. Ascorbic acid was used as positive control.

iii) Scavenging of 1,1- diphenyl- 2- picrylhydrazyl (DPPH) radicals (Shimada *et al.,* 1992)

The method was used to determine the scavenging of DPPH radicals. DPPH was prepared in 0.004% ethanol solution. 1 ml of different concentrations of biopolymer extract was added in 3 ml DPPH solution. After 30min the absorbance was recorded at 517 nm. The scavenging of DPPH radicals was calculated according to the following formula:

scavenging ability (%) = 1- (A_{sample}/ $A_{control}$)*100

where,

A_{sample}: absorbance of samples and

$A_{control}$: absorbance of control without the samples.

Ascorbic acid and butylated hydroxyltoluene (BHT) were used as the positive controls.

3.7) Application of biopolymer on conconut oil

Acid value was tested on three types coconut oil : (a) expired oil, (b) commercial oil and (c) formulated oil. Formulated oil was used for further experimentation as it does not contain any preservative or chemicals. BHT was used as positive control. Acid value was tested after 20 days, 40 days and 60 days. The sample (1) was that sample of oil which were mixed with the biopolymer again after 20 days in the equal concentrations so that the time period of biopolymer in which it act as most effective preservative can be calculated easily.

3.7.1) Acid value (Jis *et al.*, 1992)

About 0.5g of oil sample was mixed with 25 ml of fat solvent (mixture of ether and alcohol in the ratio of 1:1). 0.5 ml of phenolphathaleine was mixed in the solution. The whole solution mixture was titrated against the 0.1N KOH until the pink colour appears, again, a blank (not containing oil) was used by following the same procedure. Then the acid value was calculated according to the formula.

Acid Value = Z*5.6/weight of the oil sample,

where Z is Y-X.

Y, X are volume of KOH used for sample and for blank respectively.

3.8) Characterization of biopolymers

3.8.1) Biochemical characterization

3.8.1.1) Determination of total protein

Folin-Lowry method (Lowry *et al*., 1951) was used to determine the protein content in pure biopolymer. In this method Bovine serum albumin (BSA) was used as standard. Different concentrations of biopolymers and BSA were prepared, mix the 0.2 ml of protein solution into 2ml of alkaline copper phosphate reagent (Annexure I), mixed well. Incubated the solution for 10 min at room temperature; 0.2 ml of folin reagent was added in each test tube and incubated for 30 min. Then measure the absorbance at 660 nm. The standard curve was plotted between absorbance and concentration, determined the concentration of unknown sample using the standard curve.

3.8.1.2) Determination of total sugar

DNS method was used to determine the total sugar or carbohydrate content in biopolymer using glucose as standard. Different concentrations of glucose (0.1 to 0.9 mg/ml) and biopolymer (0.9 mg/ml) were prepared. 3 ml of DNS (3,5- dinitrosalicylic acid) was added in each test tube. The solution was incubated at 80ºC for 5 min. The absorbance was measured at 540 nm. Sugar content of unknown sample was determined by standard curve of glucose.

3.8.1.3) Determination of Uronic acid:

The method described by Haug and Larsen (1962) was used to determine the uronic acid content in biopolymer extract using D-glucouronic acid standard. Different concentrations of D-glucouronic acid (0.1 to 0.9 mg/ml) and biopolymer (0.5 mg/ml) were prepared. 250 ul of biopolymer and D-glucouronic acid were mixed carefully with ice cold reagent A (Annexure-1) with cooling and mixing in ice bath. After 10 min of heating at 100ºC the mixture was rapidly cooled in the ice bath and then 50 µl of reagent B was added with vigorous mixing. The mixture was reheated for 15 min at 100ºC and then mixture was cooled at room temperature, absorbance was measured at 525 nm. Uronic acid content of unknown sample was calculated by the standard curve of D-glucouronic acid.

3.8.1.4) Alcian blue binding

The supernatant was separated by the centrifugation of the culture, 100 µl of supernatant was mixed with the 700 µl of 0.5 M acetic acid. After that the solution mixture was mixed with the 200 µl of alcian blue dye (pH- 2.5). The solution was incubated at room temperature for 2 hrs and then centrifuged at 8000 rpm. Absorbance was measured at 580 nm using distilled water as blank instead of culture supernatant.

3.8.1.5) Sudan black binding:

BPB media (Annexure I) plates were prepared, checker board was made on each petriplate. Streaked the culture in each box with the toothpick. After the incubation at 37ºC for 24 hrs, flood sudan black dye over the petriplate for 2 minutes. Then rinsed the plates with 50% ethanol and visualized the colour of colonies. Blue colour indicated positive results.

3.8.1.6) Congo red binding :

BPB media (Annexure) with Congo red (30 µg/ml) plates were prepared and streaked with the culture, incubated overnight for the growth. Next day the plates were observed thcolour of colonies, pale yellow colour showed the positive results.

3.8.1.7) Flocculating activity:

About 4.8 ml of 0.5% kaolin light was mixed with 100 µl of 10% $CaCl_2$; 100µl of supernatant of culture which was isolated by centrifugation with the solution mixture. The mixture was vortexed thoroughly. Left the samples for five minutes to settle down. Then 1 ml of upper layer was collected in an eppendorf tube. Absorbance was measured at 580 nm by taking distilled water as reference. The flocculating activity was measured by the following formula. [absorbance of blank – absorbance of sample / aborbance of blank] * 100

3.10) Structural and functional characterization

3.10.1) Fourier Transform- Infrared spectroscopy (FTIR)

The interactions between biopolymer and iron were examined by Fourier Transform-Infrared spectrometer (Synthesis monitoring system, Perkin Elmer, USA). Wave number range 400 to 4000 was recorded to deduce the spectrum of pure biopolymer and iron bound biopolymer.

3.10.2) Zeta measurement

The charge of the particle was predicted by zeta potential. The biopolymers were dissolved in methanol to determine the colloidal particles with respect to zeta potential which were analysed by the Particle Size Analyser, Brookhaven.

3.10.3) DLS (Dynamic light scattering):

DLS was used to predict the Particle diameter of the polymer produced; a Particle Size Analyser(Brookhaven, USA) was used for this purpose. The graphs between counts and hydrodynamic diameter were plotted using Origin pro 9.0.

3.10.4) Surface characterization

3.10.4.1) Scanning electron microscopy (SEM)

Surface properties of a biopolymer were analysed by SEM. Samples of biopolymer coated with gold were analyzed by SEM (6510-LV, JOEL, Japan) at 20.0 kV.

3.10.4.2) X-Ray diffraction spectroscopy (XRD)

X-ray diffractometer (Xpert pro, Panalytical, United States) was used to determine the X-ray diffraction in pure biopolymer. Wavelength of 1.54 Aº with copper kα radiation. Between 5º to 85º was the measured rate.

4. RESULTS AND DISCUSSION

Cosmeceuticals occupy a significant part of the human care products available today, in the market such as perfumes, fingernail polish, eye and facial makeup, lipstick, shampoos, toothpaste, hair colours and deodorants. Coconut oil has several applications in cosmeceuticals since it has a shelf life of approximately two years; reports have indicated that spoilage or rancidification do not occur despite the fact it contains high amount of saturated fatty acids. Coconut oil can be used as a cosmeceutical if it is amended some beneficial molecules which can enhance the shelf life properties to quench free radicals and increase the there are many synthetic additives in market to increase the life of coconut oil like BHA or BHT but natural additives are better than synthetic ones. Biopolymers are preferred in industries owing to their reproducible physiochemical properties, novel functionality, stable cost and supply. In recent past because of increased demand of natural polymers led to the interest in biopolymer production from new sources (Avinash *et al.,* 2013).

In the present study biopolymers were tested for their antioxidant activity and applied on coconut oil to increase its life, by delaying the time of rancidification. Seven biopolymers were isolated from the seven different strains of bacteria

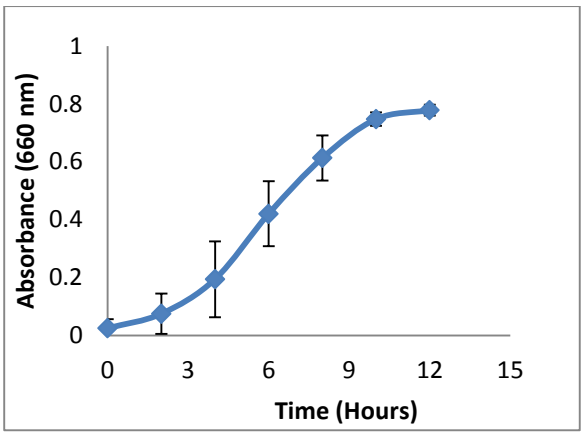
Three polymers with high antioxidant activity were selected for investigating their free radical quenching ability and rancidity of coconut oil.

4.1) Screening of biopolymer producing strains:

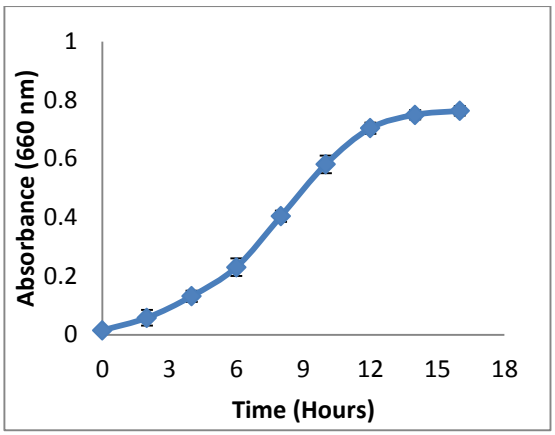
Out of the total 17 strains examined, 10 were selected for the biopolymer isolation, based on a preliminary profiling for elaboration of consistent mucoidy nature over the others; these isolates were designated as follows: HKW2C, HKW3D, HKW1A, HKW2A, PPY2, PPY3, PPY5, WP3, X3 and Y3. Variability in the morphology of the isolates: like configuration, type of strain, opacity, colour, margins, size and gram character was observed, presumably since the isolates originated from diverse sources.

4.2) Growth profile of isolates

The growth profiles of the isolates were analyzed, since exopolymers are usually produced during mid log to stationary phase where a slow down or cessation of cell multiplication occurs and the biopolymer production attains its .maximum. Figure 4.1 shows the typical profiles of isolates studied.


Absorbance (660 nm)
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15
Time (Hours)

(A)


Absorbance (660 nm)
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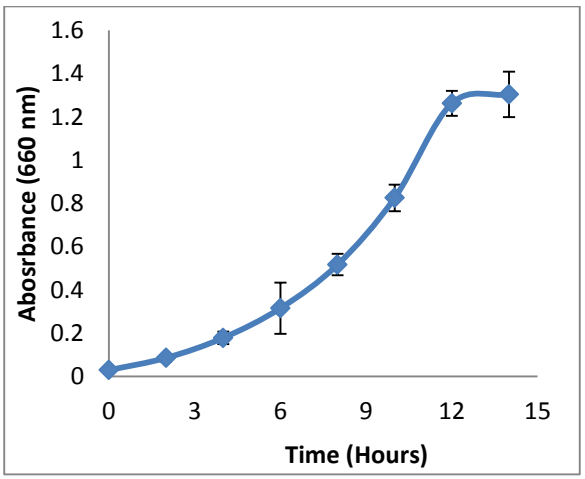
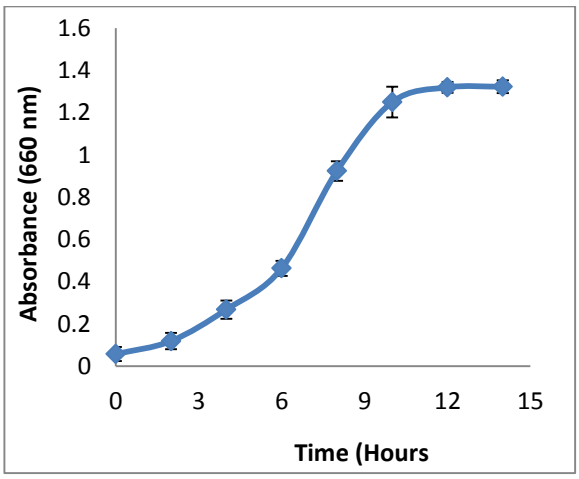
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15 18
Time (Hours)

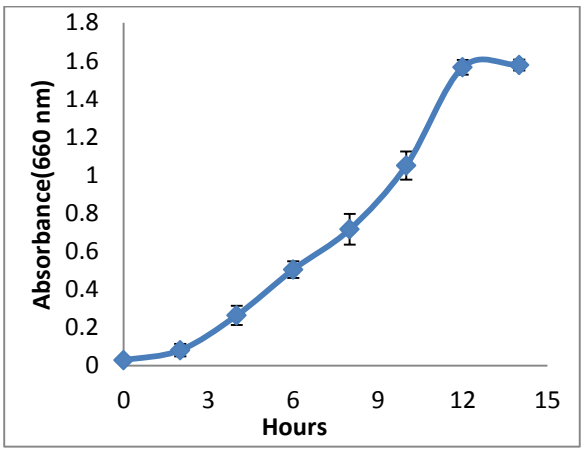
(B)


Abosrbance (660 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15
Time (Hours)

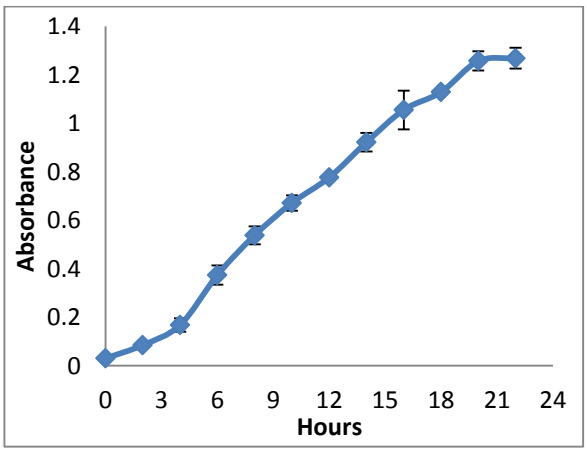
(C)


Absorbance (660 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15
Time (Hours

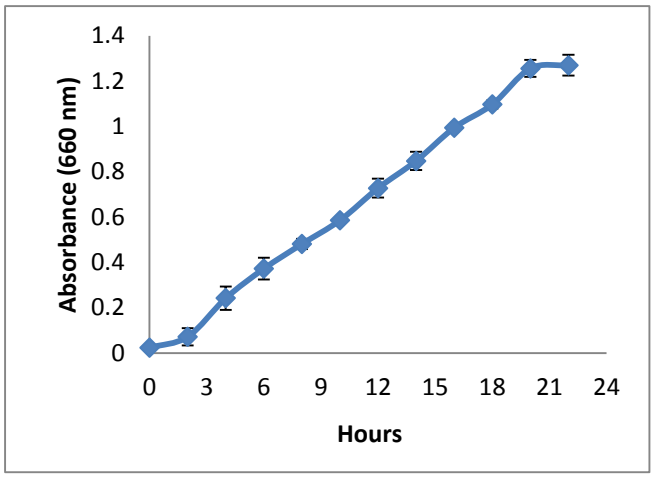
(D)


Absorbance(660 nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15
Hours

(E)


Absorbance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 3 6 9 12 15 18 21 24
Hours

(F)



(G)

Figure 4.1: Growth profiles of different bacterial strains isolated A) PPY2; B) PPY3; C) HKW3D; D) HKW1A; E) HKW2C; F) HKW2A; G) PPY5

A detailed observation indicated that the lag phase of all the bacterial strains lasted for about 2 hours, PPY2 and HKW1A has completed their log phase from 2 to 8 hours, for PPY3 and HKW2C the time was 2 to 12 hours, wheras for HKW3D 2 to 10 hours while for the rest of the strains i.e. HKW2A and PPY5 more time of 2 to 20 hours to complete the log phase in comparison to the other strains. Both HKW3D and PPY5 had highest and similar period of stationary phase, similar and highest period of achieving the stationary phase as well, other strains such as HKW3D and HKW1A reached stationary phase in the same time duration i.e. 10 to 14 hours other strains such as PPY2, PPY3, HKW2C reached their stationary phase in 10-12, 14-16 and 12-14 hours respectively. HKW2A and PPY5 took maximum time to reach stationary phase. The generation time was calculated using the equation:

$$\text{Generation time} = (\log_{10} N_t - \log_{10} N_0) * \log_{10} 2$$

and the values are depicted below in Table 4.1, different bacterial strains have different generation time, or in other words the time taken by the different bacterial strains for doubling their population, is different.

Table 4.1 Generation time of isolates

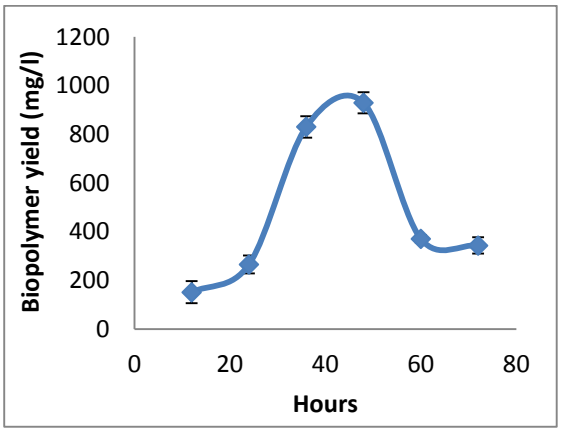
Strain	Generation time
PPY2	9 min
PPY3	13 min 14 sec
HKW3D	12 min
HKW1A	8 min 52 sec
HKW2C	10 min
HKW2A	13 min 2 sec
PPY5	10 min 38 sec

As per table 4.4 the PPY3 and HKW2A have maximum generation time that is of 13 min 14 sec and 13 min 2 sec respectively while PPY2 and HKW1A show lower generation time of 9 min and 8 min 52 sec. Other strains such as HKW3D, HKW2C and PPY5 exhibited generation times of 12 min, 10 min and 10 min 38 sec respectively. These observations reflected the variability of growth pattern of the isolates

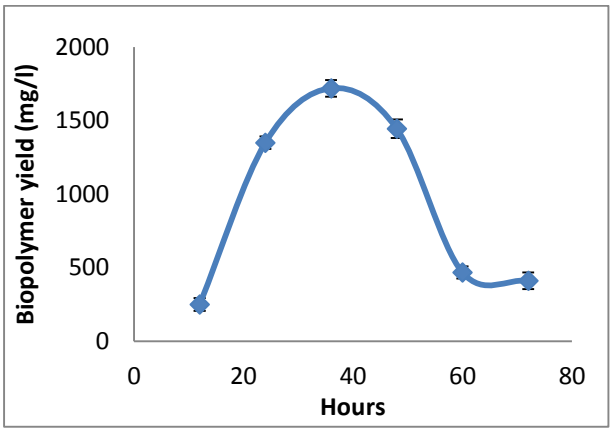
4.3) Biopolymer production kinetics

Maximum yield of biopolymer was achieved during the stationary phase of the growth. This was in line with previous observations where EPS yield were consistently higher during stationary phase (Nicolaus et al., 1993).

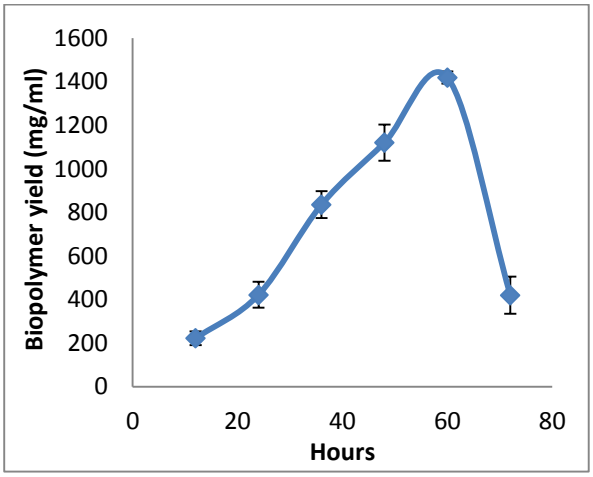
The time to attain stationary phase was used as the incubation time of bacterial strains in the BPB media for biopolymer production. Further kinetic experiments to compare the time taken to attain stationary phases of the potential isolates were carried out (Fig 4.2)


Biopolymer yield (mg/l)
1200
1000
800
600
400
200
0
0
20
40
60
80
Hours

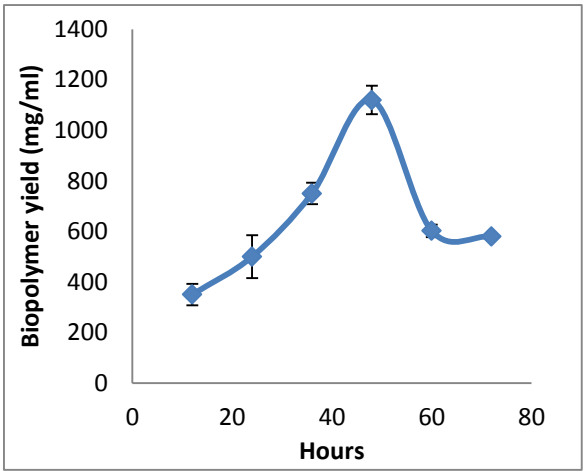
(A)


Biopolymer yield (mg/l)
2000
1500
1000
500
0
0
20
40
60
80
Hours

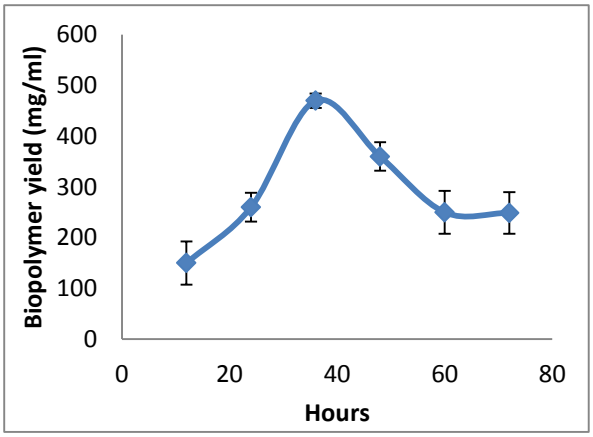
(B)


Biopolymer yield (mg/ml)
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
Hours

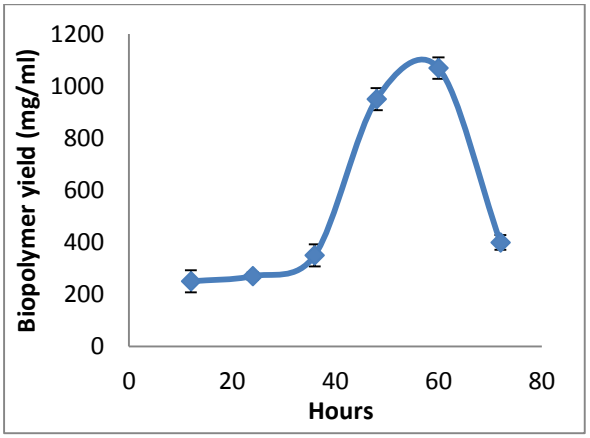
(C)


Biopolymer yield (mg/ml)
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
Hours

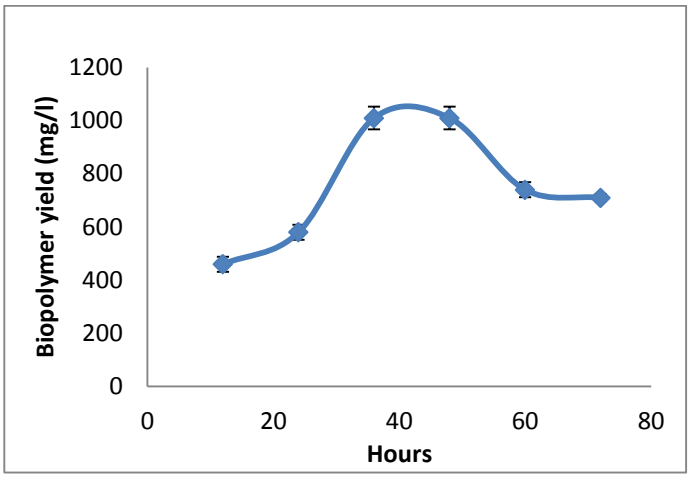
(D)


Biopolymer yield (mg/ml)
600
500
400
300
200
100
0
0
20
40
60
80
Hours

(E)


Biopolymer yield (mg/ml)
1200
1000
800
600
400
200
0
0
20
40
60
80
Hours

(F)



(G)

Figure 4.2 Biopolymer production with time by different isolates A) PPY2; B) PPY5; C) PPY3; D) HKW3D; E) HKW 1A; F) HKW2C; G) HKW2A

Clearly a variation in time period for the maximum biopolymer production during the stationary phase was observed. For instance, PPY2, PPY5 and HKW1A produced maximum biopolymer at 48 hours of stationary phase. HKW3D and HKW2A also had same time period of 60 hours, other biopolymers such as PPY3, HKW1A and HKW2C produced maximum biopolymer at 36, 48 and 72 hours of stationary phase respectively. Microbial cells generally contain various polysaccharide structures contributing to their shape and rigidity. Whereas capsule EPS are produced mainly during the log phase of bacterial growth, biopoymer is usually produced during the stationary phase (Plante and Shriver,1998). It has been suggested that the biosynthesis of biomass and biopolymer follows roughly the same metabolic pathway which results in the same metabolic control for biopolymers preproduction and growth as a function of media constituents (Kimmel and Roberts, 1998).

4.3) *In-vitro* antioxidant profiling of biopolymers

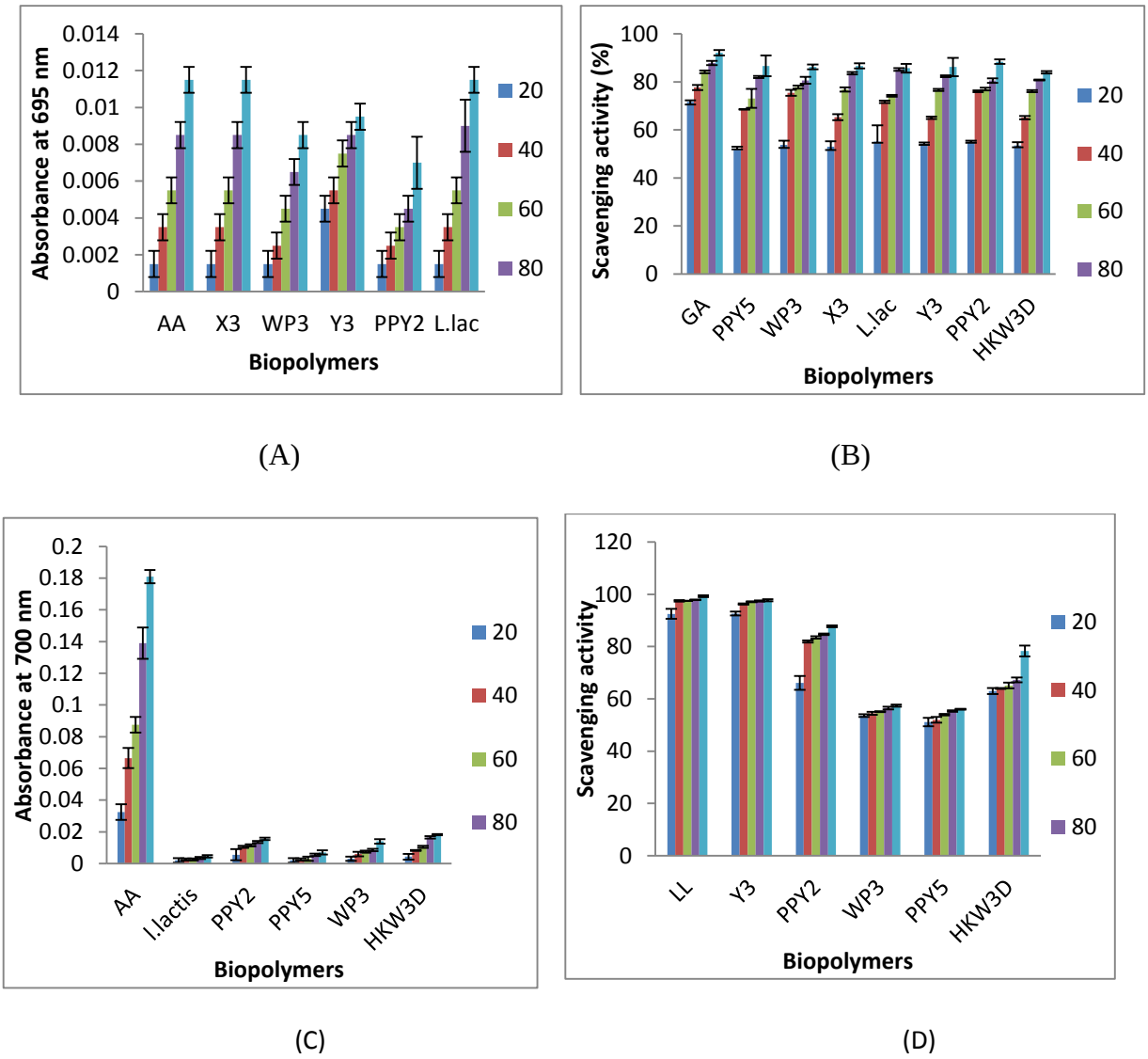


Figure 4.3 *in-vitro* Antioxidant profiling of biopolymers A) Total antioxidant capacity; B) H_2O_2 scavenging; C) Reducing power; D) DPPH radical scavenging

Ascorbic acid used as standard in all the assays except H_2O_2 scavenging where gallic acid was used as standard. Different concentrations of biopolymers were used in all the four assays.

i) Total antioxidant capacity

The measure of total antioxidant capacity (TAC) considers the cumulative action of all the antioxidants present and provides an integrated parameter rather than the simple sum of measurable antioxidants. The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, thus giving an insight into the delicate balance between oxidants and antioxidants. In fact, measuring plasma AC may help in the evaluation of physiological, environmental, and nutritional factors of the redox status in humans, determining plasma AC may help to identify conditions affecting oxidative status in vivo (e.g., exposure to reactive oxygen species and antioxidant supplementation). Total antioxidant capabilities of different concentration of biopolymer compared with the standard which implies that as the concentration of the biopolymer increases the total antioxidant activity of the biopolymer also increases. PPY5 and HKW3D was not showing any antioxidant capacity.

ii) H_2O_2 scavenging activity

As depicted in Figure 4.3 during the H_2O_2 scavenging assay, the scavenging ability of standard at 100 µl/ml was 92.11% while the biopolymers at 100 µl/ml exhibited lower scavenging ability comparatively, the maximum H_2O_2 scavenging ability was shown by PPY2 (88.38%). At concentration of 60 µl/ml the scavenging ability of standard was 84.16% while other polymers possessed scavenging ability in the range of 73 to 77 %. The results suggested that the H_2O_2 scavenging ability of the biopolymers may be concentration or dose dependent and increase linearly. Levels of ROS and H_2O_2 could attribute to the alleviation of overall free radical status, improved antioxidant defense, and/or the decrease in both oxidations of lipids. These results are in concurrence with several recent findings that reported antioxidant molecules from human beneficial bacterial isolates, such as LAB (lactic acid bacteria)

iii) Reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing capabilities of the biopolymers increased with the increasing concentrations of the biopolymer (Ye *et al.,* 2012.) Y3 and X3 have not shown any reduction potential.

iv) DPPH scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Hatano *et al.*, 1997). DPPH is one of the compound that possess a proton free radical with characteristic absorption maxima at 517 nm, which decreases significantly on exposure to proton radical scavengers. The free radical scavenging by the antioxidant samples are credited to their hydrogen-donating ability (Yamaguchi *et al.*, 1998). DPPH scavenging ability could be correlated to an increasing concentration of biopolymers. At the concentration of 20 µg/ml the scavenging ability of ascorbic acid (control) was 94.63% while the scavenging ability exhibited by biopolymers PPY2, WP3, PPY5 and HKW3D were 66.14%, 53.60%, 51.15% and 62.99% except two biopolymers LL and Y3 i.e. 92.52% and 92.65% respectively. DPPH scavenging ability of LL and Y3 was significantly ($p<0.05$) higher than other biopolymers. LL was not showing any DPPH scavenging activity.

4.5) Acid values of oil samples

Three types of oil samples were used: commercial oil, expired oil (expired in 2013) and formulated oil to compare their acid value. The acid values were measured as 2.8, 3.9 and 1.12 respectively. From the acid value it was evident that the expired oil possessed maximum acidity due to oxidation from environment, commercial oil samples were less oxidised as it contained preservatives and the formulated oil oxidised since it was made. The formulated oil was chosen for the further experiments since it lacked artificial preservatives, therefore providing scope for analyzing the antioxidant potential of biopolymers.

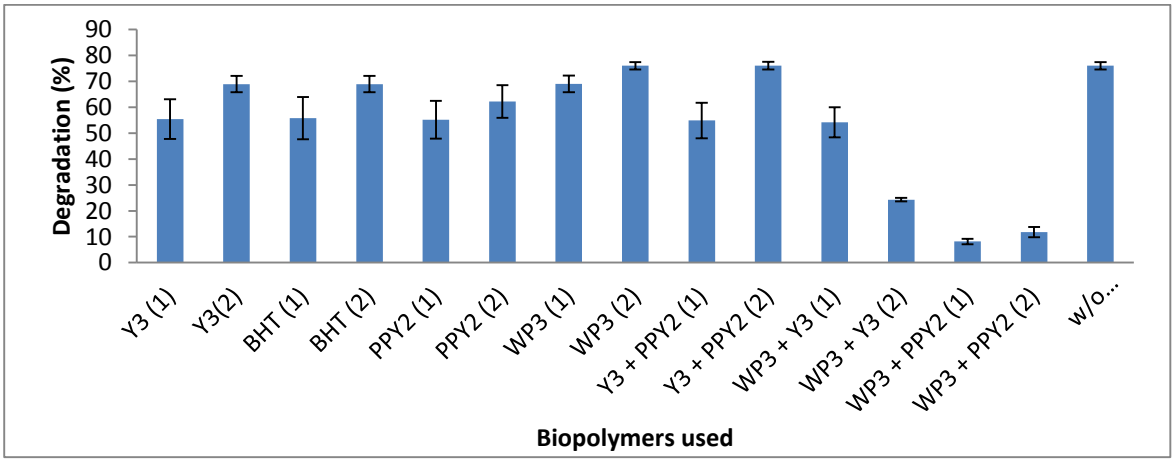


Figure 4.4 Degradation of oil containing biopolymer after 20 days of incubation

As depicted in Figure 4.4 the acid values of oil after 20 days storage at temperature of 28^0C, exhibited a marked percentage of degradation of oil. For instance, oil without biopolymer or BHT was 76% oxidised after 20 days, but the oil sample having the combination of biopolymer WP3 & PPY2 showed around 8 to 11% degradation after 20 days and in oil sample with BHT, 50% degradation was noted. It was concluded that the biopolymer combination of WP3 & PPY2 was most effective.

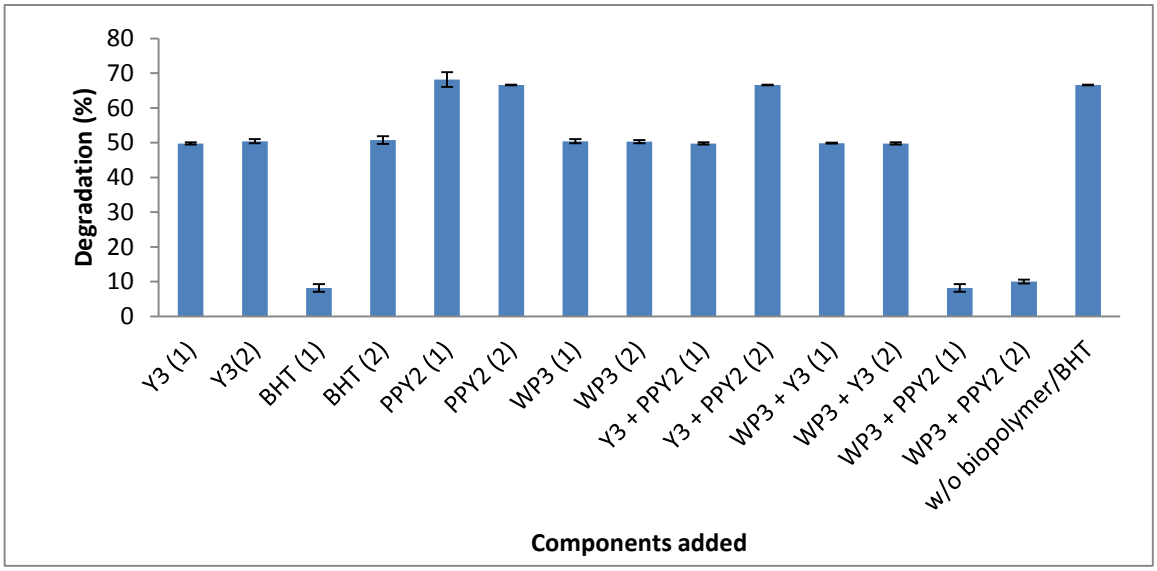


Figure 4.5 Degradation of oil sample containing biopolymer after 40 days of incubation

As shown in Figure 4.5, after 40 days the acid value of the oil decreased slowly, this meant that biopolymer acted as a preservative for the oil sample. Interestingly, after 40 days the minimum degradation remained 8.185% in the oil sample containing WP3+PPY2 (1), which indicates that the oil sample in which biopolymer was added after 20 days showed less degradation in comparison to the oil sample in which biopolymer was added initially only.

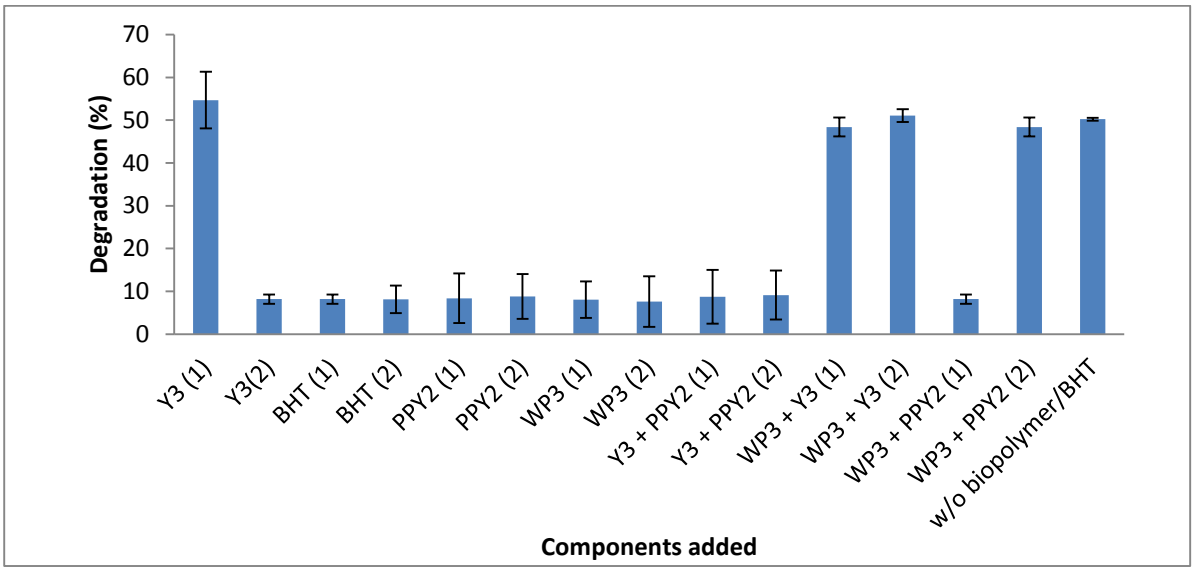


Figure 4.6 Degradation of oil sample containing biopolymer after 60 days

As depicted in Figure 4.6 the degradation of oil after 60 days in the oil sample with BHT (1) and oil sample with combination of WP3+PPY2 (1) biopolymers was equal i.e. 8.185. This suggested that the combination of two polymers i.e. WP3+PPY2 (1) could equally contribute as BHT (1) in the prevention of degradation of oil. However both biopolymer and BHT was more effective if it is added after 20 days. With the combination of biopolymers WP3+PPY2 : (1) 8.185 % degradation of oil sample throughout the experiment while with BHT (1) 50% degradation was observed in initial 20 days after that it was maintained to 8.185 % degradation. This implies that the combination of natural biopolymers i.e. WP3+PPY2 can better protect the rancidity of oil if a particular concentration of biopolymer was maintained (by addition of biopolymer subsequently after every 20 days) and can replace the synthetic biopolymer BHT. Other biopolymers also protected the oil from degradation but notably less in comparison to WP3+PPY2.

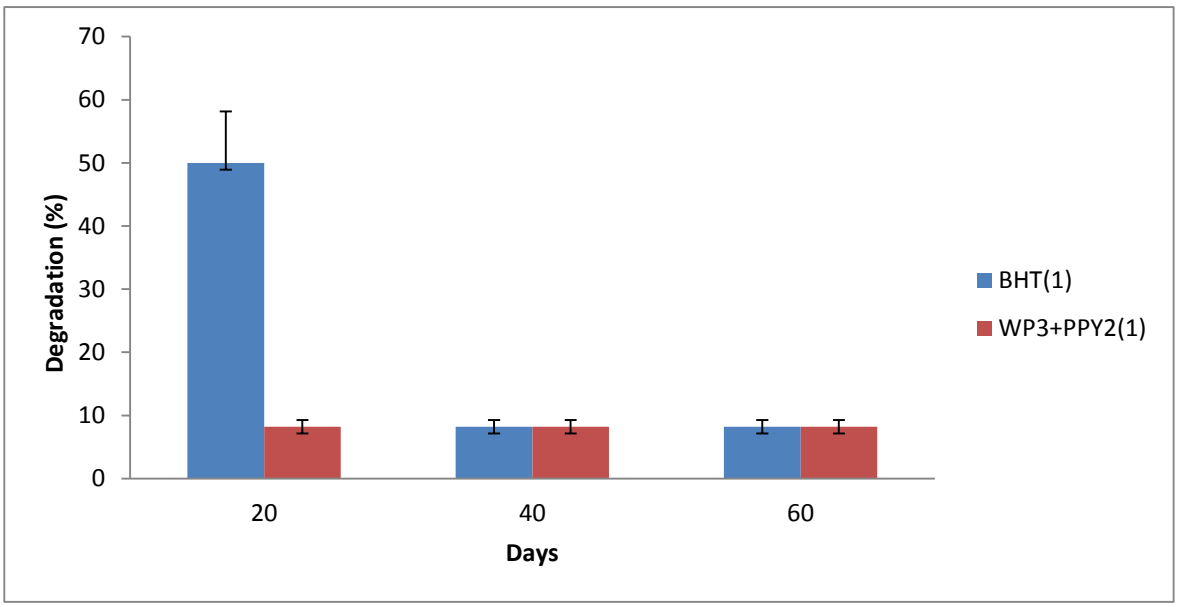


Figure 4.7 Degradation of oil containing WP3+PPY2 (1) and BHT (1)

A closer scrutiny of Figure 4.7 revealed that the biopolymers protected coconut oil from rancidity notably more than BHT. About 8.185% of degradation was observed when combination of biopolymers was used, when compared with the BHT shows 50 % degradation after 20 days. It is evident that both biopolymers and BHT have equally contributed to the protection of oil; the antioxidant capability of biopolymers therefore is comparable to that of BHT.

4.7) Characterization of biopolymers

Since WP3+PPY2 demonstrated that protection against oxidation by two polymers were chosen for further characterization, anticipating that the structural information may provide clues for further insights of the functional activities.

4.7.1) Biochemical characterization:

Biopolymers are rich in high molecular weight polysaccharides and are hetropolymeric nature (Kumar *et al.,* 2007). There are several biopolymer reported with polysaccharides, lipids and proteins (Kurane *et al.,* 1994). It is important to know the structure and composition of the biopolymers to understand their antioxidant activity.

Table 4.2: Composition of biopolymers

Exopolymers	Sugar (μgmL^{-1})	Protein (μgmL^{-1})	Uronic acid (μgmL^{-1})
WP3	87	65	88
PPY2	90	67	75

The overall composition of biopolymers is indicated in Table 4.10. PPY2 contained higher sugar components i.e. 87% then WP3 while other components such as protein and uronic acid was more in WP3 in comparison to PPY2. This indicates that the biopolymers to primarily polysaccharide in nature since sugar content higher than other components. In order to gain more insights of the chemical constituents comprising the structure of the biopolymers, selective dyes were used as rapid chemical probes.

4.7.2) Alcian blue binding

Alcian blue dye has a property of staining carboxylic acids- uronic acids and sialic acids (Green. *et al.*, 1974).Therefore a presumption of presence of these may be obtained simply by recording binding of exopolymers with alcian blue.

Table 4.3 Quantification of biopolymers through alcian blue binding

Exopolymers	Absorbance at 580 nm
Blank	1.752
WP3	0.653
PPY2	0.669

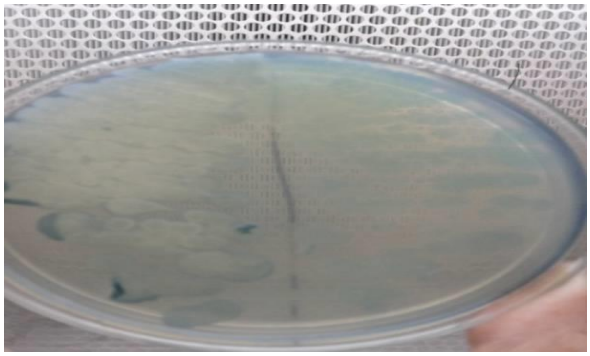
The results in Table 4.3 indicate the presence of carboxylic acid in the exopolymers. It can be observed from the absorbance that the dye has more binding with WP3 then PPY2. This implies that biopolymer WP3 contains more carboxylic acid, although significant differences in absorbances were not observed.

4.7.2) Sudan black:

Sudan black is a fat soluble dye, have high affinity for fats that stains lipids, lipoprotein and triglycerides in bacteria and gives blue-black colour; rapid identification using Sudan black staining of exobiopolymeric material is an useful approach for screening EPS.

Table 4.4 Sudan black binding

Exopolymers	Sudan black test
WP3	Positive
PPY2	Positive



(A) (B)

Figure 4.8 Sudan black binding (A) PPY2 (B) WP3

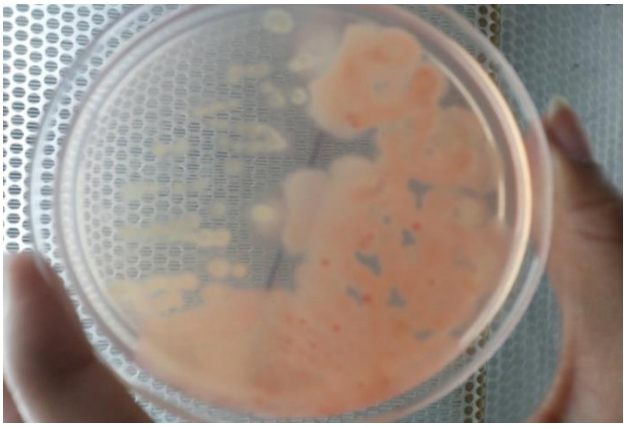
PPY2 stained creamish blue and WP3 stained greenish blue. It was observed that both the strains bind to sudan black dye concluding that the lipid and fats are the possible components of the cell wall of these two strains.

4.7.3) Congo red binding

Congo red binds to the lipopolysacchride in bacteria, and gives pale yellow colour colonies. It is a water soluble dye.

Table 4.8 Congo red binding

Exopolymers	Congo red test
WP3	Positive
PPY2	Negative



(A) **(B)**

Figure 4.9 Congo red binding (A) WP3 (B) PPY2

As noted in Fig 4.9, Congo red binding was observed in WP3 strain since the colour of the colonies of WP3 strain while PPY2 did not exhibit any binding with congo red dye. Congo red staining for biopolymers was in fact the first rapid detection method used for localizing EPS as well as understanding their nature, from *Rhizobium* sp and extended later for several other bacterial strains (Langille, 2000).

Specific probes that bind to biopolymers can be used to understand the constituents and their role in functionality. Congo red has been used to demonstrate the glycosidic linkages (α 1-4) linkages, basic and neutral nature of biopolymers.

4.7.4) Flocculating activity:

The biopolymers present on the cell surface were released in the culture medium and bind with the kaolin particles which results in the flocculation (Kurane *et al.*, 1986). Functional groups and the molecular weight in the molecular chains of biopolymers are important factors for determining the flocculating activity (Kumar *et al.*, 2004).

Activity (%) : [$(O.D._B - O.D._S) / O.D._B$] * 100

Table 4.6 Flocculating activity

Sample	**O.D. at 580 nm**	**Activity (%)**
Blank	1.023	
PPY2	0.476	53.47
WP3	0.531	92.65

Flocculation resulting from the secretions and synthesis of microorganisms, indicate that WP3 possess more flocculating activity then the PPY2 strain.To date several researchers have reported promising exocellular ppolymeric materials, many being exopolysachharides from microbial sources with promising functionalities; one important being the capability as a flocculant.

4.8) Structural and functional characterization

4.8.1) FTIR (Fourier Transform –Infrared Spectroscopy) analysis of biopolymers.

FTIR provides information about the functional groups present in the biopolymers and the functional group of the biopolymer – these being crucial for the study of antioxidant activity of the biopolymers.

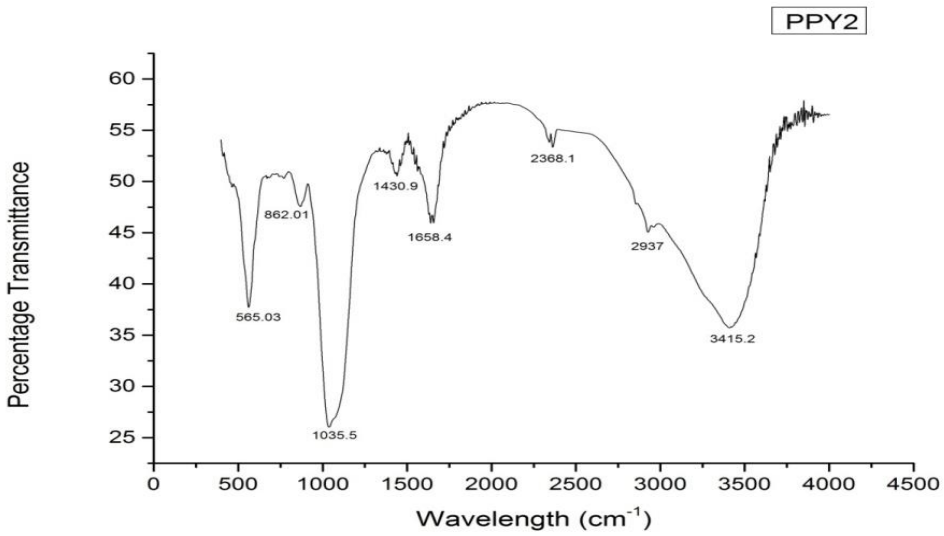


Figure 4.10 Different peaks during the FTIR analysis of PPY2 biopolymer

As depicted in Figure 4.10, FTIR analysis of PPY2 showed a medium peak at 565.03 cm^{-1} which is the characteristic of presence of alkyl halides, strong peak at 862.01 cm^{-1} indicates the presence of C-H stretch of aromatic compound, medium peak of 1035.5 cm^{-1}indicates the presence of C-N stretch of aliphatic amines. Medium peak at 1430.9 cm^{-1} denotes the C-C stretch of aromatic compounds, medium stretch of –C=C- at 1658.4 cm^{-1} indicates the presence of alkenes. Medium peak at 2937 cm^{-1}indicates the presence of C-H stretch of alkanes. Strong and broad peak of 3415.2 cm^{-1}–indicates presence of O-H stretch and hydrogen bonded alcohols and phenols.

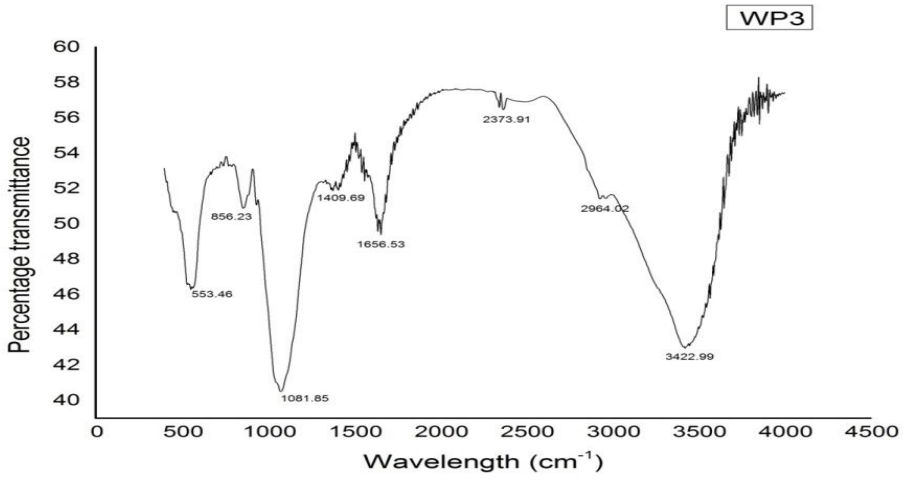


Figure 4.11 Different peaks during FTIR analysis of WP3 biopolymers

In Figure 4.11, FTIR analysis of biopolymer WP3 depicts a medium peak at 553.46 cm^{-1} which shows the presence of alkyl halides, there is a medium peak at 856.23 cm^{-1} which indicates the presence of C-H stretch of aromatics. Strong peak of 1081.85 cm^{-1} is responsible for the presence of C-N stretch of aliphatic amines. Medium peak at 1409.69 cm^{-1} is the indication of presence of C-C stretch of aromatics. Medium peak at 1656.53 cm^{-1} indicates the presence of –C=C- stretch of amines. Medium peak at 2964.02 cm^{-1} shows the presence of C-H stretch of alkanes. Strong and broad peak at 3422.99 cm^{-1} is the indicator of presence of the O-H stretch of hydrogen bonded alcohols and phenols.

Both the biopolymers contain alkyl halides which are good reducing agents and reducing agents oxidizes themselves to remove the free radical, and therefore act potentially as an antioxidant.

4.8.2) Zeta measurement

The charge on the particles of biopolymers was find out by observing zeta potential of the biopolymers.

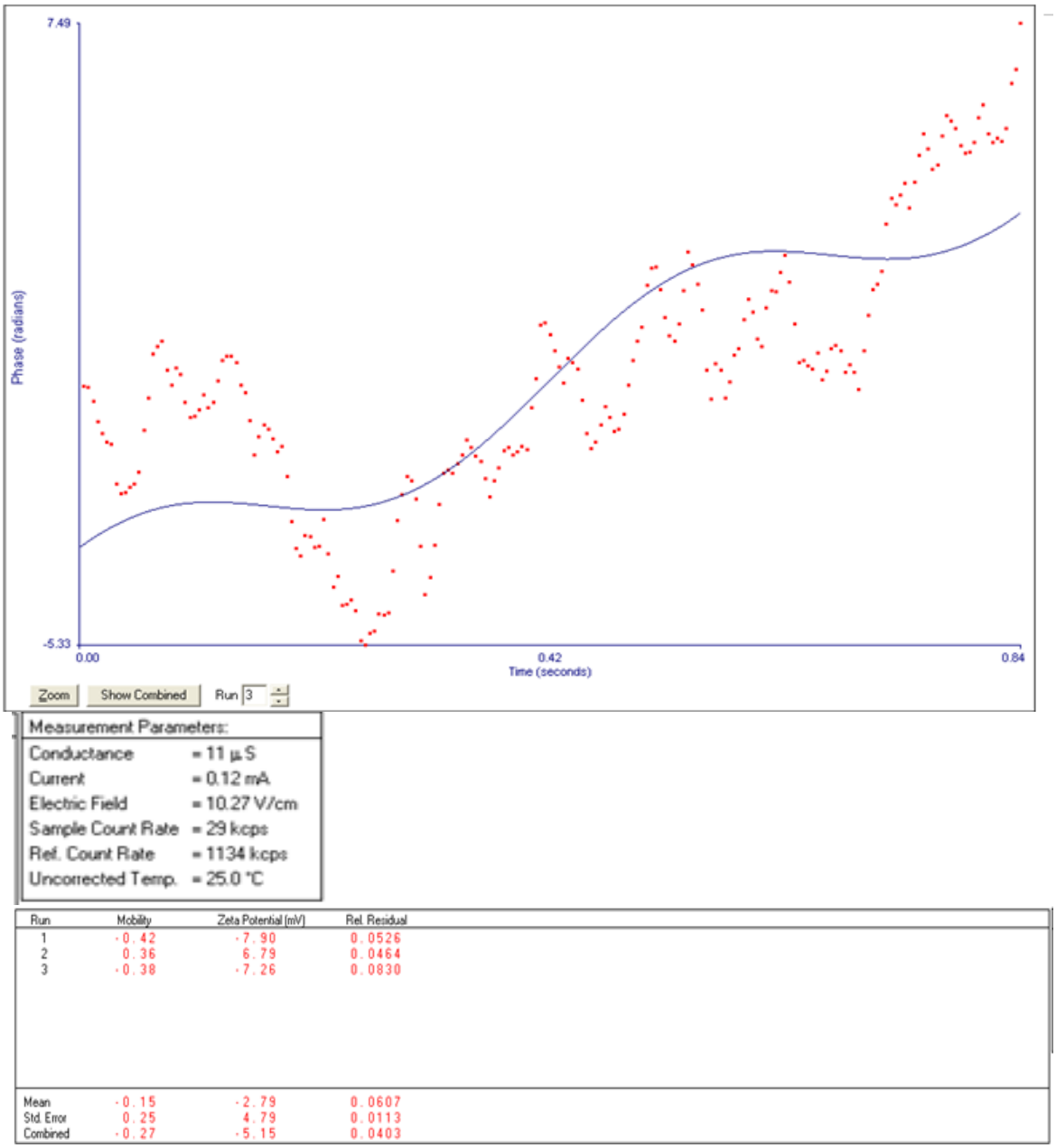


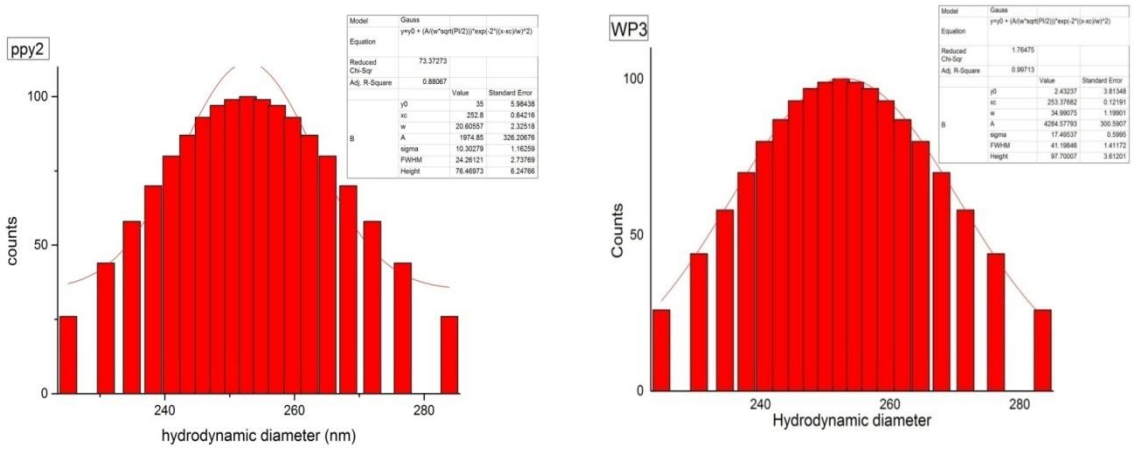
Figure 4.12 Charge of biopolymer PPY2 by zeta potential testing

The charge fluctuates between negative to positive with respect to the mobility in the zeta potential obtained, which indicated the zeta potential of biopolymer PPY2 is around 0 mV which represents the neutral charge of biopolymer.

Most of the antioxidant molecules are neutrally charged, because they donate an electron to the free radical without becoming overly reactive themselves. Antioxidant molecules have conjugated pi systems, which allow different resonance structures and electron delocalization. Thus it may be suggested that PPY2 was a good antioxidant on account of its neutral charge (Gropper *et al.* 2012). The WP3 biopolymer did not show results in zeta potential measurement.

4.8.3) Dynamic light scattering (DLS)

This technique provides us information about the shape, size and flexibility of the particles as well as nature of interactions between particles and their environment.Exopolymers were subjected to DLS.



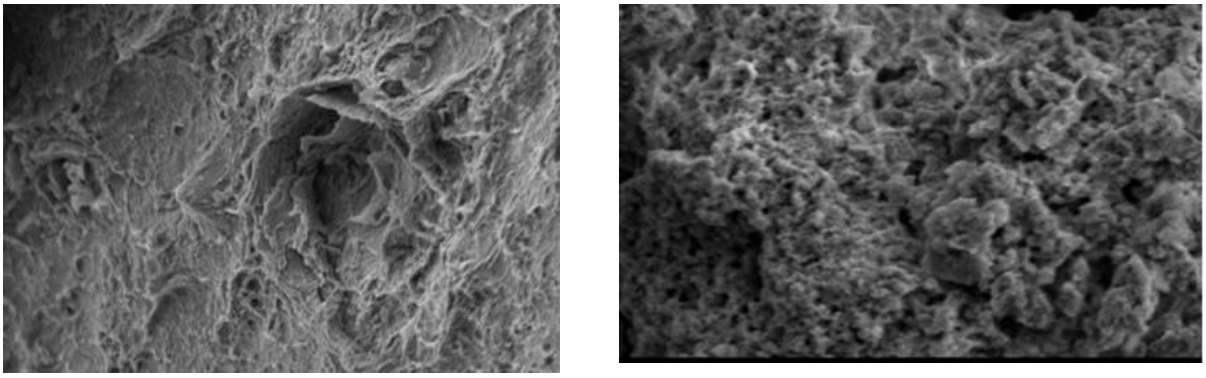
(A) (B)

Figure 4.13 Diameter of biopolymer by DLS, A) PPY2; B) WP3

Fig 4.13 depicts the diameter of the biopolymers. The graph was plotted between hydrodynamic diameter and counts, which was fit into Gaussian Curve as seen in figure to know its statistical significance. The diameter of PPY2 and is 252.8 nm and 253.37 respectively, Origin pro 9.0 was used to plot the graph.

4.8.4) Surface characterization

4.8.4.1) SEM (scanning electron microscopy)



(A) (B)

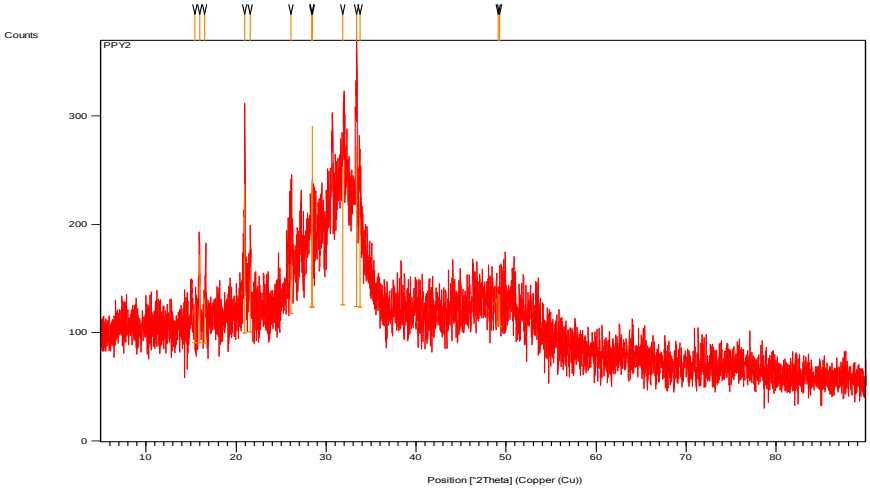
Figure 4.14 Surface of biopolymer A) WP3; B) PPY2

SEM is an important tool for visualizing conformation and surface morphology of the EPS. The SEM micrographs(Figure 4.14) revealed the porous nature of biopolymers with large pore size and surface area. Image of WP3 at 2500x the particle size is 10µm surface shows porosity and irregular size, suggesting the water retention capacity of EPS. While second image of biopolymer PPY2 shows high porosity and irregular size indicating that PPY2 have comparatively more water retention capacity that water retention potential and porosity was correlated, high capillary forces in the thin web like structure to hold water, porous structure of EPS may also be responsible for the stability of gel structure when subjected to stress or external force and for the compact structure of EPS (Mao *et al.*, 2001.). Porosity forms grooves in the polymer which is responsible for large surface area. The porous structure of biopolymers can also used as antioxidant carrier, in other words the free radical grafting technique can be used on biopolymers for better antioxidant activity without any chemical or artificial agents.

4.8.4.2) XRD (X- Ray Diffraction)

A biopolymer can be crystalline or amorphous, XRD is a very important technique for the phase identification of the biopolymers (Kavita et al., 2011; Mishra et al., 2011; Singh et al., 2011)

A)



B)

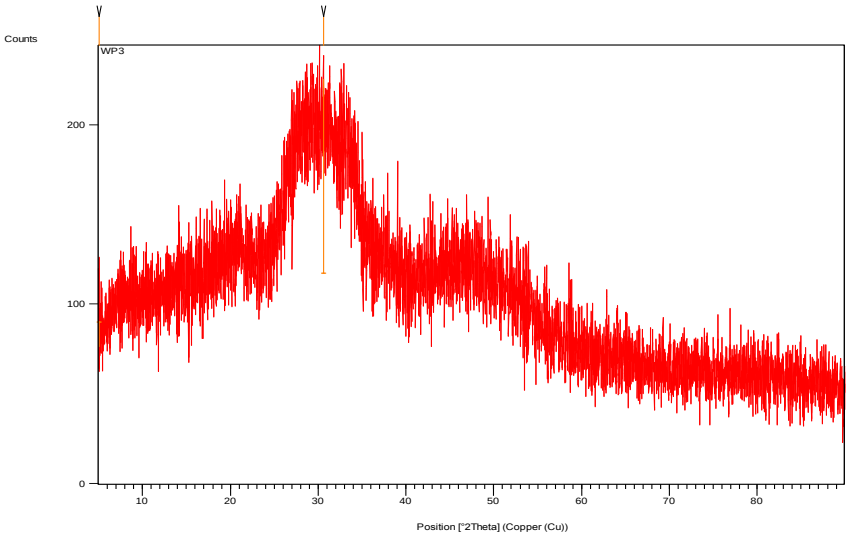


Figure 4.15 Surface studies during the XRD analysis A) PPY2; B) WP3

Both the graphs indicate broad peaks signifying the amorphous nature of the biopolymer PPY2 and WP3.Amorphous content contributes to the broad peaks while the crystalline part of the biopolymers signifies the sharp and narrow diffraction peaks (Mandal et al., 2011). It is difficult to interpret amorphous peaks (Singh et al., 2011). As observed in X- ray diffraction there is almost no crystalline peak, so it becomes difficult to calculate crystallinity index which is the ratio of sharp and narrow peak with amorphous peaks.

Amorphous nature of biopolymers increases its surface area, number of grooves which renders them suitable for the easy reaction with the free radical and acting as carrier during free radical grafting technique. It may be argued, based on the above results that the biopolymers of three bacterial isolates possessed the desired qualities for applicability, though further studies are required and could not be undertaken given the scope of the dissertation. A major functional attribute of these biopolymers being their ability to quench free radicals; to our knowledge, this is the first report of naturally occurring antioxidant biopolymer capable of enhancing shelf life and quality of coconut oil. Similar studies in other oils used for cosmetic formulations should be attempted for expanding the domain of viability. Apart from the basic advantages conferred by the biopolymers in oil based formulations, a minimal volume, maintenance of rheology, structure and other properties may prove worthy in commercializing application of biopolymers in oil based cosmeceuticals.

4. CONCLUSION

Exopolysacchrides have multifarious applications in various industries like biotechnology, pharmaceutical and cosmeceutical industries. The aim of this study was to evaluate the antioxidant activity of exopolysacchrides for the cosmeceutical applications.

Salient findings of the study:

Three biopolymers (WP3, Y3, PPY2) selected from bacterial strains were demonstrated to possess best antioxidant activity using a battery of different antioxidant assays.

These three biopolymers were applied on virgin or formulated coconut oil. The oxidation was protected by the combination of WP3 and PPY2 biopolymers throughout the observation period of 20 days at ambient temperature. Other biopolymers protected oxidation but to lesser extent than WP3 and PPY2.

The structural and functional characterisation was carried out for these two biopolymers. XRD spectrum showed the amorphous nature of the biopolymers, they have an irregular structure which can be altered for different applications.

The morphological studies by SEM revealed the porous structure of biopolymers, which helps in water retention due to large surface area and binding. FT-IR spectrum showed the presence of carboxyl, hydroxyl group, the functional group present in both the EPS are almost same. This justifies their better antioxidant activity when they are used in combination. Charge and the diameters of EPS were found by Zeta and DLS.

The results obtained in this study suggest a feasibility of extending shelf life and quality of coconut oil based cosmetics by application of biopolymers. Further studies, including toxicity/ skin patch testing/skin allerginicity are however necessary prior to advocating the use of the biopolymers. it can be calculated that the biopolymers isolated from various bacterial strains can be used as a additive in cosmeceuticals to prevent oxidation. These natural biopolymers can replace artificial antioxidants.

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