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Evaluation of *Ganoderma lucidum* strains for the production of bioactive components and their potential use as antimicrobial agents

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Abstract: In the present study, mycelial biomass of *Ganoderma lucidum* strains was produced in mushroom complete media under optimized culture conditions of temperature, pH, agitation and the fruiting bodies were raised on wheat straw supplemented with 5 per cent rice bran. Maximum biomass (16.39 mg ml^{-1}) was recorded in strain GL-3 and minimum (10.99 mg ml^{-1}) was observed in strain GL-1 after 28 days of incubation period. Maximum biological efficiency of 13.1 per cent was obtained in 57 days in GL-2 strain. Moreover, weight of fruiting bodies was highest (91 g bag^{-1}) in 5 per cent wheat bran supplemented bags in strain GL-2. Polysaccharides were extracted, characterized and analysed. The maximum yield of polysaccharides was obtained from fruiting bodies (30.06- 63.70%), followed by mycelial biomass (7.61- 21.32%) and culture filtrates (0.86- 2.86%). Non-reducing sugars were the main fraction of sugars which constituted 69.82- 70.39 per cent in fruiting body extracted polysaccharides, 62.08- 83.39 per cent in biomass and 59.75- 77.54 per cent in crude extracts. The extracts from fruiting bodies and biomass of *G. lucidum* strains also exhibited antibacterial effect against some pathogenic bacteria, *Staphylococcus* sp. and *Enterobacter* sp. The present study clearly indicated that variations existed among *G. lucidum* strains with respect to the production of bioactive molecules, yield and antimicrobial activity. Bioactive components from *G. lucidum* strains are promising antimicrobial agents that can be harnessed as potent antimicrobial toxicant.

Keywords: Antimicrobial activity, Biological efficiency, *Ganoderma lucidum*, Polysaccharides

INTRODUCTION

Ganoderma lucidum is a medicinal fungus in Polyporaceae. The fungus is saprobic in nature, growing alone or in groups on decaying hardwood logs. It is known as 'lingzhi' and was first indexed in Shen Nong's Materia Medica (206 BC-8 AD) as a longevity-promoting and tonic herb of the non-toxic superior class (Zhu *et al*., 2007). It has been used in traditional Chinese medicine for more than 2000 years to prevent and treat diseases including hepatitis, chronic bronchitis, gastritis, tumor growth and immunological disorders (Lin and Zhang, 2004). The chemical components of *G. lucidum* extracted from the fruiting body, mycelium, or spores includes polysaccharides, flavonoids, alkaloids, amino acids, steroids, oligosaccharides, proteins and mannitol (Zjawiony, 2004). Among these components, *G. lucidum* polysaccharides and triterpenes have been identified as the major bioactive components, showing multiple pharmacological effects, such as immunomodulation, anti-oxidation, hepatoprotection, anti-proliferation, and anti-angiogenesis (Xu *et al.,* 2011). Each fraction of polysaccharides and triterpenes has more than 100 molecules that have been isolated, most of these are potent immunomodulators and/or antioxidants and are also chemopreventive and tumoricidal (Gao *et al.,* 2002 and 2004). The lingzhi isolated polysaccharides are macromolecules, having a molecular weight range from 4×10^5 to 1×10^6 Dalton in the primary structure. Most of these polysaccharides are extractable with hot water, salt solutions, alkali solutions, and dimethyl sulfoxide solution. The purified molecules are less soluble in water and become more soluble in alkali (Gao *et al.,* 2004). The extracts of *G. lucidum* have also been reported to be active against *Bacillus subtilis* and *Pseudomonas syringes* which are plant pathogen (Ofodile *et al.,* 2005). Traditionally, bioactive components have been extracted from fruiting bodies. However, mycelial biomass production forms a promising future platform for fully standardized production of safe mushroom based dietary supplements containing these bioactive components. The present investigation elucidates interesting chemical compounds extracted, purified and identified from fruiting body, mycelial biomass and culture filtrate of *G. lucidum* strains and their evaluation against some pathogenic isolates of bacteria.

MATERIALS AND METHODS

Mycelial biomass and fruiting bodies: The mycelial biomass of *G. lucidum* strains (GL-1, GL-2 and GL-3) was raised in the mushroom complete medium (Glucose-20g, Peptone- 2.0g, Yeast extract- 2.0, KH_2PO_4- 0.5g, K_2HPO_4- 1.0g, $MgSO_4.7H_2O$- 0.5g) under optimized conditions of temperature (30^0C), pH

(5.0) and agitation (100 rpm) after 28 days. Fruiting bodies were also raised on sterilized wheat straw supplemented rice bran (5% w/w) substrate. Fruit bodies were harvested after maturity. Biological efficiency (B.E.) was calculated using the following formula (Chang and Miles, 2004).

Extraction and analysis of polysaccharide: The polysaccharides were extracted from the mycelial biomass, culture filtrate of three *G. lucidum* strains (GL-1, GL-2 and GL-3) and fruiting bodies of GL-1 and GL-2 strains using method by Yap and Ng, (2001). The mycelial biomass raised in MCM for all the three strains was filtered through Whatmann no.1 filter paper and washed with distilled water. About 15 g of the mycelium was homogenised in a tissue homogeniser, transferred to a 500 ml round bottomed flask and 100 ml of distilled water was added to it. The samples in the round bottomed flasks fitted with condensers, were refluxed on a hot plate at 100ºC for 10 hrs. The refluxed samples were cooled and filtered through Whatmann filter paper no.1.The filtrates obtained were used for the extraction of polysaccharides, while the residues left were discarded. The filtrates were concentrated to about $1/10^{th}$ of the original volume by evaporating water from them with the use of distillation condensers and were cooled.

To the concentrated extracts, the equal amount of pre-chilled ethanol (4ºC) was added after cooling to room temperature and the samples were kept at -20˚C for 2 hrs in order to get complete precipitation of polysaccharides. After precipitation, the samples were centrifuged in a cooling centrifuge (Remi) at 4ºC for 20 min at 6000 rpm. The residue so obtained was the first fraction of polysaccharides which was collected in a pre-weighed glass vial and stored at room temperature. The supernatant left after centrifugation was used for the extraction of second fraction of polysaccharides. Again equal volume of ethanol was added to the supernatant, kept at -20˚C for overnight. Next day, the suspension was centrifuged at 4ºC in a cooling centrifuge at 6000 rpm for 20 min. The residue obtained was collected as the second fraction of polysaccharides in glass vials and stored at room temperature while the supernatant left after centrifugation was discarded. The crude extracts left after filtration of mycelial biomass were as such concentrated without refluxing for 10 hrs and precipitated repeatedly with chilled ethanol so as to separate the polysaccharides from them. Similar procedure was followed for the extraction of polysaccharides from the fruiting bodies of GL-1 and GL-2 strains of *G. lucidum*.

The percentage yield obtained in all the samples was then calculated by the following formula:

The polysaccharides were quantitatively analysed by estimating the amount of polysaccharides obtained after extraction process. The estimation was done for total sugars by Dubois *et al.* (1956) as well as reducing sugars by Miller (1959).

Antimicrobial activity: The antibacterial activity of various extracts of *G. lucidum* strains was determined against the bacterial cultures of *Staphylococcus sp., Salmonella sp., Enterobacter sp., Pseudomonas sp., Klebsiella sp*. and *E. coli* procured from Sawai Mansingh General Hospital and Medical College, Jaipur, India. The cultures were maintained in sterile nutrient agar slant at 4 ˚C.

Preparation of mushroom extracts: Two types of extracts (Methanolic and aqueous extract) were used for the assessment of antimicrobial activity of the mushroom. The mycelium of all the three strains of *G. lucidum* was used for preparing two kinds of extracts. GL-3 strain produced no fruiting bodies hence methanolic and aqueous extracts were prepared for the fruiting bodies of GL-1 and GL-2 strains.

Methanolic extracts: The methanolic extracts of mycelium and fruit bodies were prepared using methanol as an extracting solvent according to the methodology of Kalsi (2002). 5 g of fresh mycelium of

$$\text{B.E. (\%)} = \frac{\text{Fresh wt of mushrooms}}{\text{Dry wt of substrate}} \times 100$$

each strain of *G. lucidum* were refluxed with 50ml of methanol at 40ºC on water bath for 4 hrs. The contents were filtered through pre-sterilized Whatmann no.1 filter papers and cooled. The extracts obtained were left overnight at room temperature to evaporate the methanol and were collected in pre-sterilized screw capped glass vials. Similarly, 5 g of dried and powdered fruit body of GL-1 and GL-2 strains were used to prepare the methanolic extract in a similar manner and stored at 4ºC.

Aqueous extract: The hot water extracts were prepared using mycelium and fruit body. 5 g of fresh mycelium of all the three strains of *G. lucidum* were heated with 100ml of distilled water on a boiling water bath at 100º C for 8 hrs. The contents were filtered through pre-sterilized Whatmann no.1 filter papers and cooled to room temperature. The extracts were collected in pre-sterilized screw capped vials and stored at 4ºC in refrigerator until use. The aqueous extract of the fruit body of GL -1 and GL-2 strains were also prepared in a similar way using five g of dried, powdered sample and then stored.

Antimicrobial activity of methanolic and aqueous extracts was evaluated by the filter paper disc and spectrophotometeric method. Experiment was set up in three replications. Plates and liquid medium were incubated at 37°C for 24 to 48 hrs.

RESULTS AND DISCUSSION

Liquid culture studies for biomass production: Three strains of *G. lucidum*, GL-1, GL-2 and GL-3 were grown in mushroom complete medium adjusted at pH 6.0 value. Results indicated the increase in biomass yields with increase in incubation up to 28 days. Maximum biomass yield production values were recorded in

Table 1. Biomass production of *G. lucidum* strains.

Strain No.	Incubation period (Days)			
	Dry biomass (mg g^{-1})			
	7	14	21	28
GL-1	6.17	7.17	8.80	10.99
GL-2	4.94	8.31	10.19	12.74
GL-3	10.69	11.93	15.38	16.39
CD at 5%	1.22	1.34	1.53	1.28

Average of 5 replicates; Medium: Mushroom complete medium; Incubation temperature: 30± 2˚C

strain GL-3 (16.39 mg ml^{-1}), GL-2 (12.74 mg ml^{-1}) and the least for GL-1 (10.99 mg ml^{-1}) after 28 days of incubation period. Rate of biomass production was more from 14-21 days of incubation (Table 1). Varied biomass production values have been recorded by different workers depending upon the medium used, initial pH, incubation period and type of cultivation system used. Kim *et al.* (2002) found that mushroom complete medium supported maximum biomass production in case of *G. lucidum* as compared to yeast malt extract (YM) and potato malt peptone (PMP). The dry cell weight after 15 days of incubation was 6.24 g l^{-1} for *G. lucidum.* In case of YM and PMP media, these values were 5.65 g l^{-1} and 4.20 g l^{-1} respectively after 15 days of incubation. Lee *et al.* (2007) obtained 17.8 g l^{-1}, 19.4 g l^{-1} and 18.9 g l^{-1} cell masses on the media containing glucose, fructose and maltose. Shivani (2008) had observed the same trend of biomass production using four strains of GL-1, GL-2, GL-3 and GL-4 after 21 days of incubation. Zapata *et al*. (2012) obtained mycelial biomass (23.49 ± 0.37 g l^{-1}); extracellular polysaccharides (2.72 ± 0.11 g l^{-1}); intracellular polysaccharides (2.22 ± 0.06 g l^{-1}); ganoderic acids production (299.67 ± 11.63 mg l^{-1}) in *G. lucidum* on optimal medium composition defined as (g l^{-1}): 50 of barley flour, 0.2 of KH_2PO_4, 0.1 of $MgSO_4.7H_2O$, and 1 NH_4Cl. Fraga *et al.* (2014) obtained maximum mycelial biomass and exopolysaccharide purity at low peptone level (1.65g l^{-1}) in *G. lucidum*. However, they found maximum exopolysaccharide production at higher amount of peptone (4.80 g l^{-1}).

Yield evaluation of *G. lucidum* strains: The effect of supplementation of wheat bran revealed that maximum biological efficiency of 10.3% was obtained at 5 per cent level in GL-1. Moreover, 5 per cent wheat bran supplementation resulted in more fruiting bodies (72 g bag^{-1}) and mycelia spread was also fast (53 days). Improvement in yield at higher rate of supplementation (15 % and 20 %) was not significant at 5 per cent level of significance. In case of GL-2, yield data followed the same trend as in case of GL-1 supplementation of wheat bran @ 5- 15 per cent support better growth and fruiting bodies production than at 20 per cent. Weight of fruiting bodies was highest (91 g bag^{-1}) in 5 per cent wheat bran supplemented bags as compared to others (Table 2). GL-3 strain was found non-productive on wheat straw as no fruiting body of this strain was

Table 2. Effect of wheat bran supplementation on the yield of GL-1 and GL-2 strains.

Treatments	GL-1 strain				GL-2 strain			
Supplement level (%)	DSR	NFB Bag^{-1}	WFB(g) Bag^{-1}	BE (%)	DSR	NFB Bag^{-1}	WFB(g) Bag^{-1}	BE (%)
Control	63	3	20	2.9	61	4	26	3.7
5	53	8	72	10.3	53	6	91	13.0
10	55	5	56	8.0	56	2	30	4.3
15	64	4	45	6.4	62	6	60	8.6
20	60	3	22	3.1	67	3	26	3.7
CD at 5%				6.78				0.77

DSR= Days for spawn run, NFB= No. of fruiting body, WFB= Weight of fruiting body; Dry wt. of wheat straw/ Bag: 350g; No. of replicate bags: 15; *Strain GL-3 produced no fruiting bodies

Table 3. Polysaccharide, reducing and non reducing sugar contents in *G. lucidum* strains.

Sample	Strain	Polysaccharide yield (%)	Reducing sugars (%)	Non reducing Sugars (%)
Biomass	GL-1	21.40	37.92	62.08
	GL-2	16.00	25.45	74.55
	GL-3	7.67	16.61	83.39
Crude Extract	GL-1	0.86	67.72	32.28
	GL-2	2.87	40.25	59.75
	GL-3	2.53	22.46	77.54
Fruiting Body	GL-1	30.07	30.18	69.82
	GL-2	63.70	29.61	70.39

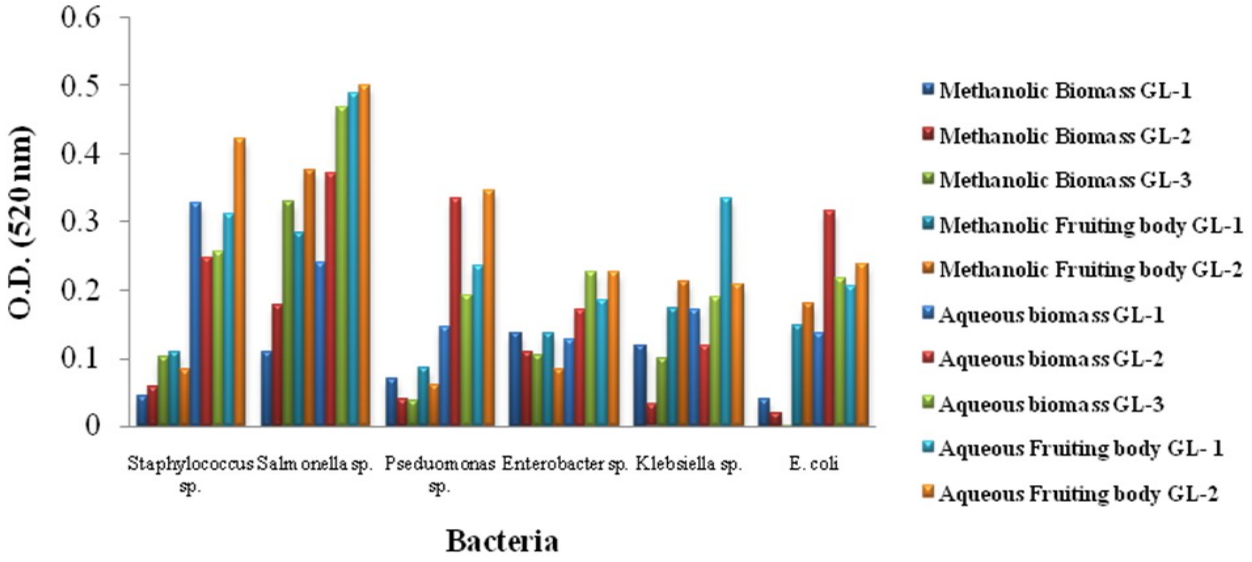

Fig. 1. *Spectro-photometric method to determine quantitative antibacterial activity of extracts of three strains of G. lucidum after 24 hrs.*

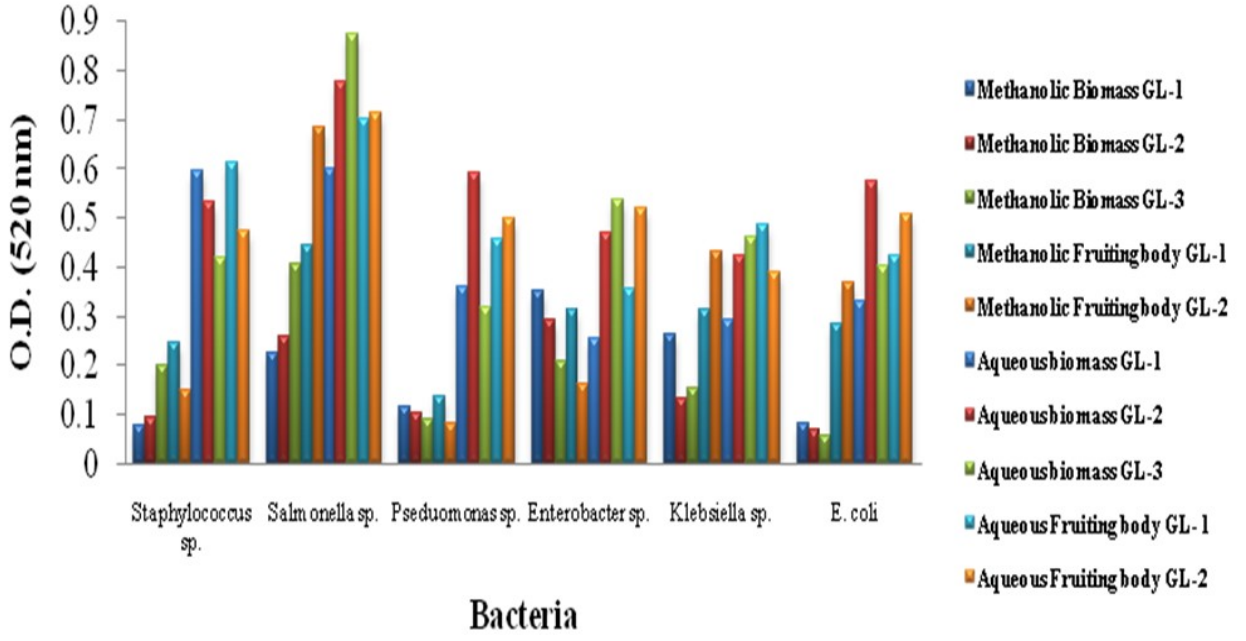

Fig. 2. *Spectro-photometric method to determine quantitative antibacterial activity of extracts of three strains of G. lucidum after 48 hrs.*

obtained.

Chen (1999) reported that rice or wheat bran addition was essential for the successful cultivation of *G. lucidum*. Addition of rice bran or wheat bran to the substrate takes care of the requirement of thiamine, which is very essential for the mycelial growth and reproduction of *G. lucidum*. Stamets (2000) reported that over supplementation with rice bran, beyond 15 per cent of the dry mass of the substrate inhibits fruit body development. Mishra and Singh (2006) reported that wheat bran supplemented with 5 per cent rice bran gave significantly higher B.E. (15-17 %) at 5 per cent level of significance than sawdust as substrate which gave only 5-14 per cent B.E. Shivani (2008) obtained maximum biological efficiency of *G. lucidum* with rice bran (10.7 %) at 5 per cent as compared to wheat bran (10.0 %) and further increase in the rate of supplementation resulted in decrease in biological efficiency which corroborate the present findings. Supplementation of both the brans @ 5-15 per cent supported better growth and fruiting body production than at 20 per cent. The best combinations for high yield (142.44 g/kg) and biological efficiency (18.68%) of *G. lucidum* were obtained by Azizi *et al*. (2012) in a combination of poplar sawdust with 5% malt extract and 10% wheat bran. The highest mycelia growth rate (10.6 mm/day) was obtained in a combination of beech sawdust with 2.5% malt extract and 10% wheat bran. Ueitele *et al*. (2014) concluded that corn cobs have the potential to be used as alternate substrate for *Ganoderma* mushroom cultivation in Namibia and

Table 4. Antibacterial activity of the extracts of strains of *G. lucidum* by filter paper disc method.

Bacteria	Chloram-phenicol (10 mg ml^{-1})	Clear zone diameter (cm)									
		Methanolic extracts					Aqueous extracts				
		A	B	C	D	E	A	B	C	D	E
Staphylococcus sp.	3.1	2.4	2.5	2.3	2.5	2.2	1.9	1.9	2.0	1.7	1.7
Salmonella sp.	2.3	1.7	1.3	1.3	1.2	1.2	1.1	0.8	1.2	1.1	1.3
Pseudomonas sp.	2.9	1.8	1.5	1.7	1.5	1.9	1.2	1.2	0.7	0.6	0.3
Enterobacter sp.	2.1	2.2	2.5	2.5	1.9	1.4	1.7	1.5	1.3	1.1	1.2
Klebsiella sp.	1.9	1.7	1.1	1.3	1.6	1.5	1.4	1.3	1.0	1.5	1.6
E. coli	2.5	2.0	1.5	1.4	0.9	0.9	0.5	0.7	1.2	1.0	0.5

A: GL-1 Biomass, B: GL-2 Biomass, C: GL-3 Biomass, D: GL-1 Fruiting body, E: GL-2 Fruiting body; Incubation Time: 24 hrs; Incubation Temperature: 37° C

obtained a higher biological efficiency (5.32%) as compared to saw dust (0.05%).

Polysaccharides, reducing sugars and non- reducing sugars: The polysaccharides obtained from fruiting bodies and mycelial biomass represents endopolysaccharides and those extracted in the culture filtrate were exopolysaccharides. The maximum yield of polysaccharides was obtained from fruiting bodies (30.06- 63.70%), followed by mycelial biomass (7.61- 21.32%) and culture filtrates (0.86- 2.86%). Endopolysaccharides were maximum in the fruit bodies of GL-2 strain (63.70%) and in the mycelial biomass of GL-1 strain (21.40%). The exo-polysaccharide production was recorded highest in GL-2 strain (2.87%) and minimum in GL-1 strain (0.86%) as given in Table 3. Cui *et al.* (2006) reported 107.34 mg g^{-1}, 83.13 mg g^{-1}, 105.45 mg g^{-1} and 98.25 mg g^{-1} of total polysaccharides in *G. lucidum* extract, by conventional vacuum drying; freeze drying, microwave vacuum and conventional vacuum drying on dry weight basis.

Non-reducing sugars were the main fraction in all the cases. Maximum non-reducing sugars were observed in GL-3 strain in biomass and crude extract. However, the non-reducing sugars were found maximum in the fruit body of GL-2 strain. Correspondingly, the amounts of reducing sugars were lesser in extracted polysaccharides from fruiting bodies, mycelial biomass and culture filtrates. Reducing sugars were 29.61 - 30.18 per cent in fruiting body extracted polysaccharides, followed by crude extracts (22.46- 67.72%) and minimum in biomass polysaccharides (16.61- 37.92%). Lee *et al.* (2007) reported that endopolysaccharides obtained from *G. lucidum* were containing 76% glucose, 8% galactose, 9% mannose and 7% xylose, whereas in case of exopolysaccharides, glucose was 81%, galactose 7%, mannose 12% and xylose was in trace elements. They also isolated a galactose rich extracellular (GLP-2) from submerged culture broth of *G. lucidum*, which was composed of galactose, mannose, glucose, arabinose and rhamnose in the molar ratios 103: 17: 12: 10:3. Narkprasom *et al*. (2013) optimized the submerged fermentation conditions for *Ganoderma tsugae* by using Plackett Burman Design and obtained maximum extracellular polysaccharide content (415 mg l^{-1}) under optimized culture conditions (31.031 g l^{-1} maltose, 14.055 g l^{-1} skim milk and an initial pH of 7.12).

Antibacterial potential of *Ganoderma* extracts on solid and liquid media: The antibacterial potential of the methanolic and aqueous extracts of *G. lucidum* strains against six pathogenic bacteria was studied by filter-paper disc and spectrophotometric methods for 24 and 48 hrs. The results of filter paper disc method are presented in Table 4.

Antimicrobial activity of methanolic extracts of biomass and fruit body of all the strains showed effective zone of inhibition as compared to aqueous extracts. The maximum antibacterial activity of methanolic extracts of biomass and fruit body of *G. lucidum* strains was found against *Staphylococcus* sp. based on zone of inhibition and minimum against *Klebsiella* and *E. coli*. The maximum inhibition zone ranging from 2.2- 2.5 cm was recorded for *Staphylococcus*. Aqueous extracts of biomass and fruit body also showed maximum inhibition of growth of *Staphylococcus* sp. and minimum of *Pseudomonas* sp. The maximum inhibition zone was ranging from 1.8- 2.0 cm for *Staphylococcus*.

In case of spectrophotometric method, increase in incubation period (24 to 48 hrs) resulted in decrease in antibacterial potential as manifested by increase of optical density (OD) Methanolic extracts were again found to be more effective against pathogenic bacteria as compared to aqueous extracts. These extracts were inhibitory to all the bacterial spp. except *Salmonella* sp. which was least inhibited by these extracts. Overall these extracts were having variable responses towards pathogenic bacteria, and these variations were also observed to different strains of *G. lucidum* (Figs. 1 and 2). The results of the present study are somewhat corroborated by the findings of Kamble *et al.* (2011) who reported that methanol, acetone, chloroform and aqueous extracts of *G. lucidum* biomass to be more effective against *Staphylococcus aureus, Bacillus subtilis,* and *Corynebacterium diphtheriae*, whereas these extracts were least inhibitory against *E. coli, Proteus mirabilis, K. pneumoniae, Salmonella typhii* and *Pseudomonas P18*. Bhosle *et al.* (2010) regarded *G. lucidum* as a broad spectrum antibiotic which were highly effective against both Gram positive as well as Gram negative bacteria. Singh *et al*. (2014) reported acetone and methanolic extract of *G. lucidum* fruiting body possessed

strong antimicrobial activity and considered as antimicrobial agent which can be used in the development of new drug for the different bacterial and fungal pathogenesis in humans.

Conclusion

Three indigenous strains of *Ganoderma lucidum* were screened for biomass, polysaccharide production and biological efficiency during cultivation indicated that strain GL-3 to be the most potential strain producing for maximum biomass production in liquid culture, whereas strain GL-1 and GL-2 can be exploited for cultivation of this fungus. The extracted polysaccharides showed potential antimicrobial activity against bacterial pathogens. Thus, bioactive components from *G. lucidum* strains are promising antimicrobial agents that can be harnessed as potent antimicrobial toxicant.

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