SURFACE ASSIMILATION OF PHYTOESTROGENS BY MICROBIAL BIOPOLYMERS

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

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CERTIFICATE

This is to certify that the thesis entitled "**Surface assimilation of phytoestrogens by microbial polymers**" submitted by Shweta Sehgal in partial fulfilment of the requirement for the award of Degree of Masters of Science in Microbiology 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 University or Institute.

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I hereby declare that the work which is being presented in the dissertation entitled "Surface assimilation of phytoestrogens by microbial polymers" in partial fulfillments of the requirements for the award of the degree of MASTERS OF SCIENCE IN MICROBIOLOGY, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is an authentic record to my own work during a period of 6 months from January 2013 to July 2013, under the supervision of Dr. Moushumi Ghosh, Associate professor, Department of Biotechnology and Environment Sciences, Thapar University.

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DEDICATED TO MY PARENTS

To my parents, for all the support and motivation they have given me throughout my life.....

Contents

List of Abbreviations

ER	Estrogen receptor
EPS	Exopolysaccharides
NOMs	Natural organic matters
FIB	Flocculant inducing broth
NB	Nutrient both
NA	Nutrient agar
LDL	Low density lipoprotein
VLDL	Very low density lipoprotein

List of Symbols

°C	degree(s) Celsius
G	gram
hrs	hours
ml	milliliter
$mgml^{-1}$	milligram per milliliter
mgl^{-1}	milligram per liter
$mgkg^{-1}$	milligram per kilogram
$mggm^{-1}$	milligram per gram
$ngdm^{-3}$	nanogram per decimeter cube
µg	microgram
$µgml^{-1}$	microgram per milliliter
$µgdm^{-3}$	microgram per decimeter cube
µl	microliter
min	minute
nm	nanometer
%	percentage
rpm	revolutions per minute
sec	second

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Abstract

In the present study the potential of microbial biopolymers to adsorb and remove phytoestrogens from the surface waters was examined. Extracellular Biopolymer producing bacteria were isolated and screened from various environmental sites, seventy one biopolymer producing bacteria were isolated of which 23 isolates showed highest flocculating activities and these were selected for their tendency to bind finally with phytoestrogens. Two isolates showing highest phytoestrogen removal activity were selected and further optimization studies were carried out. Different parameters for the effective adsorption of phytoestrogens were optimized i.e. effective dose of biopolymers and effect of time of contact on biopolymer and phytoestrogen binding. The observations from the study revealed that for coumestrol, a concentration of 1.5 $mgml^{-1}$ of biopolymer FM17 and DS1 was found to be optimum and for Formononetin, Genistein and Biochanin A the optimum concentration was 1 $mgml^{-1}$ and 1.5 $mgml^{-1}$ respectively. The biopolymer FM17 showed maximum reduction (87%) of Genistein whereas biopolymer DS1 showed maximum reduction (72%) of Formononetin which was further confirmed by Langmuir isotherm. Compositional analysis of the biopolymers revealed them to be of anionic nature and principally composed of sugars and proteins.

Keywords: Biopolymers (extracellular), Flocculating activity, Phytoestrogen, Langmuir isotherm.

1. Introduction

Endocrine disruption is a toxicological issue which is one kind of micro-pollution in aqueous environment with many short term or long term adverse effects on humans and aquatic animals. Till now lots of chemicals have been identified as endocrine disruptors and these chemical compounds when released into environment can function as hormone mimics and alter the natural endocrine systems in wildlife (Tyler *et al*., 1998). Phytoestrogens are one kind of natural plant-derived estrogen mimics that have the ability to disrupt the endocrine system (Lintelmann *et al*., 2003). These are also called as phytochemicals as they are produced by plants as their natural defense mechanism to prevent predation by grazing animals and are present in fruits, vegetables, grains, legumes, herbs, and seeds (Guhr *et al*., 1997). Biochanin A, Diadzein, Genistein, Formononetin and Coumestrol are the most commonly identified phytoestrogens in waste waters. Phytoestrogens can gain entry in organisms though the ways of bioaccumulation and biomagnification. The molecular mechanism of the estrogenic effect depends on the molecular structure of these compounds, almost all of them have a phenolic benzene-ring which is similar to that of the natural mammalian hormone (17-β-estradiol) and with this particular molecular structure, phytoestrogens can bind with the natural estrogen receptors (ERs) in the organism's body, and consequently interfere with the normal binding of hormones generated by the body with ER.

Exposure to these compounds has been shown to affect reproductive processes in many different species ranging from mice (Jefferson *et al*., 2007) to fish (Kiparissis *et al*., 2003). In fact, studies have shown that their exposure can lead to infertility and developmental problems in human and also in the womb and during early childhood, their exposure may pose risks to sexual development of the foetus. No studies have been

performed till date to determine the absolute concentration of phytoestrogens in waste water where we would expect to see negative physiological impacts on organisms. Thus, a potential environmental threshold must be estimated based on existing research so that removal strategies can be established. Research has suggested that a concentration as low as 1 μgml^{-1} can have severe impacts on humans and aquatic animals. Only one type of industry, pulp and paper, has been identified as the point source of phytoestrogens in waste water. Also the industries that use soy products are of main concern because soy is known to contain the highest levels of phytoestrogens.

Gaps in phytoestrogen removal studies

There is a need to develop strategies that could effectively remove these compounds from waste water. Many techniques have been used in removing phytoestrogens. These include ultrafiltration, reverse osmosis and biological methods that include adsorption by biomass and volatilization in bioreactors. But these strategies are ineffective because these compounds still remain in the effluent due to their incomplete removal. There is a need of finding a novel and effective means of phytoestrogen removal. Apart from all these physical and biological methods, microbial products like biopolymers can also be used for binding of phytoestrogens. Biopolymers have been extensively used for removal of many contaminants like heavy metals removal from dilute aqueous solutions (Lewandowski Z, 1992), metals removal from recovered base oil (Jamil *et al*., 2009), dye removal from colored-textile wastewater (Mousa *et al*., 2012) and many more.

Biopolymers are composed of a variety of repeating functional groups (such as carboxyl, hydroxyl, amino, etc which makes them very reactive and subject to cross-linking with other compounds (Knox *et al*., 2010). Therefore, biopolymers have high opportunity for chemical interaction with other compounds. The present study is done to explore the potential of biopolymers for phytoestrogen binding. Phytoestrogens being chemical in

nature, their tendency to bind with biopolymers has been explored in this study. The more selective adsorption performance of biopolymers can be studied by establishing the adsorption isotherms of the biopolymers with phytoestrogens. Langmuir adsorption model is an ideal model to study the interaction between adsorbate i.e. phytoestrogens and adsorbent i.e. biopolymers is explored in this study.

Objectives and Scope of the study

In view of the potential advantages of biopolymers, the following objectives were designed, in relation to phytoestrogens:

(i) Screening and characterization of potential biopolymers on the basis of their nature

(ii) Optimization and evaluation of the phytoestrogen removal efficiency of biopolymers

(iii) Modeling phytoestrogen-biopolymer adsorption.

The current study provides an innovative approach for water treatment, by affording sustainability and safety for the removal of target phytoestrogenic compounds as opposed to conventional treatment.

2. Review of Literature

2.1 Phytoestrogen

Phytoestrogens are naturally occurring compounds that are produced by plants and are found in food like nuts and oilseeds, soy products, breads, legumes, meat products, and other processed foods that contain soy. These compounds are also found in herbs and seasonings (garlic, parsley), vegetables (beans, carrots, and potatoes), fruits (date, pomegranates, cherries, and apples), drinks (coffee), alcoholic beverages and also present in hotdogs, hamburgers, sausages and other meat products as soy is used as a substitute in them. In the United States, more than 15 percent of babies are given soy formula which is very rich in phytoestrogens (Setchel *et al*., 1997). Plants make phytoestrogens as a defense mechanism to stop or limit predation by plant-eating animals (Ehrlich and Raven, 1964; Guillette *et al.*, 1995; Hughes, 1988) but because of their similarity with mammalian estradiol (17-β-estradiol) that functions as the primary female sex hormone, they interfere with the normal binding of hormone. They enter the body when these are consumed. Phytoestrogens are nonsteroidal compounds and can also be called as "dietary estrogens". These phytoestrogens are commonly recommended as dietary supplements and are also used as an alternative to estrogen replacement therapy.

The recommended level of phytoestrogen consumption per day is 1.5 – 2.0 $mgkg^{-1}$ body weight gain a protective health benefit (Wang and Murphy, 1994). Food stuffs like soybean contain upto 1 to 3 $mggm^{-1}$ of phytoestrogen, which is beyond the recommended level (Adlercreutz *et al*., 1995).

2.1.1 Classification of Phytoestrogen

There are mainly three classes of phytoestrogens: isoflavones, coumestans, and lignans. The presence of a phenol ring, molecular weights similar to those of estradiol (MW=272)

and often a pattern of hydroxylation at the 4^{th}, 5^{th}, and 7^{th} carbon positions are similar to that of estradiol (Figure 1). The distance between the two aromatic hydroxyl groups in the nucleus of phytoestrogens is also identical to the distance between the hydroxyl groups in estradiol, which also results in estrogen agonist activity (Setchell and Adlercreutz., 1988) (Figure 1).

The first class of phytoestrogens i.e. isoflavones are non-nutrient compounds found in plants. Isoflavones constitute the largest group of natural isoflavonoids, with about 364 aglycones (unconjugated forms) have been reported (Dewick, 1993). The most thoroughly investigated compounds in this group are Genistein, Diadzein, Biochanin A and Formononetin. The other group of plant phenols is Coumestans. They were first reported by Bickoff *et al.,* (1957). The most potent representative of this family is coumestrol and 4'-methoxycoumestrol. Coumestans are less common in the human diet than isoflavones (Ibarreta *et al.,* 2001). Lignans are chemically related to polymeric lignin of the plant cell wall and are found mainly in woody tissues, where they form the building blocks for the formation of lignin (Ayres and Loike, 1990). Isoflavones are primarily found in legumes such as soy, peanut and clover whereas coumestans are also found in legumes, but particularly in food plants such as sprouts of mug beans and alfalfa (Lookhart, 1980). The presence of a cyclic pattern of these phenolic compounds in the urinary excretion by humans and animals during menstrual cycle initiated interest in their physiological role. Lignans are widespread in foodstuff such as cereal, fruits and vegetables. Lignans have not been studied as thoroughly as isoflavons and coumestans (Ibarreta *et al.,* 2001).

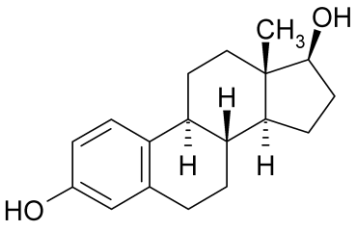


Figure 1. Structure of 17-β-estradiol.

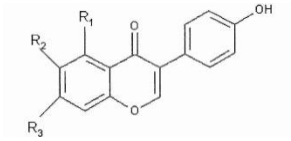
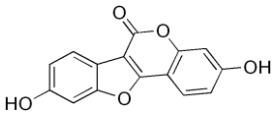
CLASS	GENERAL STRUCTURE	SUBCLASS
Isoflavone		Biochanin A (R_1=OH, R_2=OCH_3)
		Diadzein (R_1=H, R_2=OH)
		Formononetin (R_1=H, R_2=OCH_3)
		Genistein (R_1=OH, R_2=OH)
Coumestans		Coumestrol, 4'-methoxycoumestrol

Table 1. Classes and general structure of different phytoestrogen.

2.2 Phytoestrogen and Controversies

There are currently differing opinions about the role played by phytoestrogens in human health. For adults, when consumed as part of an ordinary diet, phytoestrogens are considered safe and possibly beneficial. Some studies on cancer incidences in different countries suggest that phytoestrogens may help in protecting certain cancers (breast, uterus, and prostate). These have been suggested as cancer preventatives and as treatments for menopausal symptoms and osteoporosis (Messina *et al*., 2002) and also associated with decrease in the incidence of coronary heart disease in many individuals (Anderson *et al*., 1995). Some phytoestrogens with antioxidant properties have the potential to inhibit cardiovascular disease (Wang *et al*., 1995) and are also influential in decreasing plasma low density lipoprotein (LDL) cholesterol and very low density

lipoprotein (VLDL) cholesterol in males and females (Anthony *et al*., 1996). Epidemiologic studies show that Japanese and Chinese menopausal women suffer less from menopausal symptoms than Western women do. This could be because of their higher consumption of soy isoflavons (Kao *et al*., 1995). Consumption of soy products increases the length of the menstrual cycle. A longer menstrual cycle is beneficial in lowering breast cancer risk.

As for adverse health effects, the most likely risks associated with phytoestrogens deal with infertility and developmental problems. The clover disease in Australian sheep was caused by eating grass with phytoestrogens (Kingsbury, 1964). Exposure to endogenous estrogens, including phytoestrogens in the womb and during early childhood, may pose risks to sexual development of the fetus. Humans have used plants for medicinal and contraceptive purposes for years. According to modern-day analyses, many of the plants historically noted for their ability to prevent pregnancies or cause miscarriages contain phytoestrogens and other hormonally-active substances (Riddle, 1991). Some studies showed that phytoestrogens may increase migraines. One study found that women eating a vegetarian diet during pregnancy have male offspring with an increased incidence of hypospadias which is a birth defect in boys where the penis opening is not located in the normal position at the tip of the penis, possibly due to high maternal levels of soy isoflavones (North and Golding, 2000).

2.3 Phytoestrogen as Endocrine disruptors and their mechanisms

How these chemicals affect organisms depends on the concentration of the chemical in the environment, the organism in question, and the developmental stage of the organism. In all vertebrate species estrogens are responsible for the expression and development of female physical characteristics, for the reproductive cycles of organisms, and for the fertility of organisms (Stephen L., 2009). Disorders within the endocrine system can

affect many different organs and functions in the body and can be unbearable or life-threatening. The discharge of phytoestrogens from soil and food based industries is regarded as their main source in water environment (Erbs *et al*., 2007). The major contributor of the phytoestrogens in water bodies are pulp and paper mill waste (Hewitt *et al*., 2006). Phytoestrogens concentrations in the rivers of Australia, Switzerland, Germany, and Italy were found to be 1–12 $ngdm^{-3}$ (Erbs *et al*., 2007), however, Diadzein and Genistein concentrations found in Japan were 43 μgdm^{-3} and 143 μgdm^{-3}, respectively (Kawanishi *et al*., 2004).

Regardless of the inherent difficulties in determining a concentration threshold for phytoestrogens there is a need to recognize the threshold concentration, over which negative effects may be observed. Research suggests that 1 μgL^{-1} total phytoestrogens could cause physiological impacts on fish impacts on fish (Gontier *et al*., 2007; Thorpe *et al*., 2003). Due to their incomplete removal during the waste treatment process, synthetic and natural estrogens are considered as chiefly responsible for the estrogenic activity associated with waste water treatment plant effluents (Gutendorf and Westendorf, 2001). In consequence reproductive disorders and feminization of fish populations are alarming signs of endocrine disruption in water bodies (Bern *et al*., 1991). Adverse effects of phytoestrogen consumption have been also observed in humans, such as the increasing number of endocrine responsive cancers and the decreasing reproductive fitness of men (Daston *et al*., 1997).

Because of their structural similarity with mammalian estradiol (17-β-estradiol) phytoestrogens possess the ability to cause estrogenic or antiestrogenic effects. Phytoestrogens bind to the hormone receptor with much weaker affinities than the natural hormone (Gutendorf and Westendorf, 2001; Pelessaro, 1991). There are two variants of the estrogen receptor, alpha (ER-α) and beta (ER-β) and many phytoestrogens display

somewhat higher affinity for ER-β as compared to ER-α. The phenolic ring of the phytoestrogen molecule mediates binding to estrogen receptors (Setchell, 1998). In addition to interaction with ERs, phytoestrogens may also temper the concentration of endogenous estrogens by binding or inactivating some enzymes, and may affect the bioavailability of sex hormones by depressing or stimulating the synthesis of sex hormone-binding globulin (SHBG).

Phytoestrogens bind with the estrogen receptors when their concentration increases the recommended level which is 1-3 μgml^{-1} and when they bind to the receptors they show two mechanisms that are shown in Figure 2 (a) and (b). Phytoestrogens can act as a hormone and bind to the hormone receptor, activate it, and cause an unintended cellular response, Figure 2 (a). These are called hormone mimics or agonists. In another mechanism they block the normal hormones and prevent the cellular response that was intended when the organism released the natural hormone, Figure 2 (b). These are called hormone antagonists. Isoflavons are naturally found as biologically inactive glycoside conjugates containing glucose or carbohydrate moieties. The unconjugated from (aglycone) is the bioactive form.

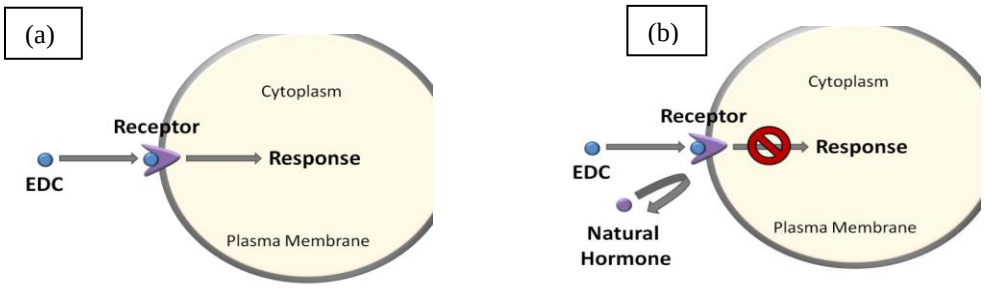


Figure 2 (a) Phytoestrogen as hormone agonists or mimics, (b) Phytoestrogen as hormone antagonists

The proportion of conjugated to unconjugated forms varies significantly among foods, but fermented soy foods, such as miso or tempeh, often contain higher levels of the aglycone than other soy-based foods. Once consumed, they are rapidly metabolized and

absorbed and enter the systemic circulation predominantly as conjugates with limited bioavailability.

The binding affinities of these isoflavons differ greatly among themselves. Genistein (4`, 5, 7-trihydroxyisoflavone) is the most active isoflavons with the highest binding affinity for the estrogen receptor (Shutt and Cox, 1972) with only 94.6 times less potency than natural estrogen. Biochanin A, which is its methoxy derivative, does not bind to the estrogenic receptor but is estrogenic in vivo (Miksicek, 1994). It's 1420 times less potent than estradiol. Diadzein (4, 7-dihydroxyisoflavone) has a higher binding affinity for the estrogen receptor than its methoxy derivative, formononetin, but both are weak estrogens in vivo (Shutt *et al*., 1972). The binding affinity of formononetin is 93.7 times less than estradiol.

2.4 Phytoestrogen removal strategies

Phytoestrogens, due to their relatively low estrogenic activity, have been understudied in the literature as potential contributors to endocrine disruption. The adverse effects of phytoestrogens have been studied by some scientists that prove toxicological effect of these chemicals. These studies includes investigation of the behavioral effects of phytoestrogen contamination on the male fighting fish, *Betta splendens* (Clotfelter and Rodriguez, 2006), effect of phytoestrogen contamination on the immune systems of fighting fish (Ardia and Clotfelter, 2006), the possibility of inhibitory effects of flavonoids on ovarian aromatase activity in rainbow trout, *Oncorhynchus mykiss* (Pelissero et al., 1996), the effects of genistein and equol on the gonadal development of the Japanese Medaka, *Oryzias latipes* (Kiparissis, 2003) and many more. These studies revealed that phytoestrogen concentration level as low as ngL^{-1} can cause severe effects in organisms. The release of such compounds in surface waters at high concentrations

might have severe impacts on health, hence removal of phytoestrogens is a mandatory task these days.

Currently removal of phytoestrogens from waste waters resorts to ultrafiltration and reverse osmosis that are the methods of concern. The filtrate obtained as a result of these processes is in very pure form and all the essential elements are removed from the water, due to this its intake in the ecosystem alters the concentrations of salts and bioelements necessary for the life (Diano *et al.,* 2007). This is the major drawback of these techniques and restricts their use in phytoestrogen removal from water. Other approaches for the selective removal of phytoestrogens are photodegradation, membrane retention and adsorption. The removal efficiency of photodegradation in removing phytoestrogens depends on the time, UV intensity employed as well as environmental factors (Lau *et al*., 2005). However, due to the refractory property of these compounds, the estrogenic effect of these compounds still remains after the photodegradation. Compared with photodegradation, membrane retention can remove most of the phytoestrogens (Thomas *et al*., 2002). However, competing substances in water stream, such as natural organic matters (NOMs), will influence the adsorption of these compounds because natural organic matters will consume most of the adsorption capacity, and as a result, the removal efficiency for the target compounds can be adversely influenced (Fukuhara *et al*., 2006). The biological treatment, which is the most important treatment process of wastewater, has been studied extensively by various researchers in removing these compounds (Ternes *et al.*, 1999; Korner *et al.*, 2000; Fred *et al.*, 2002; Anders *et al.*, 2003; Hemming *et al.*, 2004 and Urase *et al.*, 2005). It is generally recognized that biodegradation, adsorption by biomass and the volatilization in bioreactors can help to remove estrogenic compounds to some extent (Birkett *et al.*,, 2003). Unfortunately, the estrogenic effect of these compounds still remains in the effluent because estrogenic

compounds cannot be removed completely. Another biological methods that is used by many treatment plants is by the means of enzymatic reactions (bioremediation).

This approach appears more suitable, since the treatment is effective only towards the target harmful chemical and the other components present in the water remain unchanged (Georgieva *et al*., 2010). As the enzyme activity is dependent upon temperature and other physiological conditions, so the operational parameters of the treatment plant require controlled and optimized conditions. This limits the use of enzyme based removal of phytoestrogens from waste water. Selective adsorption of these compounds using biological antibody and estrogen receptor (ER) of the target molecule was also investigated by some researchers (Kuramitz *et al*., 2002; Nishiyama *et al*., 2002 and Urmenyi *et al*., 2005). This application had, however, a disadvantage that the regeneration of antibody could be a problem because the activity of the antibody or receptor would be reduced after several times regeneration due to the harsh regeneration conditions. In brief, antibody and receptor are highly selective but lack stability. Besides being costly, all these approaches have their drawbacks and disadvantages, so there is a need of finding a novel and effective means of phytoestrogen removal. Polymers are capable to bind phytoestrogens because of the presence of active sites (Zhongbo, 2010) Phytoestrogens being chemical in nature, also possess tendency to bind with biopolymers has been explored in this study. Apart from all these physical and biological methods, microbial products like biopolymers can provide a useful means for binding of phytoestrogens. The present study is done to explore the potential of biopolymers for phytoestrogen binding. Biopolymers are the microbial products that are secreted by them in the environment and are composed of polysaccharides, proteins and nucleic acids. In contrast to synthetic polymers which have a simpler and more random structure, biopolymers have complex molecular assemblies that adopt precise and defined 3D

shapes and structures. This feature is essential because this is what makes biopolymers active molecules in vivo. Some biopolymers can be complex and composed of carbohydrates containing various negatively charged groups such as sulfated sugars and ketal-linked pyruvate groups. A complex mixture of biopolymers comprising polysaccharides, proteins, nucleic acids, uronic acids, humic substances, lipids, etc. is called extracellular polymeric substance (EPS).

Exopolysaccharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties. Some are neutral macromolecules, but the majorities are polyanionic due to the presence of either uronic acids (D-glucuronic acid being the commonest, although D-galacturonic and D-mannuronic acids are also found) or ketal-linked pyruvate. Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status (Sutherland, 1990). A very few EPS may be polycationic; this contributes to the adhesive nature of exopolysaccharides. Microbial exopolysaccharides are ubiquitous and comprised of either homopolysaccharides or heteropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type: D-glucose or L-fructose. A number of lactic acid bacteria produce heteropolysaccharides. These molecules are formed from repeating unites of monosaccharides such as: D-glucose, D-galactose, l-fructose, l-rhamnose, D-glucuronic acid, l-guluronic acid and D-mannuronic acid.

Biopolymers have been extensively used in different industries till date for heavy metal removal (Jamil *et al.*, 1990), dye removal (Lozano *et al.*, 2009) etc. The main advantage of using biopolymers is that they are biodegradable and donot lead to the formation of toxic intermediate. Because of these reasons removal efficiency of biopolymers are the main focuses of this study. The feasibility of using biopolymers to remove phytoestrogens was studied using spectrophotometric analysis. However, more selective

adsorption performance of biopolymers was studied by establishing the adsorption isotherms of the estrogenic compounds. Langmuir adsorption model is an ideal model to study the interaction between adsorbate and adsorbent.

4.5 Langmuir adsorption isotherm model

Adsorption equilibrium (the ratio between the adsorbed amount with the remaining in the solution) is established when an adsorbate has been contacted with the adsorbent for sufficient time. Langmuir is a two parameter isotherm. It was originally developed to describe gas–solid-phase adsorption onto activated carbon but now it is been used to quantify the performance of different bio-sorbents (Foo *et al*., 2009). This model assumes that the adsorbed layer is one molecule in thickness (monolayer adsorption), and adsorption can only occur at a finite (fixed) number of definite localized sites (Foo *et al*., 2009). Langmuir isotherm has been extensively used in numerous studies which includes sorption of divalent ions onto peat (Ho *et al*., 2001), studies on Methylene Blue Adsorption onto Ground Palm Kernel Coat (Oladoja *et al.*, 2009), adsorption of formaldehyde on activated carbon and alumina (Aggarwal *et al*., 2011) and many more.

The linear form of Langmuir isotherm equation is given as:

$$Q_e = Q_{max} K_L C_e / 1 + K_L C_e$$

Where, C_e ($mgml^{-1}$) is the equilibrium concentration of the adsorbate, Q_e ($mgml^{-1}$) is the amount of adsorbate per unit mass of adsorbent, Q_{max} and K_L are Langmuir constants related to maximum adsorption capacity and rate of adsorption, respectively.

The plot of C_e/Q_e vs. C_e, is a straight line with slope of 1/qm and intercept of $1/Q_{max}K_L$. The characteristics of Langmuir isotherm is defined by a dimensionless constant called separation factor or equilibrium parameter, R_L:

$$R_L=1/1+K_LC_o$$

The parameter R_L indicates the shape of isotherm. Lower R_L value reflects that adsorption is more favorable. R_L value indicates the adsorption nature to be either unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$). Adsorption is irreversible when $R_L = 0$ (Foo *et al*., 2009).

4. Materials and methods

4.1 Reagents and chemicals

All the chemicals and reagents used for microbiological and chemical determinations were purchased from Sigma. Standard media components were purchased from Sigma Aldrich (USA) and Hi-media (Mumbai, India). Media solutions were sterilized by autoclaving at 121 ^{0}C and 15 psi for 15 min and were allowed to cool below 50 ^{0}C before use. Nutrient broth (Annexure 2) was used for growth of microorganisms. For screening of biopolymer producing bacteria FIB medium (flocculants isolation broth) (Ghosh *et al*., 2009) was used (Annexure 2).

4.2 Collection of sludge and soil samples from different sites

For isolation of biopolymer producing microorganisms, sludge and soil samples were collected from different sites (Table 2) of Patiala. The samples were collected in sterile plastic bags (Hi-Media, Mumbai), transported to the laboratory on ice and analyzed 6-8 hrs within receipt.

Table 2. Sample collection sites and their sources.

NAME OF THE SITE	SOURCE
Federal Mogul Goetze (India) Ltd	Fresh soil sludge
	Fresh soil sludge STP
	Dry soil
	Wet soil after sprinkling
Domestic sewage	River 1 soil
	River 2 soil

4.3 Isolation and screening of biopolymer producing bacteria

Biopolymer producing bacteria were isolated from sludge samples collected from different sites of Patiala city. Isolates were obtained by plating on Nutrient Broth (NB). Briefly, one gram of sample was thoroughly mixed in 50 ml of distilled water and kept for 5 min. After 5 min 1 ml was withdrawn from upper layer and mixed in 9 ml of saline in a test tube and marked the test tube as 10^0 dilution. Similarly dilutions were made up to 10^{-5}. Hundred microliters from dilutions 10^{-3}, 10^{-4} and 10^{-5} was spread evenly on nutrient agar (NA) plates. The plates were incubated at 37 ^{0}C for 24 to 48 hrs. Mucoid colonies were selected and isolated on NA plates using steak plate methods. A total number of 71 isolates were obtained. Further selection was done on the basis of their flocculating activities using Kaolin assay (Kurane *et al*., 1997). Isolates with high flocculating activity were selected further screened for their ability to bind the phytoestrogens and those showing highest binding phytoestrogens were selected for further studies.

4.3.1 Measurement of flocculating activity

Kaolin assay (Kurane *et al.,* 1997*)*

The flocculating activity of the cultures was determined by the methods described by (Kurane *et al.,* 1997). Kaolin clay was used as suspension material for estimating the flocculation activity. A volume of 0.1 ml culture supernatant (cell free extract) was added to 4.8 ml of 0.5 % suspension with 0.1 ml $CaCl_2$ in a test tube. The solution was thoroughly vortexed for 30 sec and allowed to stand at room temperature for 5 min. Two milliliter of aliquot was withdrawn from upper phase and absorbance was recorded at 550 nm with a spectrophotometer taking distilled water as reference. The flocculation activity was calculated in percentage.

Flocculant activity (%) = (B-A) *100 / B

Where, A-Optical density of sample at 550 nm

B-Optical density of reference at 550 nm

4.3.2 Screening of isolates with phytoestrogen removal ability

Standard concentration of all the biopolymers (1 $mgml^{-1}$) extracted from the screened isolates and phytoestrogen (1 μgml^{-1}) was prepared and mixed. To facilitate maximum adsorption the mixture was vortexed for 1 min and then shaken continuously. Then the mixture was spanned at 10,000 rpm for 1 min. The absorbance of the supernatant was measured at the absorbance maxima of phytoestrogens at the intervals of 0 min and 30 min for determining the binding abilities of the biopolymers. Isolates showing maximum reduction in phytoestrogen level were selected for further studies.

4.4 Identification and Characterization of isolates

4.4.1 Morphological and biochemical characterization

All the isolates were morphologically and biochemically as described in the Bergey's manual of systematic Bacteriology (Buchanan and Gibbons, 1974). According to this bacteria were arranged in groups mainly based on Gram-stain, morphology and oxygen requirement. Biochemical analysis of the isolates was performed as described below.

(a) Hydrogen Sulfide Test

This test determines whether the microbe reduces sulfur-containing compounds to sulfides during the process of metabolism. If sulfide is produced, it combines with iron compounds to produce FeS, a black precipitate. SIM agar (Annexure 2) tubes were inoculated with 24-48 hrs grown cultures using stab inoculation and incubated at 37 ^{o}C

for 24 hrs. After incubation strains that produced black coloration along the line of stab were positive indicators of this test. Uninoculated tube was taken as negative control.

(b) Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. An increase in pH due to the production of ammonia results in a color change from yellow (pH 6.8) to bright pink (pH 8.2). Urea broth (Annexure 2) was inoculated with 24 hrs grown cultures by means of loop inoculation and incubated the tubes at 37 ºC for 24 hrs. After incubation on the addition of phenol red (Annexure 2), strain that produces pink color indicates positive result and no change in the color of the broth was negative indication of the test. Uninoculated tube was taken as negative control.

(c) Methyl Red Test

The Methyl-Red test tests the ability of microorganisms to perform mixed-acid fermentation. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, which results in decrease in pH. Indicator Methyl Red is red at pH below 4.4 and yellow at pH above 6.0.When the culture medium turns red (pH below 4.4) after addition of methyl red, the culture gives positive result for the MR test. MR-VP (Annexure 2) broth was inoculated with 24 hrs grown cultures and incubated at 37 ºC for 24-48 hrs. After incubation few drops of methyl red indicator (Annexure 2) was added to the culture. Red color indicates the MR positive strain. Yellow color indicates negative results. Uninoculated tube was taken as negative control.

(d) Voges-Proskauer Test

VP tests are used to determine what the end products are when the test organism degrades glucose that is present in the MR-VP broth. Using sterile technique, MR-VP broth was

inoculated with 24 hrs grown cultures and incubated at 37ºC for 24-48 hrs. After incubation, few drops of Barritt's reagent A (Annexure 2) was added, with continuous shaking for 10 min. Then added few drops of Barritt's reagent B (Annexure 2). Development of a deep rose color in the culture is indicative of the presence of acetylmethylcarbinol and represents a positive result. The absence of rose coloration is a negative result. Uninoculated tube was taken as negative control.

(e) Citrate Utilization Test

This test determines whether a bacterium can grow utilizing citrate as its sole carbon and energy source. Using sterile technique, cultures were inoculated by streak inoculation on the Simmon Citrate agar (Annexure 2) slants and incubated at 37 ºC for 24-48 hrs. Citrate positive cultures were identified by the presence of blue colored growth on the surface of the slant. No change in color indicates negative result. Uninoculated tube was taken as negative control.

(f) Indole Production Test

This test determines whether the microbe produces indole from the amino acid tryptophan. If indole is produced, it will react with a chemical reagent added after incubation to produce a color change. Kovac's reagent that is added reacts with indole to produce a ring that is cherry red in color. SIM agar tubes were inoculated with 24-48 hrs grown cultures with stab inoculation and incubated at 37 ºC for 24 hrs. Cultures producing a red layer on the addition of Kovac's reagent (Annexure 2) are indole positive. Negative result is indicated by absence of cherry red colored ring. Uninoculated tube was taken as negative control.

(g) Starch Hydrolysis Test

The purpose of this test is to see if the microbe can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy for growth which is accomplished by the enzyme α-amylase. Iodine reagent complexes with starch to form a blue-black color in the culture medium. Clear halos surrounding colonies indicates the ability of microbes to digest the starch in the medium due to the presence of α-amylase. The cultures were streaked on starch agar plates (Annexure 2) and the plates were incubated at 37ºC for 24-48 hrs. After incubation, the plates were flooded with Gram's iodine solution for 30 sec and then the excess of iodine was decanted. Appearance of clear zone surrounding the colonies indicates starch hydrolyzing ability of isolates. No clear zone indicates no hydrolysis.

4.4.2 Molecular identification of isolates

All the molecular biology techniques were performed as outlined in Sambrook *et al*., (1998) unless noted. DNA modifying enzymes were obtained from Promega (Fisher Scientific, USA). PCR reactions were conducted using universal primers P0 and P6. The PCR mixture (100 µl) contained 1 µl *Taq* (10 X) commercial buffer, 5 µl purified DNA (50-100 ng), 150 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase (Sambrook *et al*., 1998). The program for 16S rDNA was as follows: The reaction mixtures were first incubated for 5 min at 95 ^{0}C and then cycled for 36 cycles according to the following temperature profiles: 1 min at 94 ^{0}C, 1 min at 55 ^{0}C, 2 min at 72 ^{0}C, followed by final extension for 10 min at 72 ^{0}C, unless otherwise specified, on a Bio Rad thermal cycler. The resultant amplicon was sent for the sequencing.

4.5 Growth profile of selected strains

To study the growth profile of the selected isolates with their biopolymer production, they were grown to mid log phase and then 1 % of the adjusted inoculum (O.D~0.3) was added to 100ml of FIB media and aseptically kept in incubator shaker at 37 ^{0}C, with shaking (120 rpm) for 60 hours. After every 4 hrs, aliquots (3 ml) were withdrawn and centrifuged to acquire the pellet. Then the pellet was washed with saline and resuspended in saline. Then the absorbance of the cells was measured at 600 nm with reference to media blank (FIB).

4.6 Extraction and purification of biopolymer

The selected isolates FM17 and DS1 were grown for 48 hrs at 37 ^{0}C and 120 rpm in FIB medium. After 48 hrs cells were removed from culture medium by centrifugation at 12,000 rpm for 10 min at 4 ^{0}C. The biopolymer was separated from the supernatant by adding double volume of absolute ethanol and precipitation at 4 ^{0}C for 24 hrs. The precipitated biopolymer was collected by centrifugation at 12,000 rpm for 10 min. The pellet was washed with deionized water and resuspended in 5-7 ml of deionized water. The crude biopolymer was purified by adding 10 % CPC (Annexure 2) drop wise with stirring till precipitates were observed. The precipitated biopolymer was collected by centrifugation at 10,000 rpm for 20 min and redissolved in equal volume of 10 % NaCl (Annexure 2). The precipitated biopolymer was recovered by adding double volume of absolute ethanol. The extracted biopolymer was dissolved in deionized water, dialyzed against deionized water, and lyophilized.

4.7 Optimization studies for maximum reduction of phytoestrogen

4.7.1 Optimization of biopolymer concentration

To study the effective biopolymer concentration, experiments were carried out using different concentrations of biopolymer i.e. 0.25, 0.5, 1, 1.5 and 2 $mgml^{-1}$.Different concentrations were prepared and added to standard phytoestrogen solution (1 μgml^{-1}) and vortexed for 1min then shaken continuously for maximum adsorption. The mixture was then spanned at 10,000 rpm for 1 min at room temperature. Absorbance of the supernatant was measured at the absorbance maxima of the respective phytoestrogens. To analyze the efficiency of the biopolymer concentration, the reduction in initial and final absorbance of the phytoestrogen was calculated.

4.7.2 Optimization of contact time between biopolymers and phytoestrogens

This experiment was conducted to analyze adsorption time of the biopolymers and phytoestrogens. The biopolymers and phytoestrogen (standard solution i.e. 1 μgml^{-1}) were mixed, vortexed for 1 min and then shaken manually for some time. At the interval of 15, 30, 45 and 60 min, 1 ml sample was withdrawn and spun down at 10,000 rpm for 1min at room temperature. Absorbance was taken at the maxima of the respective phytoestrogens. The absorbance maxima of different phytoestrogens are given below in Table 3 (Zdunczyk *et al*., 2004).

Table 3. Wavelength maxima of phytoestrogens.

Phytoestrogen	Λ_{max}(nm)(methanol)
Biochanin A	236,262
Formononetin	248,299
Coumestrol	214,242
Genistein	260,330

4.9 Adsorption capacity

To evaluate the adsorption capacity of the biopolymers, Langmuir adsorption isotherm was employed. An adsorption isotherm is a curve describing the phenomenon prevailing the retention (or release) of a substance from the aqueous porous media to a solid-phase at a constant temperature and pH (Limousin *et al*., 2009).Adsorption equilibrium (the ratio between the adsorbed amount with the remaining in the solution) is established when an adsorbate has been contacted with the adsorbent for sufficient time (Kumar *et al.*, 2007*)*. Langmuir isotherm model was assumed for studying the adsorption capacities of the polymers. Langmuir is a two parameter isotherm. It was originally developed to describe gas–solid-phase adsorption onto activated carbon but now it is been used to quantify the performance of different bio-sorbents (Langmuir *et al*., 1965). This model assumes that the adsorbed layer is one molecule in thickness (monolayer adsorption), and adsorption can only occur at a finite (fixed) number of definite localized sites (Vijayaraghavan *et al*., 2006)

Adsorption capacity of the biopolymer at equilibrium (Q_e, $mgml^{-1}$) was calculated by using the following equation:

$$Q_e = (C_oV_o-C_eV_e)/m$$

Where, C_o and C_e are initial and final sample absorbance (O.D) respectively, V_o and V_e is the initial and final volume of the sample solution and m is the weight of adsorbent added.

4.10 Adsorption isotherms

The linear form of Langmuir isotherm equation is given as

$$Q_e = Q_{max}K_L\ C_e/1+K_L\ C_e$$

Where, C_e ($mgml^{-1}$) is the equilibrium concentration of the adsorbate, Q_e ($mgml^{-1}$) is the amount of adsorbate per unit mass of adsorbent, Q_{max} and K_L are Langmuir constants related to maximum adsorption capacity and rate of adsorption, respectively.

The plot of C_e/Q_e vs. C_e, is a straight line with slope of $1/Q_{max}$ and intercept of $1/Q_{max}K_L$. The characteristics of Langmuir isotherm is defined by a dimensionless constant called separation factor or equilibrium parameter, R_L:

$$R_L=1/1+K_LC_o$$

The parameter R_L indicates the shape of isotherm. Lower R_L value reflects that adsorption is more favorable. R_L value indicates the adsorption nature to be either unfavorable ($R_L> 1$), linear ($R_L = 1$), favorable ($0 < R_L< 1$). Adsorption is irreversible when $R_L = 0$ (Weber *et al*., 1974).

4.11 Characterization of biopolymers

To determine the nature of the biopolymers, the crude polymer was dissolved in deionized water and treated with a solution of cationic salt cetylpyridium chloride (CPC). It is a cationic salt and forms precipitate with anionic substances.

4.11.1 Alcian blue assay (Bober, 2005)

Alcian blue was performed to analyze the acidic or basic nature of the biopolymers. Alcian blue is a basic dye and combines with compounds that are basic in nature. For this 100 µl of cell free extract was added to 700 µl of 0.5 M acetic acid (Annexure 2). Following the addition of acetic acid 200 µl of alcian blue dye was added and allowed the solution to incubate for 2 hrs at room temperature. After incubation excess dye was removed by centrifuging at 8000 rpm for 5 min at 4 ^{0}C. The absorbance of the supernatant was taken at 580 nm, taking distilled water as reference.

4.11.2 Sudan black assay (Phanse *et al*., 2011)

Sudan black B is a lipophilic and slightly basic dye and combines with acidic groups in compound lipids (Phanse *et al*., 2011). Sudan black assay of the isolates was performed to examine whether they produce less lipids or more. The isolates producing high amount of lipids showed intense bluish black coloration while the intensity of color decreased with the amount of lipid. The isolates were grown as single colonies on plates containing nutrient broth and were allowed to grow for 24-48 hrs at 37 ^{0}C. After incubation approximately 10 ml of 0.02 % Sudan black B (Annexure 2) solution was applied to the plates and allowed to remain undisturbed for approximately 10 min. The dye was decanted and the plates were rinsed with 10 ml of 100 % ethanol. Colonies unable to incorporate the Sudan Black B stain appeared white, while colonies able to incorporate the stain appeared bluish black.

4.11.3 Determination of total Protein (Lowry *et al.*, 1951)

Bovine serum albumin (BSA) was used as standard in different concentrations (0.1 - 0.5 mgml^{-1}) and biopolymer concentration was taken as 1 mgml^{-1}for estimation of protein content. Reagent A (50 mL) (Annexure 2) and Reagent B (50 ml) (Annexure 2) were mixed to make the reagents (A and B), 1 ml of freshly mixed complex-forming reagent was added to 0.2 ml of the sample and standard BSA solution. The solution was left undisturbed for 10 min at room temperature. The absorbance was taken at 750 nm. The amount of total protein present in the sample was calculated from the standard curve prepared by using pure BSA as standard. Standard curve of BSA is appended in Annexure 1.

4.11.5 Determination of total Sugars (Dubois *et al.*, 1986)

Two hundred microliters of phenol reagent (Annexure 2) was added to standards as well as samples. After the addition of phenol reagent 1.0 ml of concentrated sulphuric acid was rapidly added to the surface of the solution without touching the sides of the test tube. The tubes were left undisturbed for 10 min at room temperature. After incubation the tubes were shaken vigorously and absorbance was taken after 30 min at 490 nm. The total sugar content present in the sample was calculated from standard curve (Glucose, 0-1 $mgml^{-1}$). Standard curve of glucose is appended in Annexure 1.

5. Results and discussion

5.1 Isolation and screening of biopolymer producing isolates

The isolation of biopolymer producing bacteria was carried out from activated sludge samples collected from 15 industrial sites from various regions in and around Patiala. The activated sludge and industrial effluents are the main source of biopolymer producing microorganisms (Zaki *et al*., 2011). Out of 200 isolates, 71 isolates were selected on the basis of mucoid or slimy appearance of the colonies. An initial screening procedure, based on the ability of the culture to sediment kaolin suspension (Kurane *et al*., 1986) was applied to select isolates with high flocculating activities. This resulted in 23 isolates with high flocculating activity (Figure 3). It was observed that the flocculating activity of all the 23 isolates was mainly dispersed in the cell free supernatant; this indicates that the biopolymer produced is extracellular in nature. Among these 23 isolates, 5 isolates with maximum flocculating activity were further screened for their ability to maximally bind phytoestrogen. The secondary screening involved incubating a mixture of selected biopolymers with a known concentration of phytoestrogens for a particular time interval and then calculating the difference between initial and final absorbance of the solution (Figure 5). At the end of screening procedures two isolates showed maximum binding with phytoestrogens and were selected for further studies. These isolates were sourced from activated sludge sample of industrial unit named as Federal Mogul Goetze (India) Ltd and domestic sludge from Patiala, so were named FM17 and DS2 respectively.

5.2 Identification and Characterization of strains FM17 and DS1

5.2.1 Morphological and biochemical identification

Morphological identification of the isolates FM17 and DS1 were determined according to

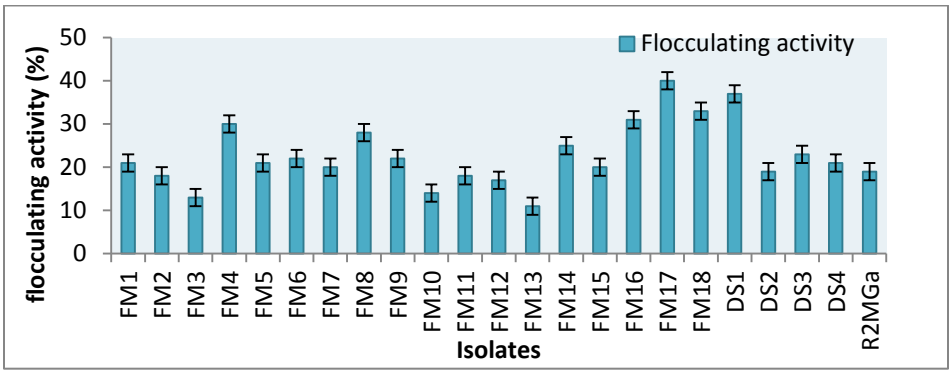


Figure 3. Flocculating activity of 23 isolates. The error bars represents ± SD of absorbance values of three replicates.

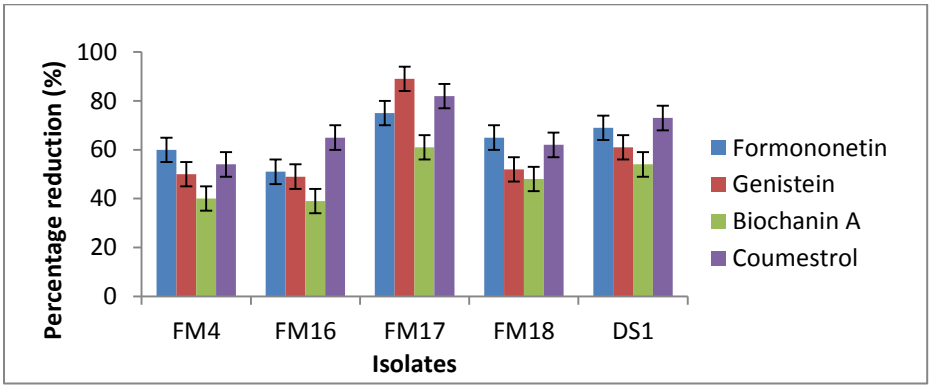


Figure 4. Percentage reduction of phytoestrogens by different biopolymers. The error bars represents ± SD of three replicates.

the identification keys described in Bergey's manual of systematic Bacteriology (Buchanan and Gibbons, 1974). According to this, the isolates were arranged in groups mainly based on Gram-staining, morphology and oxygen requirement. Morphologically, the bacterial colonies were mucoid, gram negative and motile. Biochemical tests were performed to determine physiological characteristics of microorganism, particularly in terms of bacterial enzymes. Bacteria utilize various substrates (amino acids, starch, citrate, and gelatin) as energy source. The various physiological and biochemical properties of the isolates are summarized in Table 4. From biochemical analysis, it was observed that neither the isolates DS1 and FM17 were able to reduce sulphur containing compounds, nor they possess urease enzyme. Isolate FM17 was found to be able to

hydrolyze starch when grown on starch containing media. Both the isolates showed positive results with MR tests. This indicates that they are capable to produce stable acids. From the biochemical tests result it is estimated that isolate FM17 belongs to *Enterobacteraceae* family and isolate DS1 belongs to *Pseudomonadaceae* family. Results of molecular identification of isolates are awaited.

Table 4. Morphological and biochemical identification of isolates DS1 and FM17.

Morphological identification	**Isolates**	
	FM17	**DS1**
Colony Shape	Circular	Circular
Color	White	Creamish white
Margin	Smooth	Smooth
Elevation	Smooth	Elevated
Gram's reaction	Negative	Negative
Biochemical identification		
MR test	+	+
VP test	-	-
Indole production test	-	+
Hydrogen sulphide test	-	+
Citrate utilization test	-	-
Starch hydrolysis test	+	-
Urease test	-	-

5.3 Growth profile studies of isolates

There is dependence between biopolymer production and the stage of the microbial growth cycle (Pindar *et al*., 1975). Growth pattern of the two isolates was studied by growing them in FIB medium and then suspending the cell in saline after every 4 hours then taking the absorbance (O.D) at 600 nm. The lag phase continued till 4 hrs and 8 hrs for isolate DS1 and FM17 respectively. The phase of growth which is crucial for the production of biopolymers i.e. log/stationary phase was observed to start from 28 hrs and

continued till 52 hrs for DS1 and for FM17, it is from 20 hrs and continued till 32 hrs. The plots of growth kinetics for the two isolates DS1 and for FM17 are shown in Figure 5. The biopolymer was extracted at 48 hrs of growth i.e. stationary phase of isolates which shows that biopolymer production is a characteristic of stationary phase. The specific growth rates of isolates DS1 and FM17 were 0.17 and 0.11 hr^{-1} respectively.

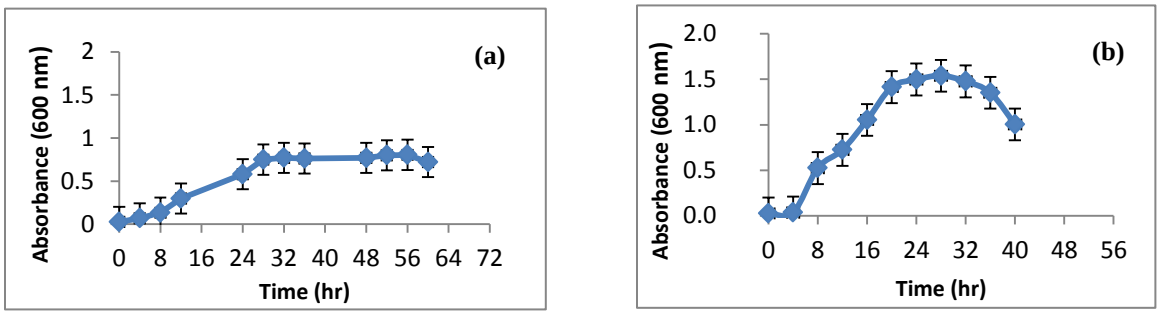


Figure 5. Growth profile of isolates (a) DS1 (b) FM17. The error bars represents ± SD of absorbance of three replicates.

5.5 Optimization studies for maximum reduction of phytoestrogen

5.5.1 Optimization of adsorbent (biopolymer) concentration

Optimization of biopolymer concentration is the most crucial step in determining the optimum conditions for the adsorption process. This study is very important in reduction of phytoestrogen levels upto maximum level. The purpose of optimization studies is to determine the most effective concentration of the adsorbent i.e. biopolymers that will effectively bind the phytoestrogens and further increase in the adsorbate concentration has no effect in the binding of the adsorbate (biopolymer) and adsorbent (phytoestrogens). The purified biopolymers were used as adsorbent for the adsorption of phytoestrogens. Experiments were conducted to determine the optimum concentration of the adsorbent i.e. biopolymers that would be used in further studies. Concentration range from 0.25-2 $mgml^{-1}$ was taken for both biopolymers, FM17 and DS1 and binding was allowed to take place for 60 min at room temperature. Figure 6 and 7 depicts the effect of adsorbent concentration on the reduction of phytoestrogen.

A significant increase in reduction of phytoestrogens was observed with the increasing concentration of biopolymer. Concentration of biopolymers optimum for the reduction of phytoestrogens was different for different phytoestrogens. Further optimum concentration of biopolymer FM17 and DS1 was same for all the isoflavones (Formononetin, Genistein and Biochanin A) i.e. 1.5 $mgml^{-1}$ and 1 $mgml^{-1}$ respectively. For coumestans i.e. coumestrol the optimum concentration was found to be 1.5 $mgml^{-1}$. This differing pattern in optimum concentrations effective for phytoestrogen removal is due to the difference in the basic structure of phytoestrogens. Also as amount of adsorbent (biopolymer) was increased i.e. from 0.25 $mgml^{-1}$ to 1 $mgml^{-1}$, number of active sites available for adsorption of phytoestrogens also increased thus percentage removal of phytoestrogens also increased. But, all active sites may not be available during adsorption due to overlapping between the active sites themselves and this is because the biopolymer is in folded form. Thus amount adsorbed of phytoestrogens decreased with further increase in biopolymer concentration.

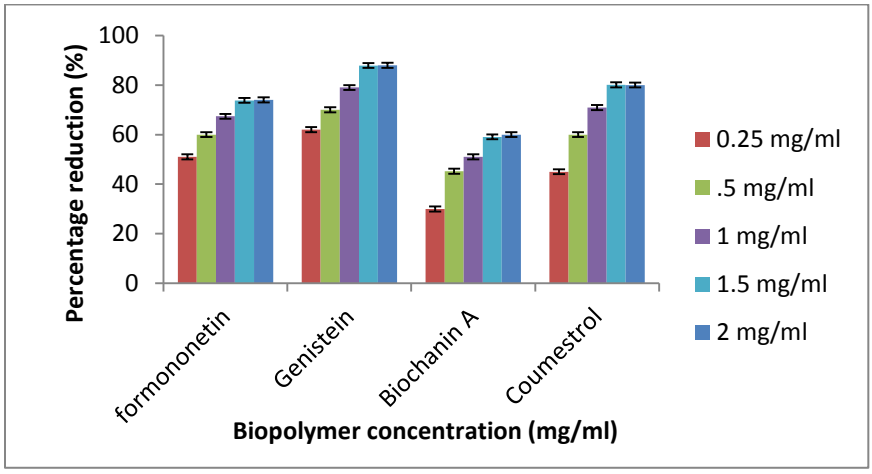


Figure 6. Effect of biopolymer (FM17) concentration on phytoestrogen removal. The error bars represents ± SD of three replicates.

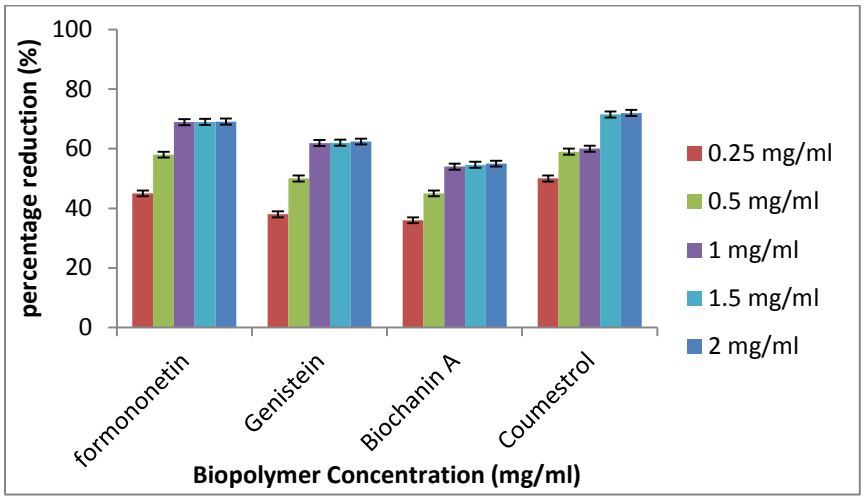


Figure 7. Effect of biopolymer (DS1) concentration on phytoestrogen removal. The error bars represents ± SD of three replicates.

Thus, the adsorption of phytoestrogens increased with the adsorbent concentration and reached an equilibrium value after certain adsorbent concentration (Patil *et al*., 2012).

5.5.2 Effect of contact time on biopolymers and phytoestrogens binding

Contact time also plays an important role in adsorption process of phytoestrogens. Adsorption requires proper time of contact between the adsorbent and adsorbate. Vortexing was done followed by shaking to increase the effectiveness of the process. Adsorption of phytoestrogens was rapid in first 15 to 30 minutes and after 30 minutes amount of phytoestrogens adsorbed was almost constant. Nearly 10 to 50 % of total phytoestrogen reduction appears to have been adsorbed in first 30 minutes of duration and at equilibrium it reached up to 75 to 80 % depending upon the adsorption ability of different biopolymers. The rapid adsorption at initial stage of adsorption was because of more number of active sites on the surface of adsorbent (Patil *et al*., 2012*)*. Effect of contact time on adsorption of phytoestrogens on biopolymers FM17 and DS1 is presented in Figure 8 (a-d) and 9 (a-d) respectively.

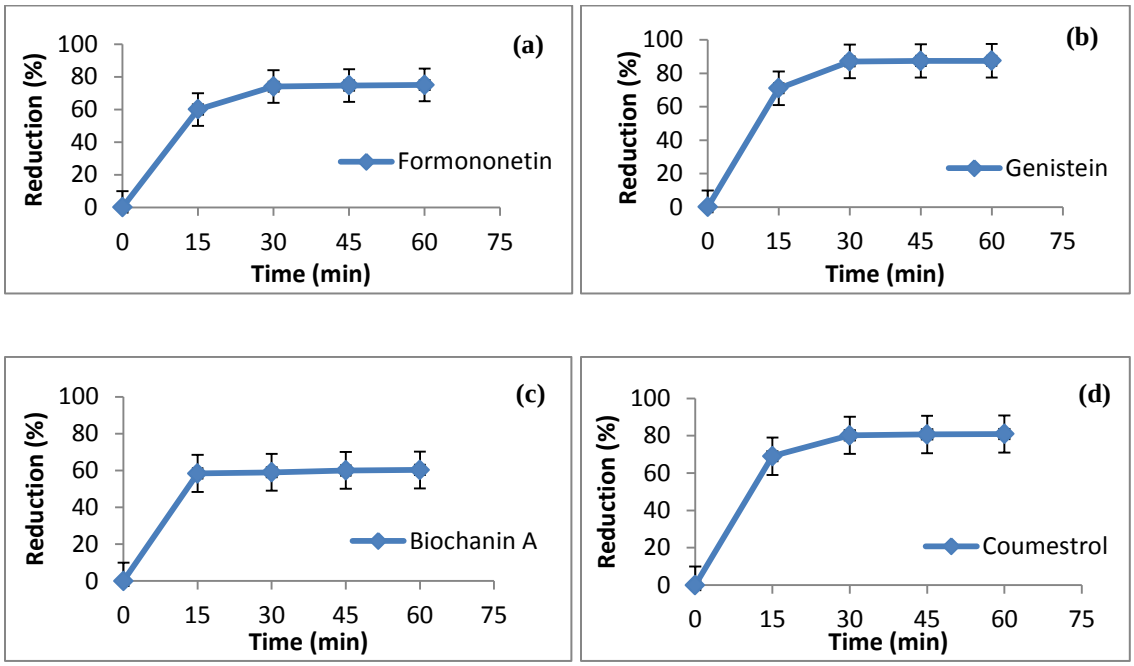


Figure 8. Effect of biopolymer contact time on removal of phytoestrogen (a) Formononetin (b) Genistein (c) Biochanin A (d) Coumestrol. The error bars represents ± SD of three replicates.

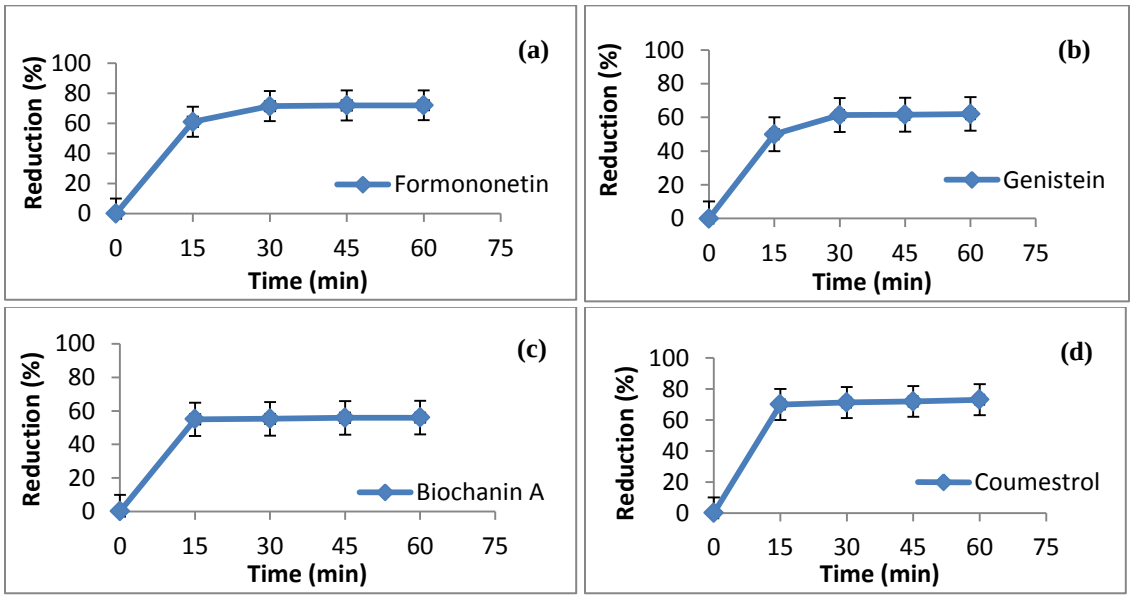


Figure 9. Effect of treatment time on adsorption by biopolymer DS1 on phytoestrogen (a) Formononetin (b) Genistein (c) Biochanin A (d) Coumestrol. The error bars represents ± SD of three replicates.

For both the biopolymers, the binding of phytoestrogens remained unchanged after 30 min. The reduction percentage of genistein was maximum with FM17 (87%) while percentage reduction of formononetin was maximum with biopolymer DS1 (72%).

5.6 Adsorption capacity

5.6.1 Langmuir isotherm model

To evaluate the adsorption capacity of the biopolymers Langmuir isotherm was used. This model assumes that the adsorbed layer is one molecule in thickness (monolayer adsorption), and adsorption can only occur at a finite (fixed) number of definite localized sites (Foo *et al*., 2009).According to Langmuir model, adsorption capacity of the biopolymer at equilibrium (Q_e, mgg^{-1}) was calculated by using the following equation:

$$Q_e = (C_oV_o-C_eV_e)/m$$

Where, C_o and C_e are initial and final sample absorbance before and after treatment respectively, V_o and V_e are the initial and final volume of the sample solution and m is the mass of biopolymer added. The plot of C_e/Q_e vs. C_e, is a straight line with slope of $1/Q_{max}$ and intercept of $1/Q_{max}K_L$. Monolayer (maximum) adsorption capacities (Q_{max}) obtained from Langmuir plots gives the idea of effectiveness of adsorbents towards the adsorbate.

The characteristics of Langmuir isotherm is defined by a dimensionless constant demonstrated as separation factor or equilibrium parameter, R_L:

$$R_L=1/1+K_LC_o$$

Where K_L (lmg^{-1}) refers to the Langmuir constant and C_o is the adsorbate initial concentration (mgl^{-1}). Lower R_L value reflects that adsorption is more favorable. R_L value indicates the adsorption nature to be either unfavorable ($R_L> 1$), linear ($R_L = 1$), favorable ($0 < R_L< 1$). Adsorption is irreversible when $R_L = 0$ (Foo *et al*., 2009)

The Langmuir adsorption isotherm for biopolymer FM17 with isoflavons (Formononetin, Genistein and Biochanin A) at equilibrium concentration of 1 $mgml^{-1}$ and for coumestans

(Coumestrol) at concentration 1.5 $mgml^{-1}$ was formed and is shown in Figure 10 (a-d). For biopolymer DS1 with isoflavons (Formononetin, Genistein and Biochanin A) at equilibrium concentration of 1.5 $mgml^{-1}$ and for coumestans (Coumestrol) at concentration 1.5 $mgml^{-1}$ is shown in Figure 11 (a-d).

Among the isoflavons i.e. Formononetin, Genistein, and Biochanin A the Q_{max} value of biopolymer FM17 and DS1 at the saturation concentration i.e. $1mgml^{-1}$and 1.5 $mgml^{-1}$ was found to be in the following order Genistein > Formononetin > Biochanin A and Formononetin > Genistein > Biochanin A respectively. As K_L is related to the energy of the adsorption process. From the Table 5 and 6 a trend was generated based on adsorption energy of the phytoestrogens and can be inferred as Genistein > Formononetin > Biochanin A and Formononetin > Genistein > Biochanin A for biopolymer FM17 and DS1 respectively. For coumestrol, at saturation concentration of 1.5 $mgml^{-1}$ of biopolymer DS1 is more effective as compared to biopolymer FM17.

The Langmuir plot of C_e/Q_e against C_e exhibited good linearity (R^2 = 0.95 to 0.99) indicating the adsorption of phytoestrogens obeys the Langmuir adsorption isotherm (Figure 10 and 11). The values of K_L and Q_{max} of biopolymers FM17 and DS1are given in the Table 5 and 6. It was found that the biopolymer which has greater adsorbent capacity Q_{max} has higher value of K_L and vice versa. Higher values of K_L represent an effected adsorption of phytoestrogens (Patil *et al*., 2009). Correlation coefficient values (R^2) showed good linearity to all biopolymers and phytoestrogens system. Thus, Langmuir adsorption isotherm fit well to these adsorption studies.

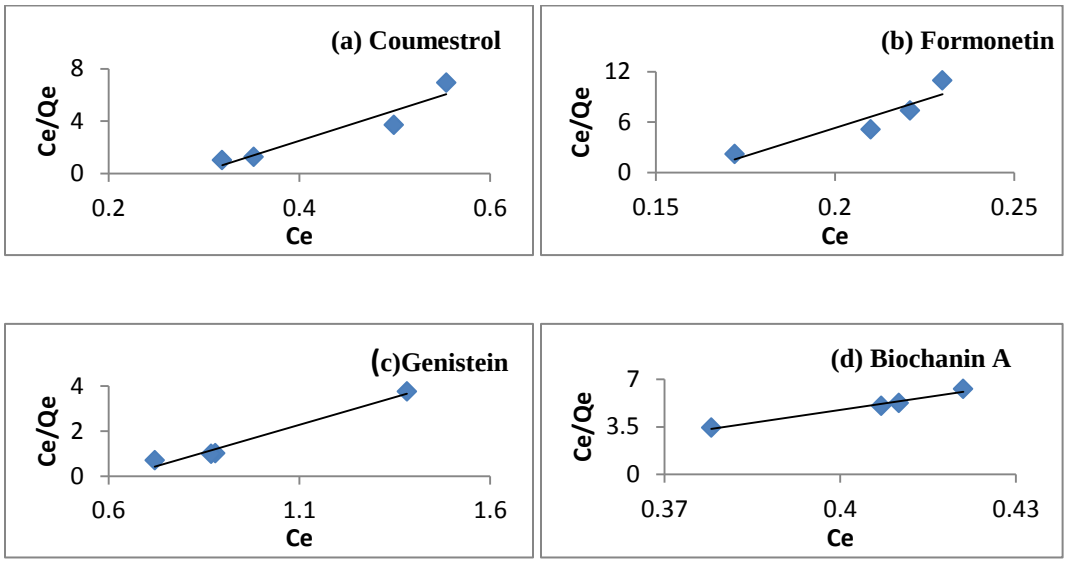


Figure 10. Langmuir adsorption isotherm on biopolymer FM17concentration 1.5 $mgml^{-1}$ for (a) Coumestrol, and biopolymer concentration 1mg/ml for (b) Formononetin (c) Genistein (d) Biochanin A. The error bars represent the mean of the values obtained from three replicates.

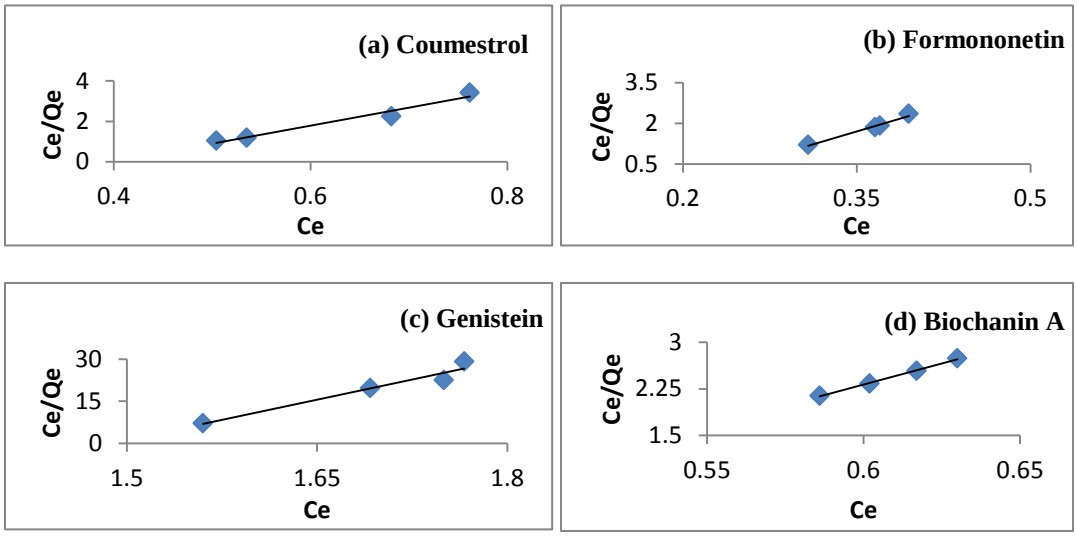


Figure 11. Langmuir adsorption isotherms on biopolymer DS1concentration 1.5 $mgml^{-1}$ for (a) Coumestrol, (b) Formononetin (c) Genestein (d) Biochanin A. The graph represents mean of the values obtained from three replicates.

5.7 Characterization of biopolymer FM17 and DS1

The biopolymers FM17 and DS1 formed precipitates when treated with a solution of cationic salt of CPC. This indicated that the biopolymers are anionic in nature. Further

Table 5. Isothermal parameters of phytoestrogen treated with biopolymer FM17.

Phytoestrogen	**Concentration**	**Q_{max}**	**K_L**	**R^2**	**R_L**
Formononetin	1 $mgml^{-1}$	0.10	0.16	0.95	0.96
Genistein		0.32	0.63	0.97	0.62
Biochanin A		0.04	0.29	0.97	0.86
Coumestrol	1.5 $mgml^{-1}$	0.14	0.32	0.91	0.84

Table 6. Isothermal parameters of phytoestrogen treated with biopolymer DS1.

Phytoestrogen	**Concentration**	**Q_{max}**	**K_L**	**R^2**	**R_L**
Formononetin	1.5 $mgml^{-1}$	4.6	5.89	0.98	0.38
Genistein		0.61	3.71	0.95	0.207
Biochanin A		0.5	2.78	0.99	0.21
Coumestrol	1.5 $mgml^{-1}$	0.52	2.81	0.96	0.16

the acidic nature of the biopolymers was depicted by the alcian blue assay (Bober, 2005). As both the biopolymers showed positive results when assayed with alcian blue dye (Table 7). The Sudan black assay (Phanse *et al*., 2011) of the isolates represented that the biopolymer that they produce contains low lipid content as indicate by the light bluish appearance of the colonies.

Further the compositional analysis of the biopolymers showed that biopolymers contained 36 and 48 $\mu gml^{-1}$of total sugars, while protein content was 120 and 70 μgml^{-1} for the strains DS1and FM17, respectively, indicating that the composition of both

biopolymers are supposed to be glycoproteins. The approximate percentages of sugars and proteins of the biopolymers are shown in table 7.

Table 7. Compositional profile of biopolymers DS1 and FM17.

Isolates	Nature of biopolymer	Acidity/basicity	Total sugars (%)	Total proteins (%)	Ratio Sugars:Proteins
DS1	Anionic	Acidic	17.6	58.6	1:3
FM17	Anionic	Acidic	26.5	38.6	1:1.4

Conclusion

The objective of this study was to identify and characterize a new biopolymer with high efficiency towards phytoestrogens. Two extracellularly produced biopolymers (FM17 and DS1) were evaluated from microbial cultures and examined for removal of phytoestrogens. Confirmation of phytoestrogen binding capabilities of the biopolymers was determined followed characterization of the biopolymers. Optimum concentration of biopolymers was found to be 1.5 $mgml^{-1}$ and 1 $mgml^{-1}$ for FM17 and DS1 respectively. In aqueous solution it was revealed that the biopolymer FM17 showed maximum reduction of genistein i.e. 87% whereas biopolymer DS1 showed maximum reduction of Formononetin i.e. 72%. Both the biopolymers showed minimum reduction of Biochanin A. The adsorption isotherms of biopolymers FM17 and DS1 indicated biopolymer DS1 to be more effective in removing coumestrol when compared with biopolymer FM17 which was in accordance with the experimental observations. Further the trend of adsorption energies i.e. K_L was found to be in order Genistein > Formononetin > Biochanin A and Formononetin > Genistein > Biochanin A for biopolymer FM17 and DS1 respectively. Results of this study provide valuable insights in further elucidating the interaction of phytoestrogens and microbial extracellular polymers.

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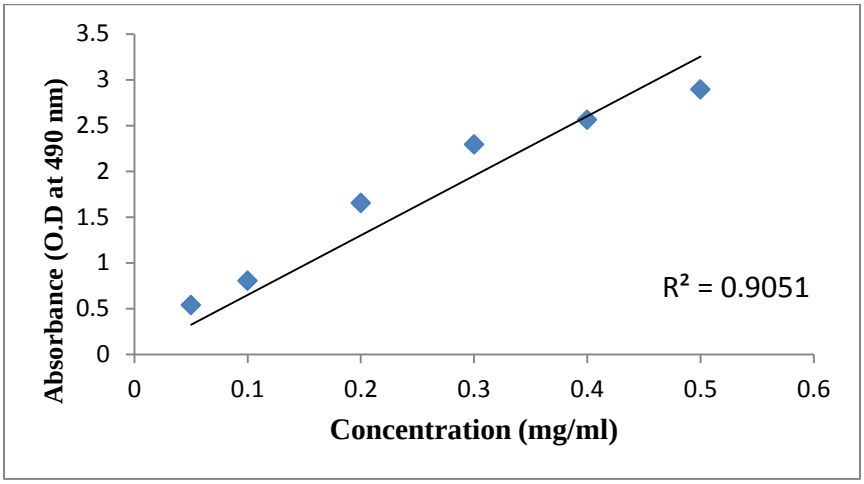
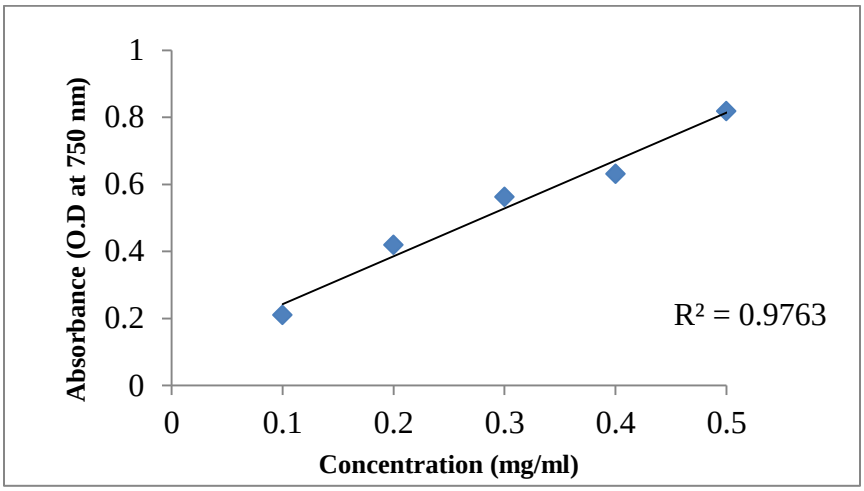
Annexure 1

1. Standard curve for glucose (Dubois *et al.*, 1986)



2. Standard curve for BSA (Lowry *et al.*, 1951)



Annexure 2

MEDIA

1. Flocculant Inducing Medium (FIB)

Composition	**gl^{-1}**
Peptone	5.0
Diammonium sulphate	2.0
$CaCl_2.2H_2O$	0.7
NaCl	0.1
$MgSO_4.7\ H_2O$	0.2
K_2HPO_4	1.0
Dextrose	1.0
Distilled water	1 lit
pH	7.0±0.2

2. Nutrient Broth Medium (NB)

Nutrient broth	13
Distilled water	1 lit
pH	7.0±0.2

3. SIM Agar

Peptone	30.0

Beef extract	3.0
Ferrous ammonium sulphate	0.2
Sodium thiosulfate	0.02
Agar	3.0
Distilled water	1 lit
pH	7.0±0.2

4. Starch Agar

Peptone	5.0
Beef extract	3.0
Starch	2.0
Agar	15.0
Distilled water	1 lit
pH	7.2±0.1

5. Trypticase soy agar (TSA)

Trypticase	15.0
Phytane	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water	1 lit

pH	7.0±0.2

REAGENTS AND CHEMICALS

1. Folin-Lowry reagent

Reagent A (Alkaline solution)

Na_2CO_3	2.0
NaOH	0.2

Reagent B

$CuSO_4$	0.25
Na-K-tartarate	0.25

2. Barritt's Reagent

Solution A

- 5.0 gm of α- napthol dissolved in 95.o ml of absolute ethanol.

Solution B

- 40.0 gm of KOH dissolved in 100 ml of distilled water.

3. Kovac's reagent

10 g of p- aminobenzaldehyde dissolved in 150 ml of isoamylalcohol and then slowly adding 50 ml of concentrated hydrochloric acid.

4. Methyl red solution

- 0.1 gm methyl red dissolved in 95 % absolute ethanol. Diluted to 500 ml with distilled water.

5. 0.5 % $CaCl_2$

- 0.5 gm $CaCl_2$ in 100 ml of distilled water.

6. 10 % CPC

- 10 gm of CPC dissolved in 100 ml of distilled water.

7. 10 % NaCl

- 10 gm NaCl dissolved in 100 ml distilled water.

8. 0.02 % Sudan Black B

- 0.02 gm of Sudan Black dissolved in 100 ml of distilled water.

9. Alcian blue solution

- 1 gm alcian blue in 100 ml of 0.5 M acetic acid.