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RESEARCH ARTICLE

ISOLATION AND PURIFICATION OF WATER SOLUBLE POLYSACCHARIDE FROM *PLEUROTUS DJAMOR* VAR. ROSEUS CORNER

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ABSTRACT

Fungi are non-photosynthetic, eukaryotic organisms which grow as single cells (Yeasts) or as multicellular filaments (Moulds / Fungi), acquiring nutrition by absorption from their surroundings. Mushrooms alone are represented by about 41,000 species, of which approximately 850 species are recorded from India. In nature, mushrooms grow wild in almost all types of soils, on decaying organic matter, wood stumps, etc. They appear in all seasons; however rain favors rapid growth when organic matter or its decomposition products are easily available. Environmental factors such as temperature, humidity, light and host trees are very important for development of fruiting body. *Pleurotus djamor* var. *roseus* is an edible mushroom, contain rich source of nutrients and is popularly cultivated for edible purpose worldwide. However, the medicinal properties of *P. djamor* var. *roseus* are not well documented. Hence, the present study was focused on isolation, purification and characterization of medicinally important polysaccharide.

Key words: Purification, Polysaccharide Chromatography and FT-IR.

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INTRODUCTION

Mushrooms are the fleshy, spore-bearing fruiting bodies of fungi, consists of a cap or pileus and a stalk or stipe but some have additional structures like veil or annulus, a cup or volva. The term 'mushroom' was defined by Chang and Miles (1992) as amacro fungus with a distinctive fruiting body, mainly basidiomycetes and some species of ascomycetes belongs to mushrooms, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand' Edible mushroom are considered as effective and balanced diet in maintaining health due to the following reasons: Mushrooms have relatively high (5-30% DW) and good quality protein. In addition mushrooms contain all essential amino acids. Mushrooms are low in total fat (2-8% DW), and have a high percentage of polyunsaturated fatty acids (80%).The carbohydrate percentage available in mushroom is around 45-85% dry wt. *Pleurotus* is an important genus of edible Basidiomycetes which are commonly called oyster mushroom. A large amount of compounds like lectins, polysaccharides, polysaccharide–peptides, polysaccharide-protein complexes have been isolated from mushroom. Many of these compounds have been found to have immunomodulatory, anticancer and antioxidant effects (Moradali *et al.,* 2007).

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Since polysaccharide are reported to have a pharmacological potential, in the present study the attempt were made to isolate polysaccharide from *Pleurotus djamor* var. *roseus.*Both proteoglucans have the same polysaccharide component but with different protein molecules bound to the polysaccharide (Hiroshi and Takeda, 1993). The basidiocarps of this variety are pink or salmon-pink when young and change from pale pink, whitish to yellow with age.

MATERIALS AND METHODS

Culture collection and maintenance of Pleurotus djamor var. roseus

P. djamor var. *roseus* culture was obtained from the culture collection, CAS in botany, University of Madras. The culture was maintained in PDA slants at 27°C ±1ºC.

Biological efficiency

$$\text{BE per cent} = \frac{\text{Total fresh weight of the fruit body}}{\text{Dry wt. of the substrate}} \times 100$$

The fruiting bodies were collected prior to over maturation (deterioration). The biological efficiency (BE per cent) was determined by the following formula (Stamets, 1993).

Estimation of total sugars and soluble protein

One g of fruiting body was ground with 5 mL of 80per cent hot ethanol. The homogenate was centrifuged at 5000 g for 20 min. The clear supernatant was evaporated to dryness over a boiling water bath and the residue was dissolved to 10 mL of distilled water. 1 mL of sample was mixed with 1 mL of 5per cent phenol (aqueous w/v), and 5 mL of conc. Sulphuric acid was added rapidly and mixed thoroughly and the tubes were incubated for 10 min, and then placed in a water bath for 20 min at 30°C. The colour development was read at 490 nm in Beckman DU-40 Spectrophotometer. The reagent without the sample served as blank. The amount of sugar was estimated using the standard graph prepared using glucose at the range of 10 µg – 100 µg/mL.

Estimation of soluble protein (Bradford, 1976)

One g of fruiting body was ground with 5 mL of phosphate buffer (pH-7.0). To this, a pinch of acid washed sand was added to facilitate grinding. The extract was centrifuged at 10,000 g for 15 min, and the clear supernatant was collected in a screw cap tube and made upto 10 mL with same buffer. 1 mL of protein extract was precipitated by adding 2 mL of 10per cent ice cold TCA for one hour and centrifuged at 10,000 g for 10 min. The pellet was collected and redissolved in 2 mL of 1 N NaOH. The amount of protein content was estimated by following Bradford's dye binding method.

Estimation

To 1 mL of the above sample, 5 mL of coomassie brilliant blue dye was added and mixed thoroughly. The absorbance was read at 595 nm in a Beckman DU-40 Spectrophotometer against a reagent blank. The amount of protein was calculated using standard graph of Bovine Serum Albumin (BSA) ranging from 10-100 µg/mL.

Isolation of partially purified polysaccharide

The polysaccharide was isolated from *P. djamor* var. *roseus* by modification method of Yap and Ng (2001). 200 g of dried powder of *Pleurotus djamor* var. *roseus* was boiled in distilled water for 4 h at 100°C. The extract was cooled and filtered through mesh cloth; the resulting filtrate was centrifuged at 10,000 g for 20 min. The supernatant was collected and precipitated by the addition of 95per cent ice cold ethanol (1:4) and kept at 4°C overnight. The precipitate was again centrifuged at 10,000 g for 20 min.

The pellet was collected, dissolved in glass distilled water and freeze dried to powder, this powder was again extracted with water at 100°C, centrifuged to collect the supernatant which has the soluble matter. To the supernatant ice cold ethanol was added and kept at 4ºC overnight for precipitation, centrifuged at 10,000 g for 20 min. The pellet was collected, dissolved in glass distilled water and treated with acetone to remove pigments, the protein contaminants was removed by addition of sevag reagent (chloroform : isoamylalcohol ; 24 : 1 v / v) the resulting aqueous portion was mixed with equal volume of 95per cent ice cold ethanol and the suspension was centrifuged at 10,000 rpm for 20 min, the pellet was dissolved in glass distilled water and dialyzed using membrane (>12500, purchased from sigma) against glass distilled water. The desalted partially purified polysaccharide obtained was lyophilized to powder.

Purification of polysaccharide

Activation of DEAE cellulose

10 g of DEAE cellulose was suspended in a beaker containing 500 mL of 0.5M HCl for removal of fines. The slurry was ultrasonicated for 2 min and allowed to stand for 30 min. at 27°C. Then the slurry was neutralized with water and suspended in 500 mL of 0.5 M NaOH. The alkaline slurry was again neutralized with water and packed into column with glass distilled water.

Experiment

The partially purified polysaccharide of *P. djamor* var. *roseus* was loaded onto the DEAE cellulose column (52 × 2.5 cm) and eluted with glass distilled water [non adsorbed fraction(NAF)] and later with 0.2 M and 0.5 M NaCl solution [adsorbed fraction (AF)] respectively. The fractions were collected at the flow rate of 0.5 mL/min. Each fraction was analyzed for carbohydrates by phenol-sulphuric acid method.

Gel permeation chromatography (Sepharose-6B)

NAF from DEAE cellulose column chromatography was loaded onto sepharose-6B (sigma, USA) column 89 × 6 cm and eluted with glass distilled water. The fraction was collected in 40 test tubes (4 mL each) and monitored at 490 nm using Beckman DU-40 UV-VIS spectrophotometer with phenol sulphuric acid reagent. The homogenous major fractions were pooled together and lyophilized to powder (purified polysaccharide). To detect the monosaccharide present in the purified polysaccharide, the sample was acid hydrolyzed with trifluroacetic acid (TFA).

Hydrolysis of polysaccharide

TFA, 1.5 mL was dissolved in 8 mL of distilled water and made up to final volume of 10 mL with distilled water.

Hydrolysis of sample

The purified polysaccharide sample (2 mg) was mixed with 1 mL of 2 M trifluroacetic acid (TFA) in a closed round bottom flask and hydrolyzed in a water-bath at 100ºC for 8 h.

Thin layer chromatography

Standard sugars such as glucose, mannitol, maltose, lactose and xylose were used. 5mg of sugar was dissolved in 1 mL of water.

Preparation of spraying reagent

2.8.2.4-aminobenzoic acid-acetic acid-phosphoric acid reagent 500 mg of 4-aminobenzoic acid was dissolved in the solution containing 9 mL of glacial acetic acid, 10 mL of water and 0.5 mL of 85per cent phosphoric acid.

TLC of hydrolyzed sample

Thin-layer chromatography (TLC) was performed on a silica gel plate. The hydrolyzed purified polysaccharide sample was spotted along with standard sugar solutions and developed using the solvent system, n-butonal: acetic acid: water (2:1:1).

The spots were visualized by spraying the plate with 4-aminobenzoic acid-acetic acid-phosphoric acid reagent and kept at 100^0C for 20 min. The R_f value of spots was calculated and compared with standard sugars.

Fourier Transmittance Infra-Red (FT-IR) analysis of purified polysaccharide

The purified moisture free polysaccharide 10 mg was ground with 300 mg of the carrier molecule, potassium bromide (IR grade) and the absorbance was recorded using a Perklin – Elmer 2000 FT – IR spectrometer from 500 - 4000 cm^{-1}

RESULTS

Mushroom cultivation

In the present study, Sorghum (*Sorghum vulgare*) grains were used for spawn preparation. The mycelia of *P. djamor* var. *roseus* covered the entire polypropylene bag containing processed grains after 14-16 days of incubation and the spawn was used for further cultivation studies.

Cultivation and yield efficiency of P. djamor var. roseus

The cultivation trials were carried out using sterilized paddy straw. Mycelium of *P. djamor* var. *roseus* covered the whole substrate after 15-18 days of inoculation. Pinheads of the fruiting body appeared after 20-22 days. The matured fruit bodies were harvested after 25 d of bagging. The yield of mushroom was more in the 1st harvest followed by 2nd and 3rd harvest. The yield from the 4th and 5th harvests was negligible. The Yield in terms of biological efficiency was determined by the fruit bodies collected from first three harvests. The biological efficiency of 120 per cent was recorded after 25 days.

Table 1. Yield efficiency of Pleurotus djamor var. roseus grown in paddy straw substrate

Harvest (g / fresh weight)			Total yield	B.E
I	II	III	(g / fresh weight)	(%)
320	190	90	600	120

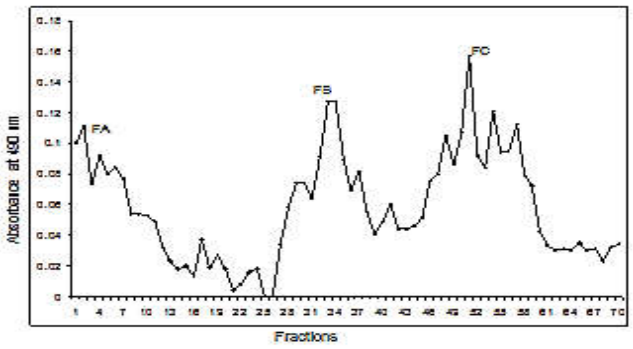
Biological efficiency (%) = fresh weight of mushroom /dry paddy straw substrate X 100.

Total sugars and soluble protein from Pleurotus djamor var. roseus

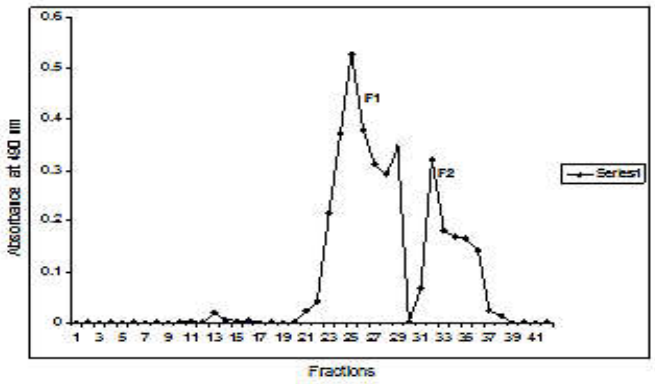
The harvested mushrooms were washed, shade dried and pulverized to coarse powder. The obtained powder of *P. djamor var. roseus* was analyzed for its carbohydrate and protein content. The mushroomcontained 51per cent of total sugar and 39 per cent of protein.

Isolation and purification of polysaccharide

200 g of dried powder of *P. djamor* var. *roseus* fruit bodies was extracted with hot water and precipitated by repeated processing with 95 per cent ice cold ethanol. The resulting crude polysaccharide was further treated with sevag reagent (chloroform: isoamylalcohol-24:1 v/v) and dialyzed against glass distilled water for removal of protein and other low molecular weight impurities. The resulting partially purified polysaccharide of *P. djamor* var. *roseus* was lyophilized and the yield was 560 mg. The overall protocol for the isolation and purification of polysaccharide was given in Fig. The obtained polysaccharide was further purified through DEAE cellulose column Fig. Three fractions (FA, FB and FC) were eluted with water, 0.2 M and 0.5 M NaCl solution respectively. The fractions were lyophilized. The yield of the fraction FA was 100 mg. The yield of FB and FC were negligible. FA was further purified through the sepharose 6B column in aqueous medium, and the two fractions F1 and F2 were obtained respectively. The yield of F1 was 30 mg, whereas F2 was negligible.



DEAE Cellulose chromatogram of the partially purified polysaccharide of *Pleurotus djamor* var. *roseus*



Sepharose 6B gel filtration chromatogram of the polysaccharide

TLC profile of the obtained polysaccharide

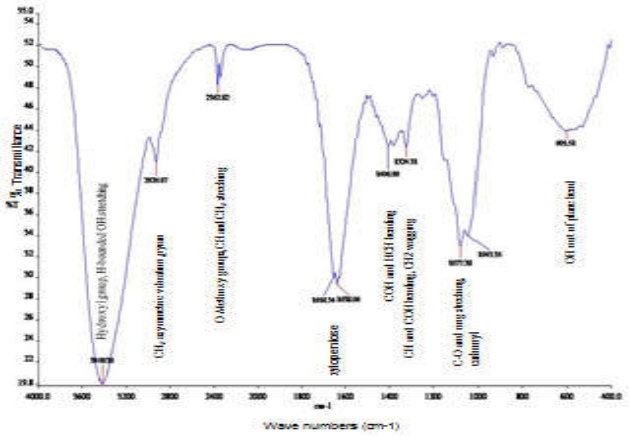
The hydrolyzed purified polysaccharide sample was spotted along with standard sugar solutions (glucose, mannitol, maltose, lactose and xylose) on pre-coated silica gel plate and develop using the solvent system n-butanol: acetic acid: water (2:1:1). The developed plate was dried and the spots were visualized by spraying 4-aminobenzoic acid-acetic acid-phosphoric acid reagent and kept at 100^0C for 20 min for spot development. Three spots were observed with R_f value of 0.308, 0.385and 0.500 respectively which correspond to the standards lactose, glucose and xylose.

Table 2. R_f values of TLC

Carbohydrate	R_f
Polysaccharide (F1)	0.308
	0.385
	0.500
Glucose	0.385
Mannitol	0.462
Maltose	0.423
Lactose	0.308
Xylose	0.500

FT-IR spectrum of polysaccharide

The chromatographed sample has been made as a KBr pellet and taken an IR spectra using FT-IR (Perklin – Elmer 2000). The spectral details were tabulated. The spectral details confirmed the presence of a polysaccharide moiety with xylopentose type of compound.



Conclusion

P. djamor var. *roseus* was cultivated using paddy straw as substrate and 600 g of fresh fruiting bodies were harvested with biological efficiency of 120per cent. The total sugar and protein content of *P. djamor* var. *roseus* was estimated as 51per cent and 39per cent respectively. Partially purified polysaccharide from *P. djamor* var. *roseus* was extracted by hot water extraction and precipitation by ice cold ethanol. It was further purified by DEAE cellulose column followed by Sepharose 6B column. 30mg of purified polysaccharide was obtained from 200 g of dried powder of *P. djamor* var. *roseus*.The hydrolysed purified polysaccharide exhibited three spots which correspond to lactose, glucose and xylose.FT-IR spectrum of purified polysaccharide confirmed the presence of a polysaccharide moiety, a xylopentose type of compound.

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