Isolation and Characterization of β-glucans with probiotic properties

A Thesis

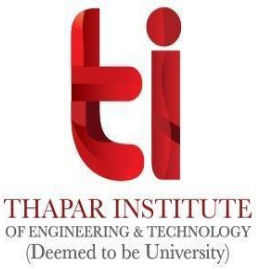
Submitted in the partial fulfilment of the requirement for

Award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY



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JULY 2019

CANDIDATE'S DECLARATION

I, the under designed, hereby declare that the research work presented in the thesis entitled **"Isolation and Characterization of β-glucans with probiotic properties"** in partial fulfillment of the requirement for the award of the degree of **Master of Technology** in Biotechnology, Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my work during the period of one year from July 2018 to July 2019,under the supervision and guidance of **Dr. Moushumi Ghosh**, Associate Professor and Head of the Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or examining body in India or abroad.

Divya Chouhan

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CERTIFICATE

This is to certify that dissertation entitled, **"Isolation and Characterization of β-glucans with probiotic properties"** submitted by Ms Divya Chouhan in partial fulfilment of the requirements for the award of Masters of Technology in Biotechnology at Thapar Institute of Engineering and Technology, Patiala is an authentic work carried out by her under my supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/institute for award of any Degree or Diploma.

Prof. Moushumi Ghosh

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ABSTRACT

The present study attempted to characterize β-glucans produced extracellularly by microorganisms. Two bacterial isolates were screened for high β-glucan producers from curd and faeces of newborn using rapid aniline blue and congo red dye binding assays. The β-glucans produced extracellularly from both these isolates at mid to late log phases of growth had yields of 31.1mg/g and 26.1mg/g dry weight respectively and optimal pH of 7.2 at a temperature of 37°C. The purified β-glucans were determined for their physicochemical attributes using HPLC, FTIR, DLS and SEM respectively. Favourable size-structure and confirmatory signatures for β-glucans could be established from these results. The isolated β-glucans demonstrated antimicrobial activities against fungal and bacterial pathogens albeit with different potencies and significant cholesterol removal in vitro. None of the β-glucans were affected functionally upon exposure to simulated gastric trials indicating their suitability for application through foods or bioactive. The producer strains were ascertained to possess beneficial properties allowing them to be classified as GRAS. Results of this study indicate an interesting possibility of further technological applications of the microbially produced β-glucans.

Keywords: β-glucan, Biopolymer, Anti-microbial activity, Fungal pathogen, Bacterial pathogen, Cholesterol

List of Abbreviations

HPLC	High performance liquid chromatography
FTIR	Fourier Transform Infrared Spectroscopy
DLS	Dynamic Light Scattering
SEM	Scanning Electron Microscopy
GRAS	Generally Recognised as Safe
LDL	Low Density Lipoprotein
EPS	Extracellular polysaccharide
C1	Curd isolate
F1	Faeces isolate
LAB	Lactic acid bacteria
PHB	Poly- β-hydroxybutyrate
PHAs	Polyhydroxyalkanoates
DEAE	Diethylaminoethyl cellulose
MRS	DeMan Rogosa and Sharpe
TGA	Thermogravimetric Analysis
EDS	Energy dispersive X-ray spectroscopy

List of symbols

ºC	Degree(s) Celsius
hr	Hour
ml	Millilitre
mg/ml	Milligram per litre
µl	Microlitre
nm	Nanometre
g/l	Gram per litre
mg/l	Milligram per litre
pH	Power of Hydrogen
mg/kg	Milligram per Kilogram
v/v	Volume per volume
ml/min	Millilitre per minute
pg/ml	Pictogram per millilitre
g/l	Gram per litre
g/ml	Gram per millilitre
cm^{-1}	Per centimetre
µg/ml	Microgram per millilitre
mm	Millimetre

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Chapter 1

Introduction

Due to unique properties of natural polysaccharides, they are used in wide range of applications that ranges from paper manufacturing to wound healing (Verma and Gu,2012).One major class of polysaccharide known as 1,3 β-Glucans which is having glucopyranose polysaccharide with 1,3 β-glycosidic linkages, moreover, they are having variety of 1,6 branches. By applying heat and humidity, 1, 3 β-Glucans has the ability to form single helical or triple helical structures which can be used for the synthesis of resilient gels. Fast growing research has been generated by the use of these polysaccharides. However, these polysaccharides are the structural agents that can provide platform for the formation of macroscopic as well as nanoscale structures (Stuyven 2010).

1,3 β-Glucans has attracted attention of researchers because of wide range of application in medicines such as immunostimulating, anti-inflammatory, antimicrobial, anti-infective, antiviral, anti-tumor , cholesterol lowering, radio protective and wound healing properties (Stone & Clarke, 1992; Bohn & BeMiller,1995; Kogan 2000; Freimund *et al*., 2003).However, It was reported that these polysaccharides are associated with many health-promoting effects. For instance, the intake of oat β-glucan of at least 3 grams per day has the ability to reduce the amount of Low Density Lipoprotein (LDL) cholesterol level and plasma total by the proportion of 5-10% in normocholesterolemic or hypercholesterolemia subjects. Moreover, glucans significantly alter the growth and development of syngenic, allogenic or autochthompus tumours in test organisms and in a diversity of human malignancies. They also have the ability to modify the function of macrophage that have strong influence on hemopoietic activity of spleen and bone marrow. In numerous studies conducted till date showed that glucans have effective functions as an adjuvant for viral, parasitic vaccines, tumour and bacterial. Broad range immunopharmacological activity of glucan coupled with the end metabolite being glucose leads to the continuous development of these polyglucose immunomodulators. That can be used for therapeutic purpose against large number of infectious and neoplastic diseases. These β-glucans

are non-digestible polysaccharides in nature that can be obtained from different sources of food and can provide many health promoting benefits as well as have potential to act as the major source of prebiotics (El Ghany *et al*., 2016).

Structure and sources:

β- Glucans are polysaccharides that are comprised of *d*-glucose monomer units which are linked through β-glycosidic bonds and these are leading components of the fungus cell wall, yeast and various types of bacterial sources (Volman *et al*., 2008). Nearly half proportion of the cell wall of fungus consists of β-glucan (Klis *et al*.,2001;McIntosh *et al*., 2005; Seviour 1992), however, there many polysaccharides in nature that are excreted into the growth media, which is further helpful for the recovery, purification and makes chemical characterization much easier and faster(Schmid *et al*., 2001; Seviour 1992). In cereals which contain β-glucans in their endosperm cell walls. Depending on their sources of their extraction, β-glucans are different in macromolecular structure as given by Volman *et al*., 2008 (Table.1).

Table 1: Different sources of β-glucan and their structures (Volman *et al*., 2008)

β-Glucan type	Structure	Description
Bacterial		Linear β1,3 glucan (i.e. Curdlan)
Fungal		Short β1,6 branched, β1,3 glucan (i.e. Schizophyllan)
Yeast		Long β1,6 branched, β1,3-glucan (i.e. WGP β-glucan, Betafectin™)
Cereal		Linear β1,3/β1,4-glucan (i.e oat, barley, rye)

Glucans are as many in numbers as their sources of their isolation which means that there are diversity of sources from where we can extract β-glucan and there are enormous methods for their purification as well as extraction (Vetvicka and Sima, 2004). The activity of glucan is impacted by their degree of branching (DB), molecular structure and size (Volman *et al*., 2008). For example, the Cell wall of baker's yeast such as *Saccharomyces cerevisiae* has many β-glucans present in it. The main chain of β-glucan consists of 1-3-linked β-D-glucopyranosyl

units that are dispersed with single β-D-glucopyranosyl units and attached with many units of 1-6 or 1-4 linkages (Figure 1.1) (Bohn & BeMiller, 1995; Leung *et al*., 2006; Zekovic *et al*., 2005). These polysaccharides has linear glucans or 6-substituted (1, 3) β-glucans which consists of branched or cyclic structures.

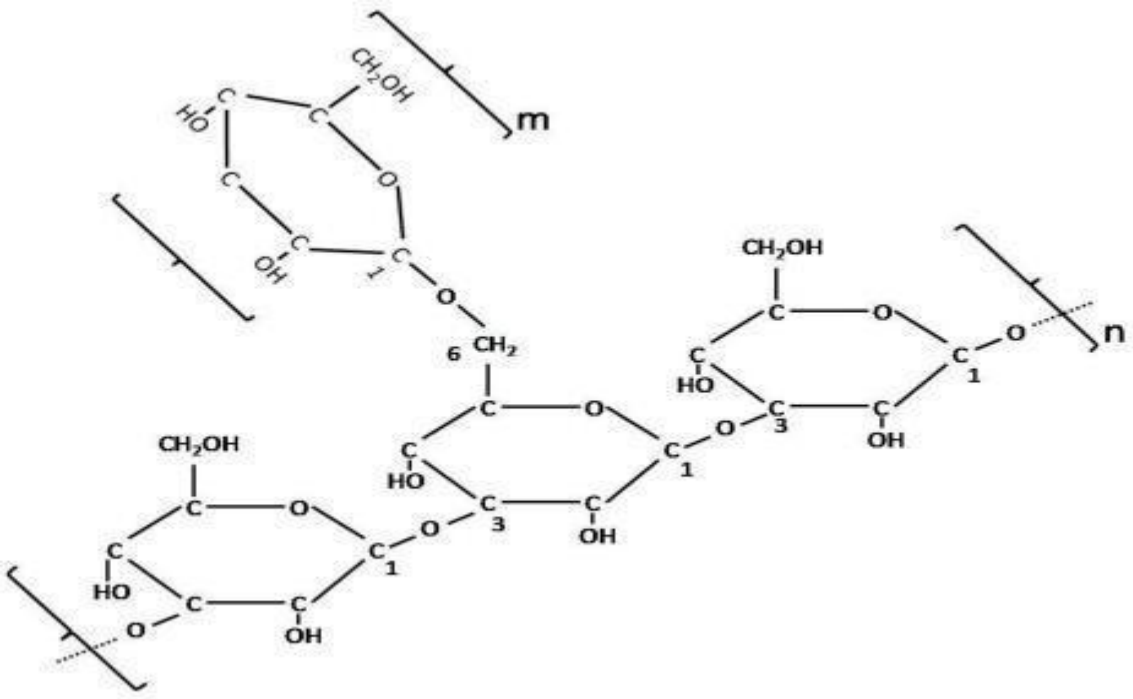

Fig.1.1 — The structure of β-1,3/1,6-glucans (Bohn & BeMiller, 1995; Leung *et al*.,2006; Zekovic *et al*., 2005)

The length as well as frequency of these branches can vary upon their source from where the β–glucan is extracted and also mainly depends on the chain length (Bohn and BeMiller, 1995; Leung *et al.*, 2006; Yoshitomi *et al.*, 2005).Consequently, β-1,3-glucans have their particular molecular conformations as they can easily converted into single helix, triple helix and random coil structures ,depicts in Fig. 1.2 (Kulicke *et al*., 1997; Falch *et al*.,2000; Norisuye 2003; Ohno *et al*.,1987; Wasser 2002; Yadomae 2000; Zhang *et al*.,2008).

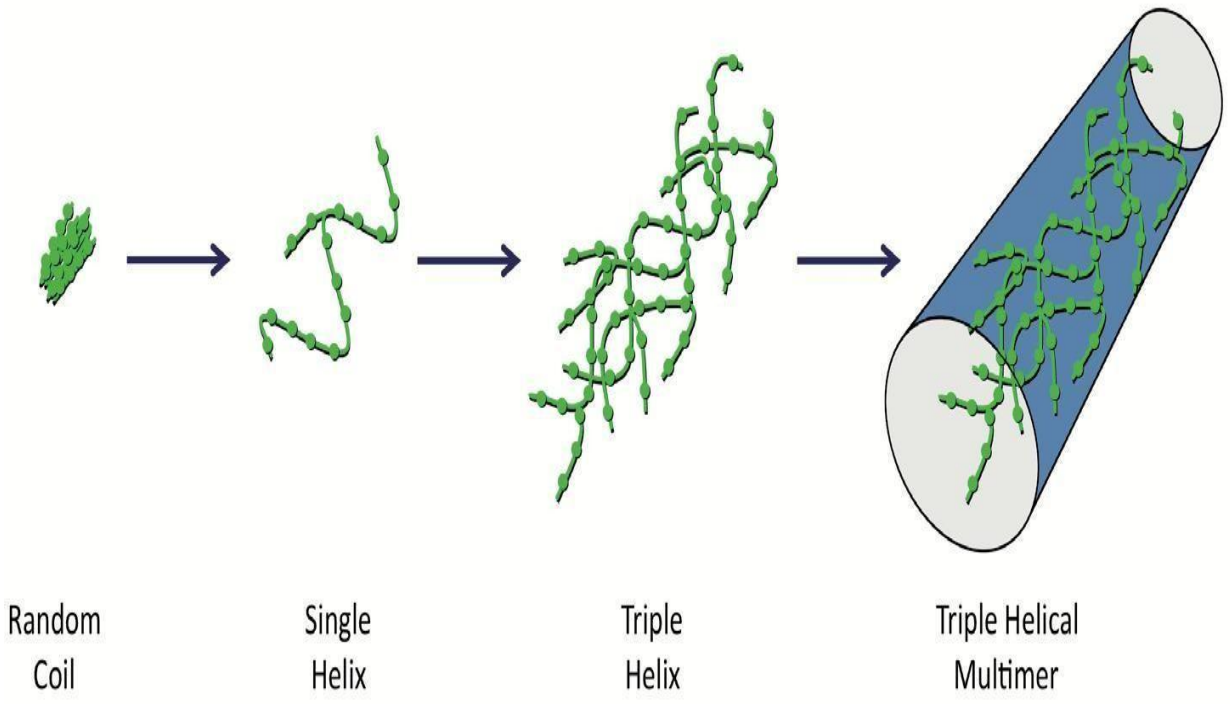

Fig. 1.2 —Random coil, single helix and triple helix structures of β-glucan (Falch *et al.*,2000).

The cell wall of fungi and yeast consist of glycopyranosyl residues (1, 3 β-linked) and it is having small number of β-(1, 6) linked branches in it (Volman *et al*., 2008). In contrast, the cell walls of barley and oats contain unbranched β-glucans along with 1,4 and 1,3 β-linked glycopyranosyl residues attached to it. Moreover, β-glucans extracted from the sources of bacteria consists of unbranched 1, 3 β- linked glycopyranosyl residues (Brown *et al*., 2003; Estrada *et al.*, 1997).

For instance, lentinan is also a type of β-glucans with high molecular weight .It was first structurally characterized as they were found as triple helical chains. However, they can be easily transformed into single random coils in aqueous NaOH solution and in water or when it is heated above 135°C temperature (Zhang *et al*., 2004; Falch *et al*., 2000; Zhang *et al*., 2005; Zhang *et al*., 2008). Many researchers revealed that high ordered structures such as triple helical structure are responsible for the immunomodulating properties of β-glucans (Falch *et al*., 2000; Hamuro *et al*., 1971; Leung *et al*., 2006; Maeda *et al*., 1988; Ohno *et al*., 1987; Yanaki *et al*., 1983; Yoshitomi *et al*., 2005).

Several forms of β-glucans(such as gel forming) has been used for various purposes such as various food additives in order to improve the physical properties like water retaining agents, thickeners, emulsifying stabilizers and fat replacers. Moreover, β-glucans extracted from barley and oat can be used as functional component of feed (Suphantharika *et al*., 2003).Some kinds of β-glucans can be used in cosmetics products. For example, film–foaming moisturizer, skin and dermatological compositions and eye drops. They can also be used as a functional food in various glutan-free bread, dairy products, yogurts and cakes. They also useful in various types of medicines such as they are used as wound dressing material, curing partial thickness burns, a bone substituting material, transparent wound dressing sheet, etc.

Generally, polysaccharides are ideal for using in drug delivery systems as they are having water soluble capacity, biocompatible, moreover, they are multifunctional (Feeney *et al*.,2009;Vinarta *et al*.,2007). For the development of polymer nanoparticles, β-glucans has not yet been used to its fullest potential**.** We can develop a nanoparticle drug delivery system. Amphiphilic copolymers self assemble to form micellar structures applicable to drug delivery due to their drug loading capability and small size, taking advantage of the enhanced permeability and retention effect.

It is clear that β-glucans have a wide amount of functionalities for human benefits. Amongst natural sources exploited thus far, microorganisms represent the current choice for β-glucans with novel applications. This is because the intrinsic properties of β-glucan offer a protective advantage to the producer living system. The present study attempts to examine bacterial isolated from the gut environment and fermented food with a view of isolating extracellularly produced biopolymers of β-glucan category. Few studies regarding this have actually been carried out and the outcomes may offer promising alternatives for human applications.

Scope of study

For over a decade now, β- glucans have proven their enormous potential due to their unique helix and gel forming capacity, other functional attributes as important molecules for health and nutritional (wellness) applications.

The present study attempted to characterize bacteria capable of producing β-glucans extracellularly and evaluate the functionality of such β-glucan molecules.

Objectives

1. Characterize β-glucan producing bacterial strains from fermented food and human samples
2. Establish the physicochemical identity of the β-glucans and optimal production conditions.
3. Examine the GRAS properties of the selected β-glucan producer bacteria
4. Evaluate the functional characteristics of β-glucans for technological applications.

Chapter 2

Review of Literature

2.1 β-glucan producing micro-organisms:

To date several microorganisms have been documented to produce extracellular polysaccharides (EPS) .These EPS can be found in capsular material or as dispersed slime in the surrounding environment which is having no association to any one particular cell. Many genera of Archaea, Bacteria, Alga and Fungi belong to mesophilic, thermophilic and halophilic groups and these are the part of EPS producing family. Lactic acid bacteria (LAB) are well-known as mesophilic group of EPS producers. Among them *Lactobacillus bulgaricus, Lactobacillus helveticus, Lactobacillus brevi, Lactococcus lactis, Leuconostoc mesenteroides,* and *Streptococcus spp*. are good EPS producers. The other potential extracellular polysaccharides producers are *Acetobacter spp*. *Acetobacter spp*. *Pseudomonas spp*. *Sinorhizobium spp*. *Escherichia spp*. *Aureobasidium spp. Aureobasidium spp.* (Singha and Kumar, 2012). Essentially, EPS producing bacteria can be examined as β-glucan producers. A lactic acid bacterium has been mostly favored amongst others due to the history of safety upon consumption. El Ghany *et al*., (2016) isolated bacteria from local Egyptian cheese, boza, yoghurt and cider. Out of the 27 isolates, only 7 were identified as Lactobacillus bacteria(LAB). Only one of them produces β-glucan and therefore, was identified as *Pediococcus parvulus*. The optimization of isolated growth was identified at different temperatures, pH and media. This study reported that the optimum temperature should be 37°Cand the pH was found to be 6 .The most selective medium for growth of β-glucan was found to be *Pediococcus* selective medium (PSM). Additionally, it also revealed that not all bacterial isolates can necessarily produce.

2.2 Screening of β-glucan producing micro-organisms:

Screening pertains to the procedure of isolation, detection, and then separating the micro-organisms of interest from the mixed population with the help of highly selective procedure.

Generally rapid and visual methods are preferable as far as possible to accord simplicity a as well as reliability.

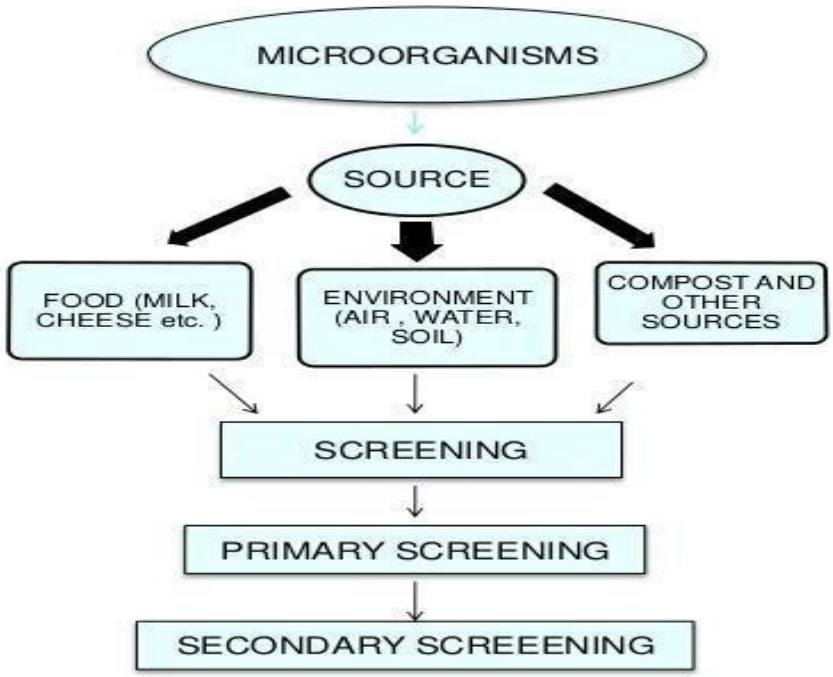

Fig.2.2: Screening of micro-organisms (Steele *et al*., 1991)

Certain aspects assume importance and needs to be considered while screening:1) Choice of source is the foremost important. In these, Samples for screening should be taken from many different sources such as soil, water, air, milk, compost, etc. 2) Choice of Substrate is followed after the chosen source. Nutrient and growth factors should be properly supplied for the growth of desired micro-organisms.3) Choice of detection is also important .Shobha *et al*.,(2012) defines the screening are of two types i.e. primary screening and secondary screening, as shown in Fig.2.2. Primary screening is based on detection and isolation of micro-organisms of our interest. It separates out only few micro-organisms which are having their commercial value and discards the valueless microorganisms. The pH indicates that dyes can be used for detecting microorganism as these are capable of producing organic acids. According to their pH, the dyes

are allowed to undergo color changes. On the other hand, secondary screening is based on sorting of microorganisms that have real commercial value. The microorganisms which have poor applicability in fermentation process are discarded. To further test the capabilities and gain information about the organisms, primary screening is followed by a secondary screening. Secondary screening is usually conducted, in flasks, on agar plates, small bioreactors containing liquid media, etc or it is conducted as a combination of these approaches. Secondary screening can be both quantitative or qualitative in approach. Screening has been used for isolating β-glucan producing bacteria with success.For example, Wu *et al*.,(2018) isolated β-1,3-glucan producing microorganisms from the soil of *Wolfiporia extensa.* It was subjected to high throughput sequencing. They found that the genera of *Streptomyces* about 1.90 percent and 0.78 percent of *Arthrobacter* belong to the order Actinomycetales in the phylum Actinobacteria, which was observed in soil for *P. cocos*cultivation. Mischler *et al*.,(2015) selected 83 strains of LAB from sourdough, fresh raw milk, raw, baker's yeast, and fermented meat, and silage isolates which was followed by MALDI-TOF MS identification. The screening for the production of β-glucan was done by a 2-phased strategy, for example, an agglutination assay with *Streptococcus pneumoniae* type 37-specific antisera which was further followed by PCR amplification of a 417-bp fragment of the glycosyltransferase (gtf) gene encoding a β-D-glucan synthase. The result obtained was a total of 8 strains belonging to the species L. *paracasei*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *L. brevis*, *L.* mali and were successfully identified as genotypic β-glucan producers.

2.3 Isolation of glucan by early workers: Baker's yeast as the primary source

Glucan, a neutral particulate made up of β -1, 3 polyglucose from the cell wall of baker's yeast *S. cerevisiae*, and it was first isolated by Hassid et *al., (*1941). The authors used baker's yeast (2700 grams) and grown it on a grain mesh .Their digestion was done in 4 liter flasks each. In each flask, 2 liters of yeast was allowed to react with 3% of sodium hydroxide. It was by heated on a boiling water bath for three to four hours. The dark brown alkaline digest was kept there at room temperature for one day and the supernatant remained there in the liquid was discarded. 3% sodium hydroxide (2 litres) was added to each flask. The flasks were kept in a shaking water bath for 2 hours. It was allowed to cool down overnight. The dark brown alkaline supernatant liquid

was decanted. The residue become acidified with concentrated HCl with *3%* hydrochloric acid (2 Litres) were added into each flask .The flasks were placed for few hours on a water bath, then the supernatant contained liquid was allowed to be cooled, discarded and again digested with 3% hydrochloric acid. The final acid digest was decanted. The residues which were remained left was washed with distilled water and it was centrifuged, followed by resuspension in water. The residues were washed well with boiling water, it was centrifuged well .The residues from both the flasks was suspended in 1 liter of alcohol and it was stored at room temperature for several days. The centrifugation of brownish-red alcohol solution was done and the residues was washed again with ether and dried at temperature 70°c in a vacuum for four hours. It was dried into a hard brownish mass and grounded it well into a fine powder in a ball mill and a grayish white powder was obtained .The moisture content of the compressed yeast obtained averages was around 70°C. A modification of the method of Hassid *et al*., (1941) was earlier reported by Di Luzio *et al*., (1979) to prepare particulate glucan from *shigella* species (*s.cerevisae*). Di Luzios *et al*.,(1979) modified procedure and briefly discussed .He explained that using a 6 liter flask containing 540 g of dry yeast was suspended in 3 litres of aqueous sodium hydroxide solution (*3%)*. The suspension was placed in a boiling water bath for 4 hrs, and allowed to cool overnight and the supernatant was discarded. This procedure was done in triplicates. The residue was then acidified with 300 ml of concentrated hydrochloric acid along with 2l of 3% hydrochloric acid and then it was placed in a boiling water bath for 4 hrs. The suspension was allowed to stand overnight and the supernatant decanted. The residue was further digested with 31 out of 33S hydrochloric acid at 100°C for 4 hrs. The *2%* hydrochloric acid digestion was repeated twice. The residues were washed again three times with distilled water. One litre of ethyl alcohol was added to the residue, and mixed thoroughly .It was allowed to stand for 24 hrs to get maximum extraction. The dark reddish brown alcohol supernatant was aspirated from the residue and discarded. The alcohol extraction procedure was repeated twice. By washing the residue with distilled water , the alcohol was removed. The preparation was then collected by centrifugation. The residue obtained was light brown in colour. The yield was 6.3% of the weight of dry yeast. Peat *et al*.,(1958) isolated glucan from fresh baker's yeast. About 6 kg of baker's yeast was dispersed in 5 liters of *6%* (w/v) NaOH and stirred at 75 °C for 24 hrs. 14 litres distilled water was then added and

insoluble material was collected by centrifugation at 1200g for 15 min. The complete material was then resuspended in 6 liters of 0.5 M acetic acid at 9^0 C for 3 hrs. After cooling, the supernatant was centrifuged. The residue was then washed properly with water in centrifuge and then suspended in 0.02M sodium acetate (pH 7.0, 2 lit.) and heated for nearly one hour at 135 °C in an autoclave. 2 lit. of water was added after cooling and the residue separated in a centrifuge. The supernatant gave an intense red colour with iodine which indicates the extraction of glycogen. After six washings of the residue with water (1.5 lit, each) the wash-liquid was remained achronic, but on further autoclaving at 135 °C in 3 lit of water which extracted more glycogen. The residue was therefore centrifuged and washed three times with water and then it was again autoclaved. The gelatinous solid was dehydrated with ethanol (3vol, each), centrifuged and washed successively with ethanol and light petroleum. The product obtained was 39 grams, a light buff-coloured powder. The total yield obtained was 0.65% of the weight of fresh baker's yeast. These studies indicate that substantial efforts as well as detailed extractive processes were employed for obtaining β-glucan from yeasts. Subsequently, several other microorganisms have been exploited as β-glucan producers.

2.4 Detection of glucan producing micro-organisms:

The location of B glucan varies in microorganisms. For example the majority of β-glucan resides in the cell wall of fungi (Fig 2.4).The application of appropriate detection methods can provide firsthand information and help decide the sequence and type of extraction process. Stone (2009) reported that at pH 8, the detection of (1, 3)-β-Glucans such as curdlan, callose and related glucans can be achieved by staining with Aniline Blue. When Congo red and fluorochromes Calcofluor White bound to 1, 3-β-glucans, it allowed UV-induced fluorescence. Other triphenylmethane dyes and the phenoxazone dye such as Resorcin Blue were also appeared to be specific for 1,3-β-glucans (Stone & Clarke, 1992).The identification of bacteria can be done by variety of ways such as staining reactions and biochemical reactions.

Bueno *et al.,* (2006) isolated six different polysaccharide-producing bacteria from soil samples and they identified them as *Arthrobacter* and *Pseudomonas*. These were further tested for the

yield of polysaccharides produced and these were grown in two different culture media, one culture containing glucose and the other containing sucrose. They reported that when the concentration of the carbon source was lower than 2%, then the yield of largest polysaccharide was obtained. In a same way, when the initial pH was increased from 5.0 up to 7.0 of the fermentation broth, polysaccharide production was increased.

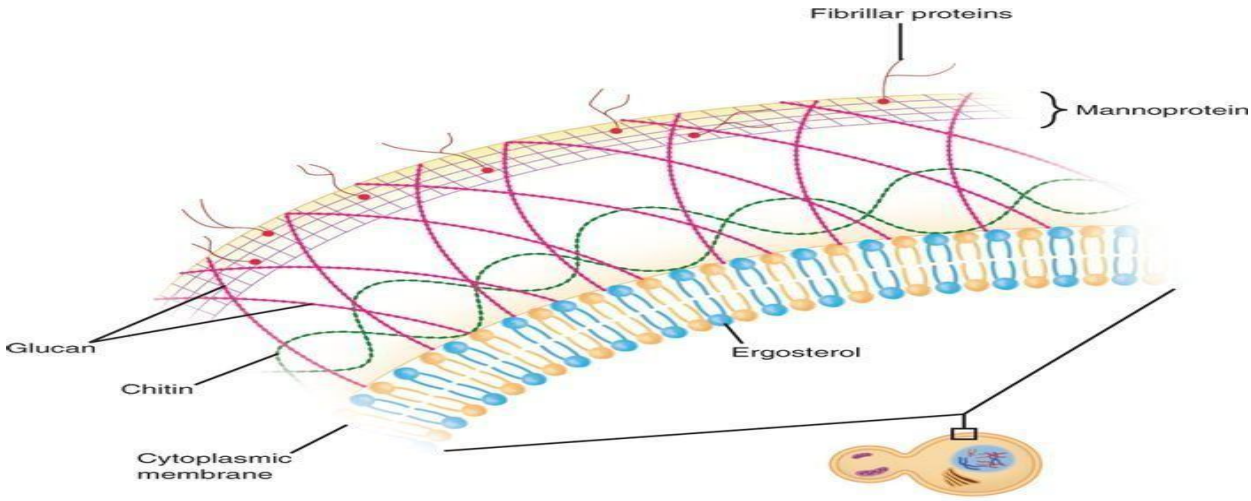

Fig.2.4: Glucan present in the cell wall of fungus (Rahar *et al*.,2011)

2.5 Polymers from natural sources:

Polymers, either synthetic or natural macro molecule of repeating units (monomers) usually arranged in the form of a chain. Synthetic polymers are synthesized in large number approximately 140 million tons around the world every year due to their stability in the environment (Premraj *et al*., 2005) eventually face environmental concerns of biodegradability. Biopolymers are produced, naturally by biological systems such as micro-organism, plants, animals, human. However, they are biodegradable and eco-friendly (Armentano *et al*., 2013). In microorganisms, biopolymers are produced by complex metabolic process under natural conditions, either inside or outside the cell. These polymers are made up of tandem repeating

units of nucleic acids, amino acids or polysaccharides (Chassenieux *et al*.,2013). Several biopolymers are having distinctive characteristics like microencapsulation, act as barrier, smart responsiveness to environmental factors (pH, temperature, light, stress etc.).Chitosan, dextran, starch, proteins, deoxyribonucleic acid (DNA), are examples of biopolymers that can be blended (Rao *et al*., 2014).

Extracellular polymers (ECP) are metabolic products of bacteria that can be accumulated on the surface of cell (Morgan et al. 1990). In 2007, Marco *et al*. purified β-Glucan extracted from *Trichoderma harzianum.* Two proteins were purified by hydrophobic chromatography that was showing β-1, 3-glucanase activity. The molecular masses were found to be 29 and 36 kDa. In 2012, Shaaban *et al*. isolated efficient poly-β-hydroxybutyrate (PHB) producing bacteria from different agricultural soil samples in Egypt .However, their extraction was carried out by Sodium hypochlorite digestion method. The morphological and biochemical analysis of these bacteria showed that they belong to *Stenotrophomonas, Bacillus, Pseudomonas Azospirillum, Azotobacter, Alcaligenes genera and P. aeruginosa*.

Pengkumsri *et al*.,(2017) extracted glucan from *Saccharomyces cerevisiae*, with the help of acids and bases such as 1.NaOH/HCl extraction; 2. Extraction of NaOH/CH3COOH; 3. NaClO extraction;4. NaOH and DMSO extraction. In 1981, Novak et al. extracted polymer from activated sludge by precipitation. They have assume that a force of 32000×G for 15 minutes can be used for quantitatively extracting polymers from activated sludges.

2.6 Polysaccharides biopolymers:

Many monosaccharides join together to form a polysaccharide. Monosaccharides are sugars similar to glucose. Special kinds of enzymes help in binding small monomers together which creates large sugar polymers, or polysaccharides. These polysaccharides are also known as *glycans*. A polysaccharide can be a *homopolysaccharide* or a *heteropolysaccharide*. Homopolysaccharides are those in which all the monosaccharides are the same, on the other hand, heteropolysaccharides are those in which the monosaccharides may vary as shown in fig. 2.6.1.Depending on which carbon sources are connected in the monosaccharides, polysaccharides can take a variety of forms. A molecule contains a straight chain of

monosaccharides is known as linear polysaccharide, while a chain having arms and turns are known as a branched polysaccharide.

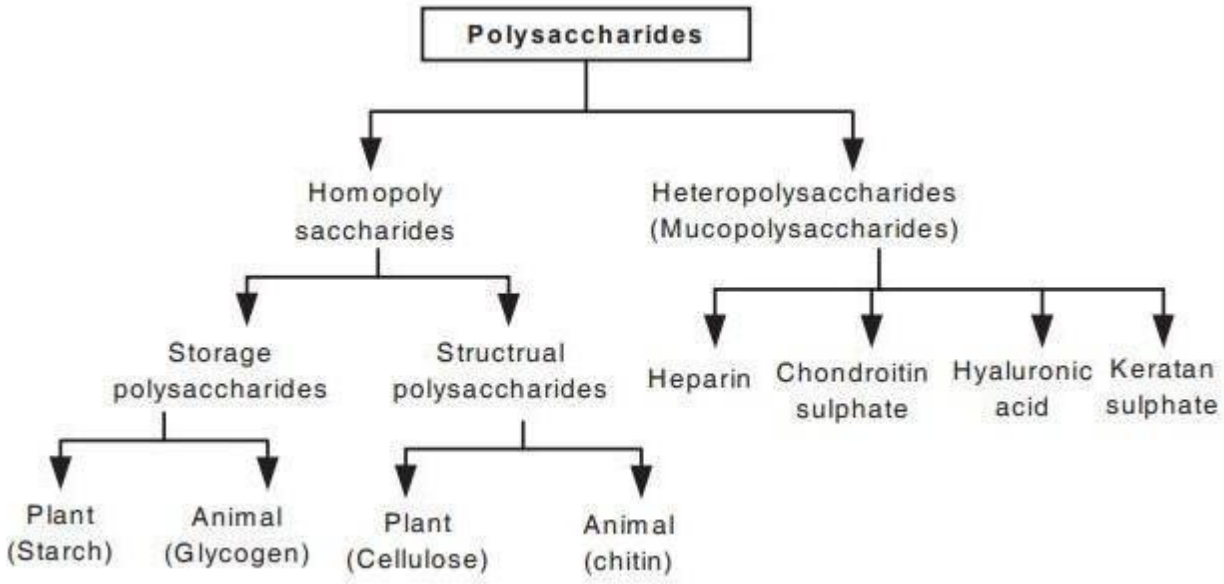

Fig.2.6.1: Classification of polysaccharides (Galan *et al.*, 2011)

Every polysaccharides are shaped by a similar essential procedure, for example, monosaccharides are associated by means of glycosidic bonds. While in a polysaccharide, singular monosaccharides are known as residues. There are numerous monosaccharides made in nature. Depending on the polysaccharide and their glycosidic bonds, any combination can be combined in series as shown in fig.2.6.2.

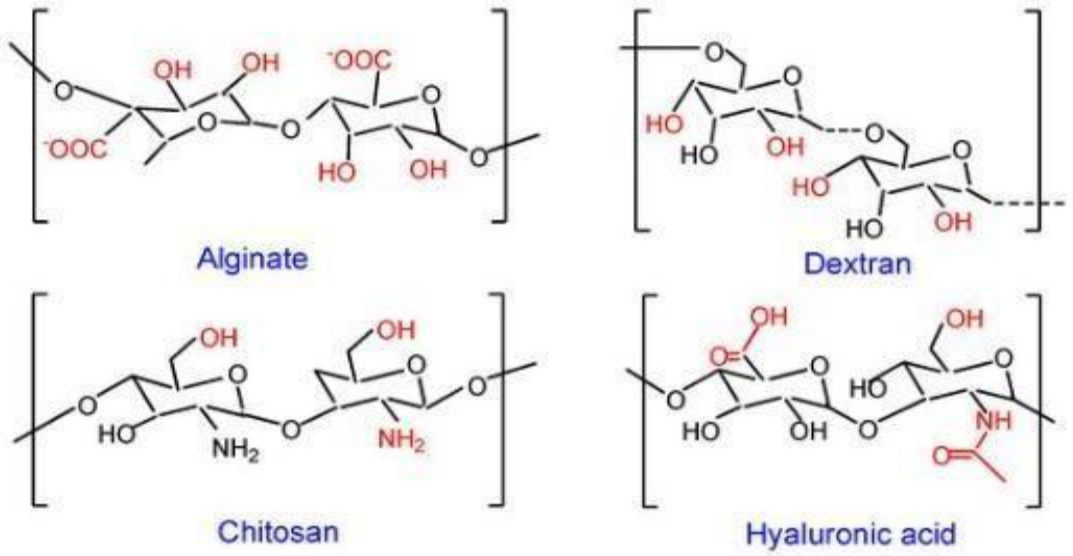

Fig.2.6.2: Structure of polysaccharides (Tiwari *et al*., 2019)

Glucan are the polysaccharides present in greatest amount in nature. There is diversity in their molecular weight of glucan depending on the source from where it is originated. It is possible to differentiate between linear as well as branched α-, β-glucan as well as mixed forms of glucans along with their various glycosidic linkages and molecular masses. The removal of ballast compound is needed for the extraction of glucans from raw types of sources that includes polyphenols, proteins, lipids and other polysaccharides (Synytsya *et al*., 2014).In 2004, Johansson *et al*. gave the information about the structural analysis of water soluble as well as water insoluble (1→3) and (1→4) β-glucans of whole wheat grain barley and oats digested with lichenase. The characterizations such as FT IR and NMR spectra were also measured. The studies concluded that no such differences were seen in NMR spectra and FT-IR during characterization.

2.7 Purification of β-Glucan:

Purification is the process of removing contaminants from the substance. An endoglucanase was mainly purified from Clostridium thermocellum by process that includes preparative gel

electrophoresis, ion-exchange Sephadex chromatography, centrifugation, selective precipitation, and ultrafiltration (Thomas *et al*., 1981). The endoglucanase which is in the purified form showed a high activity towards cellopentaose, cellohexaose, celloheptaose and carboxymethylcellulose. However, it produced low activity towards cellotetraose, microcrystalline cellulose and no detectable activity was seen towards cellotriose and showed an increased activity towards cello-oligosaccharides along with high degree of polymerization (Thomas *et al*., 1981). Notario *et al*.,(1976) isolated β-Glucanase from the cell-free extracts of Candida utilis .They purified 562 fold through adsorption on DEAE-Sephadex and filtration was done through columns of Sephadex such as G-50, G-100 and G-200.The results showed that the purified enzyme shown to be homogeneous on in ultracentrifugation studies as well as in polyacrylamide-gel electrophoresis In 1982, Woodward *et al*. purified two 1,3 and 1,4-P-glucan endohydrolases by the process known as gel filtration chromatography, ion-exchange , and ammonium sulphate precipitation from different extracts of germinating barley. However, the results showed that the amino acid compositions of 1,3 and 1,4-P-glucan endohydrolases are almost alike and the antibodies that showing cross-reactivity against the purified enzymes suggests that they are sharing a similar antigenicity .Mithöfer *et al.*, (1976) isolated β-glucan elicitor-binding protein from a soybean source by a simple, rapid and one-step purification method. They have found that the affinity-based purification technique was more effective as compared to conventional methods which preserved the binding activity to a much larger extent. It was shown by electrophoretic analyses by both the photoaffinity-labeled and purified binding protein that native protein has a molecular mass of about 240 kDa and it was an oligomer.

2.8 Medicinal properties of β-glucan:

β- glucan is a soluble fiber used classically to boost the immune system and to treat high lipids. It can alter the autoimmune mechanisms which are directed to pancreatic islets and it can also inhibit the development of diabetes in case of BB rats. The high viscosity of β-glucan may also contribute to delayed gastrointestinal absorption causing a decrease in blood glucose. It has been found by the researchers that 10% β-glucan included in breakfast cereal can helps in the 50% reduction in glycemic peak. The daily intake of 3 glucan can significantly lowers the plasma LDL cholesterol concentrations .Several controlled trials regularly examining the effect

of β-glucan on cholesterol reported a small reduction in LDL-cholesterol but not for triglycerides. Barton *et al*.,(2016) reported that β-glucan have potential to work as immunostimulator that has been used with therapeutic intent as anti-microbial and anti-tumour agents. He also claimed that range of other β-glucans with potentially beneficial effects and oral forms are immensely available. These are also potential contaminants of pharmaceutical merchandise that have been described in some blood products. Some studies investigated on β-glucans and they revealed that it can also helps in improving the immune system and therefore it increases satiety as well as it also helps in regulating the blood glucose levels. Moreover, it helps in reducing the risk of developing cancers. "Oat and barley foods have many applications such as it is shown to reduce the risk of glucose intolerance by slowing glucose absorption after a meal," says Susan M. Tosh, who a research scientist is working with Agriculture and Agri-Food Canada. While some studies suggested that β-glucans helps boosting the immunity markers occurs by consuming more amount of oats. Many researches on β-glucans have been done on mushroom extracts, than any other sources of barley or oats. *Coriolus versicolor*, also known as medicinal mushrooms, that are commonly used in making many different kinds of traditional medicines. Researchers has been investigated that a minimum of 4 - 6 grams of β-glucans are highly needed to suppress appetite. They also found that β-glucans helps in weight management. Overweight men who can consume 7 g of β-glucans per day for nearly 12 weeks can easily get rid of his body weight issues.

In 2011, El Khoury *et al.* reviewed the technical properties of β-glucans. He described that β-glucan is a soluble fiber and it that has been gaining interest due to its multiple functional and bioactive properties. It has its beneficial roles in insulin resistance, hypertension, dyslipidemia, etc. The fermentability of β-glucans may constitute the basis of their health benefits and it can form viscous solutions in the human gut. Moreover, the applicability of β-glucan as health promoting properties and is used in food ingredients which is widely considered with the purpose of increasing the fiber content of food products.

2.9 Application of β-glucans in food:

The suspension was placed in a boiling water bath for 4 hrs, and allowed to cool overnight and the supernatant was discarded. This procedure was done in triplicates. The residue was

then acidified with 300 ml of concentrated hydrochloric acid along with 2l of 3% hydrochloric acid and then it was placed in a boiling water bath for 4 hrs. The suspension was allowed to stand overnight and the supernatant decanted. The residue was further digested with 31 out of 33S hydrochloric acid at 100°C for 4 hrs. The *2%* hydrochloric acid digestion was repeated twice. The residues were washed again three times with distilled water. One litre of ethyl alcohol was added to the residue, and mixed thoroughly .It was allowed to stand for 24 hrs to get maximum extraction. The dark reddish brown alcohol supernatant was aspirated from the residue and discarded. The alcohol extraction procedure was repeated twice. By washing the residue with distilled water ,the alcohol was removed. The preparation was then collected by centrifugation. The residue obtained was light brown in colour. The yield was 6.3% of the weight of dry yeast. Peat *et al*.,(1958) isolated glucan from fresh baker's yeast. About 6 kg of baker's yeast was dispersed in 5 liters of *6%* (w/v) NaOH and stirred at 75 °C for 24 hrs. 14 litres distilled water was then added and insoluble material was collected by centrifugation at 1200 g for 15 min. The complete material was then resuspended in 6 liters of 0.5 M acetic acid at 9^0 C for 3 hrs. After cooling, the supernatant was centrifuged. The residue was then washed properly with water in centrifuge and then suspended in 0.02 M sodium acetate (pH 7.0, 2 lit.) and heated for nearly one hour at 135 °C in an autoclave. 2 lit. of water was added after cooling and the residue separated in a centrifuge. The supernatant gave an intense red colour with iodine which indicates the extraction of glycogen. After six washings of the residue with water (1.5 lit, each) the wash- liquid was remained achronic, but on further autoclaving at 135 °C in 3 lit of water which extracted more glycogen. The residue was therefore centrifuged and washed three times with water and then it was again autoclaved. The gelatinous solid was dehydrated with ethanol (3vol, each), centrifuged and washed successively with ethanol and light petroleum. The product obtained was 39 grams, a light buff-coloured powder. The total yield obtained was 0.65% of the weight of fresh baker's yeast. These studies indicate that substantial efforts as well as detailed extractive processes were employed for obtaining β-glucan from yeasts. Subsequently, several other microorganisms have been exploited as β-glucan producers.

Table 2. Presence of β-glucan in beverage and food products.

Type	**Product**	**Effects along with their health benefits**	**Reference**
Oat	Chocolate breakfast flakes	Decrease water activity; Prolong durability; Protect a gut-friendly probiotic bacteria.	Saarela *et al*.,2006
Oat	Breakfast bar	No negative effect on sensory properties; Reduced starch breakdown.	Jenkins *et al*., 2002
Barley	Bread	No negative effect on sensory properties; Reduced starch breakdown	Cavallero *et al*., 2002; Symons and Brennan, 2004
Oat	Bread	Improved Crust color, softness and taste; Increased firmness of the bread crumb	Gormley *et al*.,1999; lazaridou *et al*., 2007
Nutrim 5(oat)	Pasta	Improved the overall Strength	Inglett *et al*.,2005
Nutrim(oat)	Low-fat cheddar cheeses	Softer texture decreased melting Time	Konuklar *et al*., 2004
Oat	White low-fat cheeses	Improved Texture; Negatively affected appearance, taste and Odor	Volikakis *et al*., 2004
Oat	Low fat ice-cream;	Function as a fat	Brenn *et al*.,2002

	low-fat yogurt	replacer; Increased the viscosity; Decreased the separation of whey	
Oat	Probiotic milk based drinks	Increased stability	Angelov *et al*.,2006
Oat	Milk beverage	No effect on sensory properties	Biorklund *et al*.,2005
Oat	Beverage	Increased thickness	Lyly *et al*., 2003
Barley	Orange flavoured beverage	Increased viscosity	Temelli *et al*.,2004
Oat	Low fat beef beverages	Increasing cooking yield, moisture & fat retention, Better influence on emulsion Stability	Hughes *et al*.,1997
Barley	Reduced fat(12%),Breakfast sausages	Function as a fat replacer; No significant effect on product texture or flavor if added at the level of 0.3%(w/w)	Morin *et al*.,2002
Oat	High pressure processed chicken breast	Function as a partial salt replacer	Omana *et al*., 2011

2.10 Drug-delivery systems:

Nanoscale carriers/Drug-delivery systems occupy prominent place in array of modern drug delivery systems to selectively target tissues and deliver therapeutics. The use of nano-carriers or

drug polymer conjugates started with amphiphilic lipids in 1960's (Bangham *et al*., 1965). Further improvements were made to use them as pharmaceutical carrier and enhance the stability of the encapsulated drug (Torchilin 2005). Later in 1970, the polymer-drug conjugates based nano-carrier were used to improve the stability, circulation time, controlled-release and bio-distribution (Langer and Folkman, 1976). The first reports for targeted delivery was reported with poly (hydroxypropyl methacrylamide) (PHPMA) polymer conjugated with cathepsin B (drug) and galactose as targeting ligand. Liver cancer was targeted using galactose, an asialo-glycoprotein membrane receptor ligand for hepatocytes. In the early 1990s, other nano-scale DDS for example block copolymers, PEGylated polymeric micelles and liposomes were developed (Northfelt *et al*., 1996, Cabanes *et al*., 1998). With the beginning of 21st century, various possibilities for nano-scale DDS were explored which include dendrimers, polymers, other hyper-branched polymers, micelles, and VLPs etc. Liposomes are small lipid vesicles, widely explored as drug carriers. They are composed of phospholipids and steroids (e.g., cholesterol) bilayers, or other surfactants. They were simply synthesized by sonication of certain lipids dispersed in aqueous media. Continuous parallel packing of lipid bilayer based on hydrophobic interaction surrounds the hydrophilic head groups towards the aqueous environment. Liposomes have been reported to develop many nanomedicine by encapsulating different drugs which are available as product e.g. Doxil (doxorubicin), Myocet (doxorubicin), DaunoXome (daunorubicin) (Rosenthal, Poizot-Martin *et al*., 2002, Rivera 2003, Markman 2006) etc. However, liposomes poses many challenges such as low loading efficiency, instability in low shelf life, poor solubility of many drugs in the lipid or surfactant solution, difficulty in targeting specific tissues of the reticuloendithelial system (RES) and rapid burst release of drug (Park, Hong *et al*., 1997, Allen, Mumbengegwi *et al*., 2005, Chattopadhyay, Zastre *et al*., 2008, Zucker, Marcus *et al*., 2009). Severe side effects were associated with liposomal formulations due to their accumulation in skin tissue (Lotem, Hubert *et al*.,2000, Charrois and Allen, 2003). Polymeric nanoparticles and polymer drug conjugates were introduced almost four decades ago. These polymers were obtained from both natural such as albumin chitosan, gelatin as well as synthetic polymers such as poly-L-glutamic acid and polyacrylate (Kumari *et al*., 2010). The nanoparticles that are synthesized by these polymers vary in its size ranging from 100 to 500 nm depending on the method of preparation (nano-precipitation, emulsion diffusion and solvent evaporation) (Kumari *et al*., 2010). Polymer nanoparticles drug conjugates can be formed by

adsorption/immobilization on surface or encapsulated during synthesis or covalently conjugated in the polymer matrix (Murakami *et al*., 1999, Rawat *et al*., 2006, Bisht *et al*., 2007). Various drug molecules for different ailments had been encapsulated in various polymeric nanoparticles e.g. Abraxane (paclitaxel bound to albumin), Xyotax (Paclitaxel combined with poliglumex), CT2106 (Camptothecin poly-L-glutamate conjugate), HPMA-dox-galactosamine and N-(2-Hydroxypropyl)(Seymour *et al*., 2002, Bhatt *et al*.,2003, Singer *et al*.,2004, Gradishar *et al*.,2005) etc. However the problems faced by these nano-carriers were mainly toxicity from residual solvent, low blood circulation half-life, non-targeting and rapid clearance by macrophages of the mononuclear phagocytic system (MPS) due to opsonization (Soppimath *et al*., 2001, Owens and Peppas 2006). Dendrimers are synthetic polymeric macromolecule (5-10 nm) with well-defined size and structure. The drug may be encapsulated in the internal structure or chemically attached or physically adsorbed on dendrimers surface. The surface of dendrimers provides a good platform for attachment of specific ligands, which may include folic acid, antibodies, and cyclic targeting peptides – arginine-glycine-aspartic acid (RGD) (Wang *et al*., 2011, Yellepeddi *et al*.,2011, Mekuria *et al*.,2015). Poly (amido amide) (PAMAM) was a frequently used dendrimer in biomedical applications. This was used for encapsulation of various molecules including anticancer drugs, cisplatin, doxorubicin (Choi *et al*.,2010, Yellepeddi *et al*., 2011). The challenge for dendrimers as drug delivery system was mainly cytotoxicity leading to apoptotic cell death, haemolytic activity, immunogenic and in vivo brain toxicity (Duncan and Izzo,2005; Albertazzi *et al.*,2012). Although, there is repertoire of nanoparticle-based delivery systems under preclinical evaluation, but only few are available on the pharmaceutical market (Emerich and Thanos, 2006). Detrimental effects of nanoparticles depend on various factors such as hydrodynamic size, low drug loading efficiency/capacity, polydispersity, reactivity of the immune system and time of residence in the bloodstream. However, with respect to their size, small sized carriers have prolonged blood residence but rapid excretion based clearance. The bigger sized carriers may accumulate in the capillaries which are quickly opsonized and then it is removed from the bloodstream via the macrophages of the RES (Moghimi 1995, Moghimi and Bonnemain, 1999). Various studies established optimal size range of 20-200 nm for DDS (Moghimi *et al*., 2001). Some proteins e.g. opsonins mark the carriers as foreign and activate the complement system and facilitate phagocytic uptake by macrophages. The new research to overcome these problems using biomaterial based DDS made a big impact due to their

biocompatibility and nontoxicity (Jahanshahi and Babaei, 2008). Among the natural systems, the protein based nano-carriers showed monodisperse, well-defined robust structures to carry active ingredients and a polyvalent ligand-display surface to bind with biological moieties for cell targeting. Heat shock protein, iron storing protein (ferritin), vault proteins and virus capsids are well-known material for their use in gene and drug delivery (Ren *et al.*,2007, Nishikawa et al. 2008, Buehler *et al*., 2011, Lin *et al*., 2011). Among viruses, animal viruses posed many disadvantages such as receptor affinity, enveloped structure and accumulation in tissue (Green *et al*., 2004). However, the plant viruses are typically non-pathogenic and non-infectious in humans and their well-defined robust structure, abundance, simple in vitro assembly, stable capsids makes them a good nano-carriers for the development of drug delivery system (Young *et al*., 2008).

Chapter 3

Materials and Methods

3.1 Reagents and Chemicals

All the reagents and chemicals used were purchased from HI Media (Mumbai, India), Sigma Aldrich (Bengaluru, India) and Lobachemie (Mumbai, India).Experiments were carried using DE MAN, ROGOSA and SHARPE (MRS) agar, anaerobic flask. The composition of MRS was in grams per litre of distilled water ; Yeast extract(5.00), Proteose peptone(10.0),Beef extract(10), Dextrose(2.00), Polysorbate 80(1.0), Sodium Acetate(5.00),Magnesium sulfate(0.1),Dipotassium phosphate(2.00),Maganese Sulfate(0.050), AmmoniumCitrate(2.00),Agar(3.25).

3.2 Collection of samples:

Curd samples were collected from the local dairies (Patiala, Punjab) and faeces of newborn babies from maternity nursing home. A prior consent of maternity hospital agency and parents were obtained for collection of feces of newborn. The faecal samples were collected using sterile spatula and aseptically transferred to the sterile crew capped containers, transported into the laboratory in ice and analyzed within 2-3 hours of receipt.

3.3 Isolation of bacteria from samples:

The curd and faeces of a new born baby were aseptically weighed and homogenized by adding 225 mL of saline solution into it. Different decimal dilutions were prepared for each sample upto 10^{-3}. Aliquots were spread plate on DE MAN, ROGOSA and SHARPE (MRS) agar plate and MRS were supplemented with 0.05% L-cysteine .The plates were then placed in an anaerobic flask in the presence of gas generating kit (Anaerobic system) or nitrogen gas flushed into it and then the gas chamber was properly sealed so that it won't allow it to leave from inside. The gas chamber was then placed in an incubator at 37°C for about 24 - 48 hours.

3.4 Screening of microbial isolates:

The colonies of bacteria that were streaked, was showing different appearances on MRS agar plate were randomly subcultured and then plating on MRS agar to obtain purified isolates. The isolated colonies formed on the MRS agar plate were further identified using gram stain, biochemical tests. The isolates predominant at the highest dilution were then selected and further tested for β-glucan production as described before.

3.5 Gram Staining Test:

The isolated bacteria were stained by using gram staining kit and were further magnified under the microscope at 10x, 40x and 100x magnifications.

3.6 Catalase test:

To perform catalase test, a single isolated colony was streaked on a glass slide and one drop of 3% hydrogen peroxide was added on it. The effervescence of oxygen indicated the positive response of the bacteria to catalase test. A negative test resulted in no bubbles or only a few scattered bubbles.

3.7 Staining with alanine blue:

From freshly grown (in De Man Rogosa Sharpe broth) cultures were streaked onto solid medium. The composition of medium includes 1% glucose, 0.005% dye, 2% agar and adjusted to pH 7.2. When testing organisms produced acid, 0.3% of $CaCO_3$ was added to neutralize the medium during incubation; this is because a pH range of 5-7 is required for adequate staining with aniline blue. Staining of colonies with dye was observed by naked eye.

β- glucan producers are visualized as blue colonies or colour on plate after incubation and was selected for further study.

For the isolates, pH was optimized from 5-7, temperature range between 5-45 ℃ and carbon sources for best growth were optimized for detection of β-glucan each time using aniline blue assay.

3.8 Long term preservation of isolates:

Isolates that showing homofermentative characteristics, Gram positive and catalase negative were preserved in MRS (De Man Rogosa Sharpe) broth medium containing 20% (v/v) glycerol as frozen stocks placed at -80°C. Glycerol stock samples were prepared by mixing 0.5 mL of overnight cultures and 40% glycerol. By this, we can preserve our sample for a long duration.

3.9 Growth profile:

A growth kinetic study was carried out and β-glucan production examined after each phase over the lifespan of both isolates.

3.10 Extraction and purification of EPS:

Extraction of extracellular polysaccharide was carried out by growing cultures in MRS broth. Microbial culture in MRS broth was incubated overnight at 37°C with shaking. 1% culture was used for inoculating MRS media at 37°C for 48 hours with shaking. The overnight grown culture was harvested by centrifugation (16000rpm, 4°C for 30 minutes), supernatant was collected and concentrated to 1/10th of its original volume by lyophilizing. The glucan was precipitated by using double the amount of cold acetone for 24 hours at 4°C. The crude biopolymer sample was purified by treating with CPC (Cetyl Pyridinium Chloride) followed by dialysis. The biopolymer obtained thereafter, was stored in powdered form after lyophilization (Ghosh *et al*., 2009).

3.11 Spectrophotometric assay for β-glucan:

The assay for beta glucan was performed by the following techniques:1) β-glucan was extracted;2)Standard of β- Glucan was dispensed in distilled water at 60-100°C water bath at 1-3 hour, volume was adjusted, so that concentration of 0.1 mg/ml, was achieved to give a standard β-glucan solution;3)Congo red was taken with 0.05-0.2 mol/l in pH 6-8 phosphate or carbonate buffer solution(to give 15-120 Pg/ml final concentration of Congo red;4)The accurate standard0-1.2 ml of β-glucan was placed in separate tubes, the solution of congo red was added in each tubes, so that final concentration of β-glucan ranges from 0-20 Pg/ml, final concentration of congo-red was 10-80 PG/ml;5)It was allowed to shake for 10-60s immediately and allowed to

stand for 20-60 minutes at room temperature;6)The absorbance was taken at 550nm using congo-red as a blank ,each test was done thrice;7)Standard curve was prepared with standard β-glucan as abscissa,the absorbance as ordinate, to give Regression equation; concentration of β-glucan was calculated.

3.12 Optimal growth, production conditions and yield of Glucan intervals:

To understand the suitable temperature and pH of β-glucan production, the cultures were grown over a temperature range from 20- 45°C and pH 5-7.5. The β-glucan production was assessed using the spectrophotometric assay described before. For ascertaining the yield, extraction and purification of β-glucan was carried out and yield of the polysaccharides was determined after regular intervals i.e. 12 hrs, 24hrs, 48 hrs and 72 hrs against the dry cell mass.

3.13 Characterization of biopolymer

3.13.1 Scanning electron microscopy (SEM):

Surface properties of a polysaccharide were analyzed by SEM. The samples were ground to fine powders and were dispersed in acylonitrile /THF by agitating in an ultrasonic tank for 30 minutes. A drop of the dispersed liquid was dried on a sample holder and coated with gold by ion sputtering method (JSM541, JOEL, JAPAN) at 20.0 kv.

The microscope was coupled to an energy dispersive spectrometer (EDS). The samples were metallized to make them conductive and analysed at 15 kV voltages (Mathews *et al*., 2008).

3.13.3 Fourier Transform Infrared Spectroscopy (FTIR) :

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

3.13.4 Dynamic Light Scattering (DLS):

At an angle of 173°, the scattering of light is detected in DLS. It is an optical arrangement known as non-invasive back scatter (NIBS) optic arrangement that maximizes the detection of scattered light while maintaining signal quality. At low concentrations, an exceptional sensitivity that is required for measuring the size of entities such as polymers and nanoparticles. Measurements were carried out at a temperature of 25 °C in a polystyrene cell. The 2mg/l solutions were made such as C1 and F1 in aqueous solution. Data processing was carried out with a computer attached to the instrument. The measurements were repeated three times in order to check their reproducibility.

3.13.5 Thermogravimetric Analysis (TGA):

In thermogravimetric analysis (TGA), the change in the weight of a sample is monitored as a function of temperature or time, while it is purged with an inert gas. It can provide quantitative information resulting from any processes causing an obvious weight variation during controlled heating. For example, Chang *et al*.,(2002) used TGA to assess the amount of hydrated water and organic content in cross-linked HA/collagen nanocomposites. Moreover, TGA is a suitable technique to investigate the thermal stability of a polymer (Alexy *et al*., 2003, Mano *et al*., 2003), since the decomposition temperature represents the upper limit of the processing temperature. It also can be used to study the stoichiometry and kinetics of thermal decompositions helping in identification of the degradation mechanisms (Meenan *et al*., 2000, Mano *et al*., 2003).

3.13.6 High Performance Liquid Chromatography (HPLC):

For chromatographic analysis, samples were obtained using the previously described procedure in powdered form. The powdered samples were taken and then transferred from storage at -20 to 10°C and these were kept for approximately 12hr at 10°C. The samples were taken again then they were left at room temperature for about 4hrs before it was being analyzed. After stabilization of temperature, each sample (25 µL) was diluted by a factor of 20 with ultrapure

water, which had been filtered through an ultrafilter durapore membrane with a 0.20µm pore size (Millipore). A 20 µl volume of the diluted sample was injected into the chromatograph for analysis.(Toledo *et al.*,2013).

By HPLC separation, the samples and standard of β-glucan was analyzed with column Luna 5µ C18 with internal diameter 250×4.6 mm .The mobile phase used was acetonitrile (ACN) 100% with a flow rate of 0.5 mL/ min(Khalid *et al*.,2016) .

3.13.7 Functional characterization of β-glucan producing isolates:

In order to establish the human safe (GRAS) and beneficial attributes of the selected isolates, a standard regime comprising of phenol resistance, general antibiotic susceptibility, hydrophobicity and bile salt hydrolase activity was carried out.

For Bile salt hydrolase activity, the method of Franz *et al*.,(2001) was used. Overnight cultures were spotted onto MRS agar plates containing Sodium taurodeoxycholate (0.5%) and $CaCl_2$ and incubated at 37^0C for 18-24 hours. Zones with precipation were considered positive.

To determine phenol resistance, overnight cultures were plated on MRS media containing 0.4% Phenol and survival scored after incubation at 37^0C after 24 hours (Xanthopoulous *et al*., 1997)

The BATH test was used to evaluate hydrophobicity of the cultures. Briefly, 108 overnight grown cells were mixed thoroughly with xylene by vortexing (5 minutes) and incubated at room temperature for 1 hour. The aqueous phase was removed and absorbance measured at 660nm. Aliquots were spotted onto MRS media to confirm viability following xylene exposure. Hydrophobicity was expressed as: A0-A/A0 X100, A0 refers to Absorbance before addition and A1 after addition of Xylene.

In order to examine the general resistance to antibiotics of the isolates, the spot on lawn method was used Penicillin, Ampicillin, Streptomycin, Gentamycin, Tetracycline, Ciprofloxacin, Oxacillin and Vancomycin. The sensitivity or resistance was inferred from the zones obtained after 24 hours incubation at 37^0C.

3.14 Evaluation of β-glucan functionality:

3.14.1 Cholesterol Lowering Effect:

In cholesterol binding assay, the stock of 10 mg of lyophilized cells per milliliter was prepared and different concentrations such as 100, 200, 300, 400 and 500 µg mL of β-glucan was prepared from the stock solution .Cholesterol-ethanol solution(1ml) was prepared. In this solution, 100 µg of cholesterol was dissolved in 1 mL of 60% ethanol. It was vortexed and then it was incubated at 37°C temperature in a shaking water bath for 1 hr. The mixture was then centrifuged at 10000rpm for 10 minutes. The cholesterol which was remained unbound in the supernatant was used to determine the cholesterol lowering effect. The tests were performed in triplicates.

The resultant cholesterol was checked against blank at 517nm.The percentage of cholesterol lowering effect was determined by the equation:

3.14.2 Antimicrobial effect of β-glucan :

Anti-microbial activity of β-glucan was checked against tested spoilage *fungi* obtained from fruits, *Salmonella typhimurium, Staphylococcus aureus, Erwinia and Shigella flexneri* on the minimal media using different concentrations such as 10 µl, 20 µl, 30 µl, 40 µl, 50 µl from 1 mg/mL of β-glucan for each well. After incubation at 37°C for 24 hrs, all plates were observed for the zone of inhibition. The diameter of zones was measured in millimeters (mm). All tests were performed in triplicates and the anti-microbial activity was expressed as the mean of inhibition diameters (mm) produced.

3.14.3 Stability of β-glucan in the gastric passage:

To ascertain the stability of β-glucan in the gastric passage, a simulated gastric juice experiment was carried out. The cholesterol lowering effect was adopted as a marker for understanding the effect of passage. Artificial gastric juice prepared comprised of D-glucose(3.5g/L), NaCl (1.28g/L), KH_2PO_4(0.6), KCl (0.239g/L), $CaCl_2$(0.11g/L) OxBile (0.2mg/, Lysozyme (0.1g/L), Pepsin(0.013g/L) , pH 1.85 (adjusted with HCl). β-glucan samples equivalent to 1 g/ml was entrapped in dialysis sacs and exposed to gastric juice in beakers with gentle shaking (Orbital shakers, 45 rpm) at 37^0C for 2 hours. Samples from within the dialysis sacs were used for estimating cholesterol reduction along with appropriate control (exposed to PBS).

Chapter 4

Results and Discussion

1, 3 β-glucans as a class of polysaccharides have evoked profound scientific interest. This is due to its varied pharmacological properties such as anti-microbial and anti-tumor effects, regulation of blood glucose levels, and decreases the risk of developing some types of cancer. Moreover the ability to form single or triple helical structures that can be formed into resilient gels with the application of heat and humidity and optimal sizes MW qualify them as structural agents to provide scaffolding for the formation of macroscopic and nanoscale structure. Few studies of those ongoing have identified easily exploitable β-glucans from microbial sources apart from yeast. In view of this the current study was undertaken to explore β-glucan production from fermented food and human sources. For this, curd and faeces of newborn were considered as two most common sources for isolating glucan producing bacteria.

4.1 Identification of bacterial isolates:

The isolated bacteria from the curd sample and faeces sample of a new born were observed by compound microscope. The bacterial isolate from curd sample was gram positive, rod shaped, occurring singly or in chains. On the other hand, the bacteria isolated from faeces sample was gram negative, rod shaped as well as coccii shaped(coccobacillary) occurring singly or in chains and however, they were pink in color. The gram staining results of curd isolate showed that the isolated bacteria could be identified as gram positive non sporulating sp. whereas, the gram staining results of faeces isolates showed that the isolated bacteria could be identified as Gram negative. The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. In performing the catalase test no bubbles were observed from curd isolates, indicating that isolated bacteria is catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2. In case of faeces isolate, isolated bacteria were catalase positive and could mediate the decomposition of H_2O_2 to produce O_2. These results along with other biochemical tests (Bergeys Manual of Determinative Bacteriology, 1993) indicated that the

curd isolate belonged to *Lactobacillus* sp characteristics and the faecal isolate could be classified as *E.coli.*

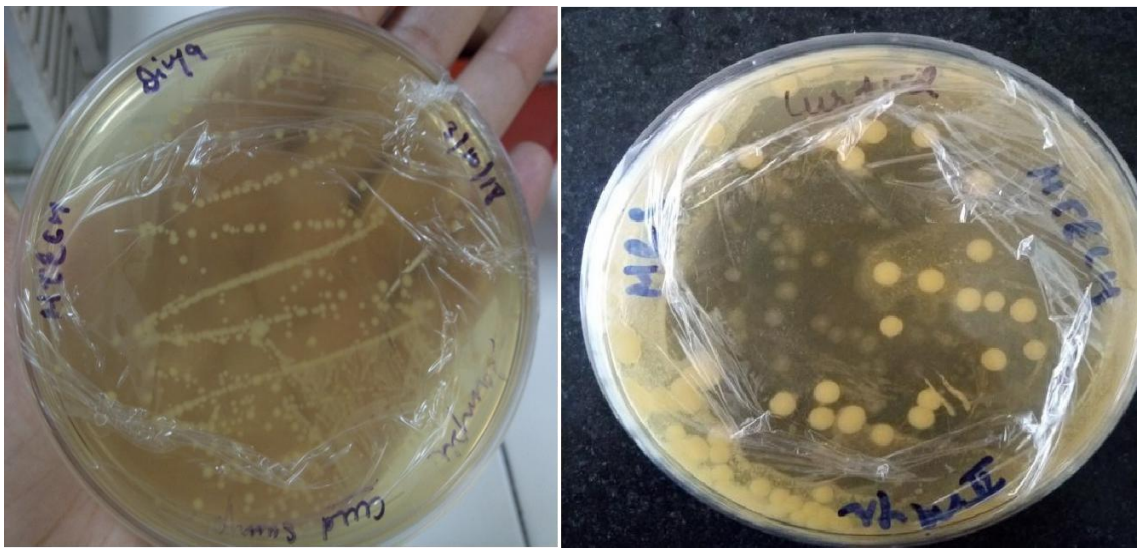

Fig.4: β-glucan producing *Lactobacillus* sp. isolated from curd sample on MRS plate.

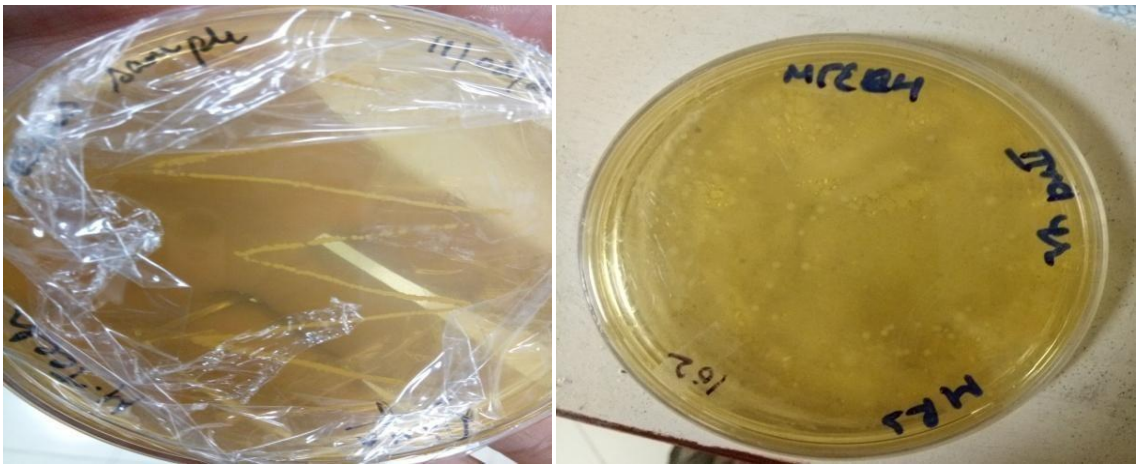

Fig.4.1: β-glucan producing *Escherichia coli* isolated from faeces sample on MRS plate.

Table 3: Morphological characteristics of curd and faeces isolated β-glucan producing bacterial isolates.

Sample	**Form**	**Elevation**	**Margin**	**Surface**	**Opacity**	**Chromogenesis**
Curd	Circular	Convex	Entire	Smooth	Opaque	White
Faeces	Circular	Flat	Raised	Smooth & dull	Translucent	Transparent White

4.2 Detection of glucan with alanine blue:

A direct detection of exopolymer producer strains offers rapidity and feasibility for further investigations. Dyes have been successfully used as these first level screening. For instance, Nakanish *et al*., (1976) showed that staining with Aniline Blue is specific for β-1,3-glucans, including curdlan-type polysaccharide, and yeast glucan, while Brilliant Blue, Trypan Blue, and Congo Red stain many kinds of polysaccharides. In this experiment, colonies forming curdlan-type polysaccharides were found to stain clearly with all the dyes. The results of experiments with Aniline Blue and Congo red on colonies of Curd Isolate and feces isolate are depicted in Fig.4.2.1, Fig.4.2.2. Aniline Blue is much more suitable than Congo Red or other dyes for detecting curdlan-type polysaccharide in colonies, since it specifically stains β-1,3-glucans, such as curdlan-type polysaccharide.

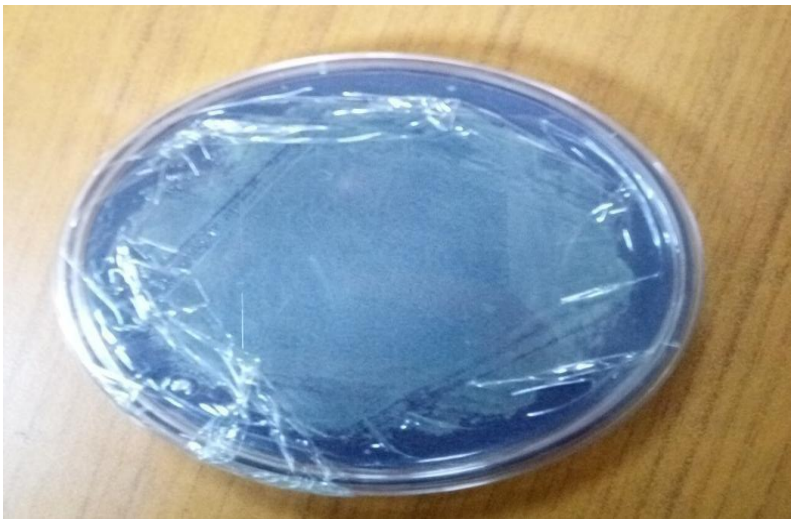

Fig. 4.2.1: Colorimetric visualization of extracellular Glucan production by curd isolate.

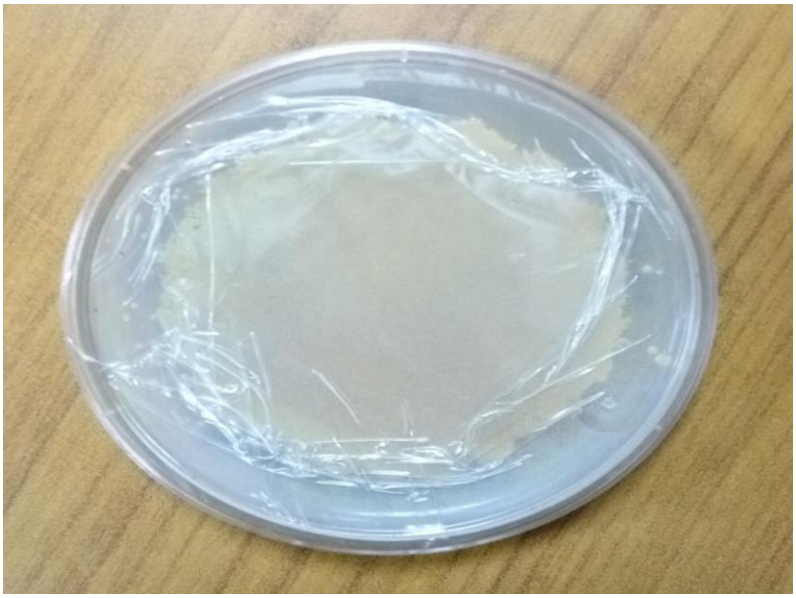

Fig.4.2.2: Colorimetric visualization of extracellular glucan production by faecal isolate.

4.3 Growth profile of bacteria:

The growth kinetic study revealed distinctive lag, log and stationary phase as well as decline phase. The initial phase is the lag phase where bacteria are metabolically active but not dividing, for both cultures the lag phase lasted approximately 10 hours. The exponential growth occurred at 12 hours. Afterwards, at 24 hrs of growth, it reaches to its stationary phase and continued to be in the stationary phase till 48 hrs. The curd isolate achieved higher cell mass in comparison to the faecal isolate. It is possible that the faecal isolate may be a facultative anaerobic which is indicated by its failure to reach a high cell density.

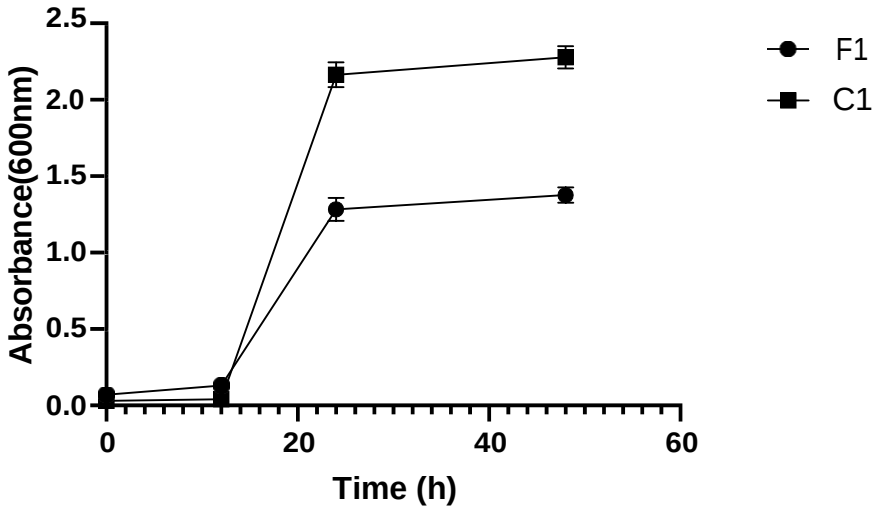

Fig.4.3: The Growth kinetics of β-glucan producing bacterial isolates from faeces (F1) and curd (C1)

4.4 Spectrophotometric assay for β-Glucan:

A colorimetric method is usually preferred for its simplicity and feasibility. The β-glucan spectrophotometric assay to determine the β-D-glucans with Congo red was adopted for this purpose (Nitschke *et al*.,2011).The underlying principle of this assay relies on the total content of

glucans and β-glucans in the form of triple helix (1,3-1,6-β-Dglucans) which bind optimally the dye Congo Red. The latter is then determined spectrophotometrically. Fig.4.4 depict that the amount of β-D glucan decrease with the increasing amount of congo-red into it.

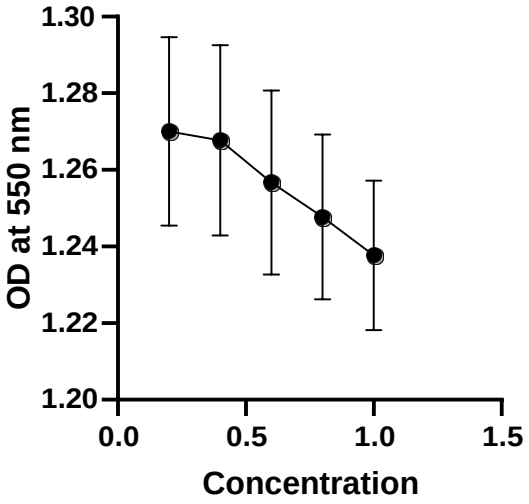

Fig.4.4: Spectrophotometric assay of β-Glucan.

4.5 Comparative yield of β-glucan of curd and faecal isolates:

In order to optimize the growth phase during which highest β-glucan is produced, The yield of β-glucan extracted from both the sources (C1 and F1) was compared over a timer period of 12hrs, 24hrs, 48hrs, 72hrs which spanned the whole period of growth. The Table depicts that highest yield was obtained from both the faecal and curd isolate after 48 hours per gram per dry weight of the cells. These results are in line with those reported earlier that exopolysaccharides are usually highest during stationary phase of bacterial cell growth possibly for protecting the cells against starvation and other stresses (Papadimitriou *et al*.,2016).

Table.4: Yield of β-glucan over complete life span of faecal and curd isolates at pH 7.2 and temperature 37^0C.

Time	Glucan from F1(mg/g) dry cell mass	Glucan fromC1(mg/g) dry cell mass.
12 hrs	23.1 mg	20.5 mg
24 hrs	27.0 mg	24.0 mg
48 hrs	31.1 mg	26.1 mg
72hrs	26.0 mg	24.4 mg

4.6 Characterization of polysaccharides:

4.6.1 SEM (Scanning Electron Microscopy) and EDS of Standard β-Glucan:

SEM has been described as an invaluable tool for understanding the architectural pattern of polymers. In order to gain insights of the structure of the architecture of the biopolymer, SEM along with EDS was carried out. EDS aids in determination of the principal chemical components.The standard Glucan was observed to be white to off white fluffy granular powder. A fibrous and porous nature was observed (Fig.4.6). The fine granules of biopolymer are irregular in shape with size <20 um in the longest dimension. Granular shapes were observed as aggregates within the range <5 um in the largest dimension. Grooves and ridges were distinguishable on its surface in range 600 um indicating a large surface area of biopolymer (Tushar *et al.,* 2014).

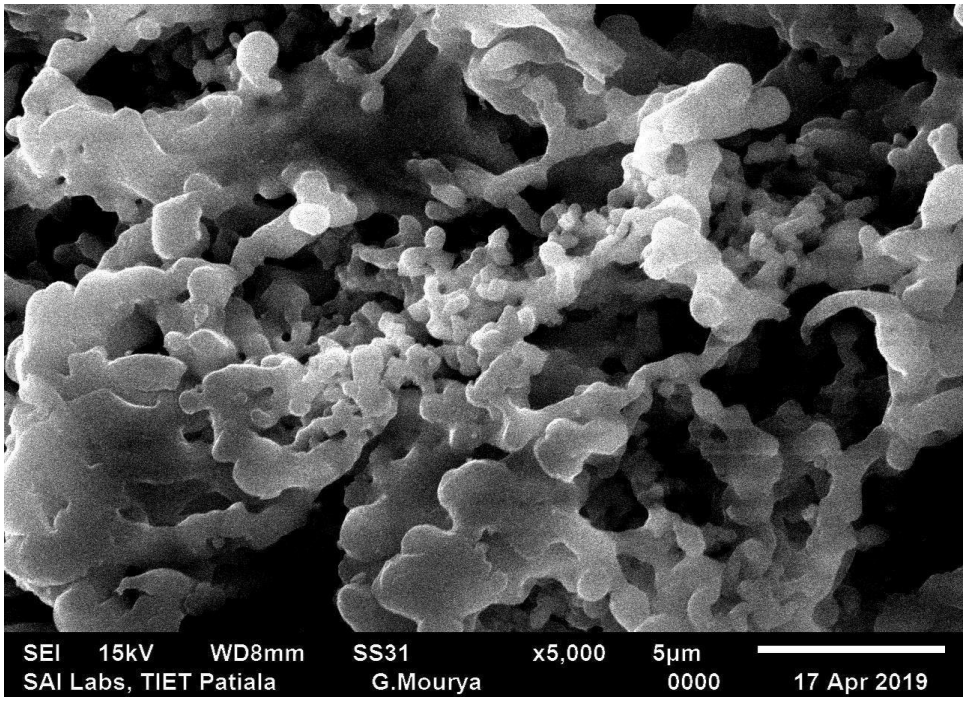

Fig. 4.6.: Scanning electron micrograph of Standard Glucan with 5000X magnification & bar size of 5µm

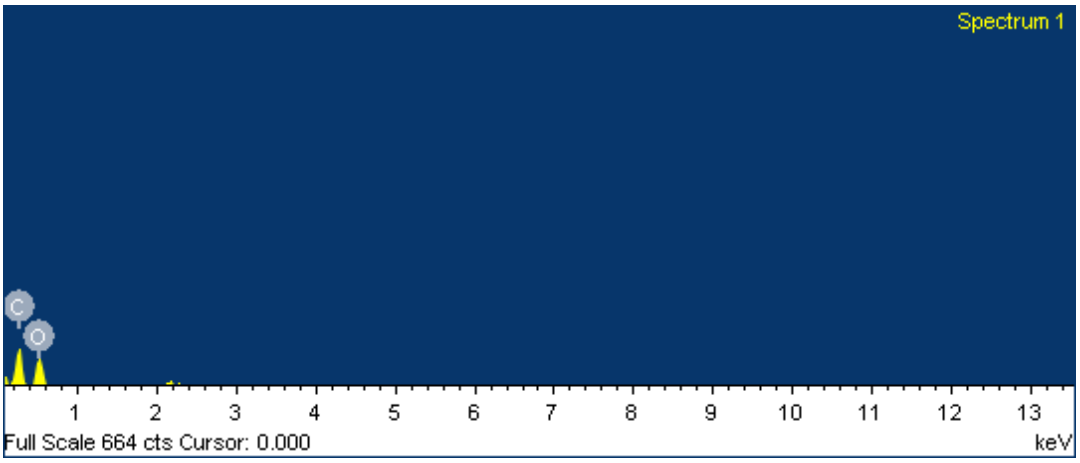

Fig.4.6.1: EDS spectra of Standard Glucan

4.6.2 SEM (Scanning Electron Microscopy) and EDS of C1 and F1 β-Glucan:

Polysaccharides and its concentration were examined under Scanning electron microscope to observe the surface topography. The SEM microphotographs showed the Glucan particles to be uniform, non-porous structures. The images also revealed the size of glucan(C1) and glucan (F1) particles was found to be approx. 2-4μm.Glucan(C1) and glucan (F1) was analyzed by subjecting the sample to scanning electron microscopy for comparing the pore size and surface properties. The electron micrograph shows a clear, small, and an average 2.4 ± 0.3nm pore size in both the samples. A homogenous porous structure was observed in the micrographs. The significant change in porosity and surface morphology in different types of Glucan extracted from different sources are depicted in Fig. 4.6.2A, Fig. 4.6.2B and Fig.4.6.2C, Fig.4.6.2D respectively. Earlier studies have indicated structural difference in β-glucans

The chemical composition of Glucan was determined by EDS along with SEM. The carbon and oxygen were found in abundant amount in Standard β-Glucan as shown in Fig.4.6.1 .In Glucan: F1 polysaccharide, the peaks are obtained which determines the presence of oxygen and manganese in large quantity and traces of phosphorous, carbon, magnesium ,calcium in the decreasing order by weight and it is shown in Fig. 4.6.2E.In Glucan: polysaccharide (C1) spectra was obtained by EDS which result in the presence of oxygen and manganese in large amount and traces of phosphorous, carbon, calcium, magnesium in the decreasing order by weight and was analyzed as shown in Fig. 4.6.2F.

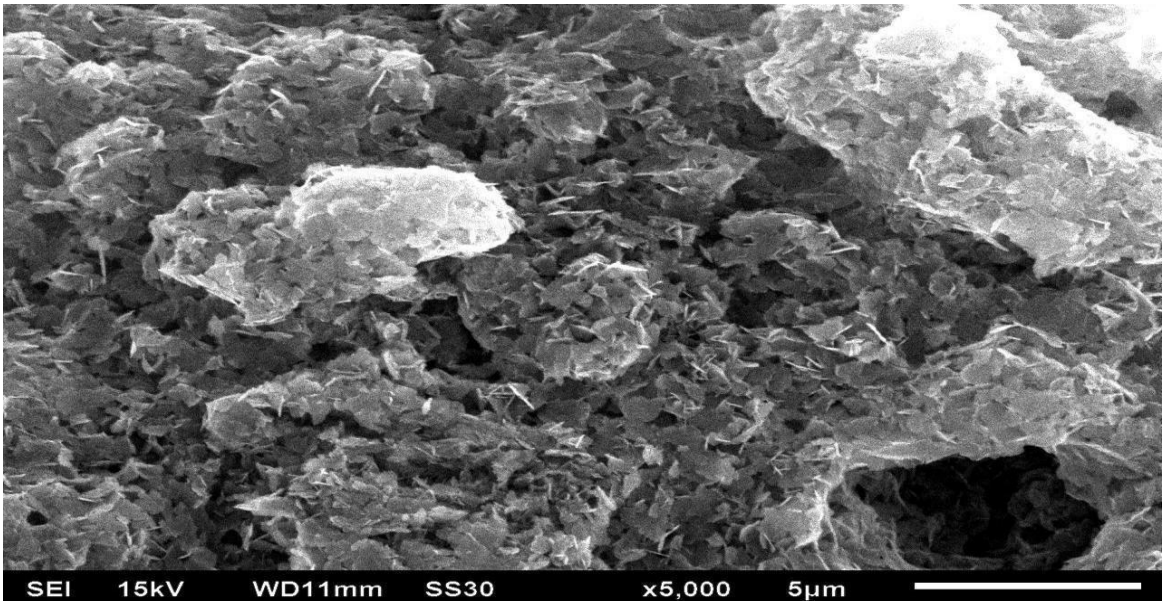

Fig.4.6.2A: Scanning electron micrograph of F1 Glucan with 5000X magnification & bar size of 5µm

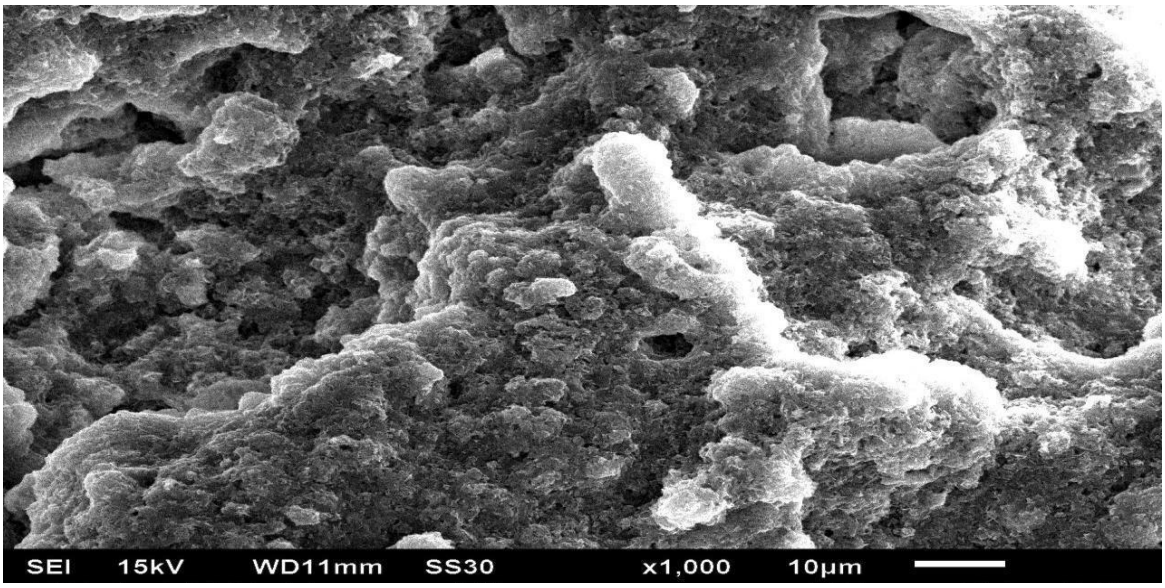

Fig.4.6.2B:Scanning electron micrograph of F1 Glucan with 1000X magnification & bar size of 10µm.

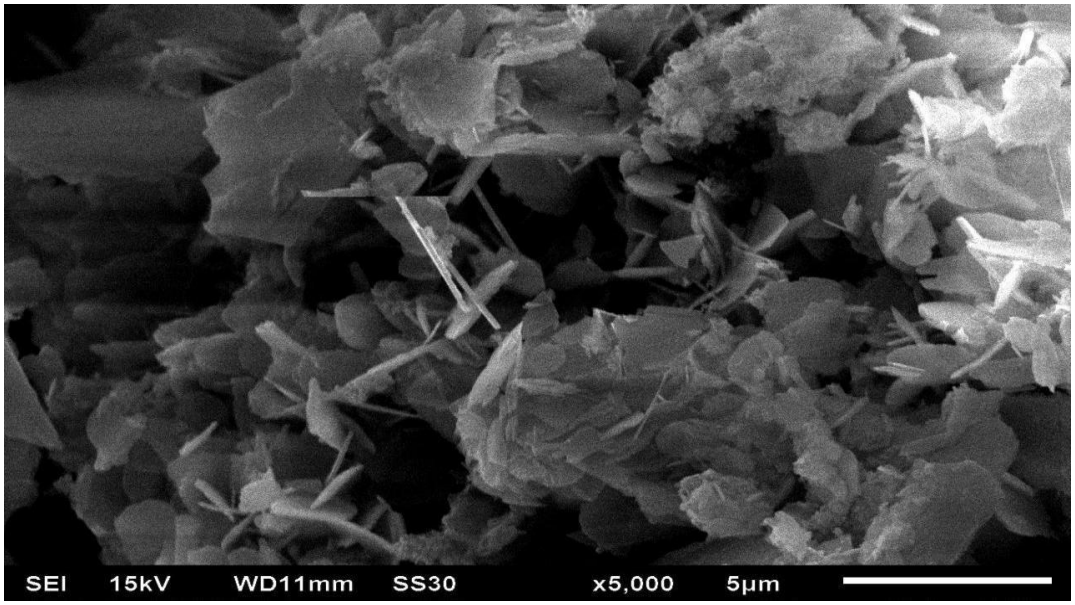

Fig 4.6.2C: Scanning electron micrograph of C1 Glucan with 5000X magnification & bar size of 5µm

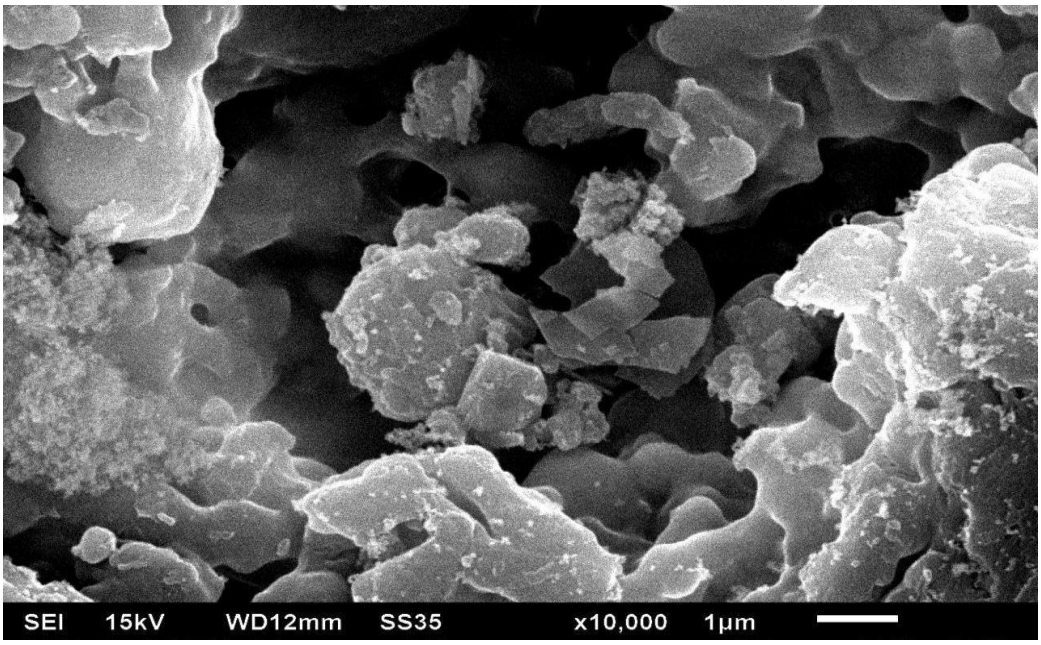

Fig.4.6.2D:Scanning electron micrograph of C1 Glucan with 10000X magnification & bar size of 1µm

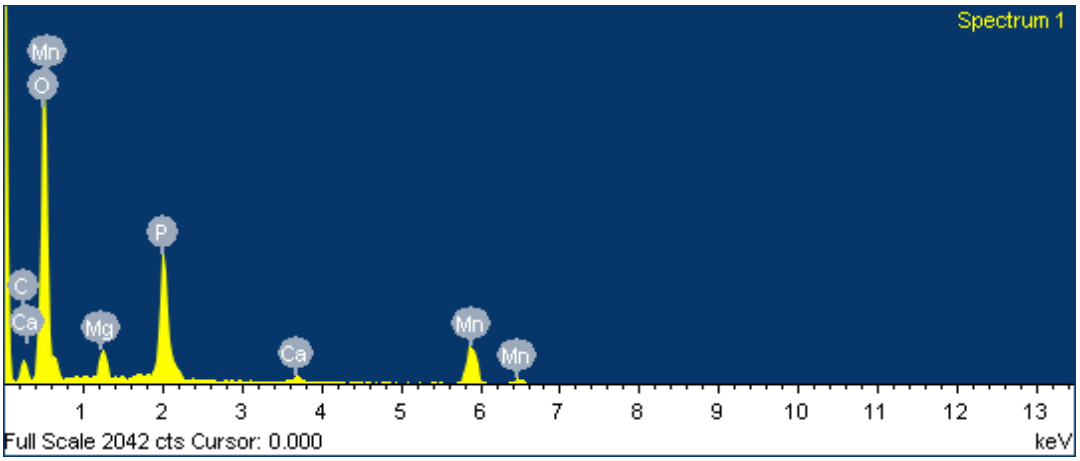

Fig.4.6.2E: EDS of F1 glucan

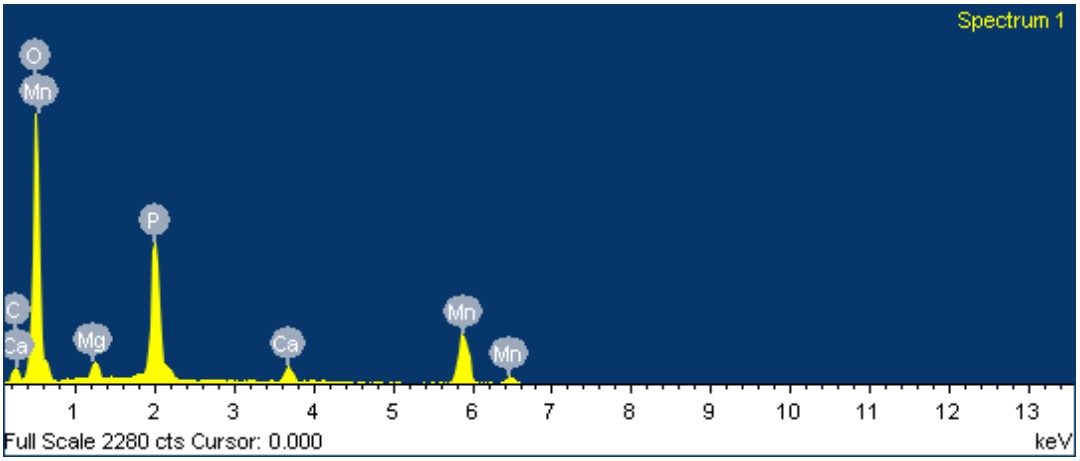

Fig.4.6.2F: EDS of C1 glucan

4.6.3 Fourier transformed infrared spectroscopy of Glucan C1:

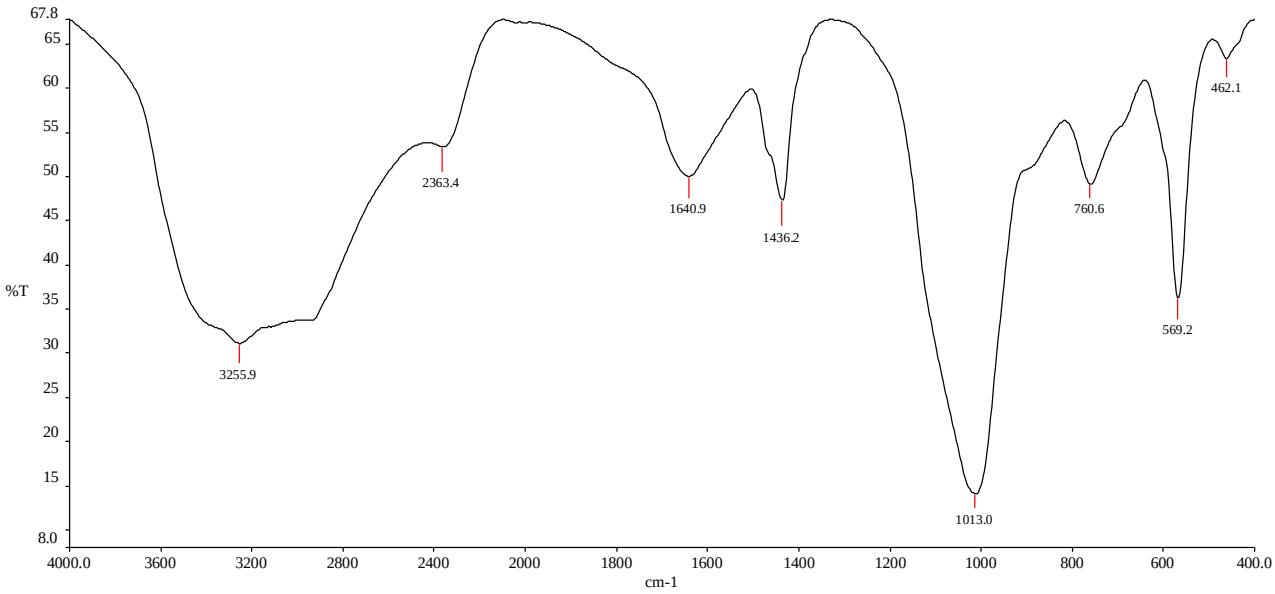

Fig.4.6.3: Fourier transformed infrared spectroscopy spectra of Glucan C1

FTIR spectroscopy was used to analyze the functional groups present in both the β-glucans. The FTIR spectra obtained was between the wavelength (cm^{-1}) and the transmission percentage. The presence of O-H stretching vibration is due to presence of alcohols and phenols at wavelength 32559.The stretching vibration is due to the presence of alkynes (-C≡C-) at 2363.The stretching vibration is observed due to the presence of alkenes(C=C) at 1640.The stretching vibration is due to the presence of aromatics (-C-C-) at 1436.2.The stretching vibration is observed at 1013.0 due the presence of alcohols, carboxylic acids, esters and ethers. N-H stretching vibration is observed due to the presence of primary, secondary amines at wavelength 760 cm^{-1} .The stretching is observed due to the presence of alkyl halides(C-Br) at 560 cm^{-1}

4.6.4 Fourier transformed infrared spectroscopy of Glucan F1:

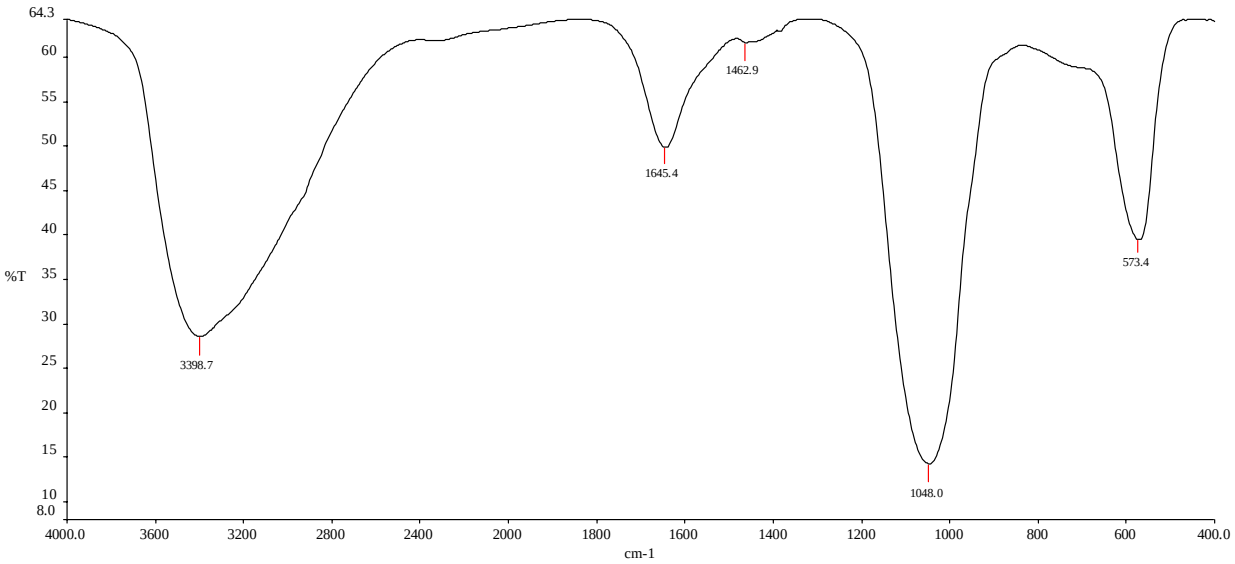

Fig.4.6.4: Fourier transformed infrared spectroscopy spectra of Glucan F1

The FTIR spectra obtained was between the wavelength (cm^{-1}) and the transmission percentage. The presence of O-H stretching vibration is due to presence of alcohols and phenols at wavelength 3298 cm^{-1} .The stretching vibration is observed due to the presence of alkenes(C=C) at 1645.4 cm^{-1} .C-H bond stretching is observed to the presence of alkenes at wavelength 1462.9.At wavelength 1048 cm^{-1} ,the stretching vibration is observed due to the presence of alcohols, carboxylic acids, esters and ethers. The stretching vibration is due to the presence of alkyl halides(C-Br) at wavelength 573.4.These results confirm the polysaccharide nature of the β-glucans and was in congruence to previous reports on FTIR analysis of β-glucans from microorganisms(Zhu *et al.*,2015)

4.6.5 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. Exopolysaccharides were subjected to DLS.Fig.4.6.5 and Fig.4.6.5A depicts the diameter of the polysaccharides. 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 C1 and F1 was found to be 0.29nm and 0.51nm, whereas, the diameter of standard β-glucan was found to be of 1.099nm.Origin pro 9.0 was used to plot the graph. Small size may render suitability especially in pharmacological or as nutritional formulations.

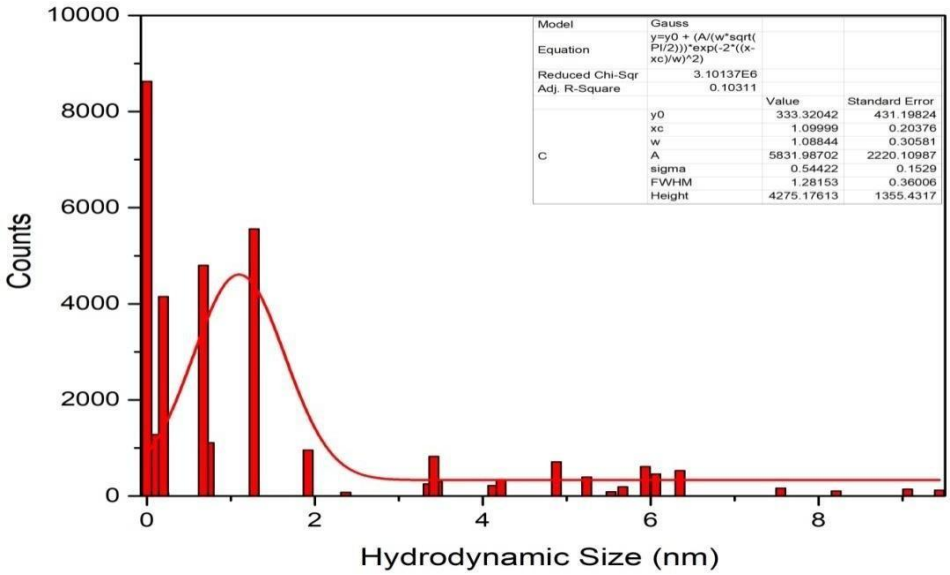

Fig.4.6.5: DLS of standard Glucan.

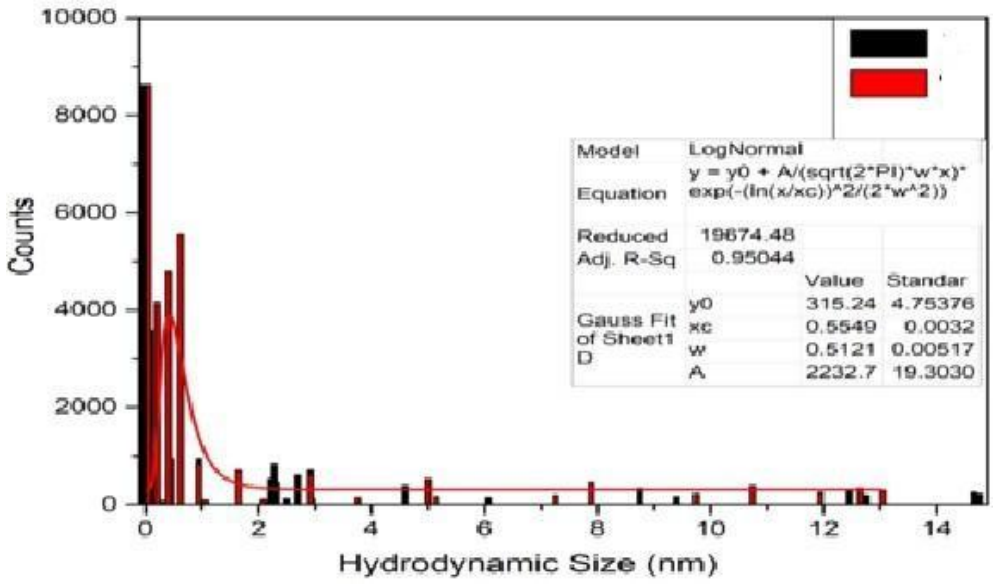

Fig 4.6.5A: DLS of Glucan from faeces isolate( ) and curd isolate( ).

4.6.6 Thermogravimetric Analysis:

Thermogravimetric analysis (TGA) is carried out to investigate the thermal performance of materials and is an important attribute for biopolymers. The thermogravimetric analysis relies on a high degree of precision in three measurements: weight, temperature, and weight change with temperature. The feature of this analysis is to determine degradation temperatures, the level of inorganic and organic components in materials, decomposition peaks temperature and residues. The polysaccharide degraded at around 625°C and continued to degrade till the temperature 850°C for the sample F1.For the sample C1, the polysaccharide degraded at around 400°C and continued to decrease at 650°C.The graph showed a sudden decrease of weight and temperature at 850°C.The TGA results clearly demonstrate the stability of both β-glucans at high temperature indicating the feasibility of application in commercial processes such as spray drying. The thermal stability may be attributed to the cross-linking in structure offering mechanical endurance.

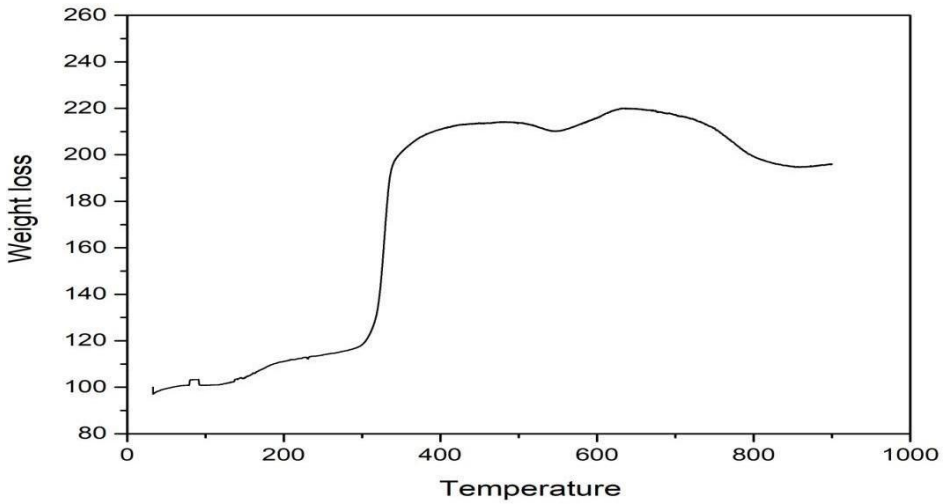

Fig.4.6.6: TGA of Glucan from curd isolate (C1)

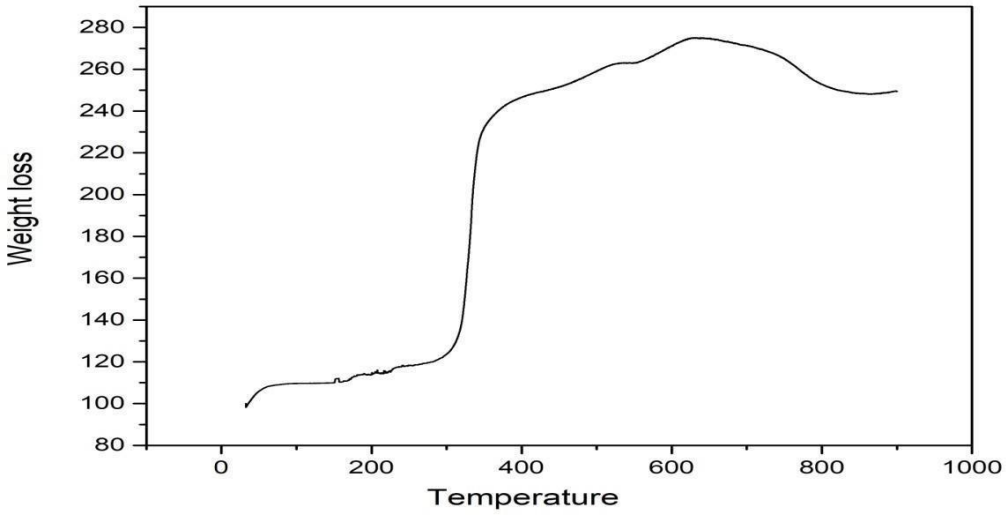

Fig.4.6.6A: TGA of Glucan from faeces isolate (F1)

4.6.7 HPLC analysis of β-Glucan:

HPLC data revealed the exopolysaccharide content of β-glucan from both the samples C1, F1 in comparison to the standard β-glucan. The β-glucan from the sample C1 was found to be at retention time 5 with area (85.84) and concentration 0.4×10^{-3}. The β-glucan from the sample F1 was found to be at retention time 5.213 with area (9.56)and concentration 0.4×10^{-3},Whereas the standard β-glucan was shown at retention time 4.527 with area(70.33) and concentration was found to be 0.4×10^{-3}. HPLC has been extensively used for determining and validating β-glucans from various sources. Based on the retention time of the standard β-glucan, a similarity in structure can be inferred. The slight difference is attributable to the bacterial source of the β-glucans while the standard β-glucan is from yeast. Such differences exist amongst β-glucans from plants (oats, barley etc.) and microorganism sources as well. Being soluble and able to sustain gastrointestinal transit as observed should enable the β-glucans apt for fermentation in the large intestine and help enable proliferation of beneficial microbiota.

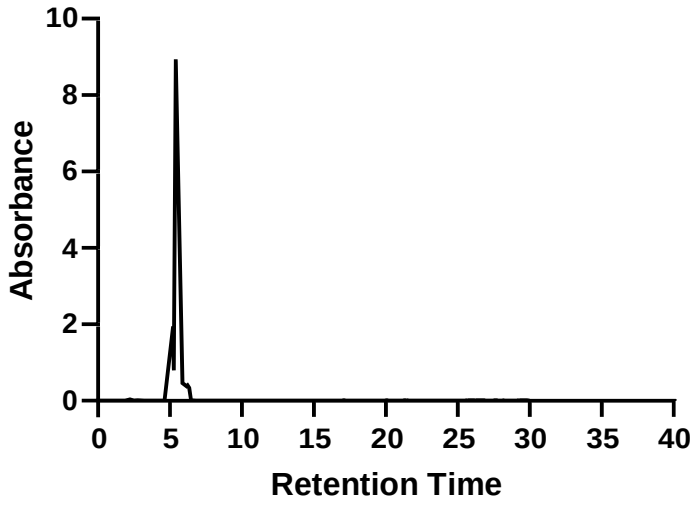

Fig.4.6.7: HPLC for β-glucan producing bacterial isolate C1.

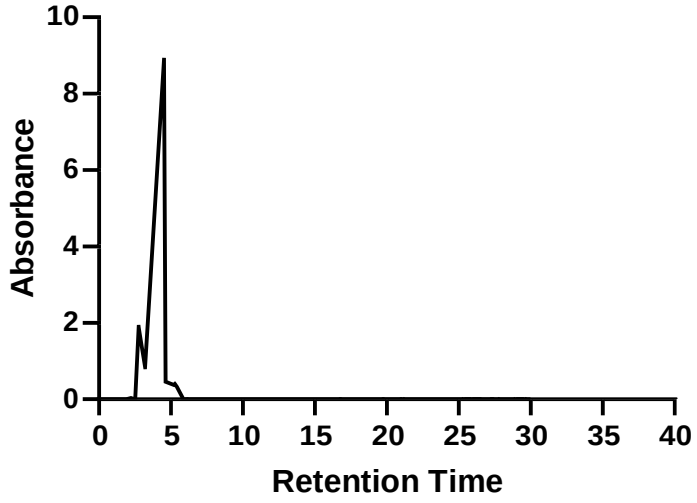

Fig.4.6.7.1: HPLC for β-glucan bacterial producing isolate F1

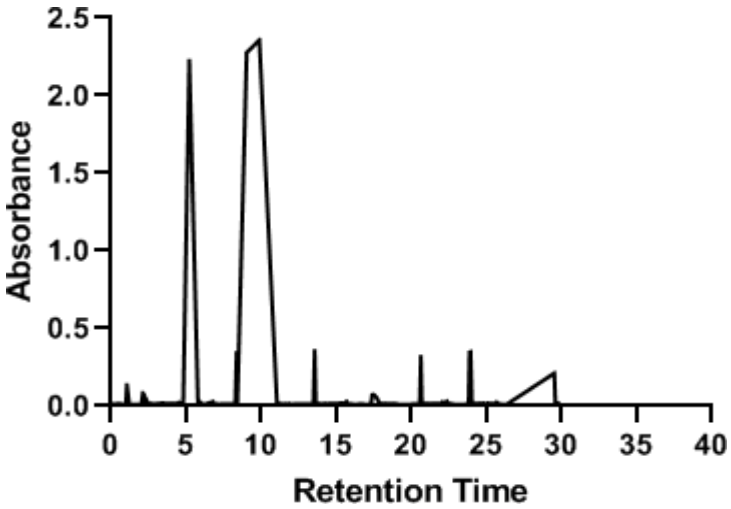

Fig.4.6.7.2: HPLC for standard β-glucan

4.6.8 Cholesterol lowering effect of β-Glucan:

Table 5: Magnitude of cholesterol reduction in two different samples.

Concentration of β-glucan(μg mL^{-1})	Reduction(%)of Cholesterol C1( )	Reduction(%)of Cholesterol F1( )
100 μg mL^{-1}	90.0	87.2
200 μg mL^{-1}	89.0	89.0
300 μg mL^{-1}	85.5	90.5
400 μg mL^{-1}	84.1	90.4
500 μg mL^{-1}	82.4	93.0

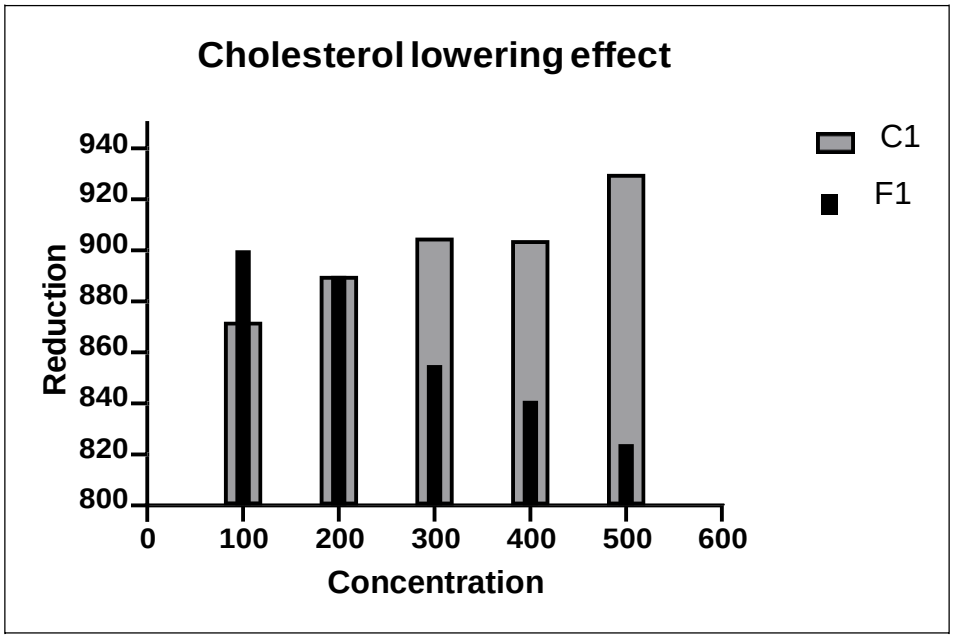

Fig.4.6.8: Cholesterol lowering effect of β-glucan produced by isolates C1 and F1

Figure.4.6.8 depicts the ability of the extracted β-glucan from two different sources for lowering cholesterol levels; cholesterol lowering effect of β-glucan from source C1 at concentration 100µg mL^{-1} resulting 90.0% in comparison to β-glucan from source F1 which showed 87.2% and the activity of β-glucan from F1 increased at a concentration of 500µg mL^{-1} at which the cholesterol lowering activities was 93%. However increasing concentrations further did not result in notable reduction. Hypocholesterolemic property of β-glucan has been extensively reported especially for those obtained from yeasts mushrooms, oats other soluble dietary fibers. It is possible that the polysaccharides are able to bind cholesterol molecules effectively leading to their removal (Sima *et al*., 2018) as observed. Few studies have investigated the glucans produced from microorganisms as potential cholesterol lowering agents. It was thought relevant at this stage to evaluate the effects of gastric passage and therefore a simulated trial was performed using artificial gastric juice. The results clearly indicate that β-glucan following exposure to gastric juice was able to reduce cholesterol without significant ($p>0.05$) loss of activity.

Table.6: Cholesterol lowering effect of β-glucan produced by isolates C1 and F1following exposure to artificial gastric juice.

β-glucan producing strains (5ooug/ml was used in both cases)	Cholesterol reduction by unexposed β-glucan (%)	Cholesterol reduction by gastric juice exposed β-glucan(%)
C1	82.2%	80.1%
F1	93%	91.2%

4.6.9 Anti-microbial activity of β-glucan:

The anti-microbial activity of β-glucan was tested against two plant pathogens including bacteria and fungi as well as common bacterial pathogens. The β-glucan from F1 inhibited plant spoilage fungi at concentration 50µL with mean inhibition zone of 40 mm in comparison with β-glucan from C1 36 mm while at minimal concentration of 10µL the inhibition zone was 13 and 17 for β-glucan from F1 and C1, respectively (Table.7A).

Table.7A:Anti-microbial activity of β-glucan against plant spoilage fungi.

Concentrations(µl)	Mean inhibition zone of β-glucan from F1(mm)	Mean inhibition zone of β-glucan from C1(mm)
10	17	13
20	21	16
30	25	20
40	33	24
50	40	36

Table.7 B: Anti-microbial activity of β-glucan against gram positive and gram negative bacteria.

Concentration Of β-glucan(µ)	Zone of inhibition of β-glucan F1 in mm	Zone of inhibition of β-glucan C1 in mm	Indicator microorganisms
50	22	24	*Staphylococcus aureus ATCC 9144*

50	14	16	*Salmonella typhimuriumATCC19585*
50	15	13	*Shigella dysenteraie2a*
50	21	19	*Listeria monocytogenes ATCC19111*
50	19	17	*Erwinia herbicola*

The antibacterial activities of β-glucans for instance laminarin obtained from marine microorganisms have been reported. The potential inhibitory activity has been described to interference in cell wall components (peptidoglycan) during growth in gram positive bacteria leading to death. In a similar fashion, cell wall of gram negative bacteria can be damaged by sulfated β-glucan (Chamidah et al. 2017). Fungal cell walls of the other hand are known to be comprised of β-glucans. The observation in our case could possibly mean that the bacterial β-glucans compete for cell wall synthesis by the β-1,3 glucan synthase leading ultimately to damage and inhibition. However this proposition requires further study relevant for designing antifungal targets.

4.6.10 GRAS and probiotic properties of β-glucan producing bacterial isolates:

Applicability of β-glucan producing bacterial strains or their production is anticipated to have technological relevance if the strains belong to GRAS category or have probiotic attributes. A series of tests established by the WHO FAO working committee (2004) was therefore used. The results of phenol tolerance indicated no significant alteration in survival following exposure for 24 hours suggesting the ability of the strains to be capable under a similar condition in the gut. Both strains exhibited a bsh positive zone (white) around the plates supplemented with 0.5% ox bile indicating their ability to produce bile salt hydrolase. The BATH test revealed hydrophobicity values of 66.4% and 65.6 % respectively. These values were in agreement with reports from other workers where Lactic acid bacteria and or other bacteria possessed similar values. The hydrophobic nature is a positive indication for binding to gut mucosal wall by probiotic bacteria and thus an important parameter. The antibiotic susceptibility tests indicated

susceptibility to Streptomycin, Gentamycin, Vancomycin, Penicillin, Ampicillin, Tetracycline, Oxacillin and Ciprofloxacin. Both strains were resistant to Chloramphenicol and Erythromycin. Antibiotic resistance is an important consideration for probiotic cultures since probiotics may serve as reservoir for antibiotic resistant genes. The findings of antibiotic susceptibility for the isolates were similar to those reported by Lee *et al*.,(2001).

In conclusion, two bacterial isolates capable of extracellular elaboration of β-glucans were characterized from curd and faeces of newborn. The β-glucan produced had high yields at 48 hours, pH 7.2 and temperature of 37^0C. Both β-glucan were purified and examined for structure and other properties by FTIR, DLC, SEM and HPLC. The yeast β-glucan was used a control in all cases. The results were in accordance to those reported for microbially produced β-glucans. The β-glucans possessed antimicrobial function as well as notable cholesterol lowering effect in vitro. Moreover, the functionality, at least the cholesterol reducing property remained unaltered upon exposure to artificial gastric juice signifying the safety during GI tract transit upon consumption. These indicated technological applicability of the β-glucans. Both β-glucan producing bacterial isolates confirmed to characteristics set forth for probiotic microorganisms and of GRAS(Generally Recognized as Safe) category. Further studies aimed in elucidating functional properties of these β-glucans will be important in designing products essential for human wellness or therapeutics.

Chapter 5

Conclusions

The present study demonstrated the β-glucan production ability of two bacterial isolates originating from curd and faeces of newborn. The β-glucan was produced extracellularly and at mid to late log phases of growth. The optimal pH, temperature and yield of the β-glucan was examined and purified β-glucan characterized using FTIR, HPLC, DLS and SEM. The analytical results indicated authenticity of a β-glucan. β-glucan from both isolates possessed notable capacity to remove cholesterol in vitro as well antimicrobial properties against common gram positive and gram negative bacterial pathogens. The intrinsic functionalities of both β-glucans were not affected following exposure to artificial gastric juice indicating their stability upon gastric transit. The bacterial isolates conformed to standard tests pertaining to GRAS or probiotic cultures suggesting safety.

Overall, the β-glucan molecules characterized in this study should provide interesting leads for further work regarding their technological aspects and application.

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