Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.7, No.6, 2017

Production of Exopolysaccharides from Submerged Culture of Antrodia Camphorata S-29

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This research work was financially supported by the Shanghai Natural Science Fund (15ZR1428900), the Hujiang Foundation of China (D15012), Shanghai College Students Innovation and Entrepreneurship Training Program (Grant number SH2016128).

Abstract
Antrodia camphorata is a unique mushroom of Taiwan, which has been used as a natural therapeutic ingredient in Traditional Chinese Medicine (TCM) for protection of diverse health related conditions. Polysaccharides produced from *A. camphora*ta have attracted much attention of research due to cytotoxic activity and miscellaneous activities. In this paper, we report on the fermentation conditions species-specific exopolysaccharides (EPS) from *A. camphorata* in submerged culture. A favorable medium for EPS production was obtained only by single-factor experiment, where Glucose and Yeast-Extracts were identified to be the most suitable carbon and nitrogen sources, with the concentration of 40 g/L and 5.0 g/L respectively. Zinc sulphate was identified to be the best salt source with the concentration of 0.4g/l. Initial pH and inoculum size for mycelial growth and EPS yield were 6.0 and 15% respectively. The maximum EPS production was 0.474 g/L in shake-flask culture, which is higher than the baseline media that was 0.351 g/L. This study provides the baseline information about production conditions for this specific specie which is crucial data to know before any further studies as it determines the properties and quantity of the desired produced specie.
Keywords: A. camphorata; Exopolysaccharide; Submerged culture.

1. Introduction

Extracellular polymeric substances/Exopolysaccharides (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances (Barbara Vu et al.2009). EPS makes the intercellular space of microbial aggregates, form the structure and architecture of the biofilm matrix. EPS major functions include mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration (Larroche et al.2007; Song et al. 2005)

EPS of microorganisms have been broadly studied because of their medicinal properties (Song et al.2005; Vuyst et al 2000) and their important applications in various fields, such as food, pharmaceutical, and cosmetics industries (Lee et al.1990; Kuo et al.1996). Most polysaccharides produced by fungi possess biological and pharmacological activities and studies on polysaccharides with hypolipidaemic effect, anti-tumor activity (Mahapatra et al. 2013), immunostimulating activity (Vuyst et al.2002) and hypoglycaemic activity (Yang et al.2001, Sugiura et al.1980) have been reported.

A. camphorata (AC), which is used as a traditional medicine in Taiwan, has been identified as a fungus of the family Basidiomycetes (Kiho et al.1993). It is a special fungus parasitic on the inner wall of the endemic species Cinnamonum kanehirai Hay (Kiho et al.1997; **En-Shyh Lin** et al. 2005). In Taiwan *A.camphorata* is well known by the names “chang-chih” or “neu chang ku.” The fruit bodies of fungi are valued as medicinal herbs and have been used traditionally for the treatment of food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer (Wu et al.1997). Some bioactive constituents from the fruiting bodies of *A. camphorata* have been isolated and characterized, including polysaccharides, steroids, triterpenoids, and sesquiterpene lactone (Chang et al.1995; 2004; Tsai et al.1985; Chen et al.1995; Cherng et al.1995; Chiang et al.1995). Polysaccharides extracted of A. camphorata exhibit biological effects in their antioxidant and free radical-scavenging activities (Cherng et al.1996), stimulating macrophage activity, and anti-hepatitis B virus activity (Chiang et al.1995).

Artificial cultivation technique of *A. camphorata* in the solid-state fermentation has been studied. The use of submerged cultures has proved to be more advantageous in higher mycelial production in a more compact space over a shorter time with a lower chance of contamination and production of other valuable metabolites such as exopolysaccharides Several edible and medicinal mushrooms that produce polysaccharides respond to environment factors directly and for some nutritional conditions determine the degree of exopolysaccharide formation (Yang et al.1996; Lee et al.2002). Studies on the influences that the cultivating conditions have on the

exopolysaccharide production by A. camphorata have been reported and it is shown that EPS is easier to obtain from submerged culture than the internal polysaccharide localized within the mycelia, but exhibits similar biological activities (Song et al.2002). In other species, mycelial growth rate, EPS yield rate, and EPS productivity have been shown to vary with environmental conditions and medium composition, including carbon source, nitrogen source, pH, etc. (Bae et al.2000; Fang et al.2002; Kim et al.2001; Papagianni et al.2004; Barbara et al.2009; Lin et al.2010).

This study will contribute to baseline information about submerged fermentation conditions for production of our specific specie of A. camphorata, where further research on optimization by statistical methods will be carried on, also Purification and structural composition.

2. Materials and methods

2.1 Microorganisms and cultural conditions

The A. camphorata S-29 strain, numbered as CGMCC No. 9590 in the Chinese common microbe bacterial preservation administration center, was maintained on glucose/soybean medium slants at 4°C. Cultivation was performed in two stages. The seed culture (pre culture) medium was consisted of the following components: 20.0 g/L glucose, 4.0 mL/L Soybean milk, 0.5g/L KH_2PO_4, 0.5 g/L $MgSO_4$^$7H_2O$ and 0.5g/L Citric Acid. The initial pH was adjusted (pH 5.0). The solution was sterilized at 115°C for 15 minutes. The pre culture that was inoculated with 10 ml mycelium was incubated on a rotary shaker at 150 r/min and 28°C for 4 days. The flask culture experiments were performed in 500 mL flasks containing 100 mL of fermentation medium, which was inoculated with 15 % *(v/v)* of the seed culture. The flasks were cultured under the same conditions as above for 10-12 days.

2.2 Effect of carbon source

To find a suitable carbon source for mycelial growth and exopolysaccharide production in A.cinnamomea, we cultivated carbon into media containing various carbon sources named glucose, sucrose, maltose, lactose, glycerol, we added each carbon source to the basal medium at a concentration of 60g/l for 14 days ,For further experiments we chose, we choose the best glucose source of carbon and we further investigate its effect by varying its concentration from (40-80)g/while keeping nitrogen constant. All the shake flask experiments were performed at 28ºC, 150 rev min) and an initial pH of 5 for 14 days.

2.3 Effect of nitrogen source

The effects of various nitrogen sources on biomass and EPS production was investigated by cultivating A.cinnamomea into media containing various Nitrogen sources which are80ml/l soybean 5g/l ammonium chloride (AC), 5g/l sodium nitrate (SN), 5g/l Corn Steep Powder (CSP), 5g/l Beef (B), 5g/l yeast-extract (YE), each nitrogen source was added to the basal medium for 14 days ,For further experiments the best nitrogen source was investigated by varying its concentration from (3-7)g/l while keeping carbon source constant, All the shake flask experiments were performed at 28ºC, 150 rev min)1 and an initial pH of 5 for 14 days.

2.4 Effect of salt

The effects of bio elements on biomass and EPS production were determined by supplementation of various mineral sources in the medium. These sources were (Fe^{2+}, $CaCl_2$, $ZnSO_4$, and MnSO4), all these were tested by the concentration of 0.2 and 0.5g/l for each salt while keeping carbon and nitrogen source chosen form above experiments constant, the best salt was then further investigated by varying its concentration form (0.2-1)g/l while keeping carbon and nitrogen source constant and experiments were performed at 28ºC, 150 rev min)1 and an initial pH of 5 for 14 days.

2.5 Effect of Inoculum amount

To investigate the effect of the inoculum amount to be used, we subjected our media which is made of the three factors from above experiments with different inoculum amount ranged from (6-18) %9(v/v), and the one which lead to high Eps and mycelial production was chosen to be used for further experiments, this experiment was also performed at 28ºC, 150 rev min) and an initial pH of 5 for 14 days.

2.6 Effect of pH

The optimal initial pH in flask cultures was studied by fermentation in different pH culture media, i.e. pH 3, 4, 5, 6, 7 and 8; the pH value, which leads to higher mycelial yield and maximum EPS production, was chosen as the best pH value for fermentation and was used for further experiments.

2.7 Fermentation in a 7 L fermenter

Fermentations were carried out in a 7-l stirred tank bioreactor for a comparison of efficiency between basal

conditions and favorable chosen (optimal) culture operations. Under basal culture conditions, the fermenter was filled with basal medium composition, pH 5 and 10% (v/v) inoculums from seed cultures. The fermentation was cultured under favorable culture conditions and compositions from the flask culture screening mentioned earlier. The stirred tank fermentations proceeded agitation speed of 150-rev min). All experiments were performed in triplicate (n = 3).

2.8 Analysis of EPS

EPS was obtained from the remaining filtrate by mixing 1litre of the filtrate with 56 mL Trichloroacetic Acid. It was standing at 4^0c overnight to eliminate proteins, 95% Ethanol was added and left overnight at 4^0c, the precipitated EPS was then collected by centrifugation at 5000RPM for 15 min and an adequate amount of water was added to collected supernatant. The solution was then put in special dialysis bags for 3 days to remove small sugar and salt molecules. The amount of EPS production was estimated calorimetrically by phenol–sulfuric acid method using glucose as a standard. The absorbance was measured at 485 nm using spectrophotometer.

2.9 Analysis of biomass

The biomass was obtained by vacuum filtration, and then dried overnight to a constant weight at 65 ^{o}C; Measured and recorded.

Statistical analysis

All data were expressed as mean ±standard deviation of triplicate results. And the values were analyzed by excel 2016.

3. Results and discussion

Different effects of culture conditions on mycelia growth and EPS production were investigated and reported as follows

3.1 Screening of carbon sources and its concentrations

Carbohydrates are a major component of the cytoskeleton and they are an important nutritional requirement for growth and development of higher fungi. However, carbon sources for biomass and EPS production are often different. All of the selected carbon sources resulted in high mycelial growth and product yield. The mycelial dry weight and EPS yield in Glucose medium were higher than those in other carbon sources *(shown in Figure 1a)*. So glucose was chosen as the carbon source for analyzing EPS from *A. camphorata ,* and our results are supported by previous research done by Subhadip Mahapatra, Debdulal Banerjee where they reported that ,in most cases composition of EPS is independent to the type of carbohydrate used for the production of that EPS, but the production intensity is very much dependent on the carbon source used and its concentration., glucose, sucrose, maltose, lactose, fructose, galactose, xylose, cellobiose, sorbitol, xylitol, mannitol, and different types of agricultural byproducts are used as carbon source in the culture medium. In most of the cases glucose, sucrose, and maltose have been chosen as the most influential carbon sources for the production of fungal EPSs. These observations indicate that there may be some effects of catabolic repression of different sugars in various EPS synthesis, which different fungal strains have different sugar uptake fascinations, or that fungi may easily metabolize these sugars. (Subhadip et al.2013). It is also reported that the concentration of selected carbon source in the culture media is critical factor for EPS production. In addition, the concentration, between 30 and 60 gm/L carbon was suggested to best support EPS production from fungi

It can be seen (*Figure 1b*) that the maximum concentration of 11.52 g/L for mycelia yield was achieved with 50g/L glucose concentration.

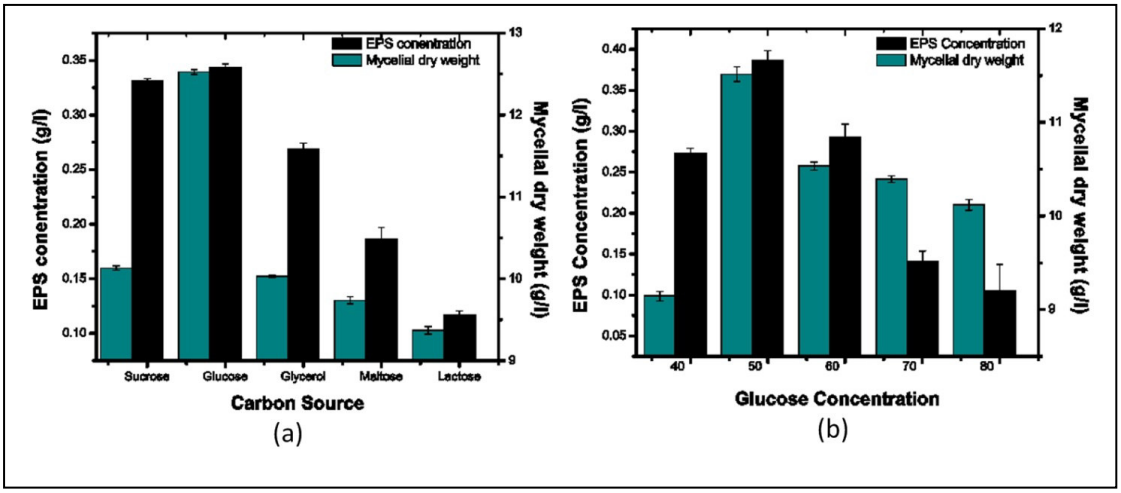

Figure: 1. Effect of carbon sources (a) and glucose concentration (b) on mycelial dry weight and EPS yield by A. camphorate

3.2 Screening of nitrogen sources and its concentrations

The effect of nitrogen sources on secondary metabolism is conditioned by many factors, including the producing organism, the type and concentration of the nitrogen sources and culture method (stationary or submerged). It has been reported from numerous research findings that, yeast extract and corn steep powder are good nitrogen supplements that induce EPS production from different fungal strains among the various inorganic sources ammonium chloride, ammonium sulfate, sodium nitrate, potassium nitrate, urea, and diammonium oxalate monohydrate are commonly studied by researchers (Subhadip et al.2013). Subhadip Mahapatra, Debdulal Banerjee said many observations suggested that in the presence of inorganic nitrogen sources, fungi produce less EPSs in comparison to organic nitrogen supplements. Among the inorganic nitrogen sources, ammonium salts are frequently more efficient than other inorganic salts. In very few studies, other inorganic salts have been found to best provide nitrogen sources for EPS production from fungi. Nitrogen sources in mushroom cultures primarily provide nitrogen incorporated into the cell mass in the form of proteins and nucleic acids. Our results indicated that yeast extracts are the best nitrogen sources and can greatly stimulate biomass and EPS production.

From our experiment *A. camphorata* could grow on a number of different nitrogen sources, but the nitrogen sources effects on EPS and biomass yield were quite distinct (*Figure 2a*). Among the six different nitrogen sources examined, yeast-extract was the most effective for enhancing the EPS yield (0.439 g/L) and biomass (10.76 g/L) by *A. camphorata*. Yeast- extract has often been used to provide necessary growth factors; however, too high a concentration of yeast extract would lower the use of other carbon sources and cause the reduction of metabolites (*Figure 2b*)

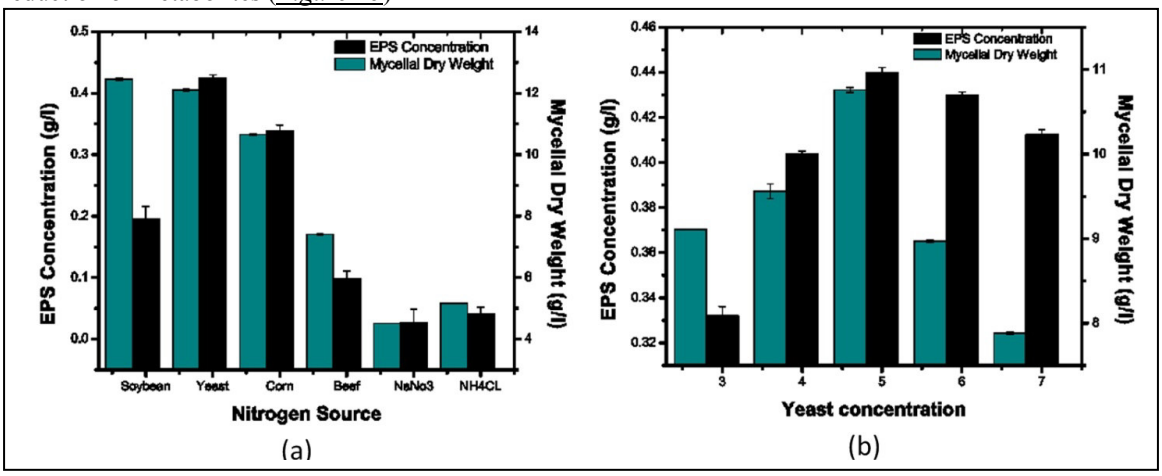

Figure: 2. Effect of nitrogen sources (a) and yeast-extract concentration (b) on the mycelial dry weight and EPS yield by A. camphorate

3.3 Influence of salts sources and its concentrations

A. camphorata had the ability to grow on a number of different salts sources, (*Figure 3*). Among the four different salts sources examined, $ZnSO_4$ was the most effective for enhancing the EPS yield (0.478 g/L) and

biomass (12.35 g/L) by *A. camphorata*

Mineral ions in a biological system are normally combined with protein as enzymes for effective biological catalysis. The present work showed that maximal biomass and EPS production was found in a medium supplemented with magnesium ions (SO^{4+}).

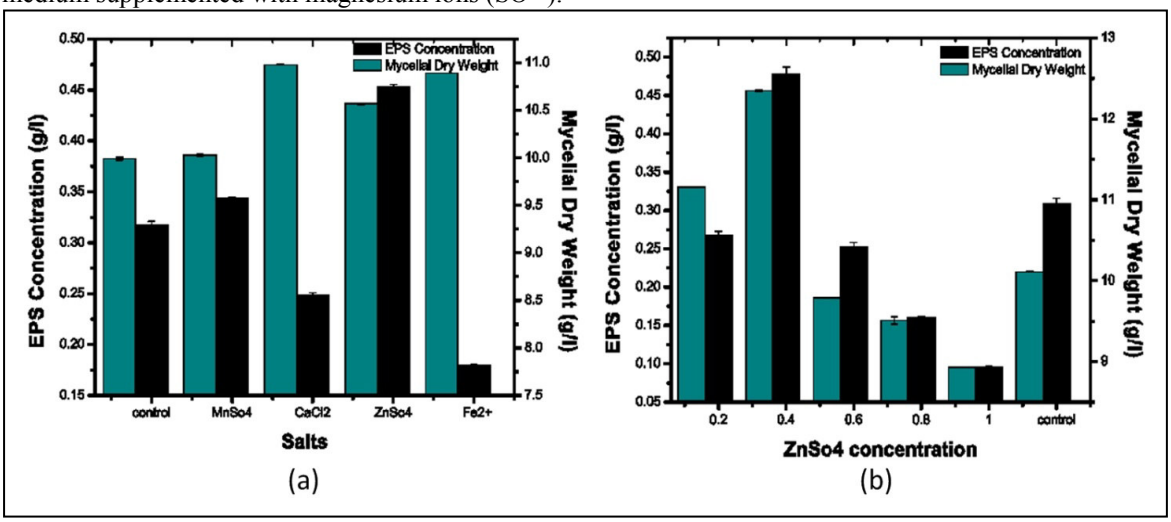

Figure: 3. Effect of salts sources (a) and zinc sulphate concentration (b) on the mycelial dry weight and EPS yield by *A. camphorate*

3.4 Influence of pH and inoculum size

Besides carbon and nitrogen sources, many growth factors also have positive impact on the yields of EPS. The pH of culture medium is another reflective factor that persuades the fungal EPS production. Generally, fungi favored low pH for EPS production with a range between pH 3.0 to 6. In 2004, Shu and Lung examined the effects of pH on EPS production by *Antrodia camphorate* and reported that variation in medium pH induces *A. camphorate* to produce EPS with different molecular weight (Mw). They noticed that relatively high Mw EPSs in low amount was produced at lower medium pH while low Mw EPS with high yield was recorded at higher medium pH (Shu et al.2004), The initial pH of the culture medium is an important factor affecting biomass and EPS production of mushrooms in submerged cultures Generally, an acidic environment will satisfy biomass and EPS biosynthesis for most mushrooms. It has also been reported that the optimal culture pH for biomass and EPS production by a few mushrooms occurred with medium or high medium culture PHS). However, our results showed that the optimal culture initial pH for biomass and EPS production was 6.,from our experiment it can be determined that the optimal conditions are pH 6.0, 15% of the inoculum size (Figure 4) Culture temperature is one of the most important parameters influencing the production of polysaccharides.Most of the fungal strains produced maximum EPSs within a temperature range 22 ºC to 30 ºC (Subhadip et al.2013), this has been well reported by different researchers, and we choose 28 ºC as the production temperature.

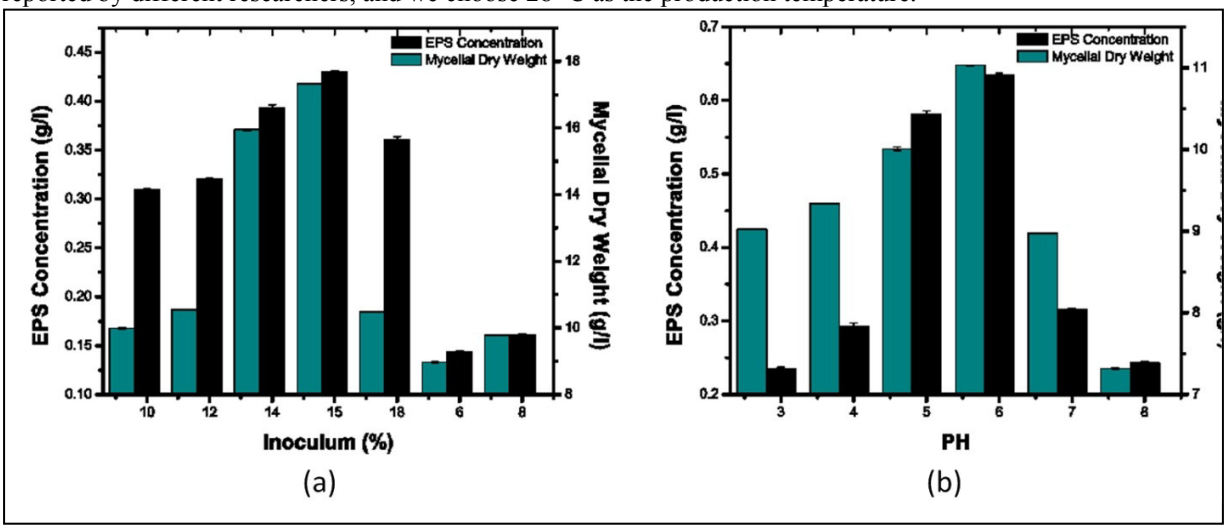

Figure 4: Effect of Inoculum amount (a) and pH (b) on the mycelial dry weight and EPS yield by *A. camphorate*

3.5 Fermentation Dynamics

For the fermentation results, the effect of the basal medium and optimized medium were compared in 7-l stirred tank fermenters under the following culture conditions: temperature 28ºC, pH 6, inoculums 15% (v/v), and agitation speed 150-rev min). The optimal environmental factors and nutritional requirements were obtained from a series of preliminary experiments in shake flasks. Figure 5a shows that the maximum biomass and EPS production were 9.25g/1 and 0.35g respectively, following fermentation in the basal medium for 12 days. In the optimized medium, the maximum biomass 13.93g/l and EPS production 0.484g/l were achieved only after 9 days of 12 days fermentation period (Fig. 5b). Therefore, a 38.29% enhancement of EPS fermentation production in the chosen favorable medium (optimized) was accomplished when compared to that in the basal medium.

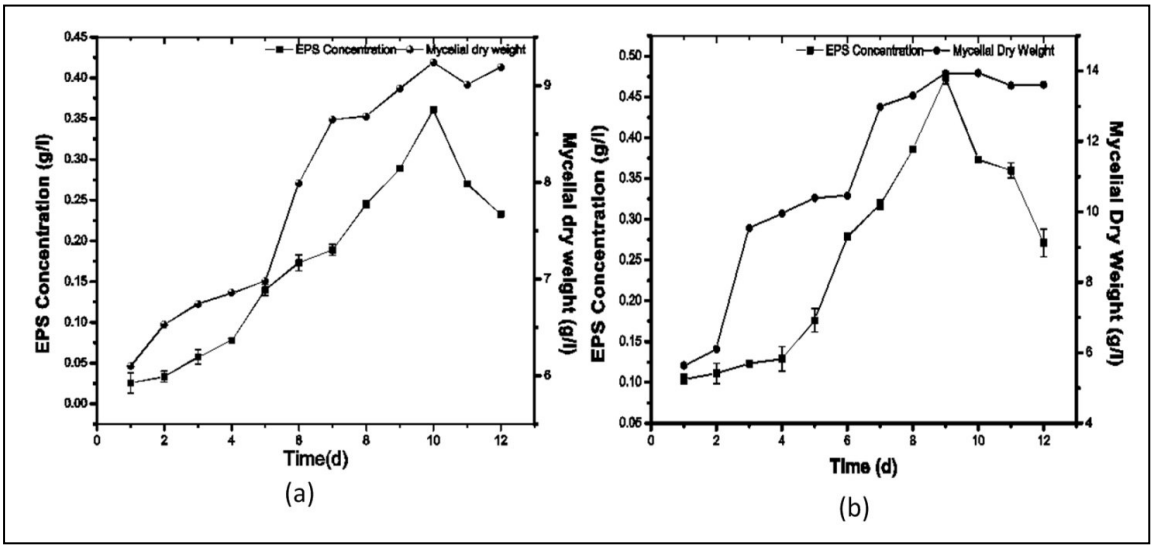

Figure: 5. typical time courses of the mycelial growth and EPS yield by A. camphorata under (a) basal media and (b) optimized media

4. Conclusion

Submerged cultures were used to identify growth-influencing nutrients of *Antrodia camphorata*. EPS and Mycelial production of *Antrodia camphorata* S-29 were higher on optimized media compared to basal media. It is important to study the basic nutrients requirements for maximum production of the EPS. Further research will be carried on and will involve purification, identification of the structure and anti-cancer activity of the pure fraction of the extracted EPS.

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