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Physiological Studies on Mycelieal Growth and Exopolysaccharide Production by *Fomitopsis feei*

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A B S T R A C T

Mycelial growth and exopolysaccharide production of *Fomitopsis feei* was studied in common production medium by changing different physiological parameters such as carbon, nitrogen sources, inoculum size, incubation time, pH, temperature, agitation. Glucose, ammonium sulphate, 6mm of inoculum, 8th and 14th days of incubation, pH 3, 30°C temperature and 150 rpm agitation were the best for the production of mycelium. Among the carbon and nitrogen sources tested glucose and malt extract were supported the high production of exopolysaccharides for both the sources respectively. They were further studied in different concentrations. Six mm of inoculum, 7 and 14 days of incubation, pH 6, 30°C temperature, 150 rpm agitation supported the maximum production of exopolysaccharide among the tested.

Introduction

In general, factors influencing the exopolysaccharide production and mycelia growth of fungi mainly include composition of culture medium and environmental conditions. The carbon source is mainly used in cellular constituent, synthesis of new cells, production of polysaccharides and as an energy source. Nitrogen comprises about 10% to 14% of cell dry weight. It is incorporated into cell mass in the form of protein and nucleic acids. Organic and inorganic nitrogen sources were investigated in order to compare exopolysaccharide (EPS) and intracellular polysaccharide (IPS) production by *Tricholoma mongolicum* in submerged cultivation (Wu *et al.,* 2012). Inoculation density (or inoculum size) is also an important factor for many cell culture processes (Wu *et al.*, 2012; Zhang and Zhong, 1997).

Appropriate harvest time selection is also an important factor to obtain the maximum fungal production in submerged culture. There will be time difference for better production of mycelia and polysaccharides. In general, the harvest time of the mycelium, in complex carbohydrate medium should not extend beyond 20 days after the inoculation in order to avoid fungal cell lysis (Wu *et al.*, 2003). The pH of the medium is very important but it is often a neglected environmental factor. The medium pH may

affect cell membrane function, cell morphology and structure, the uptake of various nutrients and product biosynthesis. Culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production. The higher fungi usually require lower pH of the culture than bacteria, but not so low as moulds. It ranges usually between pH 4.0 and 7.5.

The higher fungi usually require lower temperature for vegetation than yeast and mould (ranging 25–28°C). The optimum temperature for *Agaricus campestris* (Moustafa, 1960) ranges 25–30°C, for *Morchella hybrida* 20-25°C and for *Phlebia radiate* 28°C (Hatakka *et al.,* 1986).

Agitation is also an important parameter for adequate mixing, mass and heat transfer. Agitation creates shear forces, causing morphological changes, variation in their growth and product formation, and also damage to the cell structure (Wu *et al.,* 2012; Smith *et al.,* 1990; Pfefferle *et al.,* 2000).

Fomitopsis feei is a brown rot fungus belongs to the family Fomitopsidaceae. During the screening of exopolysaccharide (EPS) production by this fungus on 9 types of media, common production medium showed good result (data not shown) hence the present study is carried out to optimize the EPS production.

Materials and Methods

Isolation and identification of *F. feei*

Fomitopsis feei fruit bodies were collected from Pakhal forest, Warangal during rainy season and pure mycelial culture was developed on malt extract agar medium and was identified at molecular level by 28S rDNA analysis (Hima Bindu and Singara Charya, 2017).

Optimization of common production medium

One factor at a time method was followed in which 25 mL of common production broth was inoculated with 6 mm disk of actively growing *Fomitopsis feei* culture plate and incubated at 28°C for 7 and 14 days in still condition for every factor except in agitation. Isolation and determination (Kim *et al.,* 2002) of exopolysaccharides was done with every parameter with exopolysaccharides of 7 and 14 days old filtrates and precipitated with isopropyl alcohol in 1:4 ratios and incubated overnight at 4°C. After incubation, the pellet obtained by centrifugation was subjected to phenol- sulphuric acid (Dubois *et al.,* 1956) method with some modifications.

Carbon source

To find out the suitable carbon source for the exopolysaccharide production of *Fomitopsis feei*, 12 carbon sources of which three monosaccharides viz., arabinose, fructose, galactose, five oligosaccharides viz. lactose, maltose, raffinose, sucrose, xylose, two polysaccharides viz. cellulose, starch, and two sugar alcohols viz. mannitol and sorbitol were separately provided at 20 g/L instead of glucose employed in common production medium and control (without carbon source) also maintained.

Nitrogen source

In the course of optimization for nitrogen source, 12 nitrogen sources viz., asparagine, diphenylamine, hexamine, gelatine, urea, sodium nitrite, potassium nitrate, malt extract, yeast extract, peptone, glycine, ammonium chloride were supplemented at 5 g/L individually into the medium by removing ammonium sulphate used in the common

production medium. Medium without nitrogen source (control) was also maintained for comparison.

Inoculum size

The effects of inoculum size on the cell growth and exopolysaccharide production were studied by controlling inoculation density at 3–9 mm size.

Incubation time

To determine the optimal period for the production of exopolysaccharide, *Fomitopsis feei* was cultured for 6–14 days.

pH

Fomitopsis feei was cultivated in the common production medium with different values of initial pH (3.0–8.0).

Temperature

To assess the optimal temperature for the production of exopolysaccharide, *Fomitopsis feei* was cultured at different temperate conditions (20°C–45°C).

Agitation

Agitation was carried out at 50, 100, 150, 200 and 250 revolutions per minute (rpm).

Results and Discussion

Effect of carbon source on exopolysaccharide production

The mycelial growth of this fungus occurred in a variety of carbon sources, however, production of mycelia and exopolysaccharide were quite distinct. A similar observation has been reported by other investigators in macrofungal submerged cultures (Kim *et al.*, 2002; Chandra and Shoji, 2007). The pattern that the decline of pH caused by the production of organic acid resulting from high consumption of carbon source for cell growth. Among the carbon sources examined glucose (6.88 g/L, 6.04 g/L) yielded the best mycelial biomass both in 7 and 14 days respectively. The next best growth occurred in sucrose (4.92 g/L). Effect of carbon sources on dry weight of mycelial biomass and exopolysaccharide was given in figure 1. Starch, cellulose supported less on the mycelial biomass of *Fomitopsis feei* may be due to the inability of this fungus to produce enzymes to metabolise these polysaccharides.

Exopolysaccharide production by various carbon sources ranged from 0.5 to 5.5 g/L. Glucose supported the best exopolysaccharide production (5.5 g/L) after 7 days of incubation. Glucose was the most suitable carbon source for both mycelial biomass and EPS production in submerged culture of *Grifolia frondosa* (Bum *et al.*, 2004; Shih *et al.*, 2008). Arabinose, sucrose and xylose given the same production (4.5 g/L) followed by sorbitol (4.0 g/L). Minimum exopolysaccharide formation attained by galactose (1.0 and 0.5 g/L) both in 7 and 14 days. However, most of the carbon sources given good exopolysaccharide production in 7 days compared to 14 days in still condition.

Although sucrose and lactose are also good sources for the production of exopolysaccharides, glucose is a good carbon source because of its ease – of – use and low cost compared to them. However, glucose has been chosen as the best suitable carbon source for mycelium growth of a majority of mushrooms (Chang and Miles, 1989; Yang, 1986). Glucose, maltose, and mannitol were the most appropriate carbon sources for biomass and EPS production by basidiomycetes (Elisashvili *et al.*, 2009).

Effect of glucose in different concentrations

To study for the optimal glucose concentration, 5-30 g/L glucose was added to the medium and tested for exopolysaccharide production and the results were shown in figure 2. It was noted that mycelial biomass increased with the increase of glucose concentration for both 7 and 14 days whereas, exopolysaccharide production varied with concentrations of glucose. The highest mycelial growth was observed with 30g glucose in both 7 and 14 days (9.36 g/L, 9.96 g/L respectively). 15g glucose (4.0 g/L in 14 days) and 25g glucose (4.0 g/L in 7 days) supported moderate exopolysaccharide production. Whereas, further increasing glucose concentration from 25g – 30g exerted negative effect on production, for high EPS production, *C. indica* required above 15g/l glucose (Anandapandian and Eyini, 2014). Different mushrooms prefer different concentration of carbon source for best exopolysaccharide production (Chandra and Shoji, 2007; Elisasvilli *et al.*, 2009).

Effect of nitrogen source on exopolysaccharide production

Figure 3 showed the effect of nitrogen sources on mycelial biomass and exopolysaccharide production after the incubation of 7 and 14 days. The highest mycelial biomass recorded from ammonium sulphate in 7 days (6.88 g/L) followed by peptone (6.52 g/L, 6.30 g/L for 14 and 7 days respectively). Diphenyl amine did not support the growth of this fungus; this may be because of its acidic pH. In general, good mycelial growth does not seem to be a determining factor for a high production of exopolysaccharides. Maximum exopoly-saccharide production was noted in malt extract and peptone in 14 days (8.5 g/L, 7.5 g/L respectively). Similar results were reported (Shih *et al.,* 2008; Chang *et al.,* 2006). Peptone is the best nitrogen source for cordycepin biosynthesis from Cordyceps militaris (Xian and Zhong, 2006). But in our study, peptone is the second most important nitrogen source. In comparison with organic nitrogen sources, inorganic sources gave rise to relatively lower mycelial biomass. This is due to most of the basidiomycete fungi prefer complex organic nitrogen sources for their favorable submerged cultures because certain essential amino acids could scarcely be synthesized from inorganic nitrogen sources (Shih *et al.,* 2008). It is also seen that organic nitrogen sources were more effective than inorganic nitrogen sources for facilitating mycelial growth and EPS production (Yun *et al.,* 2016). Malt extract was further examined in different concentrations since it showed the highest result in both still and shake conditions although biomass production was moderate with this source. Earlier report also proved that complex organic nitrogen sources led to better growth of mushroom species (Chang and Miles, 1989).

Effect of malt extract in different concentrations

1-7 g/L concentrations of malt extract was applied to identify suitable concentration for mycelial growth and exopolysaccharide production (Fig. 4). Maximum mycelial biomass (11.84 g/L) was observed with 7 g of malt extract followed by 3g of malt extract (11.24 g/L) in 14 days. The highest exopolysaccharide production came from 4g of malt extract (8.5 g/L) followed by 2g of malt extract (7.5 g/L).

Effect of inoculum size on exopolysaccharide production

Mycelial growth was highest (6.88 g/L) with 6 mm of inoculum size. After that it was decreased even though inoculum size was increased. The maximum exopolysaccharide production was observed with inoculum of 6 mm (5.5 g/L, 4.0 g/L for 7, 14 days respectively).

Fig.1 Effect of carbon source on mycelial dry weight and exopolysaccharide production

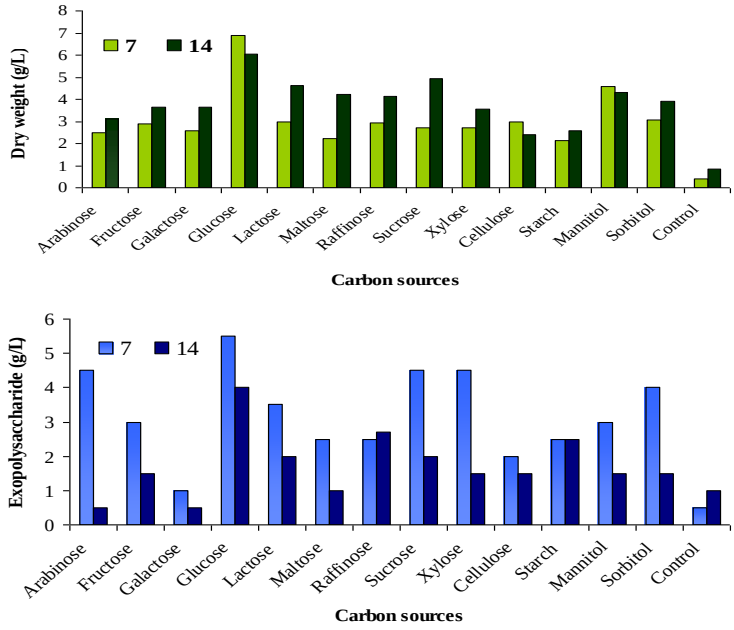

Fig.2 Effect of glucose concentration on mycelial dry weight and exopolysaccharide production

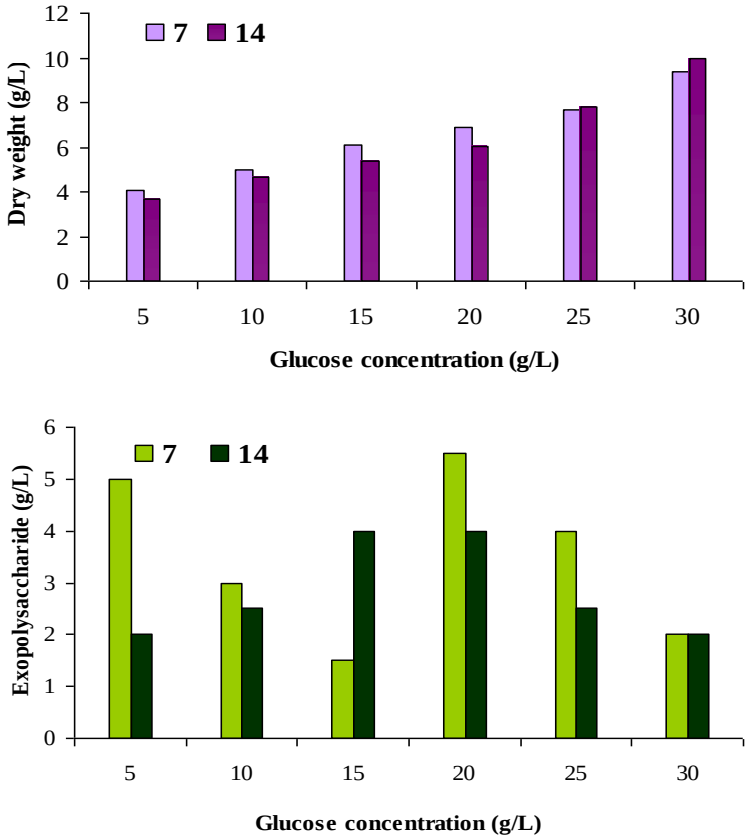

Fig.3 Effect of nitrogen source on mycelial dry weight and exopolysaccharide production

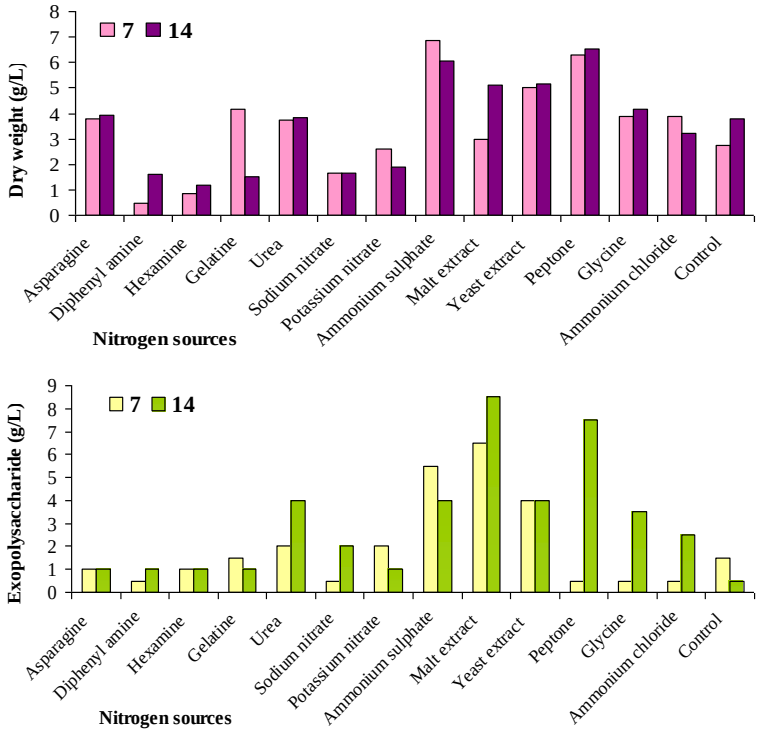

Fig.4 Effect of malt extract concentration on mycelial dry weight and exopolysaccharide production

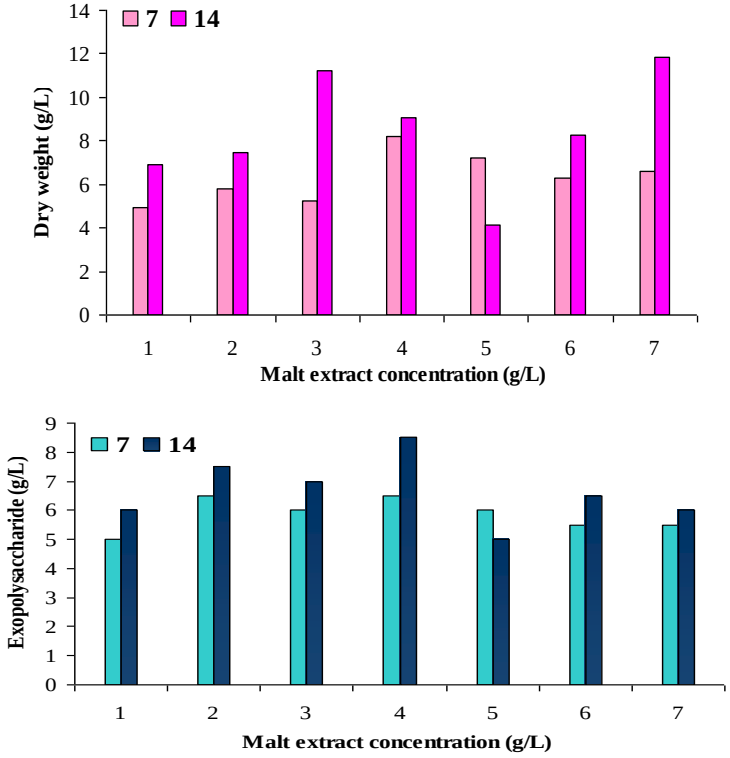

Fig.5 Effect of inoculum size on mycelial dry weight and exopolysaccharide production

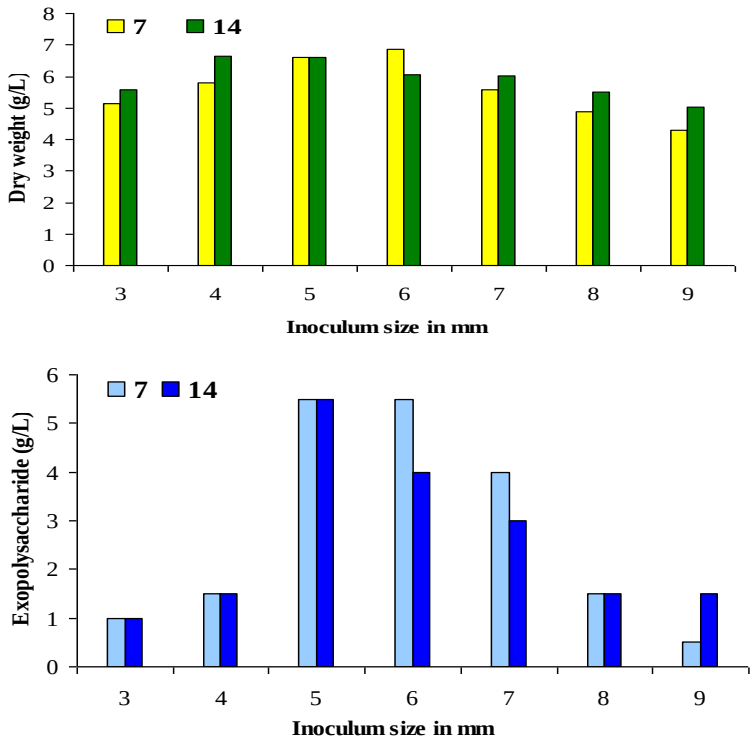

Fig.6 Effect of incubation time on mycelial dry weight and exopolysaccharide production

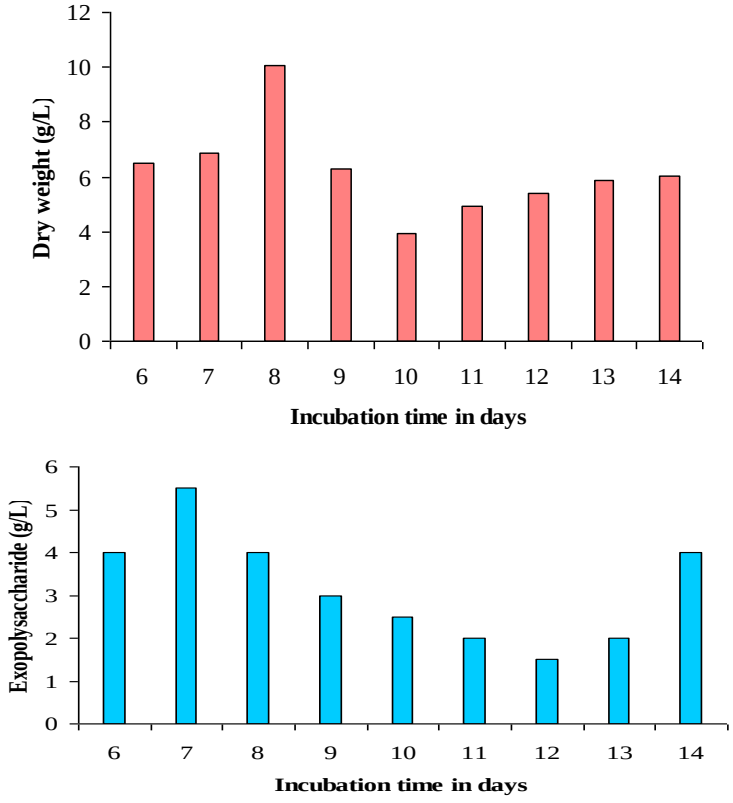

Fig.7 Effect of initial pH on mycelial dry weight and exopolysaccharide production

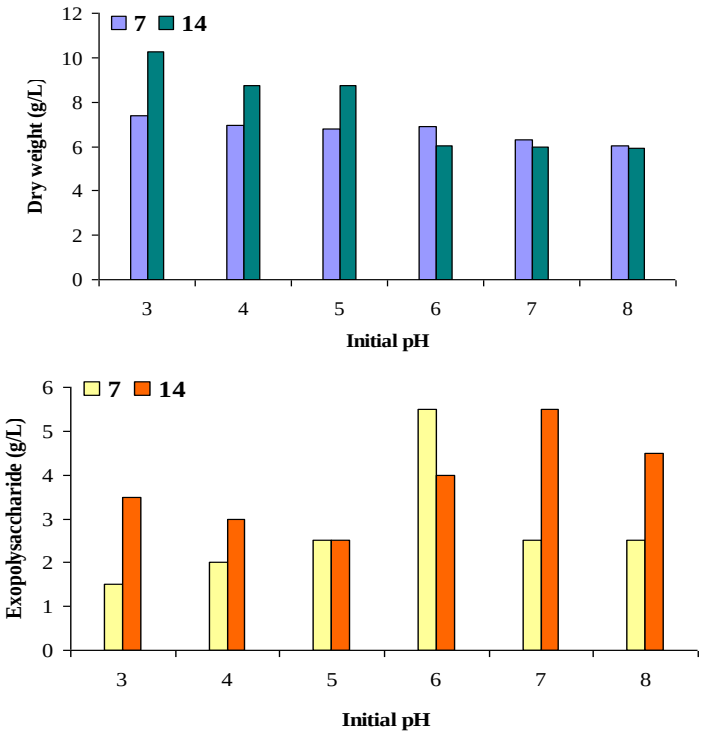

Fig.8 Effect of temperature on mycelial dry weight and exopolysaccharide production

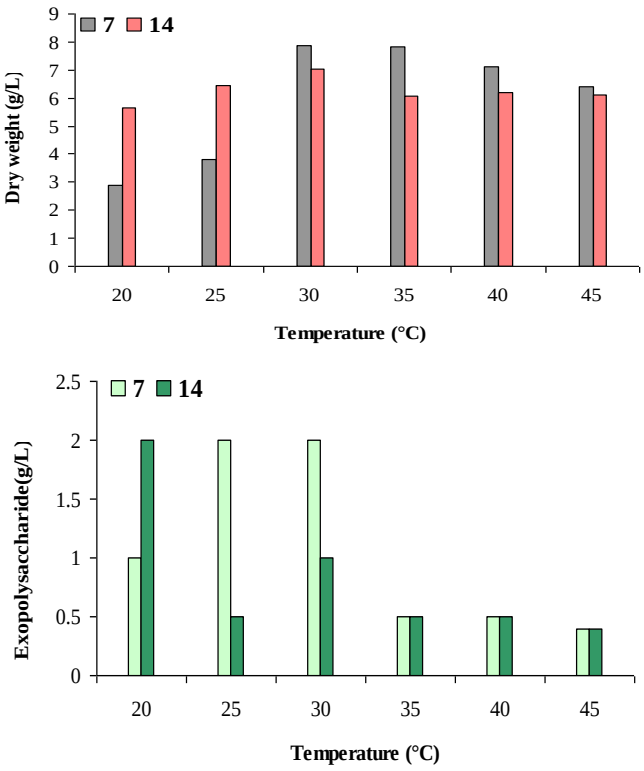

Fig.9 Effect of agitation on mycelial dry weight and exopolysaccharide production

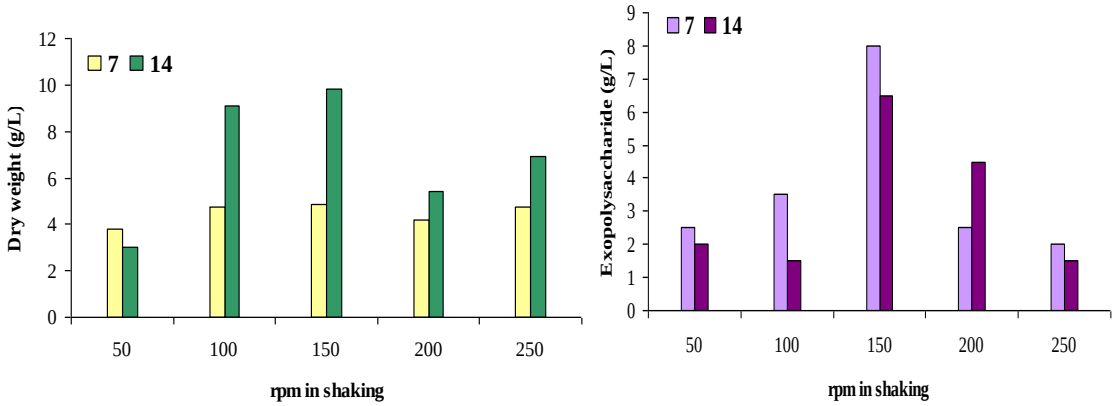

Fig.10 Growth of *Fomitopsis feei* in shaking condition

Mycelial biomass and polysaccharide production were highest (Jian *et al.,* 2006) at an inoculum size of 6%. The same decrease in exopolysaccharide production was observed with increasing inoculum size after this.

The amount of inocula could affect the length of the fermentation period. Small inoculum size may extend fermentation period, which made it susceptible to bacterial contamination; large inoculums size may quicken medium nutrients consumption, also produce large amounts of waste products. At lower inoculum levels the yield was very low (Tang *et al.,* 2008). The decrease seen with large inoculum size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture. Figure 5 showed the effect of inoculum size on biomass dryweight and exopolysaccharide production from *F. feei*.

Effect of incubation time on exopolysaccharide production

The highest mycelial growth was obtained from 8 days old culture (10.04 g/L) thereafter, these values were decreased and again increased at day 14 (6.04 g/L). Exopolysaccharide production reached a maximal value of 5.5 g/L on day 7 but decreased then onwards and again observed high on day 14 (4 g/L). Hence, a 7 and 14 day period was suitable for stimulation of mycelia

and exopolysaccharide in submerged culture of *Fomitopsis feei* and all of the following fermentations were carried out for both 7 and 14 days when optimizing the conditions in the submerged culture. Figure 6 showed that the activity of polysaccharide production was rather short-phased, even though mycelia growth continues for a longer duration. From 6th to 7th day, there was a progressive increase in EPS production, however, from 8th to 13th there was a highly significant decrease in EPS production and again increased on 14th day. Different species showed different incubation time for the production of EPS according to the previous research. Polysaccharide production by *Acremonium diospyri* was parallel with biomass over the first 10 days then ceased (Seviour and Hensgen, 1983). Mycelial biomass and exopolysaccharide concentrations were the highest at day 14 (Lin and Sung, 2006).

Effect of pH on exopolysaccharide production

The mycelial growth and exopolysaccharide production were significantly affected by culture pH of *F. feei* (Fig. 7). In general, the optimal medium pH for cell growth is around the lower range from 2-4 and the optimal medium for exopolysaccharide formation is around the high range from 5-7. The highest EPS production was at an initial pH of 5.5 in submerged fermentation of *Ganoderma lucidum* (Fang and Zhong, 2002). The final pH was mainly dropped to acidic in the media at the end of the incubation time.

The result of our investigation was also observed the same findings. The highest concentration of mycelial growth was obtained at pH values of 3 and 4 (10.24 g/L, 8.76 g/L respectively) in 14 days. This concentration dropped sharply out of this range. The maximum exopolysaccharide production was obtained at pH 6 and 7 (5.5 g/L) followed by pH 8 (4.5 g/L). It has been reported that a wide variety of mushrooms have also acidic pH optima for mycelial growth and exopolysaccharide production (Kim *et al.,* 2002). The results are similar to those reported for other exopolysaccharide synthesizing fungi such as *A. pullulans* (Lacroix *et al.,* 1985), *Sclerotium glucanicum* (Wang and Nail, 1995) for which the pH optimum for growth has been shown to be lower than that for EPS production.

Effect of temperature on exopolysaccharide production

The highest mycelial biomass obtained at 30°C (7.88 g/L) after 14 days of incubation. The maximum exopolysaccharide production was attained at 30°C (2.0 g/L) after 14 days of incubation. Exopolysaccharide production was supported (Maziero *et al.,* 1999) by the temperatures between 25 and 30°C.

Figure 8 showed the effect of temperature on mycelial biomass and exopolysaccharide production. The optimum temperature was found to be 30°C. This temperature optimum is quite similar to the results reported (Kang *et al.,* 1997) from other liquid cultures of different species of *P. linteus*. It is comparable that many kinds of mushrooms have relatively low temperature optima ranging from 20 - 25°C in their submerged cultures (Bae *et al.,* 2000; Park *et al.,* 2001). Therefore, 28-30°C was chosen to be the incubation temperature for further experiments.

Effect of agitation on exopolysaccharide production

The effect of agitation on mycelial biomass and exopolysaccharide production was showed in figure 9. The highest mycelial growth was obtained at 150 rpm (9.84 g/L)

followed by 100 rpm (9.12 g/L) after 14 days of incubation. Exopolysaccharide production was also high at 150 rpm (8 g/L and 6.5 g/L for both 7 and 14 days respectively). Growth of mycelium in shaking condition was in pellet shape, might be because of agitation (Fig. 10). The agitation speed has greatly affected the production rate and maximum concentration of polysaccharides, for which the optimal rotation speed has been reported at 150 rpm for flask cultures (Yang and Liau, 1998; Chun *et al.,* 2006). The polysaccharide production declined sharply, indicating higher rotation speed may inhibit the release of polysaccharide. The same phenomenon was observed in EPS production of *Fomes fomentarius* (Chen *et al.,* 2008).

The results revealed that same source did not support the best production of mycelium and exopolysaccharide. The result is considered helpful for further investigation on the diversity of polysaccharide formation from this medicinal fungus in bulk quantities for various purposes.

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