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Full Length Research Paper

Optimization of carbon and nitrogen sources of submerged culture process for the production of mycelial biomass and exopolysaccharides by *Trametes versicolor*

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Medicinal mushrooms have profound health-promoting benefits. Polysaccharides constitute an important percentage of fungal biomass, where the hyphal wall frequently contains more than 75% of polysaccharide. *Trametes versicolor* is a medicinal fungus producing exopolysaccharides (EPS). The media were tested with different carbon and nitrogen sources which maximize the production of EPS by *T. versicolor*. The media were optimized with different carbon (glucose, fructose, sucrose, maltose, lactose, raffinose, mannitol and xylose) and nitrogen sources (peptone, glycine, gelatin, casein, yeast extract, ammonium sulphate, KNO_3 and $NaNO_2$) for the higher yield of polysaccharides. Biomass, pH changes along with the EPS production of the broth were followed during fermentations lasting 7 and 14 days. Fructose (8 g. dr. w/l) was shown to have yielded the highest production of EPS for 7 days, and gelatin (11 g. dr. w/l) to have produced the highest biomass. An experimental design to do this was adopted, in which the effects of pH were considered.

Key words: Basidiomycetes, exopolysaccharide, biomass, submerged culture.

INTRODUCTION

Many fungi are able to produce extracellular polysaccharides. They fulfill different tasks during the growth on natural substrates, such as adhesion to surfaces, immobilization of secreted enzymes, prevention of hyphae from dehydration and increased residence time of nutrients inside the mucilage (Rau, 1999). Many of them contain α- (Pullulan) or β-linked (e.g., Scleroglucan, Schizophyllan) glucose units. The alignment and disposition of linkage and branching affect the three-dimensional structure and determine the physicochemical characteristics of the gum. The branched β-glucans are biologically active and consequently are used in medicine and biotechnology, as well as additives in food and cosmetics (Manzoni and Rollini, 2001).

Trametes versicolor, belonging to the basidiomycetes class, can produce both extracellular and intracellular polysaccharides that have received special attention due to their physiological and biological activity. These fungi are well known as a medicinal mushroom in traditional therapeutic practice in Japan, China, Korea and other Asian countries (Cui and Chisti, 2003). Their poly-saccharides have shown antitumour activity and include protein-bound polysaccharides extracted from the fungal mycelium like the Krestin (PSK) and Polysaccharopeptide (PSP) (Sugiura et al., 1980; Ng, 1998) and the extracellular polysaccharide Coriolan (Miyazaki et al., 1974).

Most of the reported studies have focused on polysaccharides isolated from the mycelium. However, a few studies on EPS from *T. versicolor* in submerged culture have been reported (Kim et al., 2002). Although a number of works have attempted to obtain the best culture conditions and EPS characterization from different fungi, the effect of medium composition on fermentations

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Table 1. Effect of carbon and nitrogen sources on biomass and exopolysaccharides production by *T. versicolor*.

Carbon source	Days	*Trametes versicolor*		
		pH	B	EPS
Glucose	7	3.88	4.0	3.0
	14	3.5	6.2	6.4
Sucrose	7	3.92	4.6	5.6
	14	3.70	8.8	3.4
Maltose	7	2.92	7.6	7.4
	14	4.39	9.2	6.2*
Lactose	7	5.70	2.6	2.8
	14	5.90	3.0	3.0
Fructose	7	3.53	4.0	8.0
	14	5.70	2.6	2.8
Xylose	7	3.40	5.0	6.0
	14	4.00	6.2	2.8
Mannitol	7	3.78	5.8	6.6
	14	3.69	6.8	4.6
Raffinose	7	3.64	6.8	3.8
	14	4.42	8.4	7.0
Nitrogen source				
$(NH_4)_2SO_4$	7	3.88	4.0	3.0
	14	3.5	6.2	6.4
Casein	7	4.48	9.2	5.2
	14	5.83	9.0	5.6
Gelatin	7	4.13	10.4	5.0
	14	5.06	11.0	3.2
Glycine	7	3.85	7.4	4.6
	14	5.00	10.2	6.4
KNO_3	7	5.22	7.2	10.2*
	14	5.82	7.8	4.4
$NaNO_2$	7	6.23	4.6	4.0
	14	6.17	6.2	7.0
Peptone	7	4.51	4.2	3.2
	14	6.03	7.6	6.4
Yeast extract	7	5.75	8.6	4.6
	14	4.96	8.0	5.4

* Precipitate, B - biomass (g. dr. w/ L), EPS - exopolysaccharides (g. dr. w/l).

and cultivation kinetics, which are important parameters to EPS production, remain relatively unexplored.

Response surfaces, based on experimental designs, have been used in several fields of bioprocesses and it has been demonstrated to be an adequate tool to evaluate the effects and interactions of the different parameters that rule a biochemical system (Box et al., 1978). However, so far experimental designs have not been applied to EPS production by *T. versicolor*.

The aim of this study was to define experimental conditions to optimize EPS production by *T. versicolor*. Firstly a fermentation broth was selected and after that an experimental design and different carbon and nitrogen sources was used to optimize the medium and the culture conditions.

MATERIALS AND METHODS

Trametes versicolor were collected from Mushroom Culture Collection Lab, Kakatiya University, Warangal, Andhra Pradesh, India, which was maintained on the malt extract medium.

Liquid culture medium (g/l): Peptone 1.0; yeast extract 2.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.2; $(NH_4)_2SO_4$ 5.0; glucose 20.0; pH 6.0. This medium was selected in preliminary studies as adequate for exopolysaccharide production. Erlenmeyer flasks containing 100 ml of sterilized culture medium were inoculated with the suspension in sterile water of fungal mycelium grown on malt extract agar slants. Incubation was done at 27°C.

The incubation times were 7 and 14 days. The culture was filtered to separate fungal biomass, which was washed twice with distilled water and quantified as dry weight (105°C to constant weight). Isopropanol was added to the culture filtrate (1:1 v/v) and after 24 h at 4°C the precipitated biopolymer was separated by centrifugation (8,000 rpm for 15 min) and also quantified as dry weight.

T. versicolor was tested with different carbon and nitrogen sources (Wasser et al., 2003; Hsieh et al., 2005). The media is optimized with different carbon (glucose, fructose, sucrose, maltose, lactose, raffinose, mannitol and xylose) and nitrogen source (peptone, glycine, gelatin, casein, yeast extract, ammonium sulphate, KNO_3 and $NaNO_2$) for the higher yield of EPS (Jonathan et al., 2006).

RESULTS AND DISCUSSION

The results were calculated with standard deviation and the graph was plotted with error bar by using SPSS software. The results were significant and shown for pH, biomass and EPS are 0.89, 2.15 and 1.87 respectively for carbon sources and for the nitrogen source, the results were significant and shown for pH, biomass and EPS are 0.89, 2.15 and 1.26 (Figure 1).

Fructose (8 g. dr. w/l) and raffinose (7 g. dr. w/l) are effective carbon sources (Table 1) and Casein (5.2 g. dr. w/l) and $NaNo_2$ (7 g. dr. w/l) are effective nitrogen sources (Tables 2a and 2b) in the production of exopolysaccharides after 7 and 14 days of incubation, respectively.

Maltose (7.6 and 9.2 g. dr. w/l) and gelatin (10.4 and 11 g. dr. w/l) are effective carbon and nitrogen sources

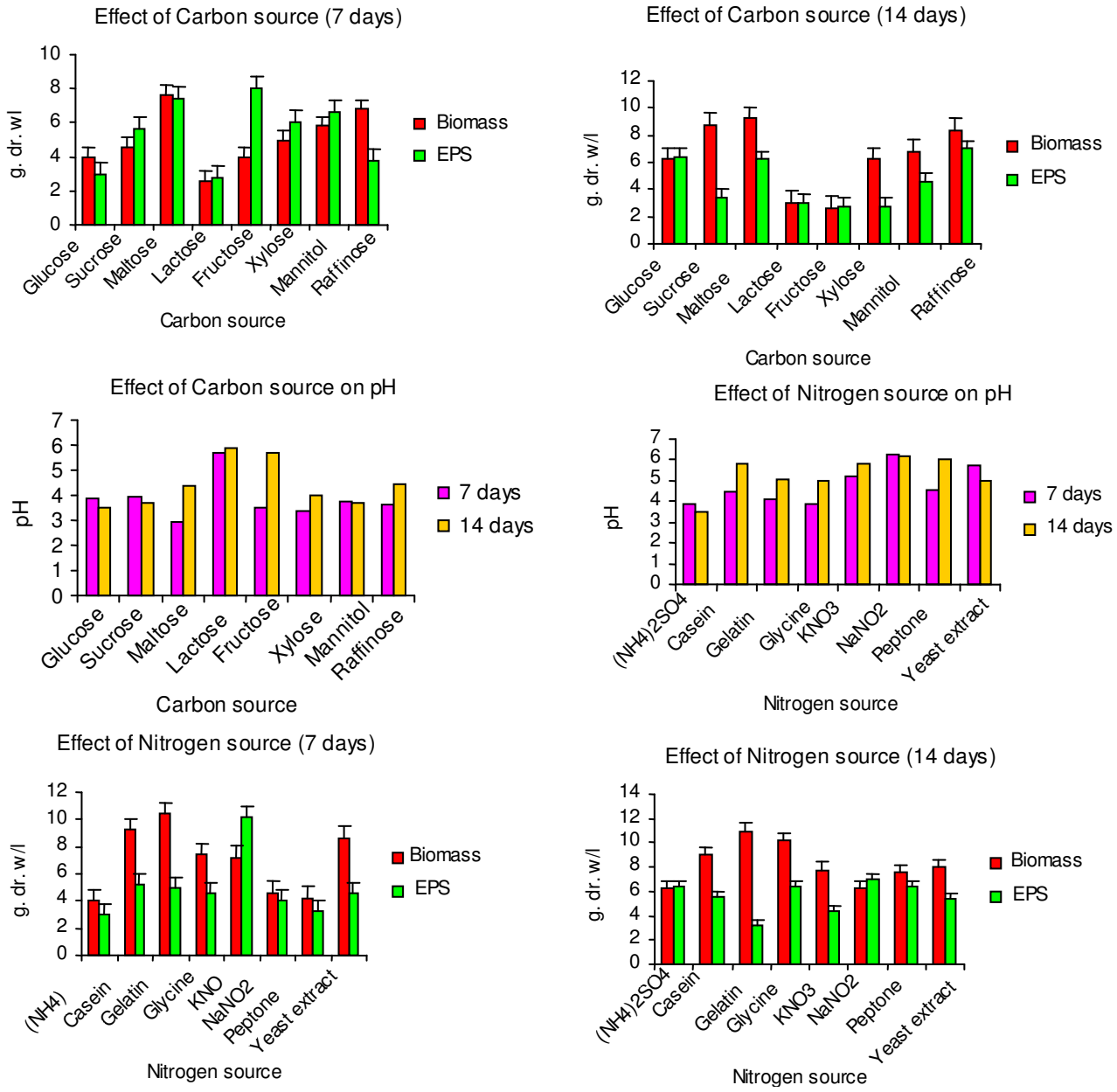

Figure 1. Effect of carbon and nitrogen source on biomass and exopolysaccharides production by *T. versicolor*.

(Tables 1, 2a and 2b) in the production of biomass after 7 and 14 days of incubation respectively.

An interesting observation was made concerning the formation of an insoluble gel when the culture filtrate was frozen prior to polysaccharide precipitation. In the Tables these strains are marked. This peculiar characteristic could aid polymer separation, since there is no need of an organic solvent such as isopropanol, ethanol or acetone for the precipitation of the polymer, thus increasing the process viability. Moreover, it is important to observe that the product obtained by solvent precipitation cannot be considered pure polysaccharide because proteins and salts present in the medium co-precipitate. The strain studied here were submitted to a lignin degradation activity test (Capelari and Zadrazil, 1997; Hou and Chen, 2008) was proved to be effective.

The conditions used for the submerged culture could be considered adequate for biomass production. Data presented in literature (Manachini, 1979; Compere et al., 1980; Masaphy and Levanon, 1992; Burns et al., 1994) showed lower production for *Pleurotus* sp. with other culture parameters. During estimation of polymer and

Table 2a. Effect of carbon source-statistics (VAR00002).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	2.92	1	6.3	6.3	6.3	.88818
	3.40	1	6.3	6.3	12.5	
	3.50	1	6.3	6.3	18.8	
	3.53	1	6.3	6.3	25.0	
	3.64	1	6.3	6.3	31.3	
	3.69	1	6.3	6.3	37.5	
	3.70	1	6.3	6.3	43.8	
	3.78	1	6.3	6.3	50.0	
	3.88	1	6.3	6.3	56.3	
	3.92	1	6.3	6.3	62.5	
	4.00	1	6.3	6.3	68.8	
	4.39	1	6.3	6.3	75.0	
	4.42	1	6.3	6.3	81.3	
	5.70	2	12.5	12.5	93.8	
	5.90	1	6.3	6.3	100.0	
		N = 16 Missing = 0	Total = 100.0	Total = 100.0		

SD for biomass-statistics (VAR00002).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	2.60	2	12.5	12.5	12.5	2.15515
	3.00	1	6.3	6.3	18.8	
	4.00	2	12.5	12.5	31.3	
	4.60	1	6.3	6.3	37.5	
	5.00	1	6.3	6.3	43.8	
	5.80	1	6.3	6.3	50.0	
	6.20	2	12.5	12.5	62.5	
	6.80	2	12.5	12.5	75.0	
	7.60	1	6.3	6.3	81.3	
	8.40	1	6.3	6.3	87.5	
	8.80	1	6.3	6.3	93.8	
	9.20	1	6.3	6.3	100.0	
		N = 16 Missing = 0	Total = 100.0	Total = 100.0		

For EPS- statistics (VAR00002).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	2.80	3	18.8	18.8	18.8	1.87506
	3.00	2	12.5	12.5	31.3	
	3.40	1	6.3	6.3	37.5	
	3.80	1	6.3	6.3	43.8	
	4.60	1	6.3	6.3	50.0	
	5.60	1	6.3	6.3	56.3	
	6.00	1	6.3	6.3	62.5	
	6.20	1	6.3	6.3	68.8	

Table 2a. Contd.

6.40	1	6.3	6.3	75.0
6.60	1	6.3	6.3	81.3
7.00	1	6.3	6.3	87.5
7.40	1	6.3	6.3	93.8
8.00	1	6.3	6.3	100.0
	N = 16 Missing = 0	Total = 100.0	Total = 100.0	

Table 2b. Effect of nitrogen- For pH: Statistics (VAR00002).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	3.50	1	6.3	6.3	6.3	.89455
	3.85	1	6.3	6.3	12.5	
	3.88	1	6.3	6.3	18.8	
	4.13	1	6.3	6.3	25.0	
	4.48	1	6.3	6.3	31.3	
	4.51	1	6.3	6.3	37.5	
	4.96	1	6.3	6.3	43.8	
	5.00	1	6.3	6.3	50.0	
	5.06	1	6.3	6.3	56.3	
	5.22	1	6.3	6.3	62.5	
	5.75	1	6.3	6.3	68.8	
	5.82	1	6.3	6.3	75.0	
	5.83	1	6.3	6.3	81.3	
	6.03	1	6.3	6.3	87.5	
	6.17	1	6.3	6.3	93.8	
	6.23	1	6.3	6.3	100.0	
		N = 16 Missing = 0	Total = 100.0	Total = 100.0		

For biomass- statistics (VAR00002).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	4.00	1	6.3	6.3	6.3	2.15283
	4.20	1	6.3	6.3	12.5	
	4.60	1	6.3	6.3	18.8	
	6.20	2	12.5	12.5	31.3	
	7.20	1	6.3	6.3	37.5	
	7.40	1	6.3	6.3	43.8	
	7.60	1	6.3	6.3	50.0	
	7.80	1	6.3	6.3	56.3	
	8.00	1	6.3	6.3	62.5	
	8.60	1	6.3	6.3	68.8	
	9.00	1	6.3	6.3	75.0	
	9.20	1	6.3	6.3	81.3	
	10.20	1	6.3	6.3	87.5	
	10.40	1	6.3	6.3	93.8	
	11.00	1	6.3	6.3	100.0	
		N = 16 Missing = 0	Total = 100.0	Total = 100.0		

Table 2b. Contd (For EPS- statistics [VAR00002]).

		Frequency	Percent	Valid percent	Cumulative percent	Std. deviation
Valid	3.00	1	6.3	6.3	6.3	1.26537
	3.20	2	12.5	12.5	18.8	
	4.00	1	6.3	6.3	25.0	
	4.40	1	6.3	6.3	31.3	
	4.60	2	12.5	12.5	43.8	
	5.00	1	6.3	6.3	50.0	
	5.20	1	6.3	6.3	56.3	
	5.40	1	6.3	6.3	62.5	
	5.60	1	6.3	6.3	68.8	
	6.20	1	6.3	6.3	75.0	
	6.40	3	18.8	18.8	93.8	
	7.00	1	6.3	6.3	100.0	
		N = 16 Missing = 0	Total = 100.0	Total = 100.0		

biomass produced it is important to consider that EPS adherent to the hyphae are also entrapped into the pellets formed during the submerged culture which means that the dry weight of biopolymer which precipitated from the culture filtrate does not correspond to the total EPS and that the biomass can be overestimated. To minimize this problem biomass was washed twice with distilled water.

The pellets formed can be regular or irregular in form and size. The form varies from spherical to cylindrical and the size from 1 to 20 mm. In some cases the formation of pellets was not observed, but rather a mycelial agglomeration without a defined form (Maziero, 1996). The pellets were smooth, hairy (with looser outer zones) or with fringes of aggregated hyphae that give the pellet a star form. The color and consistency were also different, as well as the flavour. Sometimes the culture filtrate was very clear, other times was turbid and very viscous. In most of the cultures the presence of crystals with different forms was observed, which could indicate, in some cases, the presence of excreted metabolites. When there is a depletion of glucose in the medium it was observed that pellets begin to become darker and break up. The dead hyphae are decomposed and the resulting substances are reabsorbed by the mycelium.

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