Effect of Different Carbon Sources on the Extra-cellular Polysaccharide Production by *Tremella cinnabarina* in Submerged Culture

Proma Khondkar

Department of Pharmacy, Rajshahi University, Rajshahi-6205, Bangladesh

ABSTRACT: The effect of fatty acids, emulsifiers and plant oils were studied with respect to their potential to support the growth and extra-cellular polysaccharide production of *Tremella cinnabarina* in submerged culture. The extra-cellular polysaccharide (EPS) production and mycelial growth were substantially increased by supplementation with certain fatty acids in the medium. Oleic acid, sorbitan mono-oleate and grape-seed oil significantly enhanced EPS production which were 1.5, 1.6 and 1.3 folds, respectively, in comparison to the control. In case of lipids, the results indicated that the extent of stimulation or inhibition was associated with the types and levels of lipids. However, when lipids were used as sole carbon source, poor growth and lack of EPS production were observed.

Key words: *T. cinnabarina*, extra-cellular polysaccharide, emulsifier, fatty acids

INTRODUCTION

Tremella cinnabarina (Fam. Tremellaceae) and related species (collectively known as "Jelly Mushroom") have been used in Chinese medicine since ancient times for their different pharmacological properties.[1] The properties of these 'Jelly Mushrooms' are believed to be due to the structural polysaccharides which make-up most of the fruiting body.[1] Generally, *Tremella* belongs to 'yeast-like' fungi and are characterised by dimorphism. The haploid phase of *Tremella* is yeast but upon conjugation/fusion, a dikaryotic mycelium is formed. Both phases are able to produce EPS.[2-3] Unlike polysacccharides extracted from other medicinal mushrooms, jelly mushroom polysaccharides are heteropolysaccharides in nature[1].

Correspondence to: Proma Khondkar
Tel: +88 0721 750677; Fax: +88
E-mail: proma_khondkar@ yahoo.co.uk

Tremella and other EPS have unique physical and chemical properties, which are advantageous for novel applications.[4] Microorganisms are, in principle, better suited than macroalgae or higher plants for the commercial production of polysaccharides as they exhibit higher growth rates and can be more easily manipulated for enhancing growth and/or EPS production. [5-7]

The growth and production of EPS by different microorganisms are affected by a wide range of parameters, including the composition of culture medium, incubation period, pH etc.[8] The biosynthesis of EPS requires precursors ultimately derived from catabolised sugars that are activated intracellularly.[9-13] In many microorganisms, sugar catabolism is necessary for growth and for the production of polysaccharides. Microbial polysaccharides may be present as cell wall constituents, lipo-polysaccharides (LPS) or capsular polysaccharides (CPS), which are covalently associated with the cell surface, or they

may be secreted as free EPS into the external environment.[14]

Although it has been reported that pentose sugars like xylose or arabinose fail to support growth of cells,[15] others have shown that microbial cell growth and yield of EPS are enhanced with xylose than with other pentose or hexose sugars.[16-17] A large number of microorganisms can use oils and fats as a carbon source for growth as they produce extra-cellular lipases to hydrolyse the triglycerides to glycerol and fatty acids. Plant oils have been shown to exert a stimulatory metabolic effect and accelerate the growth of some mould species.[18] It has been reported that lipids promote the production of fungal metabolites like citric acid, aflatoxins and carotenes as well as extra-cellular enzymes.[19-21] It has also been reported that the production of microbial EPS is stimulated by some fatty acids,[22] but certain antifoaming agents significantly lower the yields of extra-cellular polysaccharides.[23] In the present study, the effects of fatty acids, plant oils and emulsifying agents were investigated in an attempt to enhance cell growth and EPS production of T. cinnabarina in submerged culture.

MATERIALS AND METHODS

Microorganisms. *T. cinnabarina* (CBS-8234) was obtained from Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Carbon sources. Extra-cellular polysaccharide produced in Molt-Yeast (MY) extract medium using five and six carbon sugars have been described earlier.[16] Glucose (G7528) were obtained from Sigma-Aldrich Chemical Co. (Dorset, UK). Fatty acids (oleic acid, O-1008; palmitic acid, P-0500 and stearic acid, S-4751) were obtained from Sigma Chemical Co. Plant oils (olive, vegetable, grapeseed, rapeseed, maize, ground nut, sunflower and sunolive) used in these study were purchased from local supermarkets. Glycerol (G/0600/08) was obtained from Fluka (Dorset, UK). The emulsifying agents, sorbitan monooleate (E-494), sorbitan monostearate (E-491) and Tween-80 were supplied by Cognis (Illertissen, Germany). In all experiments, glucose was used as the main carbon source except otherwise stated.

Subculture and media composition. Malt-yeast extract (MY) media was made up as follows: malt extract 3 g (Oxoid), yeast extract 3 g (DIFCO), mycological peptone 5 g (Oxoid), glucose 60 g (Sigma-Aldrich), agar 20 g (Oxoid) and sterilised at 115^{o}C for 10 minutes. Lipids were used at 0.1%-1% (v/v) unless otherwise stated and were added to the media at the desired concentrations before autoclaving.[22] *Tremella* species was maintained on agar slants at 4°C and sub-cultured every twelve weeks in accordance with the supplier's recommendation.

Pre-inoculation and culture conditions. The pre-inocula for all the experiments were prepared in 250 ml conical flasks with 100 ml of liquid MY medium. Flasks were inoculated from the agar slants containing organism and incubated at 25^{o}C for 3 days with orbital shaking (Gallenkamp, Crawley, UK) at 150 rpm. The same conditions were used when 1% (v/v) glycerol, plant oil, fatty acid or emulsifiers were used as the sole carbon sources.

Polysaccharide recovery. After incubation, cells were removed from the culture medium by centrifugation at 48,000 g for 30 min. at 4^{o}C. The cell mass was re-suspended in water and centrifuged again for 10 min. Both supernatants were collected together and evaporated under reduced pressure at 40°C. This reduced volume was then treated with absolute ethanol (kept at 0°C for 4 h prior to adding) to precipitate the polysaccharides.[16] To facilitate the precipitation, the extracts were kept at 4^{o}C for a further 24 h and then centrifuged at 4100 g for 15 min at 18^{o}C. In case of lipid added extracts, the precipitate was extracted twice with chloroform, to remove residual oils, fatty acids and emulsifiers. All extracts were washed with 85% (v/v) aqueous ethanol and then dialysed using cellulose tubing (Medicell International Ltd., London, MW cut off-12-14KDa) against water for 72 h, to remove free sugars and organic solvents. The extracted polysaccharides were then lyophilised.

Cell dry weight (CDW). The cell mass was determined by repeated washing of the cells with distilled water and drying at 70° C in a fan assisted oven for 5 days.

Statistical evaluation. All experiments were performed in triplicate and the results shown here are presented in mean±standard deviation (SD).

RESULTS AND DISCUSSION

The influence of fatty acids as an additional carbon source on the EPS production and mycelial growth of *T. cinnabarina* was studied in submerged culture. As shown in Table 1, with the addition of palmitic acid at conc of 0.1-2% (v/v), the EPS concentration increased from 4.7 to 9.7 g l^{-1} and the mycelial biomass also stimulated from 8.71 to 16.36 g l^{-1}, respectively. Similar effect was observed when the medium was supplemented with oleic acid instead of palmitic acid. The EPS and biomass production were ranged from 5.3 to 11.8 g l^{-1} and 9.5 to 18.5 g l^{-1} respectively. The best stimulatory effect of palmitic and oleic acid supplementation on the extra-cellular polysaccharide production was observed at the level of 1.0% (v/v) which was 9.7 and 11.8 g l^{-1} respectively. Higher level (2%) of palmitic and oleic acids displayed an inhibitory effect on both extra-cellular polysaccharides and biomass production (Table 1). On the other hand, stearic acid had an inhibitory effect on both growth and polysaccharide production (Table 1) compared to other acids, which is consistent with previous reports.[22,24] The utilisation of fatty acids depends on the length of the hydrocarbon chain and can be influenced by the pH of the media and also on the capability of extra-cellular lipase production of respective microorganism.[25, 26]

Figure 1 shows the effect of supplemented glycerol and surfactants (1%, v/v) on the yield of polysaccharides and biomass produced by *T. cinnabarina* when glucose was used as the primary carbon source in MY medium. Both the glycerol and surfactants had significant effect on the production of EPS except sorbitan mono-stearate (SMS). It is noteworthy to mention that stearic acid present in SMS seemed to play a role in inhibiting the production of EPS and biomass. When glycerol, Tween-80 and SMO were added in the medium, EPS production was increased by 1.2, 1.1 and 1.6 folds, respectively. Glycerol and surfactants also increased mycelial production to 1.4, 1.2 and 1.1 fold, respectively (Figure 1). In a previous study, Wasser *et al*.,[27] reported that *T. mesenterica* produced greatest amount of polysaccharide and biomass when glycerol was used as carbon source.

Table 1. Effects of fatty acids as additional carbon sources on mycelial growth and EPS production by *T. cinnabarina* in submerged culture (primary carbon source – glucose, 6%, w/v)

Fatty acids	Conc. (%, v/v)	EPS (g l^{-1})	CDW (g l^{-1})	EPS/CDW
Palmitic acid	0.1	4.7 (±0.13)	8.71 (±0.2)	0.54
	0.5	5.3 (±0.02)	11.00 (±0.5)	0.48
	1.0	9.7 (±0.3)	16.36 (±0.5)	0.61
	2.0	3.9 (±0.5)	7.30 (±0.7)	0.53
Stearic acid	0.1	1.1 (± 0.1)	1.2 (± 0.0)	0.91
	0.5	1.3 (± 0.2)	1.5 (± 0.1)	0.86
	1.0	2.8 (± 0.3)	2.3 (± 0.5)	1.21
	2.0	2.4 (± 0.8)	2.8 (± 0.4)	0.85
Oleic acid	0.1	5.3 (±0.5)	9.40 (± 0.6)	0.57
	0.5	7.7 (±0.2)	10.20 (± 0.5)	0.75
	1.0	11.8 (±0.2)	14.50 (±0.3)	0.81
	2.0	4.5 (±0.3)	9.40 (±0.6)	0.48

CDW= Cell dry weight, EPS= extra-cellular polysaccharide

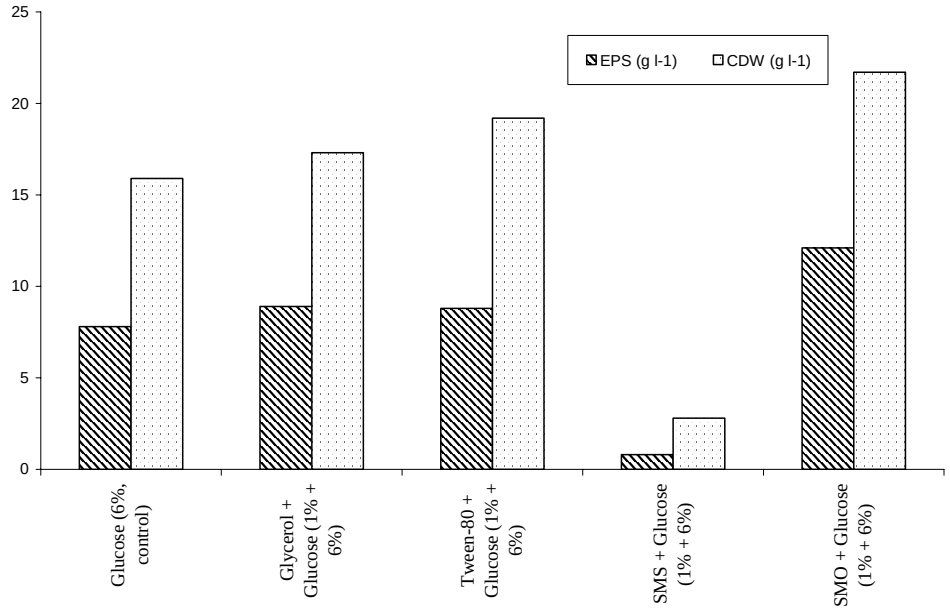

Figure 1. Effect of supplemented glycerol and surfactants on the mycelial growth and EPS production of *T. cinnabarina* in submerged culture. Each time fermentation was carried out in triplicate. (SMS= Sorbitan mono stearate, SMO= Sorbitan mono oleate)

Table 2. Effect of supplemented plant oils (1%, v/v) on the mycelial growth and bio-polymer production of T. cinnabarina in submerged culture.

Primary carbon Source (6%, w/v)	Supplementary carbon source (1%, v/v)	*T. cinnabarina* EPS (g l^{-1})	*T. cinnabarina* CDW (g l^{-1})	EPS/CDW
Glucose	No oil (control)	7.8 (± 0.3)	15.9 (± 0.5)	0.49
	Ground Nut	4.2 (± 0.1)	8.1 (± 0.1)	0.51
	Sunflower	8.4 (± 1.2)	14.9 (± 1.0)	0.57
	Sun-olive	5.9 (± 0.7)	10.9 (± 0.6)	0.54
	Rape-seed	5.5 (± 0.7)	9.7 (± 0.1)	0.55
	Maize	6.6 (± 0.8)	12.1 (± 1.0)	0.51
	Olive	9.7 (± 0.5)	17.2 (± 0.2)	0.56
	Vegetable	5.9 (± 0.5)	13.7 (± 0.5)	0.43
	Grape- seed	10.5 (± 0.6)	18.3 (± 0.7)	0.57

CDW= Cell dry weight, EPS= extra-cellular polysaccharide

Eight different plant oils were also used in this study as an additional carbon source but only grapeseed, olive, vegetable and sunflower oil promoted biomass and polysaccharide production (Table 2), which is consistent with previous reports with other medicinal mushrooms.[24,28] Oleic acid rather than other acids in these oils seem to play a role in stimulating the production of both biopolymer and biomass.[24]

When glucose, oleic acid, SMS, SMO, glycerol, olive oil and grapeseed oil were used as the sole carbon source, the growth of *T. cinnabarina* was poor and relatively small amounts of EPS were produced (Table 3). Similar results have been reported using the surfactant "Tween 80" which was unable to support the growth of *Aspergillus oryzae*[29] and *Nigrospora oryzae* var *glucanium*[30] as the sole carbon source.

Table 3. Comparison of extra-cellular polysaccharide yields using different substrates as sole carbon source

Organism	Carbon source (1%, w/v or v/v)	EPS ($g\ l^{-1}$)	CDW ($g\ l^{-1}$)
T. cinnabarina	Glucose	3.8 (± 0.2)	9.9 (± 0.5)
	Oleic acid	3.3 (± 0.2)	10.1 (± 0.9)
	SMS	NP	2.4 (± 0.2)
	SMO	3.4 (± 0.3)	11.3 (± 0.6)
	Glycerol	2.7 (± 0.3)	9.4 (± 0.3)
	Vegetable oil	1.9 (± 0.1)	5.1 (± 0.7)
	Grapeseed oil	3.3 (± 0.1)	6.3 (± 0.1)

NP= No production, CDW= Cell dry weight
EPS= extra-cellular polysaccharide

CONCLUSIONS

In the present study, the effects of several fatty acids, plant oils, surfactants and glycerol were studied in an attempt to investigate their effects on both EPS and mycelial growth of *T. cinnabarina* in submerged culture. In conclusion, it was advantageous to use either fatty acids or plant oils as supplementation with sugar carbon source, which acted as primary carbon source. It was apparent that growth of organism and production of EPS by *T. cinnabarina* under the "artificial conditions" employed were dependent on carbon bioavailability. From the results, it is clear that fatty acids and some of the oils were complementary to cell growth and production of EPS.

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