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Research Article

Optimization of fermentation media composition of *ganoderma lucidum* for improved production of antioxidant and antimicrobial compounds

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ABSTRACT: **Introduction**: To optimize the fermentation medium for *G. lucidum* by modifying the Mushroom complete medium (MCM) by three test carbon source Dextrose, Maltose, lactose and five nitrogen sources: peptone, soyapeptone, ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) and ammonium sulphate $(NH_4)_2SO_4$ to enhance the production of antioxidant and antimicrobial compounds. **Methods**: Fifteen different broth medium composition were prepared by varying the carbon and nitrogen sources in test medium (MCM). *G. lucidum* was cultivated in above test mediums for 7 days. Extraction and quantification of antioxidant compound (exopolysaccharide) and antimicrobial compounds. The antioxidant capacity was determined by three methods: Hydrogen peroxide assay, reducing power assay and hydroxyl radical scavenging assay in vitro while potential of antibacterial compound was determined by Agar well diffusion method. **Results**: High productivity were seen in medium contained maltose as carbon and soyapeptone, NH_4Cl, NH_4NO_3 as nitrogen source for both exopolysaccharide and antimicrobial compound. The samples demonstrate antioxidant capacity in dose dependent manner in all assays. Scavenging activity of hydroxyl ion ($52.7 \pm 1.55\%$) was found potent in medium contained maltose and NH_4Cl, which is above than ascorbic acid at same concentration (300 μg/mL). **Chloroform**: methanol extract shows inhibitory effect to all five human pathogenic strains, potent zone of inhibition was seen against *Shigella dysenteriae* (16.3 ± 1.69 to 19.6 ± 0.47), *Enterococcus facecalis* (16.6 ± 1.24 to 20 ± 1.63) and *Klebsiella pneumoniae* (16.6 ± 1.24 to 21 ± 1.63) which show maximum zone of inhibition (21 ± 1.63). **Conclusion**: Maltose and NH_4Cl are suitable carbon and nitrogen source for antioxidant and antimicrobial compound production.

KEYWORDS: G. *lucidum*, MCM, exopolysaccharide, antioxidant, antimicrobial compounds, carbon and nitrogen source

INTRODUCTION

Ganoderma lucidum, a medicinal fungus called "Lingzhi" in Chinese and "Reishi" in Japanese,[1] has been widely used as a tonic in promoting longevity and health in the Far East.[2]

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It has been well documented that polysaccharides from G. *lucidum* are one of the major sources which possess many bioactivities including antitumor,[3] immunomodulation[4] and antioxidation.[5]

In the last three decades the search for new therapeutic bioactive compounds that can serve as antioxidant and antimicrobial agents had increased tremendously due to multiple drug resistance in human pathogenic microorganisms. However, most of the investigations were focused on the polysaccharides from fruit bodies and mycelia.[6] Until now, various conditions of submerged fermentation and soil cultivation have been studied to improve the production of polysaccharides by *G. lucidum*.[7–9] It takes several months for the solid-culture mushrooms

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to grow into the fruiting bodies on solid substrates. Submerged culture gave rise to many potential advantages of higher mycelial biomass or EPS production in a compact space and shorter time with less chances of contamination.[10] Compared with the intracellular substances, the extracellular substances from fermentation broth with similar physiological and pharmacological functions are easily obtained.[11] In fact, food manufactures have directly employed EPS of mushrooms by fermentation to prepare drinks and capsules for sale. Recently, some studies showed that the compositions of the growth medium can affect the specific rate of EPS synthesis.[12–15]

The aim of present study was to find the optimum composition for submerged culture media of *G. lucidum* by changing the carbon and nitrogen source in tested media (Mushroom complete media), for the production of antioxidant and antimicrobial compound and analysis of the property of extracted compound by standard methods.

MATERIAL AND METHODS

I. Chemical & Media

Dextrose, Lactose, Maltose, Agar, Potassium hydrogen phosphate (K_2HPO_4), Hydrogen peroxide (3%), Magnesium sulphate ($MgSO_4.7H_2O$), Potassium ferricyanide, Potassium dihydrogen phosphate (KH_2PO_4), Bile salt, Ferric chloride ($FeCl_3$) (Rankem), Ferrous sulphate ($FeSO_4$). Yeast Extract, Peptone, Muller Hilton agar (MHA), MRS Broth, Tryptone, Beef Extract (Himedia laboratories Pvt. Ltd., Mumbai). NaCl (Merck ltd).

II. Test microorganism

The stored culture of *Escherichia coli* (MTCC 25922), *Shigella dysenteriae* (ATCC 9754), *Klebsiella pneumoniae* (MTCC 2405), *Staphylococcus epidermidis* (ATCC12228), *Enterococcus facecalis* (ATCC 29122) were collected from the Microbial Type Culture Collection (MTCC), The Institute of microbial Technology. Sector 39-4, Chandigarh, India.

III. Microorganism & culture condition

Pure culture of *G. lucidum* MTCC 1039 was obtained from Lignocellulose laboratory Department of Microbiology, Delhi University, New Delhi. The stock culture was grown on potato dextrose agar (PDA) at 25°C for regular subculture and maintained on PDA slants at 4°C.

IV. Inoculum preparation

G. lucidum was initially grown on PDA medium in a petri dish, and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized self-designed cutter.[16] The seed culture was grown in a 250-ml flask containing 100 ml of Mushroom complete media (MCM, Glucose 20 g l^{-1}, peptone 2 g l^{-1}, KH_2PO_4 0.5 g l^{-1}, K_2HPO_4 g l^{-1}, yeast extract 2 g l^{-1}) at 25°C on a rotary shaker incubator at150 revolution min^{-1} (rpm) for 7 days.

V. Tested medium and culture condition

The Mushroom complete media for the carbon and nitrogen sources testing contained 0.2% peptone powder, 0.2% yeast extract, 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$. As carbon sources dextrose, lactose and maltose were tested and supplemented to the MCM in same concentrations. As nitrogen sources, peptone powder, soyapeptone, NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$ were tested. Fifteen different medium compositions were prepared represented as medium (1 to 15) shown in table 1. The cultivation was carried out in a 250-mL Erlenmeyer flask containing 100 mL of tested medium on a rotary shaker (150 rpm) at 25°C for 7 days. The initial pH was adjusted to 6.0, and the media were sterilized at 121°C for 20 min.

VI. Analytical methods

Samples collected at various intervals from shake flask were centrifuged at 10000 g for 20 min, and the resulting supernatant was filtered by membrane filtration (0.45 µm, Millipore membrane).

Table 1: Tested medium (concentration in g l^{-1})

Carbon Source	KH_2PO_4	K_2HPO_4	$MgSO_4.7H_2O$	Yeast extract	Nitrogen source	
Dextrose 1 to 5	0.5	1	0.5	2	Soyapeptone (2,7,12)	2
		1 to 15			Peptone (1,6,11)	
Maltose 6 to 10	20				NH_4Cl (3,8,13)	
					NH_4NO_3 (4,9,14)	
Lactose 11 to 15					$(NH_4)_2SO_4$ (5,10,15)	

Extraction of exopolysaccharide

The resulting culture filtrate was mixed with four times its volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated EPS was centrifuged at 10000 g for 10 min, discarding the supernatant. The precipitate of pure EPS was air dried at room temperature and the weight of the polymer was estimated.

Extraction of antimicrobial compound

Chloroform: methanol solution was prepared in 1:1 ratio. The filtered culture fluids were extracted by repeated three times washing with chloroform: methanol solution in 9:1 ratio. The chloroform: methanol fraction was evaporated and the extracted material was dissolved in DMSO.[17]

VII. Antioxidant activity determination

Following tests were performed for evaluation of antioxidant potential of ethanolic extract of *Ganoderma lucidum*.

Hydrogen peroxide scavenging method

The ability of EPS to scavenge hydrogen peroxide was determined by standard protocol.[18] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (UV-1700, Shimadzu Corporation, Japan). Extracts (50–250 µg) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after ten minute against a blank solution containing in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of EPS and ascorbic acid was calculated using the following equation:

$$\text{Percent scavenged } [H_2O_2] = [A_o - A_1 / A_o] \times 100$$

Where A_0 = absorbance of the control, and

A_1 = absorbance in the presence of the sample.[19]

Reducing power assay

The reducing power of EPS was determined by standard protocol.[20] The different doses of EPS (50, 100 and 250 µg) in 1ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged for 10 min at 1000 × *g* (Plasto crafts industries (P) Ltd, Mumbai). The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Hydroxyl radical scavenging activity

The hydroxyl radical (OH^-) scavenging activity of Exopolysaccharides was measured by modified Fenton system.[21] Different concentrations of EPSP (0.1 ml) were added to the reaction mixture containing 1 ml of $FeSO_4$ (1.8 milligram mol^{-1}), 1.5 ml of salicylic (1.8 milligram mol^{-1}), 1.8 ml of absolute ethanol and 0.1 ml of Hydrogen peroxide (H_2O_2) (22 milligram mol^{-1}). The H_2O_2 was added into the mixture to initiate the reaction. The reaction mixture was incubated at 37ºC for 30 min and then the absorbance (A) was measured at 510 nm. The scavenging rate was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [1-(A_s-A_{sc})/A_c] \times 100\%$$

A_c is the absorbance for control (double-distilled water)

A_s is the absorbance for the reaction mixture with EPSP solution

A_{sc} is the absorbance for background (i.e. the reaction mixture without H_2O_2).

Screening of Antibacterial activity

Antibacterial activity of mushroom extracts was carried out by modified agar well diffusion method. To standardize the inoculums density of test bacterial strains for susceptibility test, a barium sulphate (BaSO4) turbidity standard, equivalent to a 0.5 McFarland turbidity standard was used.[22] The inoculums size of the test strain was 1×10^8 to 2×10^8 colony forming unit/ml. 0.02 ml inoculums of known turbidity was applied on the dried surface of prepared Muller Hinton Agar plate. The inoculated plates were left for 15–20 minutes at room temperature. Six mm diameter wells were punched into the agar using sterilized well cutter to obtain a 6mm diameter bore. 100 µl of chloroform: methanol extract Dissolved in dimethyl sulphoxide (DMSO), from 15 different modified medium was carefully pipette into each well. The plates were incubated for 24 h at 37°C and diameter of the inhibition zones around the wells were recorded in millimeters.[23] The tests were performed in triplicates and final values were expressed as mean ± standard deviation.

RESULTS

MCM, which has usually been used for the cultivation of higher fungi, were employed to modify by its carbon and nitrogen sources to select a suitable medium composition for the exo-polysaccharide, antimicrobial compound synthesis and mycelial growth.

MCM medium was served as good cultivation medium for exo-biopolymer production in *G. lucidum*. Results of experiment shows that modified MCM medium containing maltose and NH_4Cl as chief carbon and nitrogen source was suitable to achieved enhanced production of exopolysaccharide (shown in table 2) and antimicrobial compound as compared to standard MCM production in previous reports. From the aforementioned results, indicated that modified MCM (medium 8) could be used for large scale production of these two compounds as it is more cost effective than MCM.

Antioxidant capacity

The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[24] Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function.

Hydrogen peroxide scavenging assay: The ability of EPS to scavenge H_2O_2 was determined by standard method.[25] The scavenging ability of EPS extracted from different medium composition on H_2O_2 (shown in figure 1) and compared with ascorbic acid as standards. EPS from all modified medium was capable of scavenging H_2O_2 in a dose-dependent manner. Exopolysaccharide extracted from medium 8, exhibited 52.7 ± 1.55 % (300 µg/ml) hydrogen peroxide scavenging activity which was more than ascorbic acid 48 ± 0.81% at same concentration. These results indicated that EPS extracted from medium 8 posses effective H_2O_2 scavenging activity more than ascorbic acid. However, there was statistically a very significant correlation between those values and control ($P < 0.01$).

Table 2: Biomass and Exopolysaccharide produced by *G. lucidum* in different culture medium after 7 days incubation.

Culture medium	Biomass (g/L)	Exopolysaccharide (g/L)
Medium 1	7.4	0.876
Medium 2	7.7	0.910
Medium 3	5	0.650
Medium 4	6.7	0.805
Medium 5	7	1.01
Medium 6	8	1.05
Medium 7	8.2	1.09
Medium 8	7.2	0.950
Medium 9	7.3	1.05
Medium 10	6.6	1.09
Medium 11	5.5	0.548
Medium 12	6.5	0.785
Medium 13	4.3	0.496
Medium 14	6.3	0.894
Medium 15	4.9	0.542

Reducing power: The reducing property is associated with the presence of reductones and has been reported to have a direct, positive correlation with antioxidant activities of some plant compounds.[20] So, the reducing power is often used as an indicator of electron-donating activity. As it is shown in Fig. 2, EPS from modified medium possessed the ability to reduce iron (III) and also in a level- dependent pattern. The maximum absorbance was observed in EPS extracted from medium 8 (0.266 ± 0.001) followed by medium 6 (0.253 ± 0.007) than medium 9(0.248 ± 0.002) at concentration of 250 µg ml^{-1}. When compared with that of ascorbic acid, reducing power of EPSP was not so remarkable. These results suggested that maybe the EPSP from *G. lucidum* cannot act as effective electron-donators.

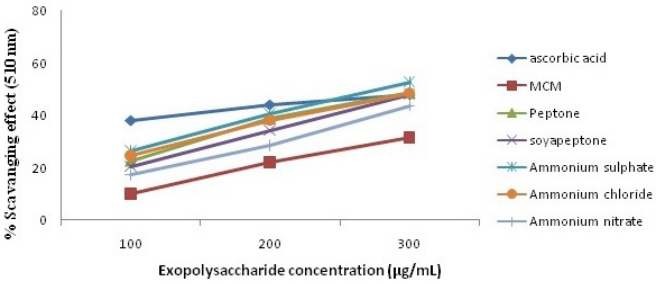

Figure 1. Scavenging effects of EPS from *G. lucidum* on Hydrogen peroxide extracted from medium contained Maltose & different nitrogen source. Data are presented as mean value (n = 3).

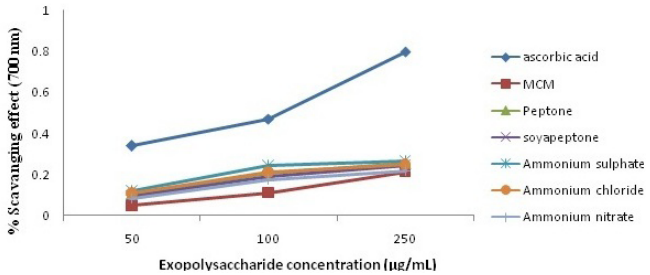

Figure2. Reducing power assay of Ascorbic acid, EPS (extracted from MCM and medium contained Maltose & different nitrogen source) from *G. lucidum*. Data are presented as mean value (n = 3).

Effect of scavenging activity of hydroxyl radical: Hydroxyl radical is the most reactive species among oxygen radicals and can directly induce oxidative damage to biomolecules resulting in destruction of cell structure.[25] So, scavenging activity of hydroxyl radical often serve as a main index for antioxidant activity evaluating of natural product. As it is shown in Fig. 4, the scavenging effect of EPSP on hydroxyl radical concentration- dependently increased and the maximum value (75.92 ± 1.56%) was achieved at the dose of 5 mg ml^{-1}. It was noteworthy that the sample showed a pronounced hydroxyl radical scavenging ability than that of ascorbic acid at each dose (range of 1–5 mg ml^{-1}). The maximal scavenging percentage of EPSP was about 2.33 times than that of ascorbic acid (32.54 ± 0.41%) at a dose of 5 mg ml^{-1}. The difference was significant ($p < 0.05$).

Antibacterial activity

Antibacterial activity against human pathogenic microorganisms was evaluated by agar well diffusion method. The results presented in table 3 shows that Chloroform-methanol extract from all 15 different medium exhibit effective Zone of inhibition against all the pathogenic strains. Maximum zone of inhibition was observed against *Shigella dysenteriae*, *Enterococcus facecalis* and *Klebsiella pneumoniae* about (16 ± 1.63) to (21 ± 1.63). Modified medium containing Maltose as carbon source, Ammonium nitrate, Ammonium chloride and Soyapeptone as a nitrogen source was found to be more potent for the production of antimicrobial compound as compared to MCM.

DISCUSSION

Mushrooms have been appreciated as sources of food nutrients for centuries and especially used for medicinal purposes in the orients for centuries.[26] *G. lucidum* are excellent natural source for antioxidant and antimicrobial compounds.[27] So, optimum compositions of fermentation medium for large scale production are prerequisite for researchers.

The nutritional requirement for EPS production in basidiomycetes and ascomycetes differs in strains and culture conditions. Moreover, different carbon source can result in the different carbohydrate compositions in polysaccharides.[28] To find the suitable medium composition for enhanced production of EPS and antimicrobial compound, the *G. lucidum* was cultivated in Mushroom culture media containing various carbon sources (dextrose, maltose

Table 3: Antimicrobial activity of G.lucidum of against human pathogenic microorganisms

Media	Zone of inhibition (mean±SD)				
	Test bacterial strains				
	E.coli	*Shigella dysenteriae*	*Staphylococcus epidermidis*	*Enterococcus facecalis*	*Klebsiella pneumoniae*
Medium 1	-	17±0.471	15±0.81	17±0.81	15±0
Medium 2	14.3±0.47	17±0.81	13.6±1.24	17±0.81	18.3±0.47
Medium 3	14±0.81	17±0.81	10.3±0.47	18±1.63	17.6±0.94
Medium 4	15.3±0.94	18±1.63	10.3±0.47	20±1.63	14.3±0.47
Medium 5	14.6±0.94	17.6±1.24	18±0.81	14±1.63	16±1.63
Medium 6	10.6±0.94	18±0.81	11.3±0.94	16.6±1.24	15.6±0.94
Medium 7	17±0	17.3±1.24	12±1.63	18±0.81	18±1.69
Medium 8	12±1.63	18±0.81	10±0.81	18±0.81	19.3±0.47
Medium 9	16.3±0.47	18±0.81	12±1.6	18.3±0.47	14±0
Medium 10	14±0.81	17.3±1.24	14±0.81	14.3±0.47	16.3±1.69
Medium 11	-	16.6±1.24	14.3±0.81	16.6±1.24	17.3±0.47
Medium 12	18±1.63	16.3±1.69	11.3±0.94	17.6±0.94	18±1.63
Medium 13	12.6±0.94	19.6±0.47	10±0.81	17.3±1.69	21±1.63
Medium 14	15.6±1.20	20.3±0.47	11.3±0.94	18±1.6	16.6±1.24
Medium 15	14.3±0.47	16.3±1.69	14±0	14±0.81	15±0.47

and lactose) and nitrogen source (peptone, soyapeptone, NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$. The suitable carbon and nitrogen source for EPS and antibacterial compound was Maltose and NH_4Cl. Maltose was known as an efficient carbon source for EPS production in liquid-cultures of mushrooms and above results was in accordance with those obtained by other investigator.[29,30]

It has been known that several complex nitrogen sources were desirable in fermentation media for higher fungus and In comparison with organic nitrogen source, inorganic nitrogen sources gave rise to relatively lower mycelial biomass and EPS production.[31] In present study modified media containing NH_4Cl with yeast extract as chief nitrogen source was seen to be suitable for production of antioxidant & antimicrobial compound as compared with MCM.

It was well accepted that the structure and chemical composition of polysaccharides influenced its bioactivity. Some researchers reported that the polysaccharide/peptide ratios of polysaccharides influenced its antioxidant property. The analysis of carbohydrate compositions in the above EPS revealed that the percentage of carbohydrate composition was significantly changed with different nitrogen sources.[32] Polysaccharopeptide obtained from mushrooms have lower polysaccharide/peptide ratios and exhibits the strongest scavenging effects.[33,34] It was found that EPSP from *G. lucidum* were complexes of polysaccharides (71.88%) and proteins (28.12%).[35] Perhaps this was why EPSP from *G. lucidum* had more powerful free radical scavenging capacity and reducing power. The present study need to be further analysis for more pronounce results so that large scale cultivation would be achieve from these natural bioactive metabolites.

CONCLUSION

The present study revealed that modified media containing Maltose as carbon source and soyapeptone, NH_4Cl and NH_4NO_3 as nitrogen source could be suitable sources for enhanced production of antioxidant and antimicrobial compound from submerged cultivation of *G. lucidum*. Compounds extracted from modified media demonstrate potential antioxidant and antibacterial effect in their assay.

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