DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATION OF EXOPOLYSACCHARIDES PRODUCTION IN SUBMERGED AND SOLID STATE FERMENTATIONS

by

Seda ILGIN

December, 2016
İZMİR

INVESTIGATION OF EXOPOLYSACCHARIDES PRODUCTION IN SUBMERGED AND SOLID STATE FERMENTATIONS

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Seda ILGIN

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"INVESTIGATION OF EXOPOLYSACCHARIDES PRODUCTION IN SUBMERGED AND SOLID STATE FERMENTATIONS"** completed by **SEDA ILGIN** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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INVESTIGATION OF EXOPOLYSACCHARIDES PRODUCTION IN SUBMERGED AND SOLID STATE FERMENTATIONS

ABSTRACT

Exopolysaccharides (EPSs) are macromolecular carbohydrates synthesized by various organisms, predominantly microorganisms. EPS productions by *Pleurotus* spp. were investigated in submerged fermentation (SmF). The best EPS producer and its incubation period were determined as *Pleurotus sajor caju* and on 5 days, respectively. Then culture conditions for EPS production by *P.sajor caju* in SmF were optimized. The optimal initial pH, temperature and agitation rate of culture were determined as 5.0, 25 degree Celsius and 150 rpm, respectively. The optimized medium composition was as follows (in per liter): 90 g of glucose, 10 g of yeast extract, 10 g of peptone, 100 mM of magnesium sulphate. Under the optimized culture conditions, the maximum concentration of EPS was determined as 33.32g in per liter. Maximum EPS production by solid state fermentation used wet potato peel as a substrate was determined as 0.82 g in per liter. After isolation of EPS from optimized submerged culture conditions, one EPS fraction was obtained by gel filtration chromatography. FT-IR spectra showed bands characteristic of C-H, C-O-C and C-H-O groups that are present in the structure of partially purified EPS. TLC analysis determined that the partial purified EPS was mainly composed of glucose. The proton NMR spectrum of the partial purified EPS was supported this data. Furthermore, TGA indicated that the degradation temperature of the partial purified EPS was 276.91degree Celsius. Finally, hydroxyl, superoxide and DPPH radical scavenging activities of the partial purified EPS fraction were also investigated.

Keywords: Fermentation optimization, *Pleurotus sajor caju*, EPS production, partial purification and characterization, antioxidant activity.

DERİN KÜLTÜR VE KATI HAL FERMENTASYONLARIYLA EKZOPOLİSAKKARİT ÜRETİMİNİN İNCELENMESİ

ÖZ

Ekzopolisakkaritler (EPS), çeşitli organizmalar özellikle mikroorganizmalar tarafından sentezlenen makromoleküler karbohidratlardır. *Pleurotus* spp. türleri tarafından EPS üretimi derin kültür fermentasyonu ile incelendi. En iyi üretici ve onun inkübasyon periyodu sırasıyla *Pleurotus sajor caju* ve 5. gün olarak belirlendi. Ardından SMF de *P. sajor caju* tarafından EPS üretim koşulları optimize edildi. Optimal başlangıç pH, sıcaklık ve karıştırma hızı sırasıyla 5; 25 derece Celcius ve 150 rpm olarak belirlendi. Optimize edilen ortam bileşenleri (litrede); 90 g glukoz, 10 g maya ekstraktı, 10 g pepton, 100 mM magnezyum sülfat. Optimize kültür koşulları altında maksimum EPS miktarı litrede 33,32g elde edildi. Yaş patates kabuğunun substrat olarak kullanıldığı katı hal fermentasyonu ile maksimum EPS üretimi litrede 0,82 g olarak belirlendi. Optimize derin kültür koşullarından EPS'nin izolasyonunun ardından jel filtrasyon kromotografisi ile bir EPS fraksiyonu elde edildi. FT-IR spektrumu ile kısmi saflaştırılan EPS'nin yapısında mevcut olan karakteristik C-H, C-O-C ve C-H-O grup bantları gösterildi. TLC analizi ile kımi saflaştırılan EPS'nin ağırlıklı olarak glukozdan oluştuğu belirlendi. Kısmi saflaştırılan EPS'nin proton NMR spektrumu, bu bilgileri destekledi. Ayrıca, kısmi saflaştırılan EPS'nin degradasyon sıcaklığı TGA da 276,91 derece Celcius olarak belirlendi. Son olarak, kısmi saflaştırılan EPS fraksiyonun hidroksil, süperoksit ve DPPH radikal sönümleme aktiviteleri de incelendi.

Anahtar kelimeler: Fermentasyon optimizasyonu, *Pleurotus sajor caju*, EPS üretimi, kısmi saflaştırma ve karakterizasyon, antioksidan aktivite.

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CHAPTER ONE
INTRODUCTION

1.1 General Information of Microbial Biopolymers

The term “biopolymers” generally identifies polymers produced in a natural way by living organisms (Sutherland, 1996). Historically, biopolymers have been mainly used by people as food, or for making clothing and furniture. Since the industrial time, fossil fuels such as oil are the largest source in the development and manufacture of almost every commercial product, such as the plastic, which is used at a very wide scale. But these fuels are not limitless resources, and environmental concerns general aspects of using fossil fuels for production and energy must be taken into account. We must act in a sustainable way, which means that the resources must be consumed at a rate such that they can be organized by natural cycles of our planet. Therefore, at present, the renewable nature of biopolymers causes them a rebirth and a new attention. In the last 40 years, this attention in sustainable products has driven the development of new biopolymers from renewable feedstocks. Biopolymers have to compete with polymers produced by fossil fuel not only because of their functional properties but also in terms of cost. In this regard, biopolymers are competitive when the price of oil is high and the price of feedstocks, such as starch from corn, is low.

Biopolymers are a macromolecule in a living that is formed by linking together several smaller molecules (Sutherland, 2002). The synthesis of biopolymers involves enzyme, chain growth polymerization reactions of activated monomers, which are typically formed within cells by a complex metabolic process. They contain various carbohydrates, proteins, oils and fats (lipids), and nucleic acids (Figure 1.1).

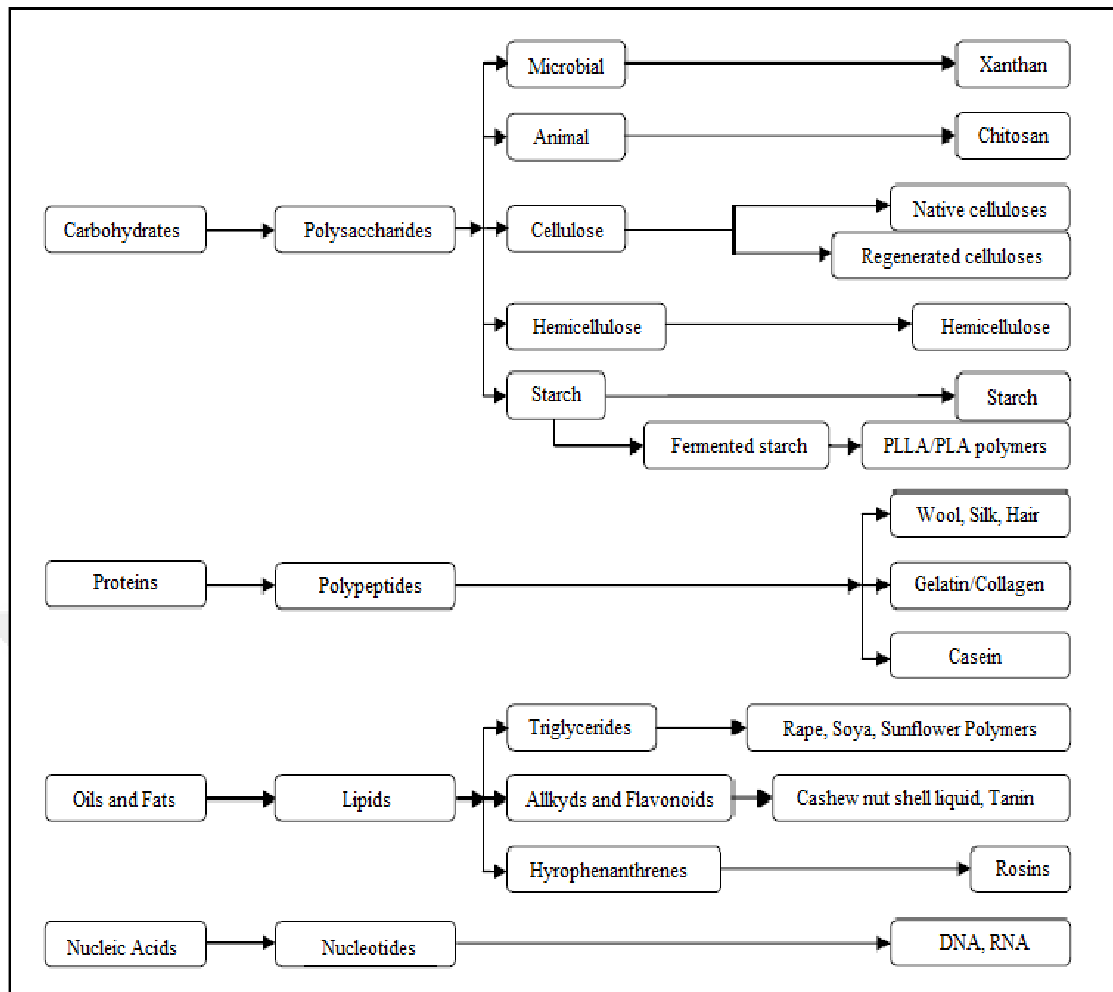


Figure 1.1 Categorization of naturally occurring biopolymers (Johnson, Mwaikambo, &Tucker, 2003).

Carbohydrates are naturally occurring and the most important building blocks of the biosphere. These evolutionary and biologically significant organic materials are present on Earth in various forms. Traditionally, on the basis of the number of sugar units, carbohydrates are classified into three groups: monosaccharides, oligosaccharides, and polysaccharides (Mahapatra, & Banerjee, 2013). Monosaccharides are the most basic units of biologically significant carbohydrates. Although glucose is the most common monosaccharide, other simple sugars (galactose, fructose, and mannose) are also found in living organisms. The condensation reaction of two monosaccharides, which includes the elimination of water, from the functional groups gives a disaccharide. Sucrose, maltose and lactose are common disaccharides. Polysaccharides are biopolymers, composed of monosaccharides linked together through glycosidic bonds. These structures can be linear or include branched side chains. Polysaccharides have a general formula of

$C_x(H_2O)_y$ where x is usually a large number between 200 and 2500. Considering that the repeating units in the polymer backbone are often six-carbon monosaccharides, the general formula can also be represented as $(C_6H_{10}O_5)_n$ where $40 \leq n \leq 3000$ (Zong, Cao, & Wang, 2012).

Polysaccharides derived from different sources and by different chemical manipulations exist in a variety of chemical compositions, molecular weights and structures. The polysaccharides possess various physicochemical properties such as gelation, solubility and low osmotic effect depending on their composition and architecture (Chandra, & Rustgi, 1998).Many microorganisms synthesize extracellular polysaccharides with commercially significant physiological and therapeutic activities. Recently, much interest has been considered to the microbial exopolysaccharides due to their numerous health benefits.

Polysaccharide biosynthesis and accumulation usually take place after the growth phase of microorganism. The polysaccharides produced by microorganisms can be classified into three main groups according to their location in the cell: (1) cytosolic polysaccharides, which provide a carbon and energy source for the cell; (2) polysaccharides that make up the cell wall, including peptidoglycans, teichoic acids and lipopolysaccharides and (3) polysaccharides that are emitted into the extracellular environment in the form of capsules or slime, known as exopolysaccharides (EPSs) (Donot, Fontana, Baccou, & Schorr-Galindo, 2012).

1.2 Exopolysaccharides

EPS term was first used by Sutherland (1972) to identify high molecular weight carbohydrate biopolymers produced by many marine bacteria. Since that time, EPS also has been used to indicate more generally described extracellular polymeric substances (Nichols, Bowman, & Guezennec, 2005).

Many microorganisms synthesize EPS that either attached to the cell surface or are found in the extracellular medium in the form of amorphous slime. EPSs help the

cell in various functions. EPSs protect against biotic stress as competition, and abiotic stresses that might contain nutrients limitation, temperature, light intensity or pH. For example, in the events of acidophilic or thermophilic species and *Archaea*, EPSs help in adapting to extreme conditions (Antón, Meseguer, & Rodriguez-Valera, 1988).

EPSs are water soluble polymers and may be ionic or non-ionic. The repeating units of these EPSs are very regular, branched or unbranched, and are connected by glycosidic linkages (Beshay, Daba, & Gohar, 2009). These structures are often linear, but may involve various degrees of branching. There are two types of polysaccharides:

- Homopolysaccharides (e.g., cellulose, dextran, levan)
- Heteropolysaccharides (e.g., xanthan, gellan)

Homopolysaccharides compose of one monosaccharide and are usually produced in large amount from sugars by the action of glycansucrases e.g. dextran, levan, alternan etc. The sample forms of branched and unbranched homopolysaccharides are given respectively, in Figure 1.2.

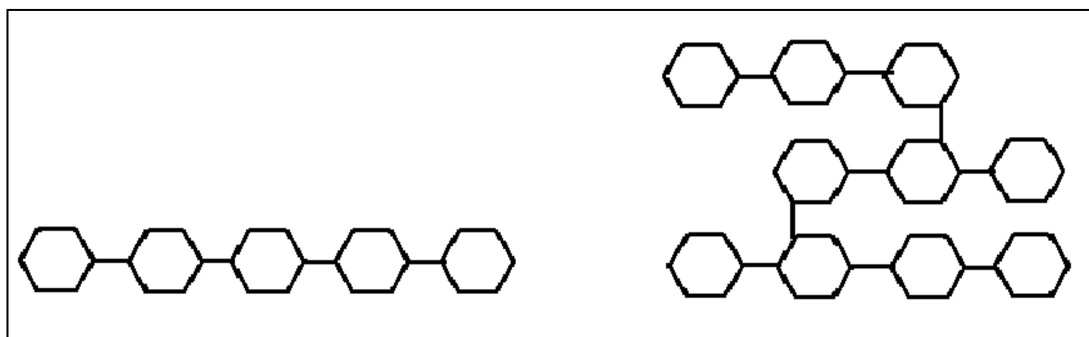


Figure 1.2 Unbranched and branched form of homopolysaccharides (Beshay, Daba, & Gohar, 2009)

On the other hand, heteropolysaccharides are mostly composed of identical repeating units consisting of two or more monosaccharides such as galactose, glucose and fructose. Several linkages can occur at the same time in one

polysaccharide. Sugar nucleotides act an essential role in the synthesis of heteropolysaccharides due to their function in sugar inter-conversions as well as sugar activation (Jaiswal, Sharma, Sanodiya, & Bisen, 2014). Both unbranched and branched forms of heteropolysaccharides, different colors represent different monosaccharides, are shown in Figure 1.3.

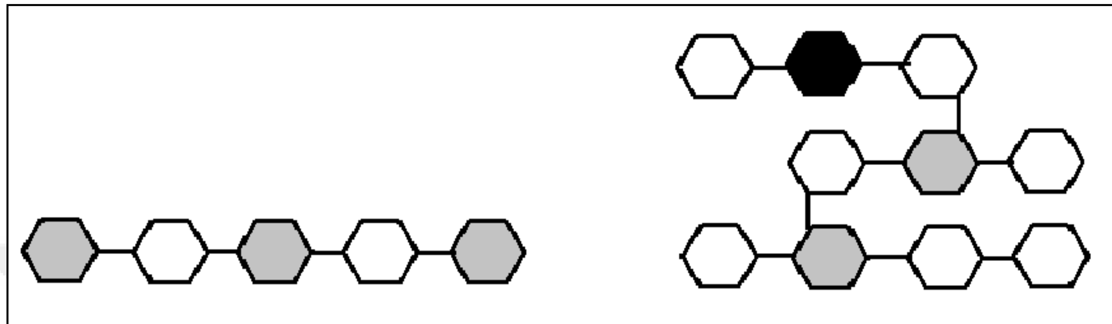


Figure 1.3 Unbranched and branched form of heteropolysaccharides (Beshay, Daba, & Gohar, 2009)

1.3 EPS Production Process

Fermentation is a versatile process technology for producing value added products such as microbial biopolymers and since fermentation parameters have a high impact upon the viability and economics of the bioprocess. Especially, microbial polysaccharide production is greatly influenced by fermentation conditions such as pH, temperature, oxygen concentration and agitation rate as well as by the composition of the culture medium (Nicolaus, Kambourova, & Toksoy Öner, 2010; Sutherland, 2007). Moreover, besides the fermentation conditions, the chemical structure, monomer composition, and physicochemical and rheological properties of the final product also change with the type of strain.

1.3.1 EPS Production in Submerged Fermentation

Submerged Fermentation (SmF) evaluates free flowing liquid substrates like broths. The bioactive compounds produced by microorganism are secreted into the fermentation broth. The substrates are evaluated quite rapidly; hence need to be constantly supplemented with nutrients. SmF is best suited for microorganisms such

as bacteria that require high moisture content. Besides, in SmF purification of products and measure of process parameters like pH, temperature, oxygen concentration is easier. On the other hand, the disadvantages of SmF are large reactor need, high cost and contamination risk (Couto, & Sanromán, 2006; Subramaniyam, & Vimala, 2012).

EPS production are batch, fed-batch or continuous processes depending on the microbial process used. Some examples of industrial cultivation process for EPS production are shown in Table 1.1. Fed-batch fermentations may be preferable to the use of high initial sugar concentrations. After fermentation of the culture broth, recovery by precipitation with isopropanol is followed by drying and grinding to yield a fine powder. Filtration or centrifugation and other downstream processing add to the final cost (Sutherland, 2002).

Table 1.1Industrial productions of principal exopolysaccharides (Mironescu, 2003)

Exopolysaccharide	Cultivation System	Cultivation Conditions
Alginate	Continuous	30°C; pH 7.2; Aeration; Agitation
Dextran	Batch or feedbatch	25°C; pH 6.0; No aeration in the phase of EPS stimulation.
Xanthan	Batch	28°C; pH 7.0; Aeration

1.3.2 EPS Production in Solid State Fermentation

Solid State Fermentation (SSF) utilizes solid substrates, such as bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and stably, so the same substrate can be used for long fermentation periods. Accordingly, SSF supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content (Bhargav, Panda, Ali, & Javed, 2008). However, it

cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria (Babu, & Satyanarayana, 1996).

SSF is seldom used in polysaccharides production. SSF is defined as the fermentation type in which microorganisms grow on solid materials without the presence of free liquid (Bhargav et al., 2008). Many researchers make an effort polysaccharides production in SSF but they didn't succeed, because agitation rate is an essential factor on polysaccharide production.

1.3.3 Pleurotus spp.

Pleurotus species belong to the genus *Pleurotus* (Quel.) Fr., tribe Lentineae Fayod, family *Polyporaceae* (Fr.) Fr., and they are largely throughout the Northern Hemisphere, such as Europe, North Africa, Asia and North America (Singer, 1986). *Pleurotus* species are saprophytic, white rot fungi that are cultivated on substrates including lignin or cellulose like agro-industrial wastes. *Pleurotus* spesies secrete enzymes such as peroxidases, laccases, cellulases, hemicellulases and xylanases (Cohen, Persky, & Hadar, 2002).

The genus *Pleurotus* is widely cultivated and commercialized on the world. It involves many bioactive materials, such as polysaccharides, proteins, enzymes, and vitamins (Wang, Sakoda, & Suzuki, 2001). Recently, fungi derived polysaccharides have emerged as a significant class of bioactive compounds, two which unlimited medicinal, antioxidant and therapeutic properties have been attributed (Ooi, & Liu, 2000). The species of the genus *Pleurotus* produce large amounts of biomass and EPS in submerged cultures, which may be used for biotechnological areas. For instance, water soluble polysaccharide of *P. ostreatus* could be a potential immuno-stimulating agent for use in functional foods or medicine against both pathogens and cancer. However, EPS of *P. pulmonarius* showed in vitro antioxidant activity (Shen, Shi, & Xu, 2013). Likewise, EPS produced by *P. eryngii* observed that this EPS has been antioxidant and antitumour activities (Jing, Mao, Geng, & Xu, 2013).

During the recent years, attention of EPS produced by *Pleurotus* spp. in submerged cultures has been increased due to its antioxidant and antitumour activities.

1.4 Parameters Affecting EPS Production

EPS production from microorganisms (bacteria, fungi and algae) generally depends on the type of microbial strain used, physical conditions (such as pH, temperature and agitation rate) maintained during fermentation, and type of medium components applied for the production.

Among the EPS producers, bacteria and fungi are most common. Bacterial EPSs have been studied extensively (Freitas, Alves, & Reis, 2011). EPS production from fungi has been studied sufficiently over the last two decades. At present, a considerable number of fungi including higher basidiomycetes, lower filamentous fungi, and yeasts from different ecological niches were known for their ability to synthesize EPSs in laboratory culture conditions. Besides, many still remain uninvestigatedor under explored. The important genera include *Pleurotus*, *Auricularia*, *Flammulina*, *Grifola*, *Hericium*, *Lentinus*, and *Tramelle*. EPS producers are represented in Table 1.2.

Table 1.2 Major types of EPS and producer microorganisms (Donot, Fontana, Baccou, & Schorr-Galindo, 2012)

Homopolysaccharides	**Microorganisms**
Pullulan	*Aureobasidium pullulans*
Scleroglucan	*Slerotium sp.*
Schizophyllan	*Schizophyllum commune*
Heteropolysaccharides	**Microorganisms**
Xanthan	*Xanthomonas campestris*
Carrageenan	*Chondrus ocellatus*
Alginat	*Pseudomonas aerugina, Azobacter sp*
Hyaluronic acid	*Streptococcus sp.*

Carbon and nitrogen sources generally act a significant role in microorganisms, because these nutrients are directly linked to the cell proliferation and metabolite biosynthesis (Kim et al, 2003; Lopez et al., 2003). Also, the nature and concentration of the carbon source can regulate the secondary metabolism like catabolic repression (Görke, & Stülke, 2008).

As the carbon source is essential for energy and structural molecules, the intensity and the type of carbon source are important for all bioprocesses. The carbon source and its concentration have an effect on the yield of EPS. In general, glucose, sucrose, maltose, lactose, fructose, galactose, xylose, cellobiose, sorbitol, xylitol, and mannitol are used as carbon source in the culture medium (Mahapatra, & Banerjee, 2013). In most of the cases glucose, sucrose, and maltose have been selected as the most essential carbon sources for the production of fungal EPSs (Kim et al., 2005; Zhang, & Cheung, 2011; Gao, Wang, Su, Zhang, & Yang, 2012). These observations indicate that there may be some effects of catabolic repression of different carbon sources in various EPS synthesis, that different fungal strains have different carbon source uptake fascinations, or that these carbon sources may be easily metabolized by fungi.

The nitrogen source is another variable that is reported to induce EPS production. Both inorganic and organic nitrogen sources were tested by several investigators to find the suitable one. Among the organic sources peptone, yeast extract poly peptone, Soybean meal, and corn steep powder were tested mostly (Gao et al., 2012; Zou, 2005).From numerous studies, it was noticed that yeast extract and corn steep powder are good nitrogen source that induce EPS production from different fungal strains (Kim et al., 2002; Pavlova, Koleva, Kratchanova, & Panchev, 2004). Among the various inorganic sources ammonium chloride, ammonium sulfate, sodium nitrate, potassium nitrate, urea, and diammonium oxalate monohydrate are studied by scientists mostly (Farina, Sineriz, Molina, & Perotti, 1998; Sudhakaran, & Shewale, 1988; Schmid et al., 2001). Many findings indicated that in the presence of inorganic nitrogen sources, fungi produce less EPSs in comparison to organic nitrogen sources. Sutherland (2007) reported that EPS production mainly occurred in nitrogen limiting

conditions. Researchers suggested that for different fungal strains, different concentrations of selected nitrogen favored maximum EPS production. Except for a few reports, investigators found that in comparison to carbon sources very little nitrogen is required by fungi for EPS production and concentrations between 1–10 g/L is are sufficient (Yuan, Chi, & Zhang, 2012).

Another effective factor in EPS production by microorganism is ions and concentrations. Among the different additional ionic salts, many describe magnesium sulphate ($MgSO_4 \cdot 7H_2O$) as most suitable for EPS production by different fungal strains while in some cases calcium chloride ($CaCl_2$), sodium chloride (NaCl) are also needed by the fungi for optimal production (Mahapatra, & Banerjee, 2013; Lin, & Chen, 2007).

Physical conditions such as pH, temperature, and agitation rate are quite important in the yield of the EPS produced. Many microorganism produce EPS in media buffered at neutral pH (Dassy, Stringfellow, Lieb, & Fournier, 1991). The pH of culture medium is important factor that induce the EPS production (Wang, & McNeil, 1995). In general, fungi favored low pH for EPS production with a range between pH 3.0 to 6.5 (Feng, Li, Wu, Cheng, & Ma, 2010). A few fungi preferred neutral or alkaline pH for maximum EPS production (Pokhrel, & Ohga, 2007). In 2004, Shu and Lung studied the effects of pH on EPS production by *Antrodia camphorate* and indicated that variation in medium pH induces *A. camphorate* to produce EPS with different molecular weight (Mw) (Shu, & Lung, 2004). They noticed that relatively high Mw of EPSs in low amount was produced at lower medium pH while low Mw of EPS with high yield was recorded at higher medium pH.

Temperature affects biochemical reactions in microorganism cells such as EPS production. The EPS production by microorganisms can occur in a wide range of temperature. For example, scleroglucan production can occur in the range of 20-37°C by *Sclerotium glucanium* (Halleck, 1967). Many microorganisms produce maximal

amounts of EPS at temperatures slightly lower (e.g., 30°C) than optimal growth (37°C) as observed for *Klebsiella sp.* (Farres, Caminal, & Lopez-Santin, 1997).

Additionally, aeration and agitation are important factors as ease the oxygen transfer and enable nutrient transport to microorganisms. The presence of oxygen and the rate at which the culture medium is mixed might have direct effect on the production polysaccharide (Dassy et al., 1991) and in increase in EPS production often results from the increase in oxygen supply (Bayer, Eftekhar, Tu, Nast, & Speert, 1990; Suh, Herbst, Schumpe, & Deckwer, 1990). The EPS surrounding the cell may act as a barrier for nutrient and oxygen transfer, and mixing of the culture at high speeds may increase the availability of both nutrient and oxygen.

Viscous fermentation products such as EPS produced especially in the late stages of microbial growth result in severe oxygen limitation and limited mass transfer (McNeil, & Harvey, 1993). Although most reported EPS producing microorganisms are aerobic, it has been suggested that oxygen demand during the EPS production phase, which is not always growth associated, may be low. High oxygen-transfer rates may actually reduce the rate of pullulan formation by *Aureobasidium pullulans* (Wecker, & Onken, 1991), and oxygen limitation stimulated (1→3) β-D-glucan formation by *Fusarium solani* (Rau, Müller, Cordes, & Klein, 1990).

Carbon, nitrogen and oxygen limitations are factors affecting the conversion of the carbon source into polysaccharide. For instance, C, N and O limitations affected the conversion of glucose into alginate and proportion of mannuronate to guluronate residues in *Pseudomonas mendocina* (Sengha, Anderson, Hacking, & Dawes, 1989). EPS production is favored by a high carbon/nitrogen (C/N) ratio (Sutherland, 1983) and ratio of at least 10/1 is most favorable for optimal EPS yield. Culture conditions affecting the production of EPS vary from one microorganism to the other, obviously, but the only parameter which appears to increase the production of many microbial EPS is a medium with a high C/N ratio.

1.5 Recovery, Purification and Chemical Characterization of EPS

Recovery of microbial EPS is critical in determining their costs and their functional properties. The main steps to obtain EPS produced by fermentation are extraction, precipitation, and purification (Brown, & Lester, 1980). Vander Waal forces, electrostatic interactions, hydrogen bonds, hydrophobic interactions and covalent bonds such as disulfide bonds in glycoprotein, etc., are generally the main forces between EPS and the cell surface (Wingender, Neu, & Flemming, 1999).

There are numerous methods that have been developed for the extraction of EPS, each appropriate under different scenarios. Researchers suggested that methods have been reported to achieve the task of separating EPS from the cell (Comte, Guibaud, & Baudu, 2006) and they can be divided into three categories: physical methods, chemical methods, and a combination of the two, (Nielsen, & Jahn, 1999). Microbial EPSs are occurring in the form of capsules or slime. Extraction methods of EPS depend on form of EPS. 'Slime' EPS may be isolated from the producing microorganisms by centrifugation. The speed of centrifugation depends on the nature and viscosity of EPS (Sutherland, 1972). When working at the laboratory scale, ultracentrifugation may be used to remove of the microbial cells from culture broths (Roger, & Colonna, 1993). Generally, the physical methods use for extraction of Slime EPS.

'Capsular' EPS must first be removed from the cells. The selection of the method tends to nature of the association between the cells and EPS. Centrifugation, as described for the slime EPS, enables the separation of weakly associated capsular EPS (Glaudemans, & Treffers, 1967). When the capsular EPS is strongly associated to the cells, more severe conditions, such as alkaline treatment with alkali (e.g., NaOH) prior to centrifugation and alcohol precipitation (Cohen, & Johnstone, 1964) are needed. Other drastic methods, for example, dialysis, filtration, sonication (King & Forster, 1990), cation exchange (Frølund, Palmgren, Keiding, & Nielsen, 1996), and heating (Sheng, & Greenberg, 1990) were used. Chemical methods utilize chemical reagents, like ethylenediaminetetraacetic acid (EDTA), formaldehyde

(H_2CO), sodium hydroxide (NaOH), and ethanol (C_2H_5OH) to fulfill the objective of separating EPS from the cells (Liu, & Fang, 2002). In the literature, the chemical methods have been reported to yield more than physical methods; however, with higher probability of contamination from the reagent or cell lyses (Nielsen, & Jahn, 1999). Although physical methods usually yield less than chemical methods, the fact that they usually have minimal contamination and cell lyses also makes them effective approaches for EPS extraction (Comte et al., 2006).

Purification of EPS will remove various interference contaminants, like cell debris, protein, nucleic acid, ions, etc, for further composition and structure analysis. In general, the complication of isolation and purification methods is determined by the culture medium used for microorganism growth. Usually more than one method is used in order to achieve the highest recovery of EPS, elimination of the contamination and the least modification of EPS structure.

EPSs are mainly recovered by solvent precipitation of the fermentation broth (Sutherland, 1983). The polysaccharide can be precipitated from the supernatant by the addition of polar organic solvents miscible with water, like lower alcohols or acetone. The precipitation yield increases concomitantly with the increase of solvent volume. The EPS/solvent ratio is variable and one, two or three volumes of solvent are currently used, although two volumes are often used. Organic solvents permit separation by lowering the EPS solubility. They may also serve to decolorize and to extract low molecular mass fermentation products and medium components. When solvent precipitation is used to recover EPS, proteins and salts of the culture medium are also precipitated along with the wanted EPS.

There are many techniques available for analyzing and quantifying the components of EPS, including colorimetric methods (Lowry, Rosebrough, Farr, &Randall, 1951; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), total organic carbon (TOC), high performance size-exclusion chromatography (HPSEC) (Omoike & Chorover, 2004), gas chromatography-mass spectrometry (GC-

MS), ^{1}H-NMR and ^{13}C-NMR (Harding, & Hintikka,2003), high-performance liquid chromatography (HPLC) (Garrote, Bonet, Merino, Simon-Pujol, & Congregado, 1992), Thermo-gravimetry (TG) (Yao, Wu, Lei, Guo, & Xu, 2008) and thin layer chromatography (TLC) (Thombre, & Paraag, 2015). To quantify polysaccharides content in EPS, one traditional method is the phenol sulfuric acid (PSA) method (Dubois et al., 1956). Lowry et al. (1951), Bradford (1976), and Smith et al. (1985) methods are the typical approaches for bulk protein content quantification in polysaccharide.

1.6 Biotechnological Applications of EPS

The industrial use of polysaccharides was based until recently on materials extracted from plants (starch, cellulose, pectins, galactomanans, and gums) or algae (carragenan, alginates, and agar) (Ruas-Madiedo, & Reyes-Gavilán, 2005). Recently, biotechnologies for the microbial production of extracellular polysaccharides were improved. Microbial EPSs have gained significant from the last few decades as several studies showed different applications that not only indicate the alternative source of marketed plant or seaweed polysaccharides but also have some new and interesting bio-applicability (Sutherland, 2002). Furthermore, upstream and downstream processing of these EPSs are easier and one can produce a much larger amount in a shorter time when compared to plant or algal polysaccharide production.

While dextran was the first microbial polysaccharide to be commercialized and to receive consent for food use, several such polymers now have a variety of commercial uses. The achievement is because of their properties and to the diversity of producing microorganisms and synthesized polysaccharides.

Microbial polysaccharides are a diverse class of polymers that have obtained enormous importance in recent times due to their possible bioactive roles (Kim, Shim, Kim, & Jang, 1999), rheological properties (Sutherland, 2002), high stability (Wang, & McNeil, 1995) at wide range of temperature and because they possess

great applicability in cosmetic, food and pharmaceutical industries (Kumar, Mody, & Jha, 2007).

Due to their multiple and enhancing physicochemical and rheological properties, the microbial EPSs play as new biomaterials and find themselves broad areas of applications in many industries like food (Dlamini, Peiris, Bavor, & Kailasapathy, 2009), medicine (Miranda et al., 2008; Novak, Bryers, & Reichert, 2009) textiles (Fijan et al., 2009), detergents (Friedemann, Stallmach, & Karger, 2009), adhesives (Hoffmann et al., 2009), microbial enhanced oil recovery (MEOR) (Singh, Van Hamme, & Ward, 2007), wastewater treatment (Pant, & Adholeya, 2007), dredging (Yim, Kim, Ahn, & Lee, 2007), brewing (Alsteens et al., 2008), downstream processing (Maina, Tenkanen, Maaheimo, Juvonen, & Virkki, 2008), cosmetology (Im, Song, Kang, & Kang, 2009), and pharmacology (Yan et al., 2009; Kumar, Joo, Choi, Koo, & Chang, 2004).

EPS may function as thickeners, viscosifying agents, stabilizers, emulsifiers, gelling agents, or water-binding agents in food (Van Kranenburg, Boels, Kleerebezem, & de Vos, 1999). Large number of polysaccharides used in foods is of plant origin. Most of them are chemically or enzymatically modified in order to improve their rheological properties, e.g. cellulose, starch, pectin, alginate and carrageenan. EPSs produced by microorganism have unique rheological properties because of their capability of forming very viscous solutions at low concentrations and their pseudo plastic nature. Most of the EPSs used are employed because of their ability to thicken or to cause gel formation (Table 1.3).

Table 1.3 Exopolysaccharide properties used in food (Sutherland, 2002).

Function	Application
Adhesive	icing and glazes
binding agent	pet foods
Coating	Confectionery
emulsifying agent	salad dressings
Encapsulation	powdered flavours

Both microbial and non-microbial polysaccharides find a wide range of non-food industrial uses. In such usage, the polysaccharides may compete with synthetic organic polymers, but in some applications only natural products are acceptable because of biodegradability and lack of toxicity.

Surfactants and emulsifiers from bacterial sources create an attention because of their biodegradability and possible production from renewable resources. In the oil industry, usage of water-soluble biopolymers or microbial EPSs has importance among microbiological methods of oil extraction. Application of the EPSs seems to be ecologically proven (ecologically safe method). Table 1.4 shows the properties of EPSs for use in oil industry.

Table 1.4 Properties of EPSs for use in oil industry (Sutherland, 1990)

High viscosity in water
High shear stability
Pseudoplastic
Stable over wide pH range
Stable for sustained periods when exposed to high temperatures
Freedom from particulate material; high injectivity

On the other hand, one of the cosmetics uses for EPSs is in deodorant gels (Palaniraj, & Jayaraman, 2011). Gels are also used in the preparation of microbiological culture media. The traditional gelling agent for this is agar but it has the disadvantage of problems of supply and provides a gel which is not water clear.

Polysaccharides constitute a structurally diverse class of biological macromolecules with a wide range of physicochemical properties, which are the basis for the different applications in the broad field of pharmacy and medicine.

A more generally inclusive and constative name for the group that are abundant in nature, present in many plant sources are polysaccharide hydrocolloids; only a limited number is of commercial importance of substances. Functional properties of

polysaccharide hydrocolloids are; stabilizer (suspending agent), thickener (film-forming agent), coagulant (water retention agent), and colloid (lubricant or friction reducer) (Franz, 1989). Many interesting areas have been opened in the past which involve their role in drug delivery, in wound treatment, in cancer therapy, and the diagnosis and treatment of bacterial and viral diseases (Moscovici, 2015).

Within the field of pharmacologically active biopolymers, the area of immune-stimulating polysaccharides is a rather new and charming area of tumor therapy (Dumitriu, 2004). The attention in polysaccharides as antitumor substances came from the dissatisfaction with current cancer chemotherapy. Numerous numbers of chemical compounds have been tested as cytostatic agents with possibly a high specificity for the cancer cell. Figure 1.4 summarizes the correlation between the most associated polysaccharide properties with their main fields of application.

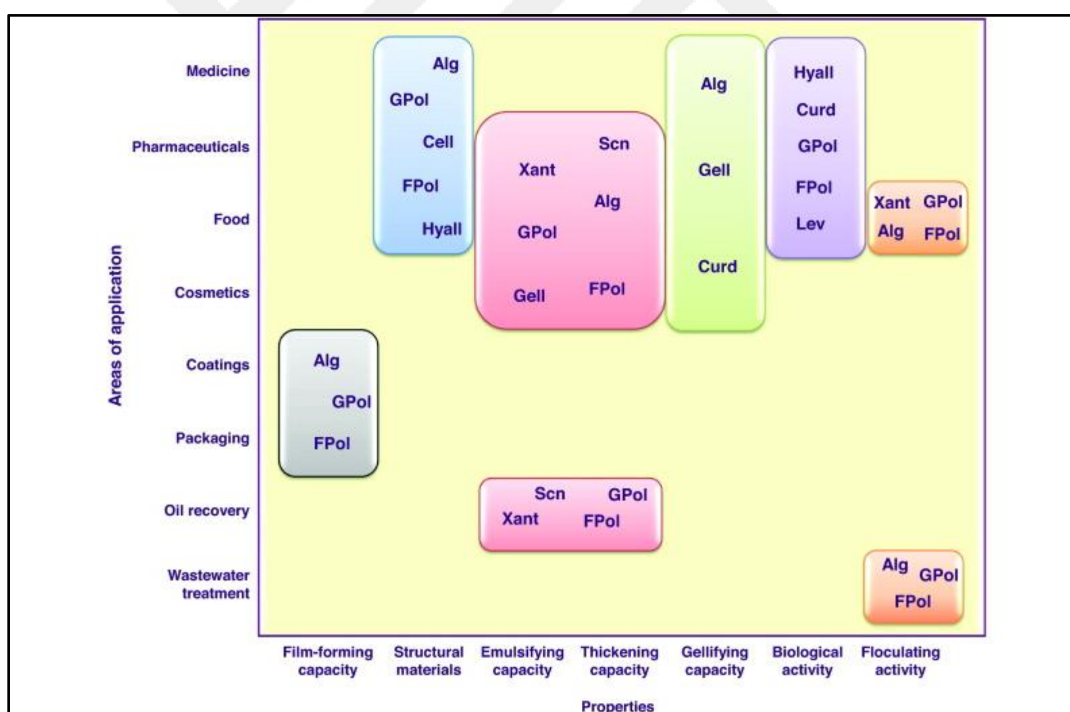


Figure 1.4 Diagram representing the correlation between the most associated polysaccharide properties with their main areas of application. Abbreviations: Alg, bacterial alginate; Curd, curdlan; FPol, FucoPol; Gell, gellan gum; GPol, GalactoPol; Hyall, hyaluronan; Lev, levan; Scn, succinoglycan; Xant, xanthan gum (Freitas, Alves, & Reis, 2011).

The aim of this study is EPS production with high productivity by white rot fungus *Pleurotus* spp. in SmF and SSF. The first step of this study was determination of the best producer strain and its incubation period. In the second step, effects of carbon and nitrogen sources, their concentration, Mg^{2+} ion concentrations, pH, temperature, and agitation rate were investigated to optimize EPS production conditions in SmF. In the third step of the study, for EPS production in SSF, three different solid substrates and optimum conditions in SmF were studied. After production at optimum condition in SmF, EPS was extracted and partial purified by Sepharose CL-6B column. In the last step of the study, partial purified EPS was further characterized by chromatographic (TLC), spectroscopic (FT-IR and NMR) and TGA analyses. Also antioxidant properties, such as OH^-, O_2^- and DPPH radical scavenging activities and reducing power and chelating activity of the partial purified EPS fraction were investigated.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganism

Four different *Pleurotus* strains were used in this experiment as follows: *P. djamor* (Rumph. Ex Fr.) *Boedijn* (MCC15), *P. ostreatus* (Jacq.) *Pleurotus Kumm.* (MCC16), *P. sajor caju* (Fr.) *Singer* (MCC29) and *P. eryngii* (DC.) *Gillet* (MCC58).

2.2 Methods

2.2.1 Microorganisms'Maintenance

P. djamor, P. ostreatus and *P. sajor caju* were grown on potato dextrose agar (PDA) (39 g/L, pH 5.6) at 25°C for 7 days while *P. eryngii* was grown on malt extract: peptone: agar (MPA) (30: 3: 15 g/L, pH 5.6) at 25°C for 14 days.

To prepare the medium, the contents of medium were stirred, then sterilized at 121°C for 20 minutes. After the medium was cooled, it was transferred to Petri dishes. When the medium solidified, inoculation was performed and incubated at 25°C then prepared stocks were maintained at 4°C, transferred every month to fresh medium.

2.2.2 Submerged Fermentation (SmF) Media

Pleurotus spp. were grown on PDA and MPA slants and then transferred to basal medium by 1cm^2 of the five agar plates with a sterilized cutter. Fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of basal medium. The composition of basal medium was as follows in (g/L): glucose, 10; NH_4NO_3, 0.724; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 1.0; KCl, 0.5; yeast extract, 0.5; $FeSO_4.7H_2O$, 0.001;

$ZnSO_4.7H2O$, 0.0028; $CaCl_2.2H_2O$, 0.033; peptone, 10 (Bazalel, Hadar, & Cerniglia, 1997). The pH of medium was adjusted to 6 and sterilized at 121°C for 20 minutes then cooled. Inoculated flasks were incubated on the rotary shaker at 150 rpm and 25°C for two weeks.

2.2.3. Production of EPS by Different Pleurotus Strains

To determine the best *Pleurotus* strain which produce high level of EPS and the incubation day of maximum EPS production, we used four *Pleurotus* strains (*P. djamor, P. ostreatus* and *P. sajor caju* and *P. eryngii*). They were incubated on the rotary shaker at 150 rpm and 25°C for two weeks.

After estimation of the best strain and incubation day for EPS production according to yield of EPS, components of the liquid medium and fermentation conditions were optimized.

2.2.4 Optimization of EPS Production Conditions

2.2.4.1 Effect of Different Carbon Sources

To determine the effects of different carbon sources on the yield of EPS produced, selected best strain was grown in basal medium supplemented with sucrose, glycerol and sorbitol at the concentration of 10 g/L. Each carbon source was added to the basal medium instead of glucose. Samples were collected on the 5^{th}day of incubation to analyze EPS production.

2.2.4.2 Effect of Carbon Source Concentration

To determine the effects of suitable carbon source concentration on the yield of EPS, determined best strain was grown in basal medium supplemented with glucose at concentrations of 5, 10, 20, 40, 60, 90, 120 and 180 g/L. Samples were collected on the 5^{th}day of incubation to analyze EPS production.

2.2.4.3 Effect of Different Nitrogen Sources

To determine the effects of different nitrogen sources on the yield of EPS produced, selected best strain was grown in basal medium supplemented with glucose at its optimum concentration and various combinations of peptone, yeast extract and ammonium nitrate at concentrations of 10 g/L. Samples were collected on the 5thday of incubation to analyze EPS production.

2.2.4.4 Effect of Nitrogen Source Concentration

To determine the effects of different nitrogen source concentration on the yield of EPS, determined best strain was grown in basal medium supplemented with glucose at its optimum concentration and peptone, yeast extract, ammonium nitrate, urea at various concentrations (0.5, 0.724, 1.5, 3, 6, 10, and 12 g/L). Samples were collected on the 5thday of incubation to analyze EPS production.

2.2.4.5 Effect of Mg^{2+} Ion Concentration

To determine the effects of Mg^{2+} ion concentration on the yield of EPS, determined best strain was grown in basal medium supplemented with glucose, peptone, yeast extract, ammonium nitrate at their optimum concentrations and Mg^{2+} at concentrations of 4, 7, 10, 30, 50, 100, and 250 mM. Samples were collected on the 5thday of incubation to analyze EPS production.

2.2.4.6 Effect of Initial pH

To determine the effect of initial pH of cultivation medium on the yield of EPS, determined best strain was cultivated in basal medium supplemented with glucose and peptone, yeast extract, ammonium nitrate, magnesium ion at their optimum concentrations and adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0.Samples were collected on the 5thday of incubation to analyze EPS production.

2.2.4.7 Effect of Fermentation Temperature

To determine the effect of cultivation temperature on the yield of EPS, determined best strain was cultivated in basal medium supplemented with glucose and peptone, yeast extract, ammonium nitrate, magnesium ion at their optimum concentrations and pH 5.0 in rotary shaker set at 20, 25 or 30°C constant temperature. Samples were collected on the 5th day of incubation to analyze EPS production.

2.2.4.8 Effect of Agitation Rate

To determine the effect of cultivation temperature on the yield of EPS, determined best strain was cultivated in basal medium supplemented with glucose and peptone, yeast extract, ammonium nitrate, magnesium ion at their optimum concentrations and optimum pH and temperature in rotary shaker set to 100, 150 or 180 rpm agitation rate. Samples were collected on the 5th day of incubation to analyze EPS production.

Optimum condition parameters were determined according to the yield of EPS as explained above.

2.2.5 Solid State Fermentation Media and EPS Production

For EPS production in SSF, three different solid substrate and optimum conditions in SmF were used (Table 2.1). To prepare production medium, 5 g solid substrate was weighed in 250 mL Erlenmeyer flasks. Optimum medium (Table 2.1) was the same for all the solid substrates and optimum pH was adjusted (Bazalel, Hadar, & Cerniglia, 1997). Potato peels and grape wastes were dried at 60°C for 24 h.

Table 2.1 Optimum medium components in SmF and solid substrates used in SSF

Optimum Medium (g/L)		**Solid Substrate (5 g)**
KH_2PO_4,	1.0	Potato peel (wet and dried)
$MgSO_4.7H_2O$,	4 mM	Grape waste (wet)
KCl,	0.5	
Yeast extract,	10	
$FeSO_4.7H_2O$,	0.001	
$ZnSO_4.7H_2O$,	0.0028	
$CaCl_2.2H_2O$,	0.033	
peptone,	10.0	

Solid substrates and liquid medium were sterilized at 121°C for 20 min. After sterilization, 10 mL liquid medium was added into solid substrate (humidity 70%) and 7 agar plugs (1 cm^2) were inoculated. Flasks were incubated at 25°C and samples from flasks were harvested after 15 days. After production period of SSF, for extraction each flask was received 25 mL of distilled water and was agitated for 1 h at 150 rpm, 25°C. The suspension was centrifuged at 10000 rpm and 4°C for 20 min and supernatant was used for analysis below.

2.2.6 Mycelial Dry Weight and Isolation of EPS

Samples collected from shake flasks were centrifuged at 10000 rpm for 20 min, and the supernatant was filtered through a 0.45µm membrane filter. The supernatant was used for analysis below. The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70°C overnight to obtain constant mass. The resulting culture filtrate was mixed with four volumes of absolute cold ethanol, stirred vigorously and left for 24 h at 4°C. The precipitated crude EPS was obtained by centrifugation at 10000 rpm for 20 min and then dried at 60°Covernight to obtain constant mass.

2.2.7 Determination of Chemical Composition of Production Medium

After production period of SmF and SmF, cell free supernatant was prepared as above and chemical composition analyses were performed.

2.2.7.1 Determination of Protein Content

Protein content of cell free supernatant of production culture media was specified by Bradford (1976) method using bovine serum albumin as standard in the range of 0-250 ppm. The Bradford reagent was used in this method. 100 mg of Coomassie Brillant Blue G-250 was dissolved in 50 mL 95% ethanol so Bradford reagent prepared. The solution was added to 100 mL 85% H_3PO_4, and diluted to 1000 mL total volume with water. 100 µL samples was added to 900 µL reagents and mixed. This solution was waited for 2 min at room temperature and the absorbance was measured at 595 nm against a reference sample. The standard function was y= 0.0063x, R^2 = 0.9826.

2.2.7.2 Determination of Reducing Sugar Content

For determination of reducing sugar content, 3,5-Dinitrosalicylic acid (DNS) method was used (Miller, 1959). To prepare DNS reagent, solution B (30 g of sodium-potassium tartarate/ 50 mL distilled water) and solution A (1 g DNS/ 20 mL, 2N NaOH) were mixed with 100 mL total volume. To determine reducing sugar content, after mixing 500 µL supernatant (500 µL distilled water for reference) and 500 µL DNS reagent the mixture were boiled for 10 min and cooled to room temperature. After cooling, 5 mL distilled water was added and mixed. The absorbance was measured at 546 nm against a reference sample. D-glucose was used as standard in the range of 0-165.1 µg/mL, the standard function was y= 0.009x, R^2 = 0.9986.

2.2.7.3 Determination of Nitrogen Content

Nitrogen content of cell free supernatant was determined by phenol-hypochlorite method (Weatherburn, 1967). To prepare phenol reagent, solution A (5 g phenol/ 50 mL distilled water) and solution B (25 mg sodium-nitroprusside/ 50 mL distilled water) were mixed in equal volumes. Alkaline hypochlorite solution was prepared by mixing equal volume of solution C (5 g sodium hydroxide/ 100 mL distilled water) and solution D (26 g/L NaOCl). 2 mL supernatant (2 mL distilled water for reference), 500 µL phenol solution and 500 µL alkaline hypochlorite solutions were mixed and incubated for 5 min at 60°C. The absorbance was measured at 630 nm against a reference sample. Standard was prepared in the range of 0-500 µg/mL of ammonium sulfate and the standard function was $y= 0.1299x$, $R^2 = 0.9923$.

2.2.8 Determination of Chemical Composition of Crude EPS

After production of EPS at optimum conditions in SmF, as above prepared crude EPS was dissolved in distilled water and then its chemical composition was determined by protein, reducing sugar, nitrogen, total carbohydrate, pyruvate and uronic acid assays.

2.2.8.1 Determination of Total Carbohydrate Content

Total carbohydrate content was determined according to phenol-sulfuric acid method (Dubois et al., 1956). To determine total carbohydrate content, 1 mL cell free supernatant (1 mL distilled water for reference) was stirred with 1 mL 5% phenol solution and 5 mL concentrated H_2SO_4. After incubation for 20 min at room temperature the absorbance was measured at 490 nm against a reference sample. Glucose was used as standard in the range of 0- 250 µg/mL and the standard function was $y= 0.0041x$, $R^2 = 0.9857$.

2.2.8.2 Determination of Pyruvate Content

The method for the determination of pyruvate involves the reaction with nitrophenylhydrazines (Maughan, 1982). Samples (600 μL) were stirred with reagent (2,4 dinitrophenyl hydrazine) and incubated for 5 min then added 2N NaOH (1.2 mL) and incubated for 10 min the absorbance was measured at 520 nm against a reference sample. Sodium- pyruvate was used as standard in the range of 0- 1 μmol/ mL, the standard function was y=0.00359x, R^2 = 0.9972.

2.2.8.3 Determination of Uronic Acid Content

Uronic acid content of EPS was determined with borate-sulfuric acid- carbazole assay (Healy, Devine, & Murphy, 1996). 0.5 mL dissolved EPS (0.5 mL distilled water for reference) and 3 mL cold borate-sulphuric acid reagent (3.82 g sodium borate was dissolved in 10 mL hot water and 390 mL well-cooled concentrated sulphuric acid) were stirred and heated in boiling water bath for 20 min. after cooling to 0°C, 0.1 mL 0.2% carbazole solution were added. The solution was shaken and heated again in the boiling water bath for 10 min. After cooling at room temperature for 15 min the absorbance was measured at 530 nm against a reference sample. Glucuronolactone was used as standard in the range of 0-1 μmol/ mL, the standard function was y= 0.0037x, R^2 = 0.9880.

2.2.9 Partial Purification of EPS

After production at optimum condition in SmF, the ethanol precipitates of the crude EPS treated with Sevag reagent (1:4 n-butanol/chloroform, v/v) to remove most of proteins. After removing the proteins and Sevag reagent by centrifugation, water phase was ultra-filtered (10kDa) and filtrate was mixed with absolute cold ethanol, the precipitated EPS was centrifuged and dried. The EPS (100 mg) were dissolved in 0.2 M NaCl buffer, and loaded onto a Sepharose CL-6B column (1.6 cm×90 cm). The column was eluted with the same buffer at a flow rate of 0.6 mL/min. Protein concentration was determined according to the Bradford method

(Bradford, 1976) using bovine serum albumin as the standard. The total sugar content in the EPS was determined by PSA method using glucose as the standard (Dubois et al., 1956). The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, while the carbohydrate moiety was monitored at 490 nm.

2.2.10. Determination of Molecular Weight of EPS

The molecular weight of the partial purified EPS was determined using gel filtration on a Sepharose CL-6B column (1.6 cm×90 cm). Five dextrans (25000, 80000, 270000, 670000 and 1100000 Da) were used as molecular-weight standards. The dextran standards (2.5 mg) were dissolved in 0.2 M NaCl buffer, and loaded.

2.2.11 Partial Characterization of Produced EPS

After the partial purification of EPS, collected partial purified EPS fractions were lyophilized.

2.2.11.1 Chemical Composition of EPS

Chemical structure of partial purified and lyophilized EPS was determined by protein and total carbohydrate assays as explained above.

2.2.11.2 Chromatographic and Spectroscopic Analyses of Partial Purified EPS

To determine monosaccharide composition of the partial purified EPS and standard sugars (glucose, mannose, galactose, rhamnose and xylose) were analyzed by thin layer chromotography (TLC) (CAMAG). Ethyl acetate: acetic acid: water (2:2:1) was used as mobile phase on TLC silica gel 60 F_{254} plates (MERCK, Germany) with solution as staining reagent (%2 naphtoresorcinol in %10 ethanolic phosphoric acid). EPS (50 mg) was hydrolyzed with 2 N H_2SO_4 (4 mL) at 100°C in oil bath for 2 h then it was neutralized with NaOH (1 g) and loaded on plates and analyzed.

For spectroscopic analyses, FT-IR and ^{1}H-NMR were used. The FT-IR spectrum was recorded on the Perkin Elmer Spectrum BX, in the 4000- 400 cm^{-1} spectral region and was employed using the KBr disc (back ground reference) for the analysis and detecting functional groups. All samples were dried at 70°C overnight before analysis. Approximately 1 mg of the sample (EPS fractions) was mixing with approximately 300 mg of dried KBr and then pressed to form a pellet for measurement.

For NMR spectroscopy, the sample was recorded as solution in dimethyl sulfoxide ^{1}H- spectrum was recorded on a MERCURY Plus-AS 400 spectrometer at 400 MHz and 30°C. The chemical shifts were expressed in ppm.

2.2.11.3 Thermal Gravimetric Analyses (TGA)

TGA of partial purified EPS was carried out with Perkin Elmer- Diamond TG/DTA. About 3-5 mg of dry purified EPS sample was loaded on a platinum pan and its energy level was scanned in the ranges of 30- 600°C under a nitrogen atmosphere with a temperature gradient of 10°C/min. The analyses were carried out under gradual increase in temperature by plotting the weight loss percentage and differential weight percentage against temperature.

2.2.12 Evaluation of in vitro Antioxidant Activity of Partial Purified EPS

2.2.12.1 Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity of partial purified EPS was measured according to the method of Halliwell, Gutteridge, & Aruoma (1987). One milliliter of the final reaction solution consisted of aliquots (100 µL) of various concentrations of the EPS, 1 mM $FeCl_3$, 1 mM EDTA, 1 mM H_2O_2, 1 mM L-ascorbic acid, and 0.028 mM deoxyribose in 20 mM potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37 °C, and further heated in a boiling water bath for 15 min after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1%

(w/w) 2- thiobarbituric acid. The color development was measured of 532 nm against a blank containing phosphate buffer.

2.2.12.2 Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity of partial purified EPS was determined according to the method of Liu, Ooi, &Chang (1997). Superoxide radicals were emergent in 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0), which contained 78 µM nicotinamide adenine dinucleotide (reduced form, NADH), 50 µM nitroblue tetrazolium (NBT), 10 µM phenazin methosulfate (PMS), and various concentrations of EPS. The color reaction of superoxide radicals and NBT was determined at 560 nm. Vitamin C (Vit C) was used as the control in this assay.

2.2.12.3 DPPH Radical Scavenging Activity

The method was based on Kumaran (2006). 1.5 mL of 1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was mixed with 500 µL various concentrations of EPS in a test tube. Samples were stored in dark at room temperature for 30 minutes. Absorbance was measured at 517 nm wavelength against ethanol. Vit C was used as the control (Duh & Yen, 1997). The capability to scavenge DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = 100 - [(A_1 / A_0) \times 100] \qquad (2.1)$$

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

2.2.12.4 Reducing Power

The reducing power of EPS was determined according to the method of Oyaizu (1986). EPS solutions in water at various amounts were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. After 2.5 mL of TCA (10%) was added, the mixture was centrifuged at 3000 rpm for 10 min. Supernatant (2.5 mL) was mixed

with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1%) and the absorbance was measured at 700 nm. Vit C was used as the control. Higher absorbance of the reaction mixture indicates greater reducing power.

2.2.12.5 Chelating Activity on Fe^{2+}

Chelating activity method was based on Decker & Welch (1990). One milliliter sample containing various concentrations of EPS was mixed with 3.7 mL of deionized water and then 0.1 mL of $FeCl_2$ (2 mM) and 0.2 mL of ferrozine (5 mM) were added. This mixture was incubated at room temperature for 20 min and the absorbance at 562 nm was determined.

Chelating activity of samples on Fe^{2+} was calculated as follows:

Chelating activity (%)= (A*B)/A 100, where A was absorbance the control and B was absorbance the test sample. Ethylenediaminetetraacetic acid (EDTA) was used as the positive control.

2.2.13 Statistical Analysis

The SPSS 13 statistical program was used for statistical significance analyses. The values were the mean of three separate experiments. Comparisons were also made with Pearson's correlation.

CHAPTER THREE
RESULTS AND DISCUSSION

3.1 Optimization of Fermentation Conditions

3.1.1 Determination of the Best EPS Producer Strain

EPS production capacities of four different *Pleurotus* spp. were analyzed in order to determine the best EPS producer in basal medium in SmF and the incubation day of maximum EPS production. They were incubated on the rotary shaker at 150 rpm and 25°C for two weeks.

As shown in Figure 3.1., the results indicated that *P. sajor caju* was the best strain for the EPS production (2.5 ± 0.4 g/L, 5th day of incubation period). The maximum EPS productions by *P. eryngii, P. djamor*, and *P. ostreatus* were achieved on 6th, 3rd and 7th days of incubation period as 2.1 ± 0.35, 1.4 ± 0.3 and 1.2 ± 0.25g/L, respectively. Our results showed that EPS production depends on microorganisms strain. Likewise, Nehad, & El-Shamy (2010) reported that yield of EPS produced by different fungal strains was different.

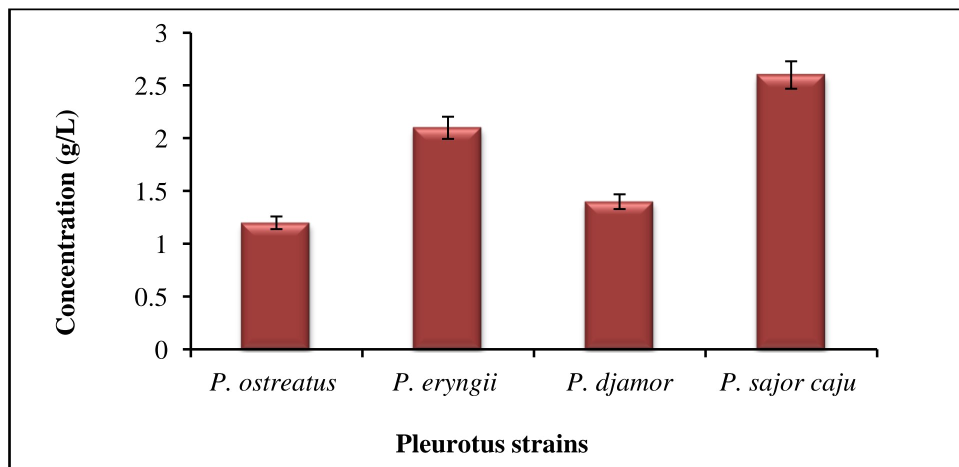


Figure 3.1 Production of EPS by *Pleurotus* strains

After determination of the best EPS producer as *P. sajor caju*, incubation time and pH of medium were investigated. Figure 3.2 showed that maximum EPS production

was determined as 2.62 ± 0.43 g/L on 5th day of incubation while maximum cell dry weight (CDW) was obtained as 4.06 ± 0.4 g/Lon 7th day.

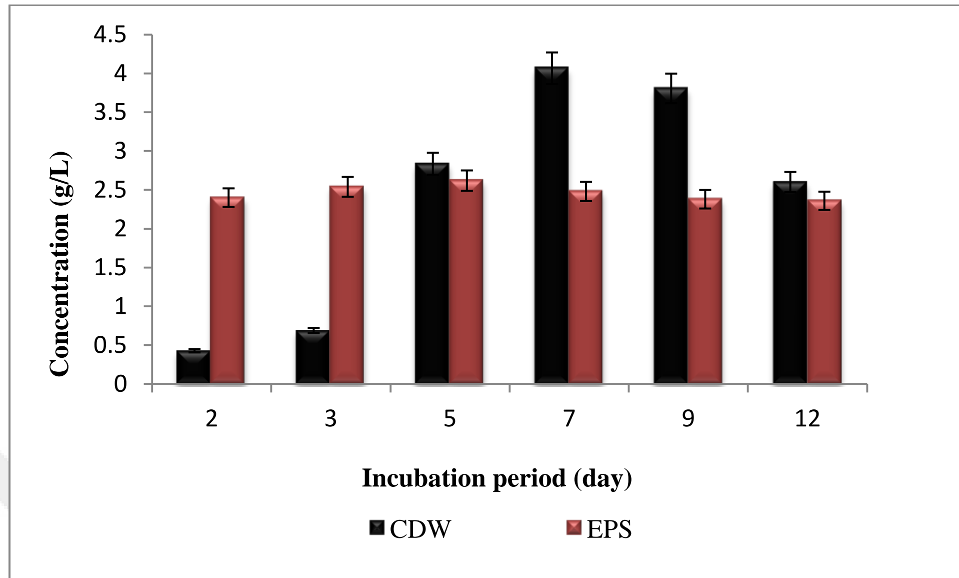


Figure 3.2 Production of EPS by *P. sajor caju* and CDW changes during incubation period.

As shown in Figure 3.3, pH of medium was not a significant change up to 7 days, and then it slightly increased to 8.17 ± 0.07on 12th day.

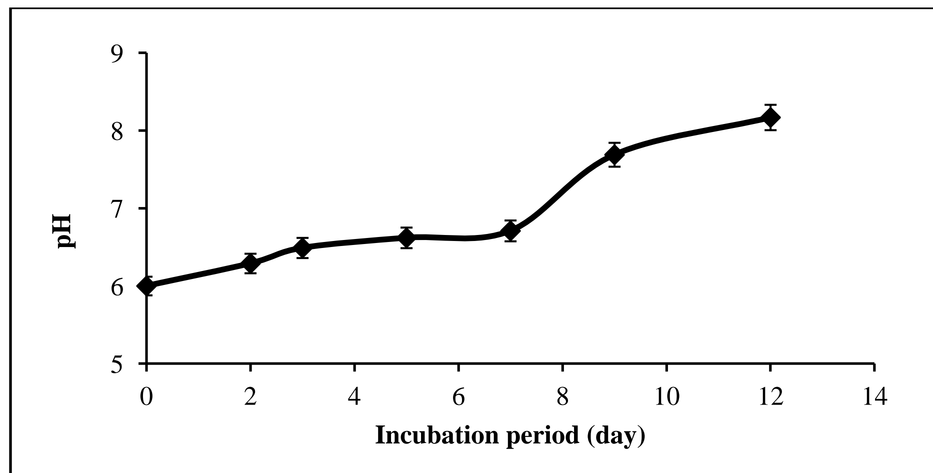


Figure 3.3 pH changes during EPS production period by *P. sajor caju*

Chemical composition analyses of medium were carried out during incubation period (Figure 3.4). Maximum protein concentration was detected as 78.09 ± 3.2 ppm on 12th day of incubation. The maximum nitrogen concentration was recorded

on 7th day of incubation as 331 ± 17 ppm. Concentration of reducing sugar on 2nd day of incubation was determined as 593.4 ± 29 ppm.

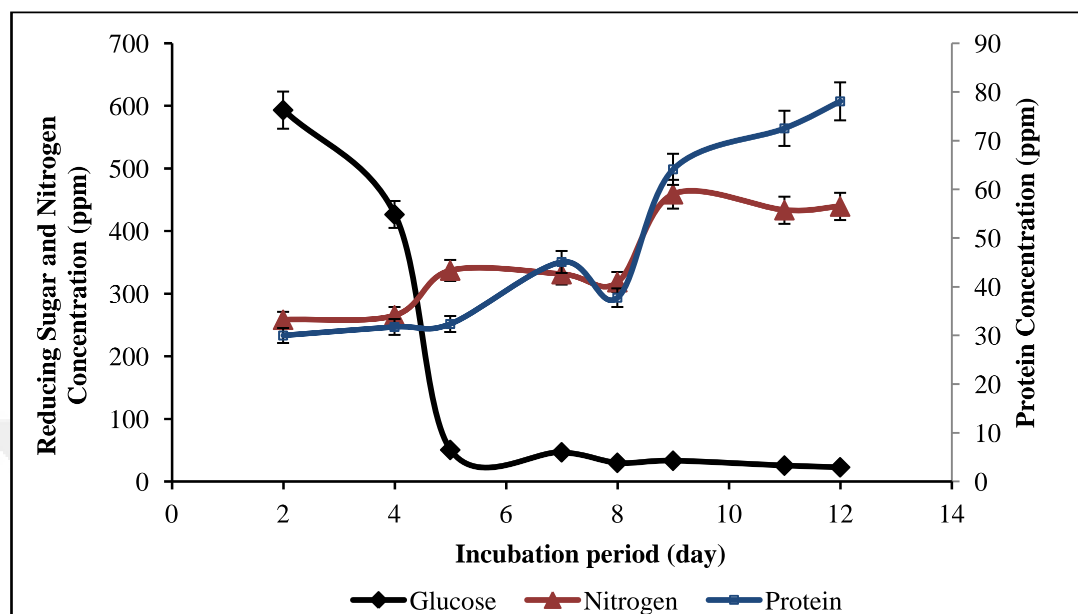


Figure 3.4 Glucose, nitrogen and protein content of medium during incubation period of *P. sajor caju*

After the determination of best EPS producer as *P. sajor caju* and incubation day (5th) for EPS production according to yield of EPS, components of the liquid medium and fermentation conditions were optimized.

3.1.2 Effect of Carbon Source and Concentration on EPS Production Capacity of P. sajor caju

Carbohydrates are a major component of the cytoskeleton and an important nutritional requirement for the growth and development of higher fungi (Xiao et al., 2006). However, different fungal species vary in their ability to utilize various carbon sources. It is possible that different carbon sources might have different effects of catabolic repression on the cellular secondary metabolism. Such a phenomenon was also claimed in submerged cultivation of many kinds of fungi (Hwang, Kim, Xu, Choi, & Yun, 2003; Kim et al., 2003). To find suitable carbon sources for EPS production by *P. sajor caju*, three different carbon sources named sorbitol, sucrose, and glycerol were separately provided at 10 g/L in instead of

glucose employed in the basal medium. Samples were taken from shaking flasks on the 5th day of incubation analyzed for EPS production. Among the carbon sources tested, maximum EPS production and mycelial CDW were obtained in glucose medium as 2.62 ± 0.16 g/L and 0.9 ± 0.04 g/L, respectively (Figure 3.5). In submerged cultures of fungi, different carbon sources, such as sucrose (Bae et al., 2000), glucose (Xu et al., 2003), and starch and maltose (Xu, Wang, Jin, & Yang, 2009) have been reported for maximum EPS formation. From the practical point of view in the industry, glucose is a good medium ingredient considering its ease in use and low material cost compared to other nutrients.

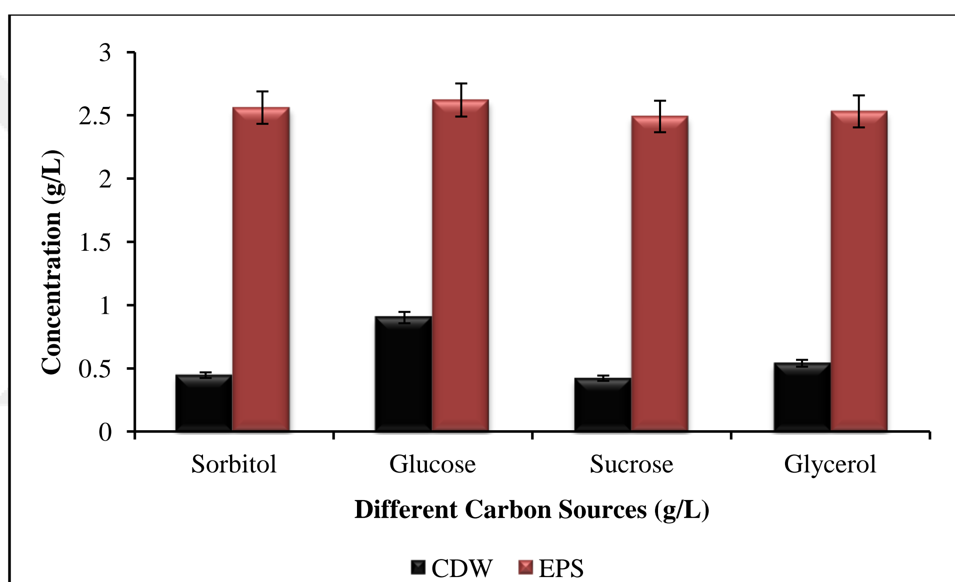


Figure 3.5 Effect of carbon sources on EPS production by *P. sajor caju*

Our results were similar with the findings by the other investigators, who demonstrated that glucose is clearly a good carbon source for EPS production in submerged cultures of fungi (El-Dein, El-Fallal, El-Shahat, & Hereher, 2004; Xu et al. 2003). Also, Hammond, G. W., Lian, C. J., Wilt, J. C., Albritton, W. L., & Ronald (1978) has been reported that glucose was as a good respiratory substrate.

After the glucose was selected as the best carbon source for EPS production, its optimum concentration was determined for EPS production (Figure 3.6). For this purpose, various glucose concentrations were used in medium and the results showed

that optimum glucose concentration for EPS production (3.97 ± 0.15 g/L) and CDW (6.03 ± 0.32 g/L) was 90 g/L. These results clearly show that growth and other metabolic activities could happen in a specific concentration of glucose or other medium. In addition, the maximum CDW accompanied by a higher production of polysaccharides was specifically noted only in the case of glucose concentration, but not any other nutrient sources.

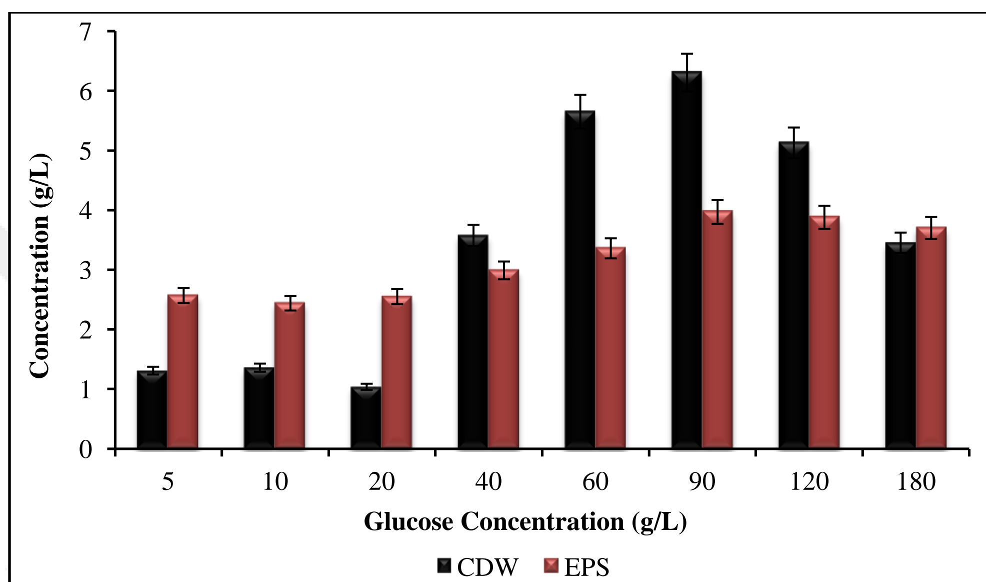


Figure 3.6 Effect of glucose concentration on EPS production by *P. sajor caju*

3.1.3 Effect of Nitrogen Source and Concentration on EPS Production Capacity of P. sajor caju

Nitrogen may be supplied as ammonia, nitrate or organic compounds, such as amino acids and proteins. Therefore, the omission of nitrogen in the medium greatly affects fungal growth and metabolite production. In this study, the ability of *P. sajor caju* to use different nitrogen sources for EPS production was analyzed. For this purpose, cells were grown in basal medium containing glucose (90g/L) as a carbon source and four different nitrogen sources, namely, peptone, yeast extract, ammonium nitrate, and urea were added at various concentrations. Samples were taken from shaking flasks on the 5thday of incubation. Figure 3.7 showed that maximum EPS production was achieved in 10 g/L peptone, 10 g/L yeast extract and 0.724 g/L NH_4NO_3medium as 4.01 ± 0.38 g/L, 3.56 ± 0.3 g/L and 3.09 ± 0.27 g/L,

respectively. Figure 3.8 indicated that maximum CDW was determined in 12 g/L peptone and 10 g/L yeast extract medium as 5.93 ± 0.5 g/L and 8.51 ± 0. 77g/L, respectively.

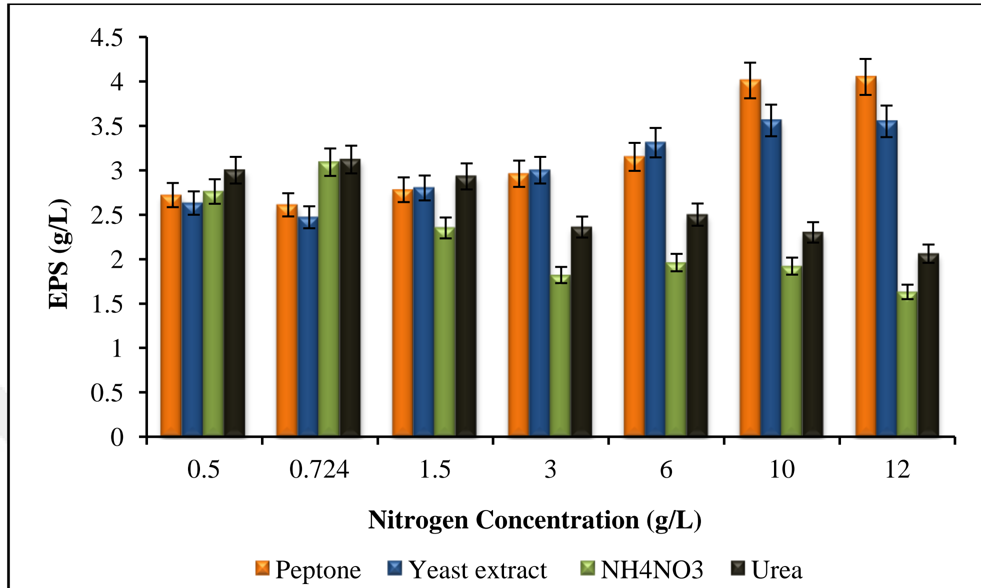


Figure 3.7 Effect of nitrogen sources and concentrations on EPS production by *P. sajor caju*

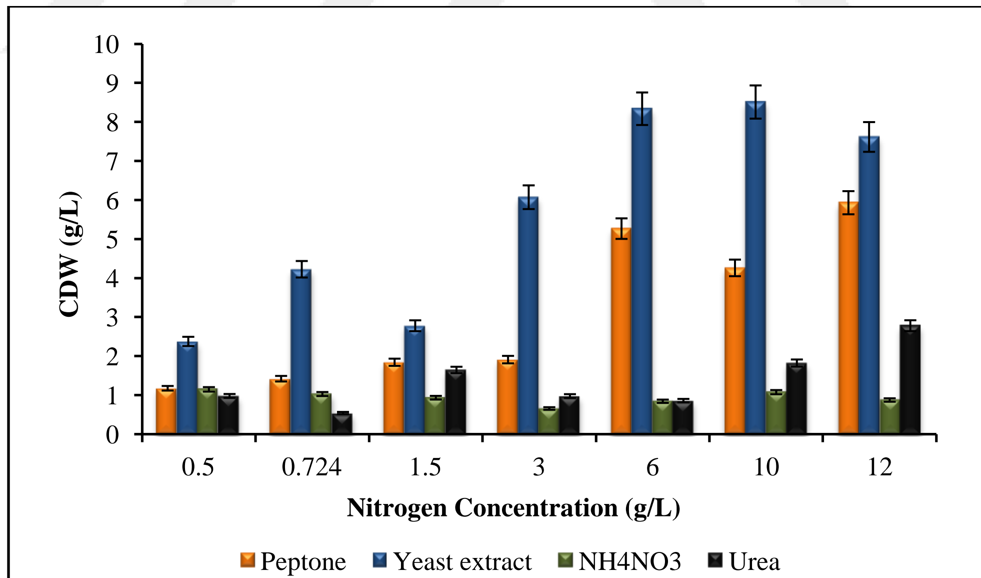


Figure 3.8 Effect of nitrogen sources and concentrations on *P. sajor caju* CDW

After the peptone, yeast extract and NH_4NO_3 were determined as the best nitrogen sources for EPS production, their optimum concentration combinations were studied for EPS production (Figure 3.9). The maximum EPS production and CDW were

achieved in 10 g/L peptone and 10 g/L yeast extract medium as 5.86 ± 0.48 g/L and 12.74 ± 1.25 g/L, respectively.

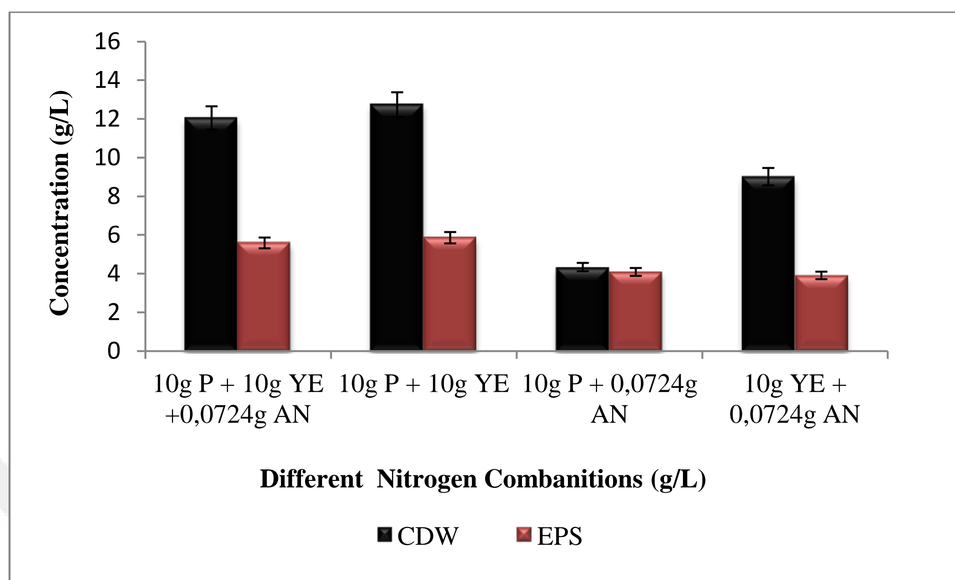


Figure 3.9 Effect of suitable nitrogen concentrations on EPS production by *P. sajor caju* and CDW (P: peptone, YE: yeast extract, AN: ammonium nitrate)

Our results were similar with the findings by the other researchers, who demonstrated that in comparison with organic nitrogen sources, inorganic nitrogen sources frequently yield relatively lower mycelial growth and EPS production (Yang, Huang, & Yang, 2003).

3.1.4 Effect of Mg $^{2+}$ on EPS Production Capacities of P. sajor caju

Mg^{2+} is essential to all fungi. It is a cofactor in enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent (Kim et al., 2005). The positive action of Mg^{2+} on mycelial growth and EPS production was obvious in the present submerged cultures (Hwang et al., 2003). The effect of Mg^{2+} mineral source on EPS production and mycelial growth was investigated at various concentrations (Figure 3.10). Samples were harvested on the 5th day of incubation and analyzed for EPS production. The optimum Mg^{2+} ion concentration for EPS (31.08 ± 3.0 g/L) was 100 mM. The maximum CDW (19.25 ± 1.89 g/L) was achieved in 50 mM $Mg^{2+.}$

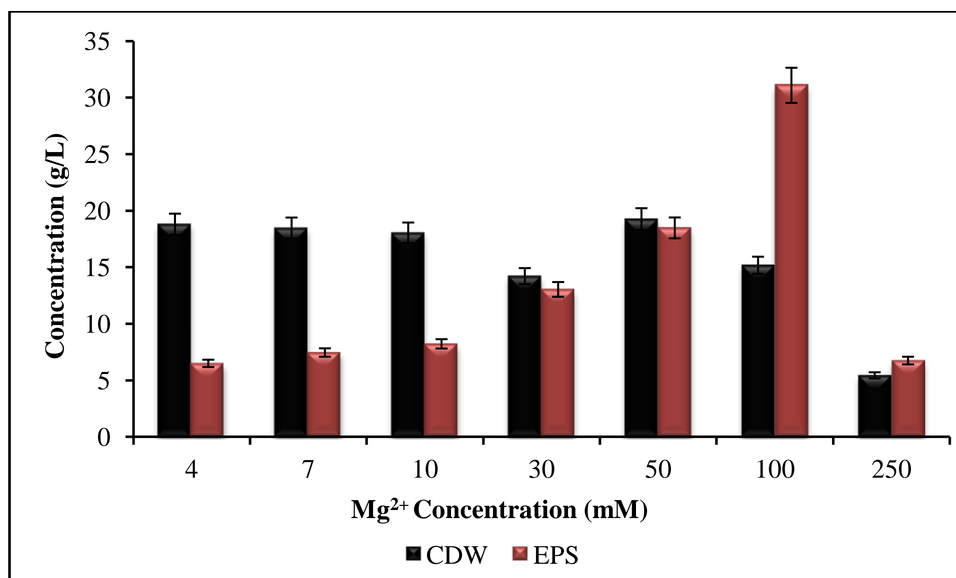


Figure 3.10 Effect of different Mg^{2+} ion concentrations on EPS production by *P. sajor caju*

Many investigators suggested that this bioelement has usually been recognized as favorable for mycelial growth and EPS production in liquid cultures of several basidiomycetes (Chardonnet, Sams, & Conway, 1999; Hwang et al., 2003; Okba et al., 1998).

3.1.5 Effect of Initial pH on EPS Production Capacities of P. sajor caju

The pH of the culture medium is vital factor that governs mycelial growth and EPS production. In order to investigate the effect of initial pH on the EPS production, *P. sajor caju* was cultivated in media with different initial pH (3.0-9.0) in SmF, after 5 days it was analyzed for EPS production (Figure 3.11). The results showed that the optimal pH for EPS production (33.32 ± 1.6 g/L) and CDW (7.49 ± 0.5 g/L) was 5 and 7, respectively. It is comparable that most of the enthomopathogenic fungi have pH optima within the range of 8–9 for EPS production (Bae et al., 2000; Xu et al.,2003; Xu et al., 2009).Likewise, other researchers have reported optimum pH of 5.5 for other EPS synthesizing fungi, such as *P. pulmonarius* (El-Dein et al., 2004).

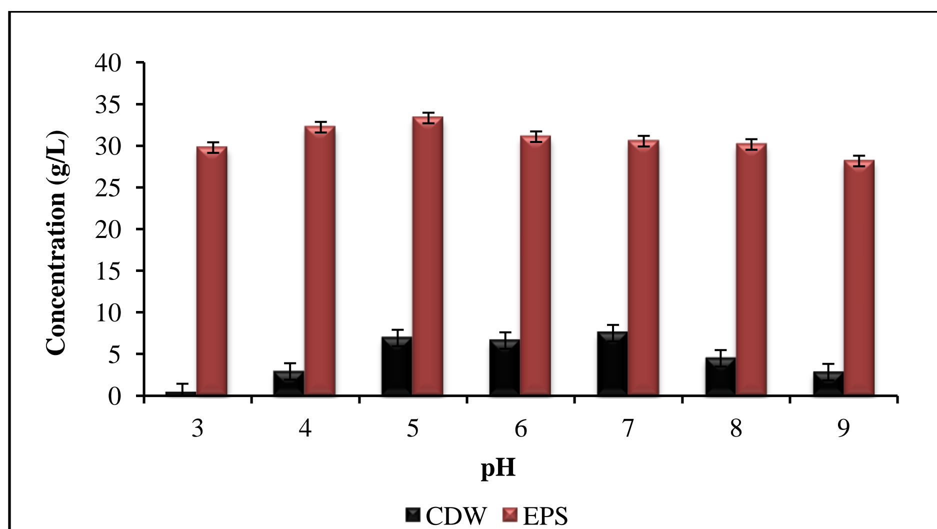


Figure 3.11The effect of initial pH on EPS production by *P. sajor caju*

In the study of Li, R., Jiang, X. L., & Guan, H. S. (2010), it is suggested that the initial pH of the culture medium is an important factor affecting mycelial growth and EPS production of *Hirsultella pat* in submerged cultures. They found the optimum pH for EPS production as 5.5.

On the other hand, Kim et al. (2005) indicated that the different morphology of fungi mycelia under a different initial pH value was the critical factor in biomass accumulation and metabolite formation. The pH level of medium may affect cell membrane function, cell morphology and structure, uptake of various nutrients, and product biosynthesis.

3.1.6 Effect of Temperature on EPS Production Capacities of P. sajor caju

Temperature is associated with the activation of enzymes that act in the polymerization process of the synthesized material. To find the optimal temperature, *P. sajor caju* was cultivated at various temperatures in the temperature controlled shaking incubator for 5 days, the optimum temperature was detected as 25°C for EPS production (Figure 3.12). The maximum CDW was achieved at 30°C.Many researchers have reported different optimal values of temperature for EPS production; it seems that changes in the environmental factors lead to certain changes in the EPS yield that vary depending on the fungal species (Ruas-Madiedo,

Hugenholtz, & Zoon, 2002). Prasertsan, Wichienchot, Doelle, & Kennedy (2008) indicated that incubation temperature is often acritical factor in polysaccharide biosynthesis. The optimum temperature for polysaccharide production depends on the type of microorganism.

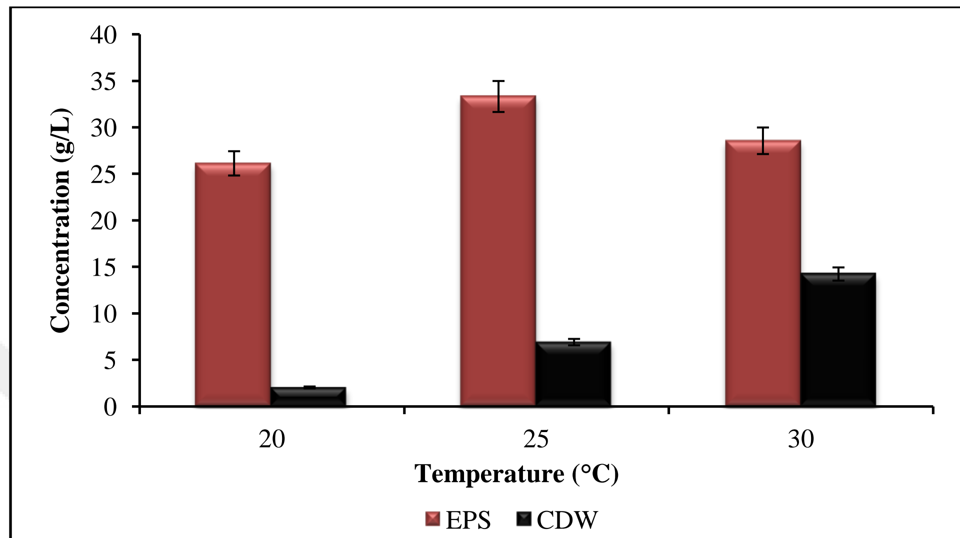


Figure 3.12 The effect of different temperature on EPS Production by *P. sajor caju*

Similarly, *Agrocybe cylindracea* was cultivated at various temperatures ranging from 15 to 30°C (Kim et al., 2005). The maximum EPS was observed at 25°C, which is comparable to many kinds of fungi that have relatively low temperature optima (e.g. 20–25°C) in their submerged cultures (Bae et al., 2000; Kim et al., 2003). In contrast, many kinds of fungi have relatively low temperature optimum, ranging from 20 to 25°C (Xu et al., 2003; Xu et al., 2003). Culture temperature is one of the most important parameters influencing the production of polysaccharide. In this study, it is claimed that the optimal culture temperatures in flask cultures for mycelial growth and EPS production are often different; for instance, the optimal mycelial growth was detected at a higher culture temperature, but the maximum EPS production appeared at another lower culture temperature. In this study, the optimal culture temperatures of mycelial growth and EPS production were dissimilar.

3.1.7 Effect of Agitation Rate on EPS Production Capacities of P. sajor caju

Agitation rate is closely related to oxygen supply and some rheological properties during the cultivation processes. Oxygen supply can influence the formation and accumulation of bioactive metabolites in submerged cultivation. So, agitation rate might play an important role for the mycelial growth and production of EPS. In order to investigate the effect of agitation rate on the EPS production, *P. sajor caju* was cultivated in a medium with different agitation rate (100, 150,and 180 rpm). Figure 3.13 showed that maximum EPS production and CDW were obtained at an agitation rate of 150 rpm.

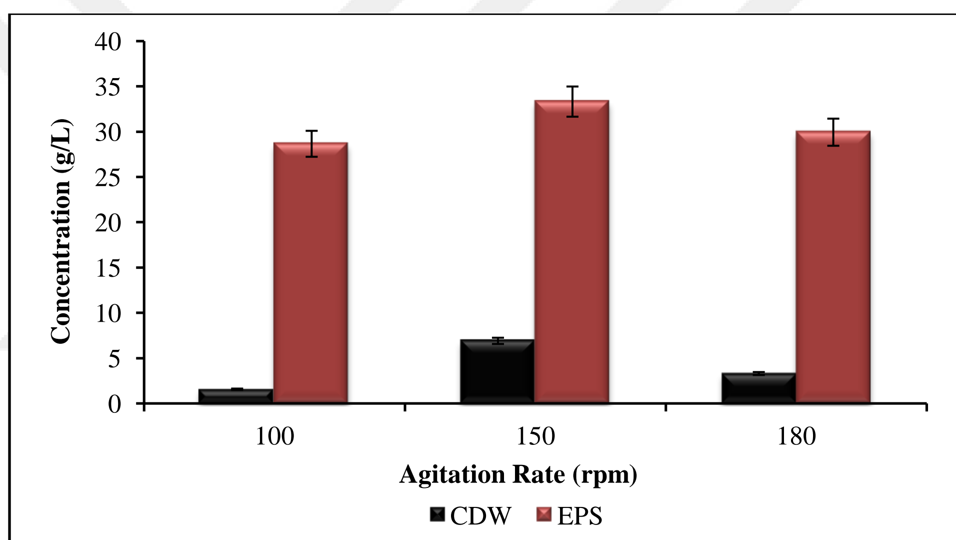


Figure 3.13The effect of agitation rate on EPS production by *P. sajor caju*

Martin & Bailey (1985) observed that higher agitation rate caused an increase in mycelial growth associated with high frequency of filamentous mycelia but a decrease in the pellet size of *Agaricus* mycelium. To investigate the agitation effect in stirred-tank bioreactor, Cho, Oh, Chang, & Yun (2006) cultured the *Tremella fuciformis* under three different agitation speeds. The maximum CDW (7.03 g/L) and EPS production (2.00 g/L) were achieved at 200 rpm.

After optimization of EPS production conditions, the produced EPS was isolated and its chemical characterization was analyzed. The amount of maximum EPS was

33.32 ± 1.6g/L. The chemical composition of 1 mg isolated EPS was formed 2.26 ± 0.41 µg protein, 219.75 ± 10 µg total carbohydrate, 28.59 ± 0.32 µg reducing sugar, 15.73 ± 1.1 µg nitrogen, 1.01 ± 0.01 µg pyruvate and 6.7 ± 0.5 µg uronic acid. Results of chemical composition analyses demonstrated complex structure of produced EPS.

3.2 Production of EPS in SSF

After optimization of SmF conditions, SSF were carried out for EPS production. For this purpose, SSF production was carried out in optimum SmF conditions. As shown in Figure 3.14, EPS production by *P. sajor caju* in SSF conditions was obtained as low levels. Maximum EPS production was achieved on wet potato peel as 0.82 ± 0.06 g/L. Although, several investigators have made efforts to cultivate fungi on solid media rather than submerged culture they did not obtain high yield (Kim et al., 2005).

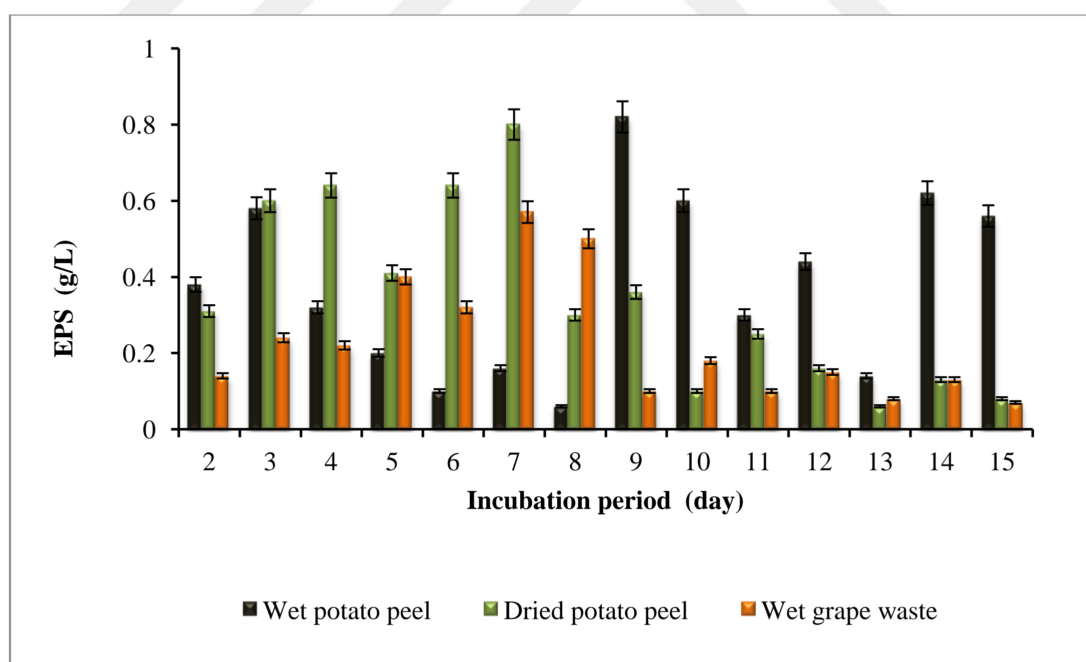


Figure 3.14 Production of EPS by *P. sajor caju* in SSF

3.3 Isolation and Partial Purification of EPS and Determination of Molecular Weight

The produced EPS in optimum SmF conditions by *P. sajor caju*, 90 g glucose, 10 g peptone, 10 g yeast extract, 100 mM Mg^{2+} per liter, pH 5, at 25°C and 150 rpm, was isolated. Isolated EPS was mixed Sevag reagent then ultrafiltrated and applied on gel filtration chromatography (Sepharose CL-6B). Three fractions were obtained. One fraction of EPS (Fr-I), which consisted of polysaccharides and proteins, were co-eluted as shown in Figure 3.15. Fr-I was revealed to be glycoproteins based on preliminary data. A similar result was reported by Shen, Shi, & Xu (2013), and one fraction was isolated by purification of polysaccharide from *P. pulmonarius.* Many research findings indicated that EPS produced by fungi generally occurs glycoproteins (Kalogiannis, Iakovidou, Liakopoulou-Kyriakides, Kyriakidis, & Skaracis, 2003; He et al., 2012; Hwang et al., 2003).

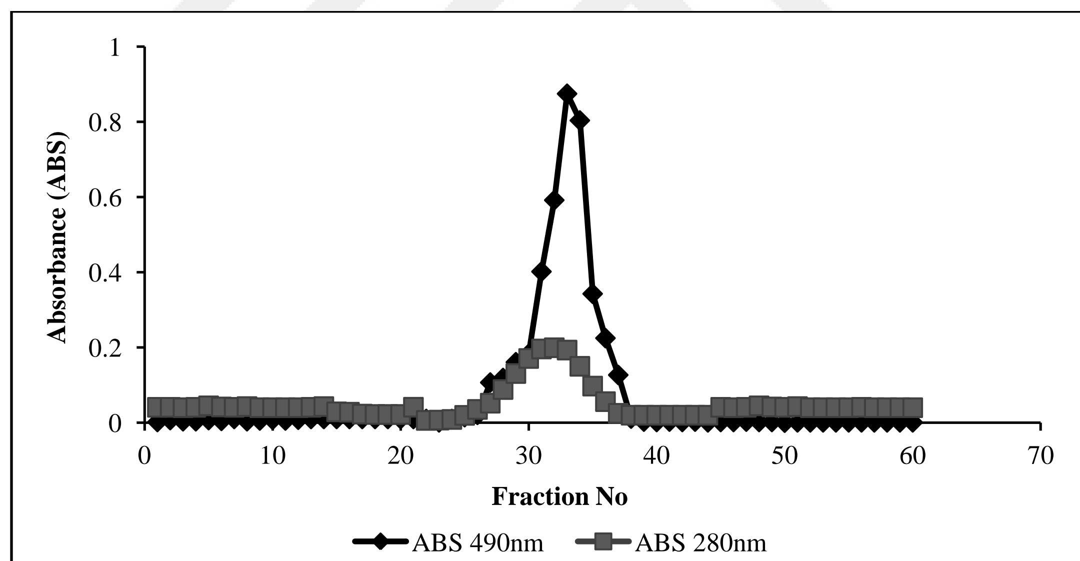


Figure 3.15 Elution profiles of Fr-I in sepharose CL-6B chromatography

After partial purification of EPS, dextran standards were used to determine for molecular weight of EPS. The dextran standards were loaded on Sepharose CL-6B column (Figure 3.16). The molecular weight of the produced EPS was detected approximately 25± 0.3 kDa (Figure 3.17).Similar observations were made by Oh et al (2007) and Cheng, Demirci & Catchmark (2011) in other fungi fermentations.

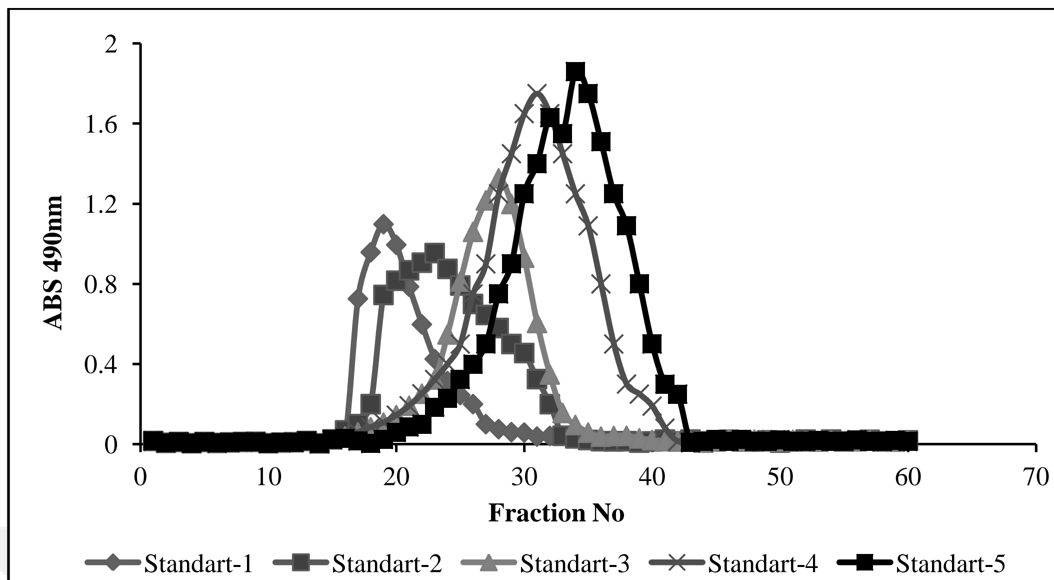


Figure 3.16 Elution profiles of Dextran standards in Sepharose CL-6B Column

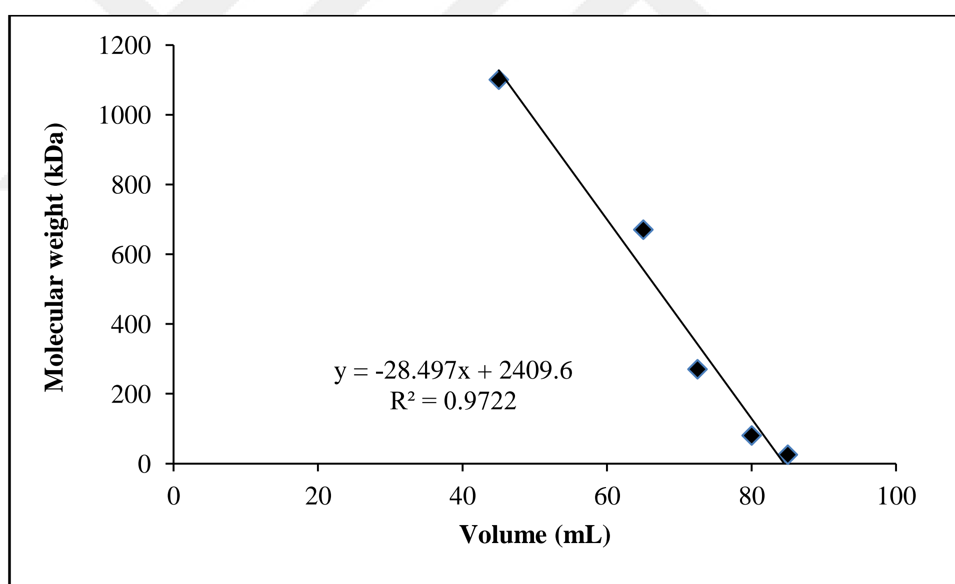


Figure 3.17 Determination of molecular weight of Fr-I in Sepharose CL-6B Column

3.4 Partial Characterization of Purified EPS

TLC is an effective chromatographic method for detecting monosaccharide composition. On TLC analysis, the Rf values of the standards were: glucose 0.456 ± 0.04, galactose 0.445 ± 0.05, mannose 0.503 ± 0.05, xylose 0.551 ± 0.06 and rhamnose 0.563 ± 0.04 (Figure 3.18). Our results showed that Fr-I mainly included glucose. Many fungal EPSs common occur glucose monosaccharide (Sutherland 1996; Lin, & Chen, 2007).

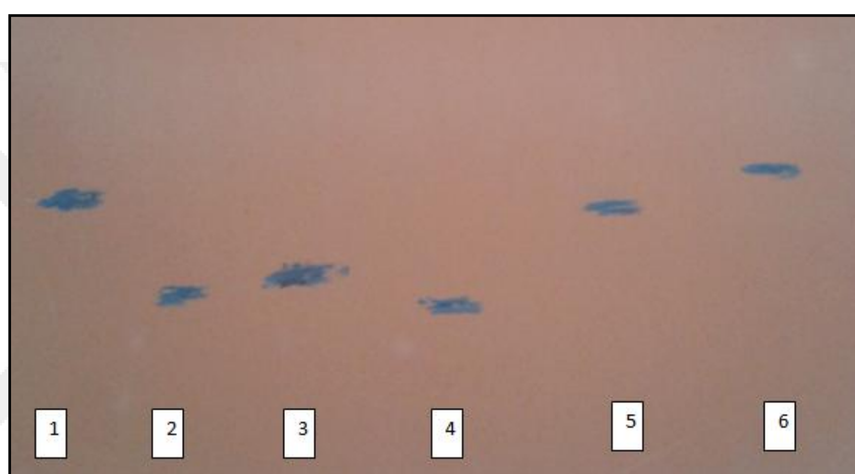


Figure 3.18TLC Analysis of Fr-I and standards. Solvent system-ethyl acetate butanol:aceticacid: water (2: 1: 1), Spray reagent; %2 naphtoresorcinol in %10 etahanolic phosphoric acid. Spot (2) Fr-I; (1), (3), (4), (5) and (6) Mannose, Glucose, Galactose, Xylose and Rhamnose, respectively

FT-IR spectrum is an effective instrument method for detecting functional groups and characterizing covalent bonding information. Typical IR spectrum for Fr-I is presented in Figure 3.19. Fr-I exhibited a stretching intense characteristic peak at approximately the region of 3388 cm^{-1} for the carbohydrate ring. Small band at 2948 cm^{-1} was assigned to the stretching vibration of the methylene group (C-H), usually present in hexoses. A characteristic peak absorption band appeared at 1638 cm^{-1} was assigned to the stretching vibration of the carboxyl group (C=O). A small peak around 1110 cm^{-1} corresponding to the glycosidic linkage (C-O-C) was also observed in the spectrum of polysaccharide (Copikova, Cerna, Novotna, Kaasova, & Synytsya, 2001). The stretching vibration peak of 1040 cm^{-1} suggested the presence of C-H-O link bond position.

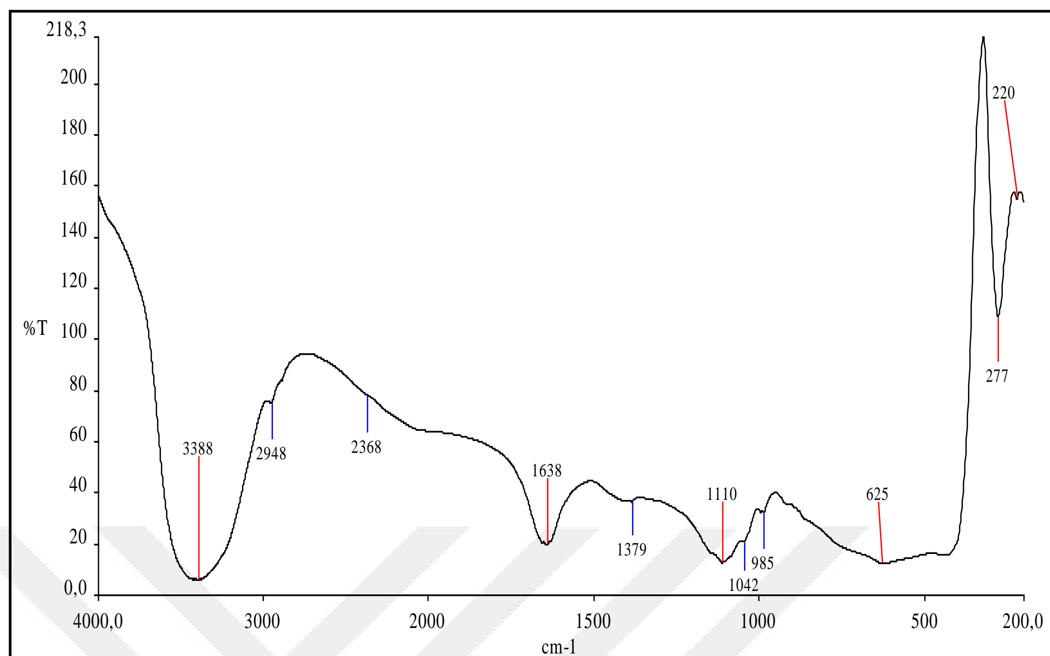


Figure 3.19 FT-IR spectrum of Fr-I

According to ^{1}H-NMR spectrum (Figure 3.20) of Fr-I, signals at 2.2 -2.8 ppm were attributed to peaks of C-H. The C-C signals were located in the region 3.0-4.2 ppm, which were attributed to protons of the C-2-C-6 of hexosyl glycosidic ring. The NH signals at 7.1-7.5 ppm indicated amino acid and peptide structure.

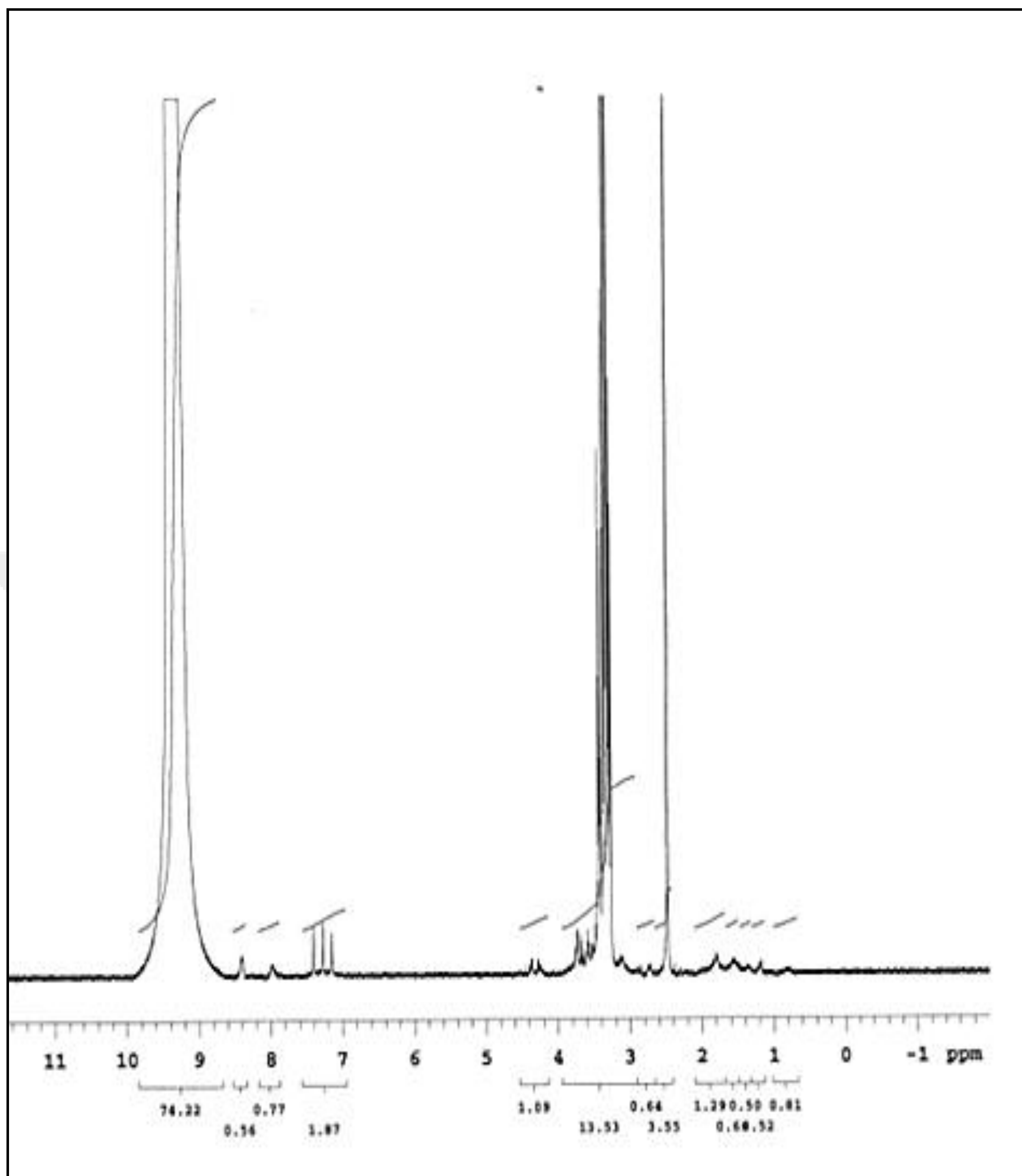


Figure 3.20 ^{1}H-NMR spectrum of Fr-I in DMSO

The obtained results demonstrated that Fr-I of produced by *P. sojar caju*, had functional groups, bonds and structures which are presence in glycoprotein type polysaccharide. It was concluded that Fr-I had a complex structure by having carbohydrate and protein contents.

The thermal stability of polysaccharide is an important characteristic with respect to its applications. Degradation of Fr-I occurred by two steps as observed in TG analysis (Figure 3.21). In the first step 6.769% of weight loss was recorded from

38.16 to 78.74°C due to loss of alcohol molecules and moisture. In the second step degradation 11.196% weight loss was observed between 169.94 to 369.44°C. Decomposition temperature of Fr-I was around of 276.91°C. Degradation temperature of the purified EPS by *P. pulmonarius* was 217°C (Shen, Shi, & Xu, 2013).

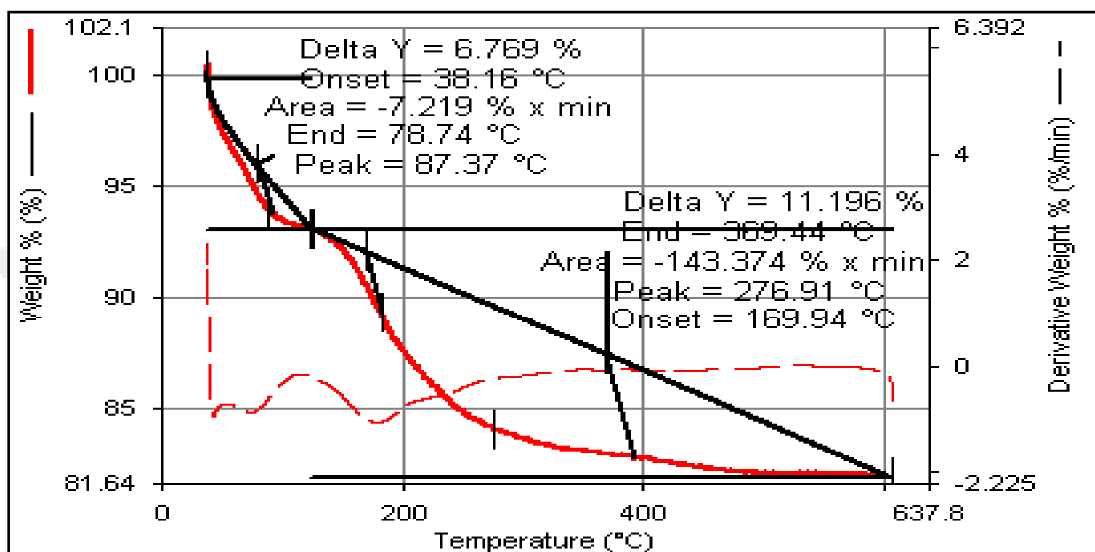


Figure 3.21 TGA curves of Fr-I

3.5 Antioxidant Activities of Partial Purified EPS from *P. sajor caju*

Antioxidant activities have been attributed to kind reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion catalysts (Frankel, & Meyer, 2000).In this work, the in vitro antioxidant capacity of Fr-I of EPS produced by *P. sajor caju* were evaluated using different biochemical methods including hydroxyl, DPPH and superoxide radical scavenging assays and reducing power and chelating activity on Fe^{2+} assays.

Hydroxyl free radicals and their derivative radicals are highly potent oxidants, which can react with most biomacromolecules in living cells and induce severe biological damage and lipid peroxidation. As shown in Figure 3.22, the Fr-I had an obvious hydroxyl radical scavenging activity increased from 10 ± 1.08% to 61.3 ± 5.4% in a dose-dependent manner, which was higher than that of 50.8 ± 4.8% for

Boletus edudis, 49.4 ± 4.2% for *Pholiota adipose*, 26.2 ± 2.4% for *Antrodia camphorate* at 5 mg/mL (Lin et al., 2012). It has been proposed that the hydrogen or electron abstraction mechanism might be the best explanation of why polysaccharides can inhibit the formation of hydroxyl radicals (Chen, Wang, & Qu,2009; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006).

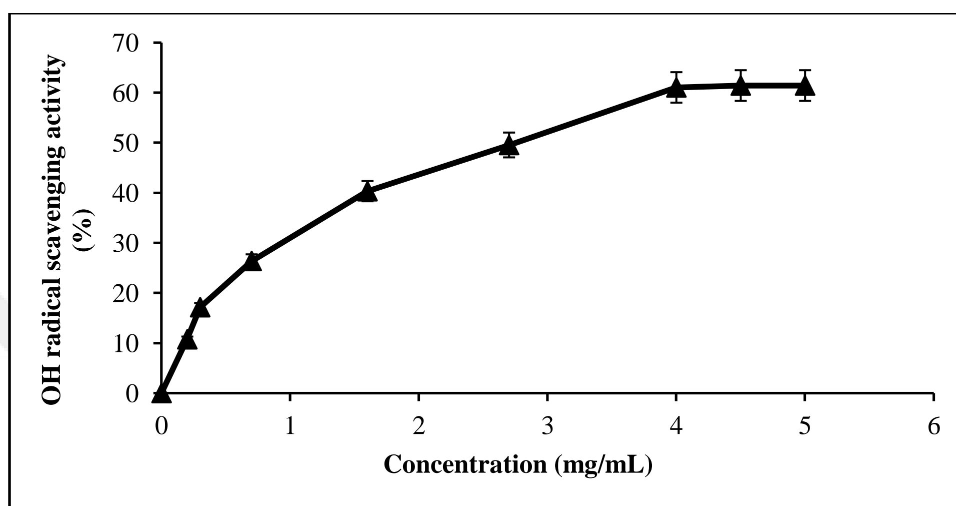


Figure 3.22 OH radical scavenging activity of Fr-I of produced EPS by *P. sajor caju*

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol and can easily undergo scavenging by an antioxidant. DPPH free radical is largely used to evaluate the free radical scavenging activities of natural substance (Leong, & Shui, 2002). As shown in Figure 3.23a, the Fr-I had an obvious DPPH scavenging ability increased from 7.4 ± 0.3% to 41.83 ± 4.0% in a dose-dependent manner. The scavenging rate of the Fr-I was much higher than 20 ± 0.3% of *Boletus aereus* EPS (Zheng et al. 2014). Vit C was used as a positive control (Figure 3.23b).

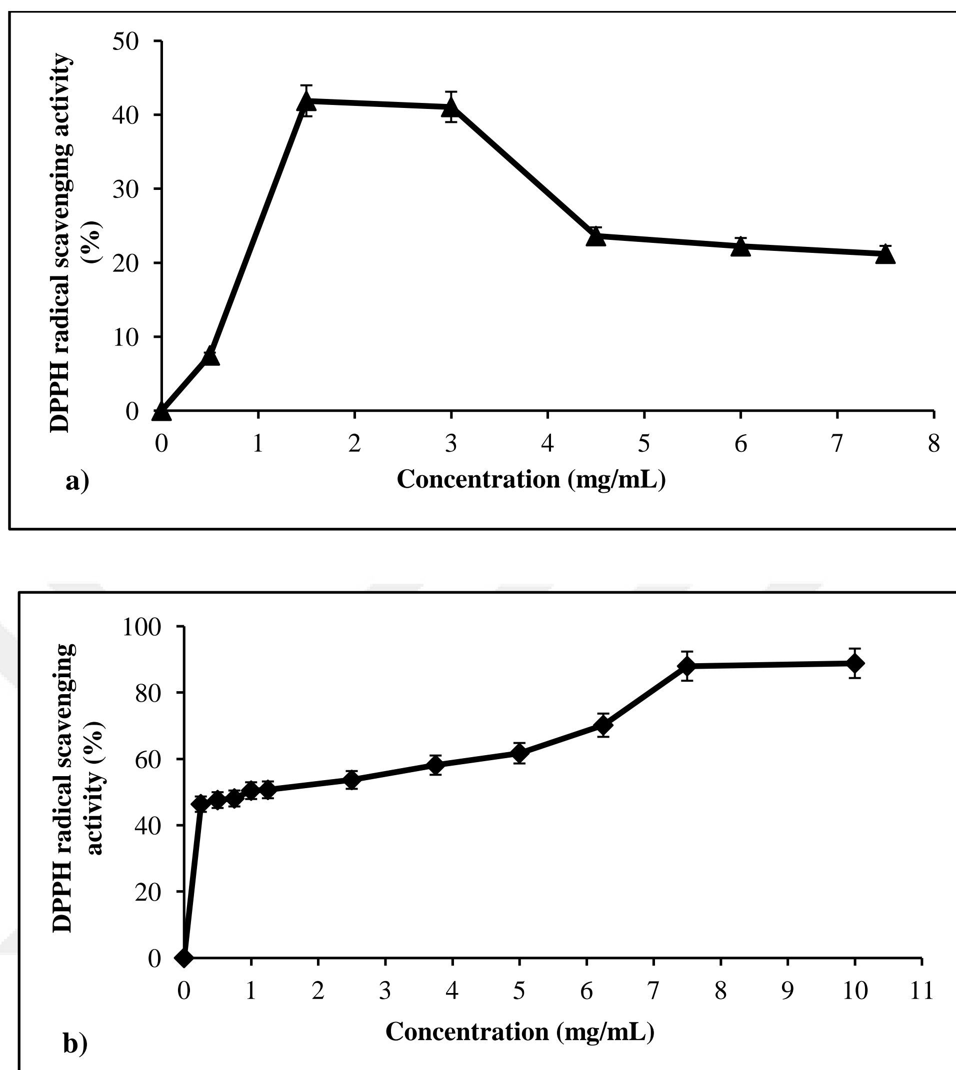


Figure 3.23 DPPH radical scavenging activity of Fr-I of produced EPS by *P. sajor caju* (a) and Vit C (b)

Superoxide radicals are harmful free radicals for cellular components, and the presence of superoxide anions can magnify cellular damages, as they produce other types of free radicals and oxidizing agents (Athukorala, Kim, & Jeon, 2006). Therefore, the superoxide radical scavenging ability is of great importance to its potential antioxidant activity.

The scavenging activities of superoxide radical by Fr-I of produced EPS by *P. sajor caju* are shown to be dose-dependent in Figure 3.24. The superoxide radical scavenging effect of Fr-I ranged from 1.31 ± 0.2% at 0.25 mg/mL to 18.83 ± 1.5% at 5 mg/mL, which was similar to *Cordyceps militaris SU5-08* and *Bacillus edudis* (Lin

et al., 2012). It has been reported that the mechanism of superoxide anion scavenging may be associated with the dissociation energy of O–H bonds (Tsiapali et al., 2001).

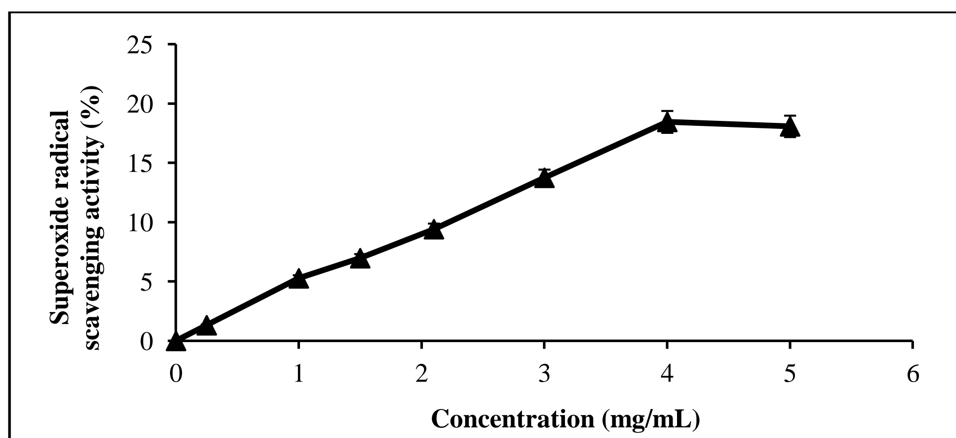
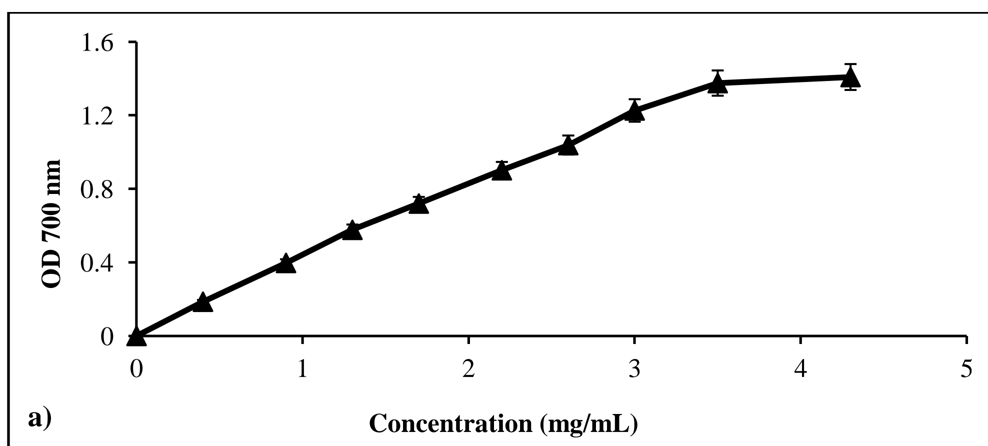


Figure 3. 24 Superoxide radical scavenging activity of Fr-I of produced EPS by *P. sajor caju*

The reducing power of compound may serve as an important indicator of its potential antioxidant activity (Kumar et al., 2004).As shown in Figure 3.25a,our results suggested that Fr-I had stronger reducing power. BHA, α-tocopherol and Vit C was used as positive controls (Figure 3.25b). Compared with other studies, the reducing powers of Fr-I at 3.5 g/mL were also higher than some researcher's data (Ma, Chen, Zhu, & Wang, 2013; Li et al., 2013; Gao, Wang, Wang, & Wang, 2013).



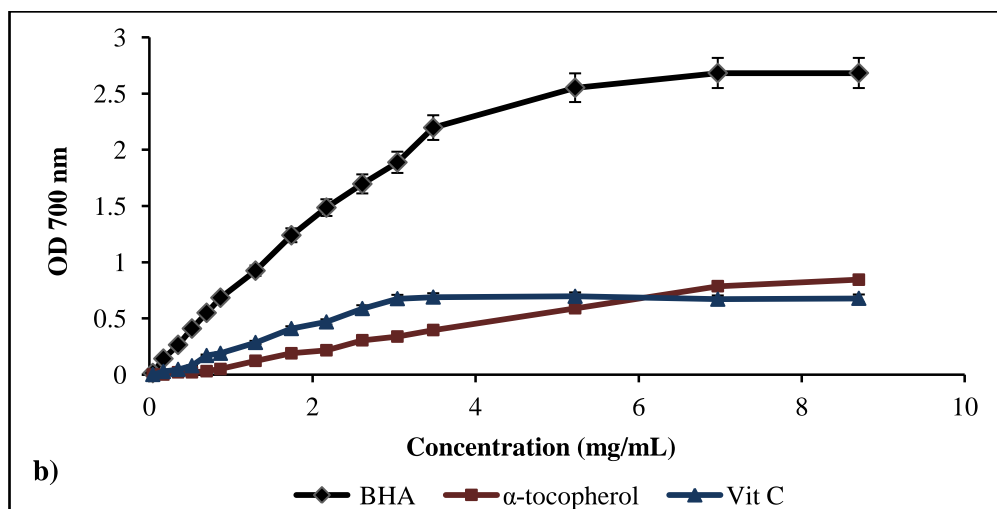


Figure 3.25 Reducing power of Fr-I of produced EPS by *P. sajor caju* (a)and BHA, α-tocopherol, Vit C (b)

Iron is the most powerful pro-oxidant among the various metal ions and catalyzes the oxidative changes in lipids, proteins and other cellular components (Kohgo, Ikuta, Ohtake, Torimoto, & Kato, 2008). The results regarding the Fe^{2+}-chelating activity of Fr-I revealed that the EPS was a less effective chelating agent (Figure 3.26a). Metal chelating capacity is significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. EDTA was used as positive control (Figure 3.26b).

The iron chelating ability of the EPS found to be related with the concentration of sample. The iron chelating ability of EPS was determined as 19.8 ± 1.7% in this study.

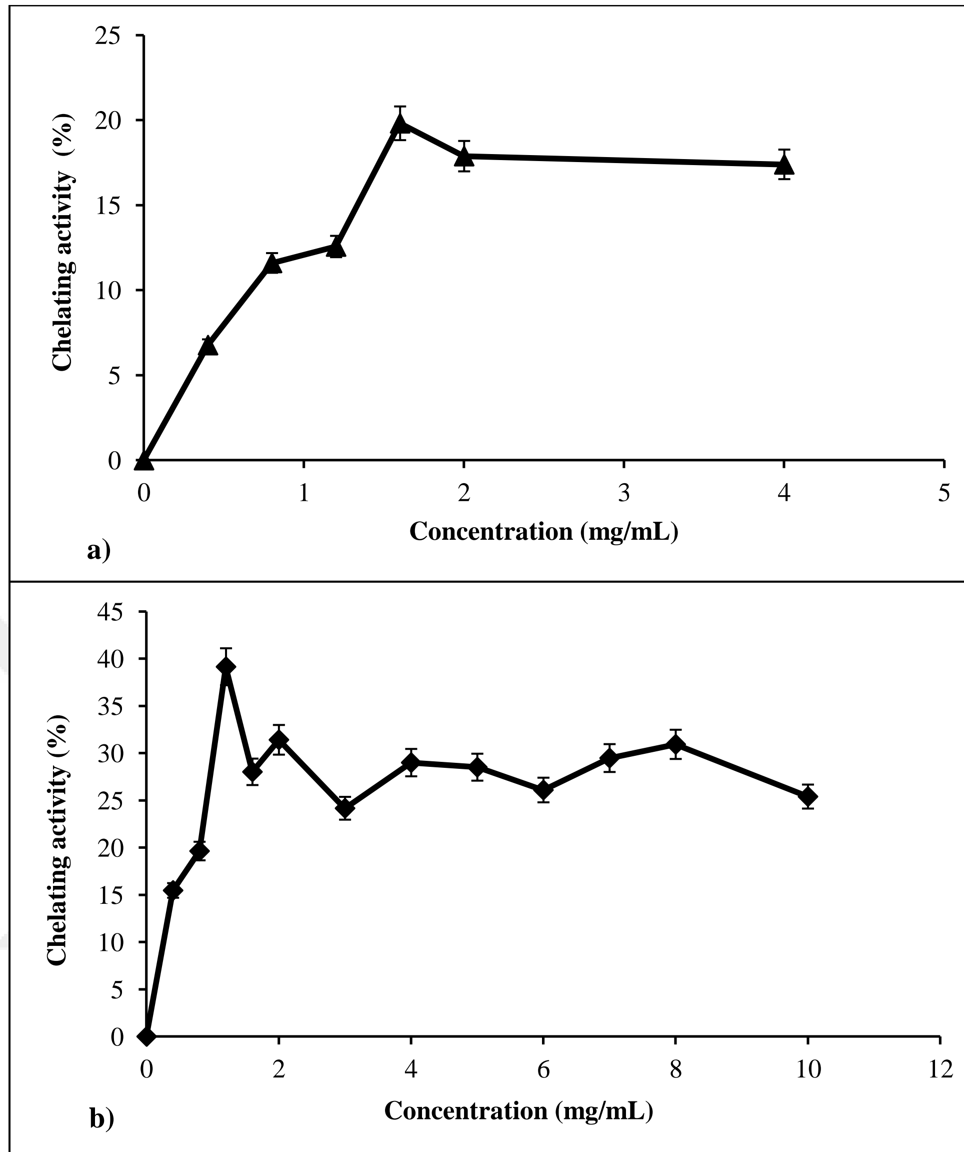


Figure 3.26 Chelating activities of Fr I of produced EPS by *P. sajor caju* (a) and EDTA (b)

CHAPTER FOUR
CONCLUSION

During the recent years, much interest has been generated in EPS produced by submerged cultures of numerous fungi because they have biological and pharmacological activities including anti-tumor, antioxidant, anti-cancer activities, etc. (Han et al., 2006; Li, Chen, Wang, Tian, & Zhang 2010; Song, Lin, Yang, & Hu, 2008; Zhao et al., 2012). Submerged cultures of medical fungi obviously give rise to potential advantages of effective EPS production in a compact space and shorter time without significant contamination risk (Kachlishvili, Penninckx, Tsiklauri, & Elisashvili, 2006).

In this work, the optimization of SmF conditions for EPS production by *P. sajor caju* was studied. One partial pure fraction of EPS was obtained by gel filtration chromatography and characterized by TLC, FT-IR, ^{1}HNMR and TGA. Furthermore, the antioxidant capacity experiments showed that the partially purified EPS might be a valuable compound for medicine production.

In this present study, EPS production by four different *P. sajor caju* using potato peel or grape waste in SSF with optimized basal medium in SmF were carried out. But, EPS production by *P. sajor caju* in SFF has been not occurred successfully. We know research about EPS production by *Pleurotus* spp.is limited to this study.

After determination of the best EPS producer strain as *P. sajor caju* and the its incubation period as 5th, optimization of carbon and nitrogen source and their concentration, Mg^{2+} ion concentrations, temperature, pH and were carried out. The optimal initial pH, temperature and agitation rate of culture were determined as 5.0, 25°C and 150 rpm, respectively. The optimized medium composition was as follows: 90 g/L of glucose, 10 g/L of yeast extract, 10 g/L peptone, and 100 mM of $MgSO_4$.The produced EPS in optimal condition was determined as33.32 ± 1.6 g/L. The chemical composition of 1 mg isolated EPS was formed 2.26 ± 0.41 µg protein, 219.75 ± 10 µg total carbohydrate, 28.59 ± 0.32 µg reducing sugar, 15.73 ± 1.1 µg

nitrogen, 1.01 ± 0.01 µg pyruvate and 6,7 ± 0.5 µg uronic acid. Results of chemical composition analyses demonstrated complex structure of produced EPS. In the present study, high yield EPS produced by SmF was carried out.

Produced EPS by *P. sajor caju* in optimum SmF conditions was extracted and partial purified by gel filtration chromatography and the molecular weight of the EPS was determined as 25± 0.3 kDa.

The partial characterization of the purified EPS was studied by HPTLC, FT-IR and ^{1}H-NMR. When compared HPTLC chromatograms of purified EPS and standards (glucose, mannose, rhamnose, galactose, xylose) were clear that EPS mainly occurred glucose monosaccharide. However, peaks of produced EPS in FT-IR spectrum showed that structure of EPS looks like glucose units. Lastly, according to ^{1}H-NMR spectrum of produced EPS in optimum conditions by *P. sajor caju* had functional groups, bonds and structures which are presence glycoprotein type polysaccharide. It was demonstrated that produced EPS had a complex structure and chemical composition analyses of produced EPS also supported this result. On the other hand, thermal stability of produced EPS was investigated by TGA analysis. The produced EPS observed high thermal stability with the maximum degradation temperature at 276.91°C.

Additionally, the in vitro antioxidant capacity of partial purified EPS was investigated using different biochemical methods including hydroxyl, DPPH and superoxide radical scavenging assays and reducing power and chelating activity on Fe^{2+} assays. The in vitro antioxidant assay showed that strong antioxidant properties, especially hydroxyl free radical scavenging ability and reducing power. These results suggest that partial pure EPS from *P. sajor caju* may be a natural alternative to conventionally used food or medicine production with potential antioxidant activity. Besides, produced EPS has been high antioxidant capacity so it can use in biotechnological applications, such as cosmetics, medicine, pharmaceutics and food.

Microbial EPS have found out standing medical applications since the mid-20th century. Other EPS entered medicine firstly as conventional pharmaceutical excipients (e.g., xanthan – as suspension stabilizer, or pullulan – in capsules and oral care products).

The numerous potential applications still wait to be developed into commercial pharmaceuticals and medical gadgetry. Based on previous and recent results in important medical-pharmaceutical domains, one can undoubtedly state that EPS's medical applications have a broad future ahead.

Due to EPS has antioxidant activity at same level as compared to synthetic antioxidants and they are natural and non toxic, generally EPS is more preferred.

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