JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

THE EVALUATION OF SELECTED BIOACTIVITIES OF FUNGAL MYCELIUM OF A WILD *Schizophyllum commune* Fr. FROM SUBMERGED CULTURES

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https://doi.org/10.55251/jmbfs.3250

ARTICLE INFO

Received 10. 6. 2020
Revised 8. 7. 2022
Accepted 2. 8. 2022
Published 1. 10. 2022

Regular article

OPEN ACCESS

ABSTRACT

Schizophyllum commune is a widely distributed macrofungal species assorted with bioactivities. The specific objectives were evaluation of antibacterial and antioxidant properties, synthesis of exopolysaccharides and carbohydrates contents estimation of mycelial extract of *S. commune* under submerged condition. Methanolic extracts of identified *S. commune* showed highest FRS (92.59%), chelating effect on ferrous ion (79.36%), reducing power (1.844) activities at 16, 1.5, 16 mg/ml concentrations respectively (p<0.05). *S. commune* showed potent antibacterial activities (p<0.05) against four bacterial strains except against *P. aeruginosa* where no activity was observed. The maximum inhibition zone was exhibited against *S. aureus* (20.2 mm) and minimum inhibition was found against *B. subtilis* (5.0 mm). This study recorded the highest dry weight (9.33 g/L) and rate of average growth (1.33 g/L/day) of *S. commune* in glucose (p<0.05) medium but highest EPS (4.10 g/L) production was observed in xylose medium. Out of seven nitrogen sources, maximum mycelial yield (2.7 g/L), rate of average growth (0.38 g/L) and EPS (4.26 g/L) were recorded in yeast extract medium (p<0.05). In glucose (0.597 g/100g) and yeast extract (0.006 g/100g) medias, the maximum amount of total carbohydrate was obtained from the EPSs of *S. commune*. That's why it may be an excellent source of human medicines.

Keywords: Gillies or Split Gills; exopolysaccharides; bioactivities; antibacterial; antioxidant activity

INTRODUCTION

Wild mushrooms are crucial component of forest ecosystem and it contains a numeral of diverse secondary metabolites, proteins, carbohydrates, vitamins, minerals, fibres, fats and having different medicinal properties like, antimicrobial, antifungal and antioxidant **(Chowdhury *et al.*, 2015)**. Microorganisms have capacity to produce extracellular exopolysaccharide **(Mahapatra and Banerjee, 2013)** and t They have a number of physiological activities and prospective uses in a variety of industries like food engineering, agriculture, biodegradable polymers, fuels, pharmaceuticals, and so on hey have a number of physiological activities and prospective uses in a variety of industries like food engineering, agriculture, biodegradable polymers, fuels, pharmaceuticals, and so on **(Joshi *et al.*, 2013)**. Mushroom species can serve as possible indicators of the health or development of an ecosystem, and they are crucial for the growth and establishment of plants in forests as well as the recycling of nutrients (**Tapwal *et al.*, 2013**). India has a long history of using mushrooms for food and medicine, some of which date back to 1700–1100 BC (**Wasson, 1971**). India is a diversified nation that is home to numerous ethnic and tribal groups, each of which has its own strategy for handling the natural resources they use on a regular basis (**Debnath *et al.*, 2019**).
Schizophyllum commune is known as Gillies or Split gill macrofungi which was widely distributed species found mostly in tropical forest and it is cultivated commercially especially in Thailand. This species is also used as food by the different tribes of the world and has various medicinal benefits in different countries of the world **(Preecha *et al.*, 2016)**. The production of *S. commune* has increased due to their various medicinal, nutritional properties and its popularity **(Wasser, 2002)**. Schizoplyllan, a polysaccharide present in *S. commune*, is a therapeutic drug with antitumor, anti-inflammatory, immunomodulating, and anticancer effects **(Ooi and Liu, 1999; Wasser, 2002)** and additionally, it has a wide range of potential uses, including thickening agents for cosmetics and films that are oxygen-impermeable for food preservation. **(Teoh and Don, 2012)**.
Therefore, the objectives of this study were to assess the antibacterial and antioxidant qualities as well as the exopolysaccharide production employing various nitrogen and carbon sources. Estimation of total carbohydrates content from superior exopolysaccharides synthesized from carbon and nitrogen sources.

MATERIAL AND METHODS

Site of Collection and Characterization

The specimen, which grows on dead trees, was found in the mixed forest area of Mandwi (N 23°19.903' E 091°29.192'), West Tripura, Northeast India. Both morphological and molecular methods were used to characterize the wild *S. commune*.

Bioactive Properties

Fungal mycelia production

The PDA medium was used to cultivate the mycelium. Fungal mycelia were grown in a sterilised erlenmeyer flask (250 ml) containing 50 ml of Basal Synthetic Liquid (BSL) Medium in order to examine the antioxidant properties **(Saha, 1985)**. Then, fungal mycelium (6 mm) from a 5-8 day-matured culture on PDA media was put into the BSL medium. Under static conditions at 27°C, the growth was accomplished (Incubator: LSI 4018R). After 30 days, the mycelia was eliminated from the broth by filtering it with Whatman No.4 paper **(Kalyoncu *et al.*, 2010)**.

Fungal mycelia extraction

Mycelial extracts was done by the technique of **Mau *et al.* (2004)** with some modification. The fresh dried mycelia (5g) were extracted by crushing with 50 ml of methanol. After fine crushing the sample was extracted (Whatman No. 4) and the sieved crude mycelia was then re-extracted two times (50 ml). The extract's methanol was then dried off in a Rotavap (Model No: PBV-7D) by evaporating it at 30 to 40°C. The estimation of several bioactivities was done using the produced crude extract.

Antioxidant Activities

DPPH-based Free Radical Scavenging (FRS) effect

A modified **Shimada *et al.* (1992)** technique was used to investigate the FRS action **(1992)**. Inhibitions of FRS by percent of DPPH were intended as follows:

Percentage of inhibition: $[(A^{Blank}–A^{Sample})/A^{Blank}] \times 100$, Where A^{Blank} and A^{Sample} represents, respectively, the absorbance of the control and test composites. The graph was also used to get the EC_{50} (mg/ml) figure.

Chelating effect on ferrous ion

The **Decker and Welch (1990)** method was applied for the determination of the chelating effect. The development of the Ferrozine Fe2+ complex was then created with the following inhibitory percentage:
Metal Chelating Activity (%) = (A0-A1/A0)100%, wherein A0 and A1 stand for the absorbency of the samples and the control, respectively. The graph of ferrous ion inhibition percentage versus fungal extract concentration was used to get the EC_{50} (mg/ml) value.

Reducing power activity

A minor adjustment to Oyaizu's formula was used to estimate reducing power (**Oyaizu 1986**). The useful concentration (EC50, mg/ml), at which the wavelength was 0.5, is used to reduce power.

Phenol estimation

Total phenolic content in methanolic extracts was determined by the method of **Swain and Hillis (1959)** and tannic acid served as the baseline.

Flavonoids estimation

The method of Kosalec et al. (2004) was applied to calculate the total flavonoid content in mg of quercetin equivalents/g of methanolic extract (2004).

Test for antibacterial action

Microorganisms used in tests

Two Gram-positive bacteria (*Bacillus subtilis* MTCC 619 and *Staphylococcus aureus* MTCC 96) and three Gram-negative bacteria (*Pseudomonas aeruginosa* MTCC 424, *Xanthomonas campestris* MTCC 2286 and *Escherichia coli* MTCC 40) were examined for antimicrobial activity. We bought test bacterial strains from IMTECH Chandigarh in India.

Test for antibacterial effectiveness

The disc diffusion method (**Collins and Lyne, 1987**) was used to measure the antibacterial activity at concentrations of µg per ml.

Optimisation of Exopolysaccharides (EPSs) Production in Submerged Production

Test Media formation

For the best biomass development and EPSs content production, mushroom mycelium was cultivated in the BSL medium (**Saha, 1985**).

Inoculum and culture medium preparation

The inoculum was prepared by the modified method of **Saha (1985)**. Fungal was determined by measuring the mycelial dry weight **(Kim, 2005)**.

The use of various carbon sources

The BSL medium containing each of the seven carbon sources (30 g/L) namely glucose, maltose, sucrose, fructose, starch, *carboxymethyl cellulose* (CMC) and xylose in separate triplicate sets were evaluated. The inoculated culture flasks were cultured for 7 days at a temperature of 25°C in a shaker incubator at 120 rpm with 30 mL of medium that had a pH of 6.0.

The use of various nitrogen sources

The BSL medium at 6.0 pH containing each of the different sources of nitrogen at 3 g/L (yeast extract, peptone, beef extract, urea ammonium sulphate and calcium nitrate) in separate sets were used and starch used as common carbon source. Inoculated flasks were incubated at 25°C temperature.

Analysis Techniques

Measurement of mycelial weight (dry cell weight) and average rate of growth

Mycelial dry weight was calculated as dry weight. The mycelium was obtained by centrifuging it at 10,000 g for 10 min, rinsing it three or four times, and then drying it at 105°C for up to 12 hours to maintain a constant dry weight.

Exopolysaccharides (EPSs) measurement

The EPS of fungus was calculated using the **Fang and Zhong (2002)** method, and quantification was carried out using the **Dubois et al. (1956)** method.

Total carbohydrate content of exopolysaccharides (EPSs)

Using the Hedge and Hofreiter (1962) method, the total amount of carbohydrates was estimated.

Statistical Evaluation

The Past software version 2.17c was used to perform all of the calculations (**Hammer et al., 2001**). Experiments were carried out in triplicate, and all values were reported as mean standard error (SE). To examine the significance between the various parameters of the mushroom sample at $p<0.05$, Tukey's honestly significant difference (HSD) test was used to analyse the experimental results using one-way ANOVA (analysis of variance).

RESULTS

Identification

Morphological and molecular identification revealed that characteristics showed similarity with *Schizophyllum commune*. The NCBI received the *S. commune*'s ITS sequence after submission and assigned the accession number MH105058.

Biological Activities

Antioxidant property of the methanolic extracts of mycelium from submerged fermentation

The results of different antioxidant properties of *S. commune* and standard (positive controls) were reported in Figs. 1A to 1C for FRS activity, chelating effect and reducing power, respectively. To find out the significant effect of selected wild mushroom with controls on different concentrations, the analysis of variance (ANOVA-One Way) had been performed at 5% level of significance ($p<0.05$). The ANOVA (One Way) had been found that there were notable variations ($p<0.05$) present among the different concentrations of three different antioxidant properties of mushroom samples with controls. Now to find out the effectiveness of mushroom samples on different concentration over various antioxidant properties, the Tukey's HSD test had been performed.

Free Radical Scavenging (FRS) property

When exposed to radical scavengers, 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical that exhibits a distinctive wavelength at 517 nm and becomes a permanent diamagnetic compound, its absorbance at that wavelength considerably reduces.The scavenging effect rapidly increased from 0.5-8.0 mg/ml (Fig. 1A). *S. commune* (92.59%) showed its highest activity at 16 mg/ml concentration and found equally effective with respect to both the standard BHA and ascorbic acid (Fig. 1A). At 0.125 mg/ml, BHA and ascorbic acid had scavenging rates of 97.83 percent and 95.37 percent, respectively.

Chelating effect on ferrous ion

This test indicated that *S. commune*'s mycelial extract interfered with the production of ferrous and ferrozine complexes, indicating that they have chelating action (Fig. 1B). Statistical analysis showed that *p* values of both the studied mushrooms were significantly effective (p-value<0.05) with standard but activity were lower in comparison to standard. Comparing EDTA to the investigated mushroom, ferrous ion chelating properties were highest at 91.11 percent.

Reducing power activity

When measuring reducing power, antioxidant chemicals change the oxidised ferricyanide (Fe3+) ion of iron into the ferrous (Fe2+) ion. Because of its electron transferability, the reducing power can be a useful predictor of possible antioxidant action. Statistical analysis showed that *p* values of the studied mushroom with standard BHT were significantly effective (p-value<0.05), but activity was lower than the standard. The capacity of S. commune to reduce demonstrated outstanding activity and steadily increased from lower to higher concentrations (0.5–16 mg/ml), as shown in Fig. 1C.
The effective concentration of EC_{50}, is the level at which DPPH radicals are scavenged by 50% and antioxidant activity is 50%. By extrapolating from linear regression analysis, EC_{50} was determined (TABLE 1). The powerful EC_{50} of FRS and Chelating Effect on Ferrous Ion activities had been found in *S.commune* at 1.49 and 1.89 mg/ml concentrations. In comparison to the researched mushroom, synthetic antioxidants (ascorbic acid and BHT) had a higher (p-value<0.05) EC_{50} (0.049 mg/ml) value. At a concentration of 1.847 mg/ml (BHT), which was higher

than the maximum value of *S. commune*, the EC50 value of BHT's reducing power was observed to be significant ($p<0.05$). Total phenol and flavonoid content of *S. commune* were depicted in TABLE 1.

Antibacterial activity

Tested *S. commune* showed more or less antibacterial property against both Gram-negative and Gram-positive bacteria except *P. aeruginosa* (Fig. 2E). Table 2 and Fig. 2A–E show the antibacterial activity of a methanolic extract of the mycelia from the wild edible mushroom *S. commune*. An initial analysis of variance (ANOVA-One Way) was conducted. As a result, Tukey's HSD test was used to determine which concentration of mushroom sample had the most significant effect compared to controls once the significant effect of at least one concentration of mushroom sample had been found at the 5% level of significance (i.e., *p*-value 0.05).

Out of the five bacterial strains, the mycelial sample solution of *S. commune* was found to have antibacterial properties against four of them (Fig. 2A-E and Table 2) and observed that significant differences between concentrations of mycelial extracts of *S. commune* and positive control (Streptomycin) were present. Then the Tukey HSD test had been conducted to find out the most significant concentration of mushroom sample over different antibacterial strains. The crude mycelial extract of *S. commune* showed the highest (20.2±0.5 mm) inhibition zone against *S. aureus* (Fig. 2A) at 100 µg/µl concentration, whereas the least (5.0±0.00 mm) inhibition zone was exhibited against *B. subtilis* (Fig. 2B) at 100 µg/µl concentration.

Exopolysaccharides (EPSs) Production

In order to determine the optimal chemical conditions for growth and exopolysaccharide synthesis, various carbon and nitrogen sources were tested in this study. The analysis of variance (ANOVA-One Way) had been done initially to determine the significant impact of carbon and nitrogen sources on a particular wild edible mushroom (*S. commune*) that was reported in Fig. 3A through 3B. As a result, the Tukey's HSD test was used to determine which carbon and nitrogen sources in *S. commune* had the most significant effects if the significant effects of at least one carbon and nitrogen source had been detected at the 5% level of significance (i.e., *p*-value 0.05). The ANOVA (One Way) had been (<0.05) found that in average growth rate, dry cell weight and EPSs production, there were significant variation present on studied submerged culture condition of each selected mushroom. Now to find out the most significant submerged culture condition, the Tukey's HSD test had been performed and findings were reported as below.

Mycelial Growth Rate and EPSs Production by Using various Carbon Sources

To identify the ideal carbon source for *S. commune* submerged cultivation's mycelial development and EPS generation. Fig. 3A compares the outcomes of mycelial growth, average growth rate, and EPS production in different carbon sources. We tested 7 distinct carbon sources, with no carbon source acting as a control. The *S. commune* produced the most EPS (4.100.26 g/L) when grown on xylose medium, although its highest dry weight (9.330.66 g/L) and average growth rate (1.330.09 g/L/day) were recorded in glucose medium (Fig. 3A). It was noted that the supplementation of different carbon sources significantly affected average growth rate, dry weight and EPSs production of both the studied wild edible mushrooms (*p*-value < 0.05). It has been noted that different sources of carbon have a significant effect (*p*-value < 0.05) on higher production dry weight, average growth rate and EPSs as compared to control.

Mycelial Growth Rate and EPSs Production by Using various Nitrogen Sources

In Fig. 3B, the outcomes of cell expansion, growth rate, and EPS generation under varied nitrogen sources are displayed. Present findings showed that *S. commune* produced highest mycelial yield (2.7±0.36 g/L), average growth rate (0.38±0.5 g/L) and EPS (4.26±0.11 g/L) in yeast extract medium (Fig. 3B). It was also noted that organic and inorganic sources of nitrogen (*p*-value<0.05) substantially assisted the appropriate production of dry weight, average growth rate and EPSs of both the studied mushrooms. It has been also recorded that different sources of carbon have a significant effect (*p*-value<0.05) on higher production of dry weight, average growth rate and EPSs as compared to the control of studied wild edible mushroom (*S. commune*).

Total Carbohydrates Content from EPSs

The maximum content of total carbohydrate from EPSs of *S. commune* was obtained in glucose (carbon source) and yeast extract (nitrogen source) medium which was 0.597±0.06 g/100g and 0.006±0.006 g/100g, respectively (Fig. 4). Carbohydrates are significant (*p*<0.05) carbon and energy producers for cultivated EPSs.

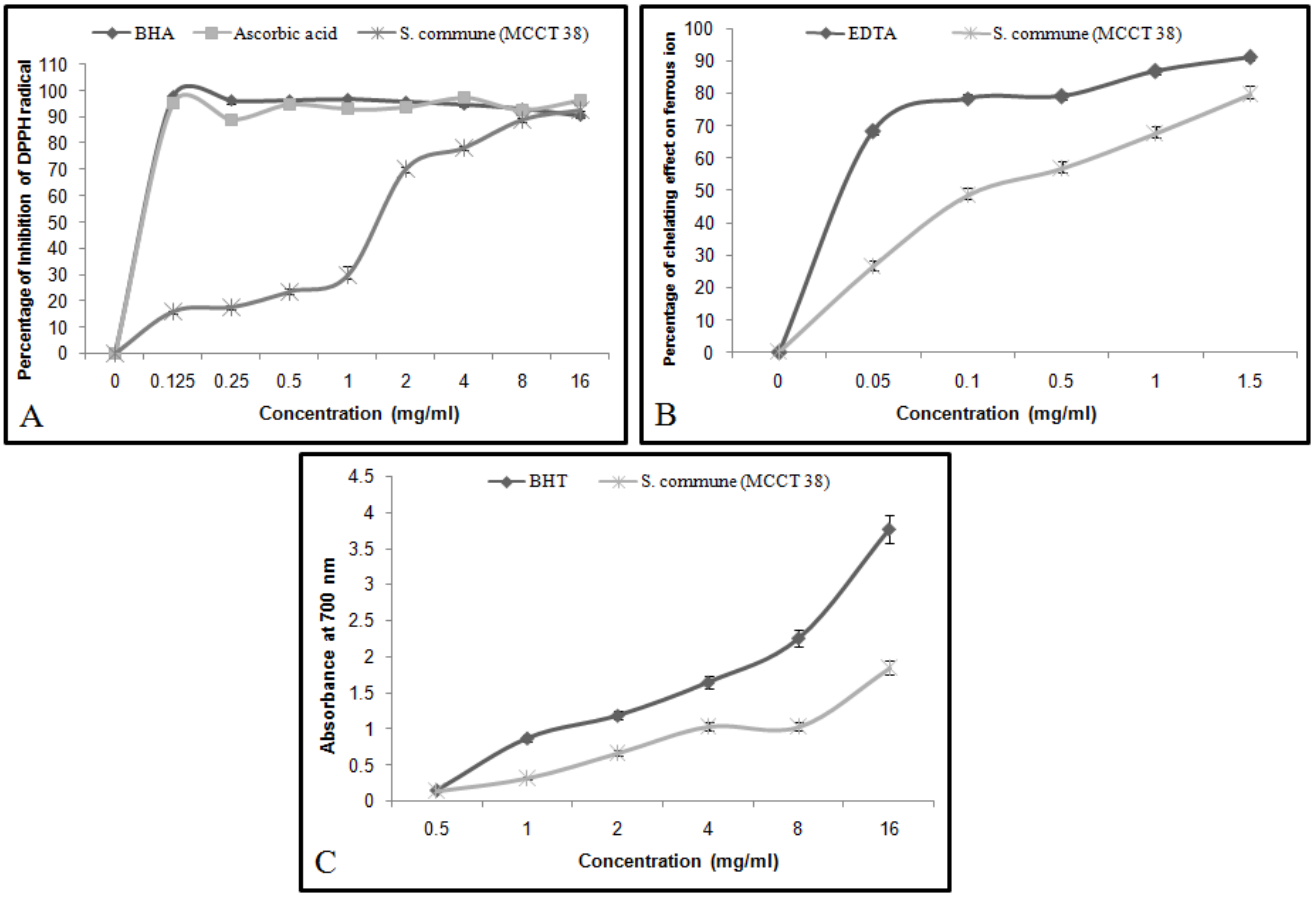

Figure 1 Different antioxidant properties namely, **A**: DPPH radical scavenging activity, **B**: Chelating effect on ferrous ion and **C**: Reducing power ability of mycelium of a wild edible *S. commune* produced in submerged culture

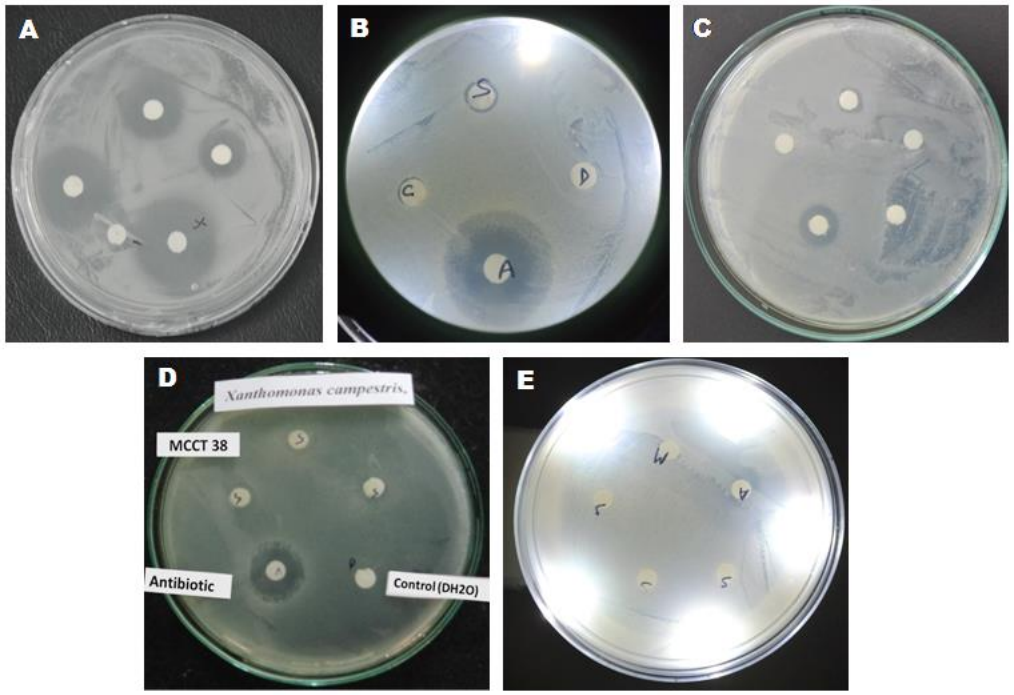

Figure 2 Antibacterial property of *S.commune* (**A-E**) verses **A** of *S. aureus*, **B** of *B. Subtilis*, **C** of *E. coli*, **D** of *X. campestris* and **E** of *P. aeruginosa*

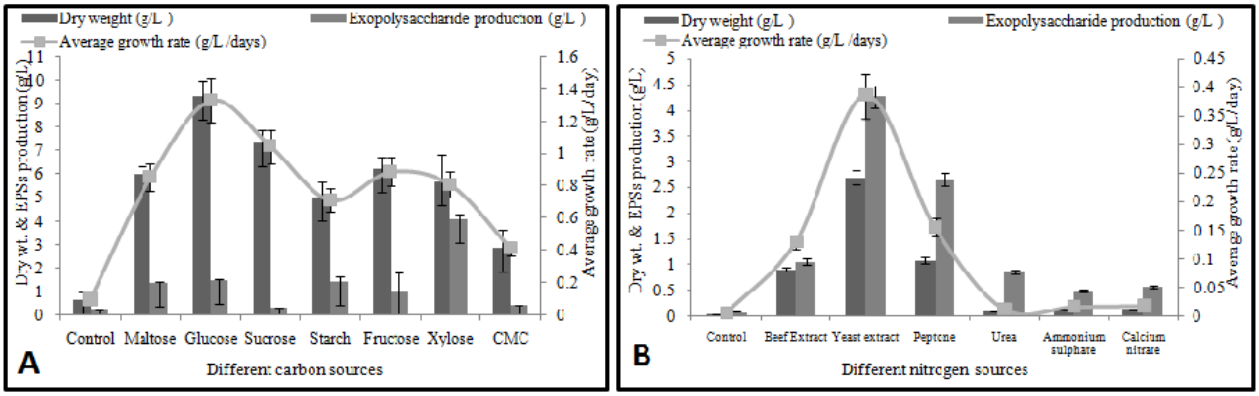

Figure 3 *S. commune* production of EPSs, average mycelial growth rate, and cell biomass in submerged cultivation using various carbon (**A**) and nitrogen sources (**B**)

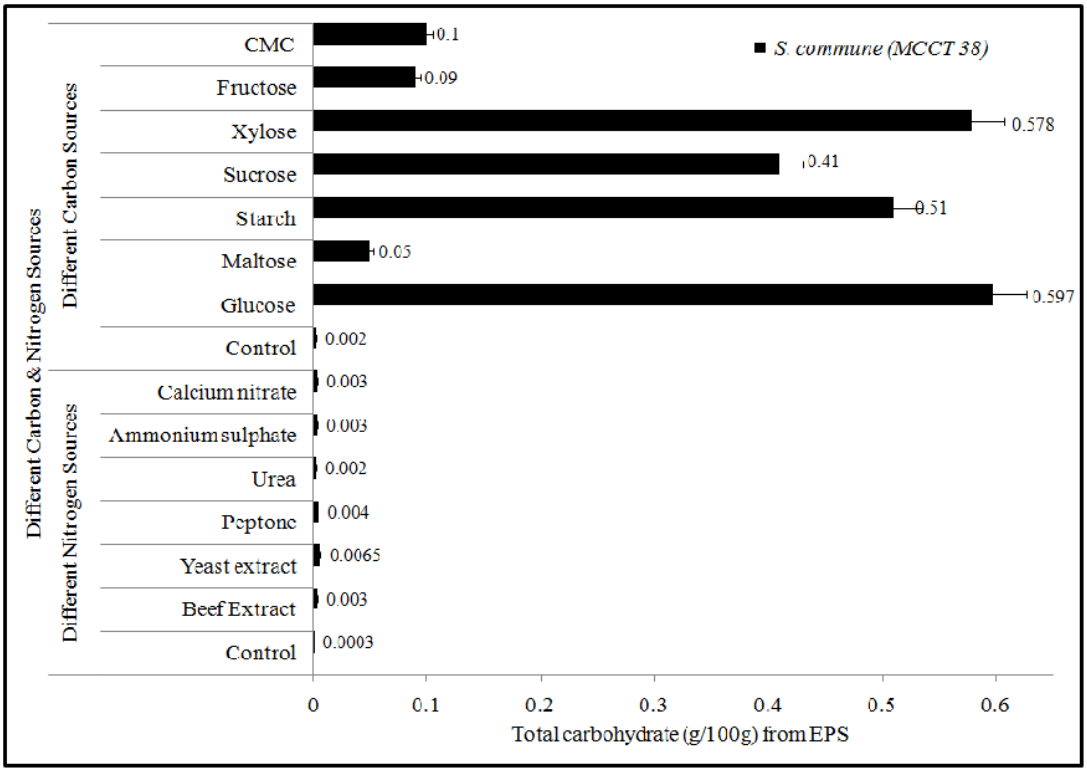

Figure 4 Total carbohydrate contents (g/100g) from EPSs produced from various carbon and nitrogen sources of *S. commune*.

Table 1 EC_{50} of FRS activity, reducing power, metal chelating effect, total phenol and total flavonoid contents of *S. commune* and standards

Mushroom sample	EC_{50} value (mg/ml) in percentage			Total phenol (mg GAE/g)	Flavonoid content (mg QE/g)
	Free Radical Scavenging (FRS) activity	Reducing power ability	Chelating effect of ferrous ions		
BHT	0.05±0.02	1.84±0.11	-	-	-
Ascorbic acid	0.05±0.04	-	-	-	-
EDTA	-	-	0.03±0.12	-	-
S. commune (MCCT 38)	1.49±0.06	< BHT	1.89±0.10	2.00±0.05	0.04±0.00

The mean SE of all values (n = 3) is used to express them.

Table 2 The mycelial extract of the Tripuran wild edible mushroom *S. commune*'s inhibition zone (mm) against two Gram-positive and three Gram-negative bacteria

Mushroom sample	Concentration (µg/ml)	Inhibition zone (mm)					*p*-value
		Gram-positive bacteria		Gram-negative bacteria			
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Xanthomonas campestris*	*Pseudomonas aeruginosa*	
Streptomycin	10 µg/ml	26.0±0.5[a]	27.0±0.2b	12.5±0.00c	16.5±0.20d	6.7±0.50e	3.807E-15
Negative control (DH_2O)	100 µl	0	0	0	0	0	-
S. commune (MCCT 38)	10 µg/ml	9.5±0.00a	0	0	6.0±0.5b	0	0.0002867
	50 µg/ml	14.0±0.00a	0	0	6.0±0.5b	0	5.241E-06
	100 µg/ml	20.2±0.5a	5.0±0.00b	7.1±0.50c	7.0±0.0c	0	5.492E-11

The mean SE of all values (n = 3) is used to express them. The *t-test* significance threshold of $p<0.05$ applies to values with differing letter values.

DISCUSSION

Our previous study documented that wild *S. commune* is an edible mushroom which contains a good amount of nutrients with potentiality of antibacterial and antioxidant activities. Suitable temperature, incubation time and pH for the mycelial growth were 25ºC, 21 days and pH 6.0 respectively **(Debnath *et al.*, 2017b)**. Comparatively to other extracts, the methanol extract had a higher level of DPPH radical scavenging activity and positive control showed higher activity in comparison to macrofungal extracts **(Debnath *et al.*, 2017a)**. Documentation also showed antioxidant capabilities were positively associated with total phenolic content in *in vitro* and *in vivo* studies of FRS **(Vidovic *et al.*, 2010; Debnath *et al.*, 2017a)** and most of the medicinal macrofungi contain phenolic compounds which are naturally existing antioxidant ingredients **(Cheung *et al.*, 2003; Mau *et al.*, 2004)**. Various researchers claimed that *S. commune* has anticancer, hepatoprotective and antioxidant properties of various extracts **(Yim *et al.*, 2011; Tripathi and Tiwary, 2013; Arbaayah and Kalsom, 2013; Debnath *et al.*, 2017b; Kumar *et al.*, 2019)**. According to **Devi *et al.* (2014)** ethanolic extract of *S. commune* had potent EC_{50} values of FRS and reducing power activities at 0.883 mg/ml and 0.825 mg/ml concentrations which indicates slight differences with our finding. *S. commune* contained 82.42 mg GAE/g of phenol in its ethyl acetate extract and also exhibited the maximum DPPH radical scavenging activity of 70.52%, CUPRAC of 0.38 and metal chelating activity of 81.29%, at concentrations of 0.1 to 1.0 mg/ml **(Mayakrishnan *et al.*, 2013)**, whereas our findings revealed that the highest FRS activity of 92.59±0.07%, reducing power of 1.84±0.01 absorbance and metal chelating effect of ferrous ions of 79.36±2.78 mg/ml were recorded at 16.0, 16.0 and 1.5 mg/ml concs., respectively. Our findings observed total flavonoid and phenolic contents were very much lower in the studied mushroom **(Arbaayah and Kalsom, 2013)**, which was slightly different from findings of other researchers but the present finding of phenolic content of methanolic extract showed similarity with **Mirfat *et al.* (2010)**. Previous research findings revealed that various extracts of macrofungi were stronger at preventing the Gram-positive bacteria in comparison to Gram-negative ones which showed similarity with our findings and it was already reported by various researchers from different parts of the world **(Bitew, 2010; Fagade and Oyelade, 2009; Barros *et al.*, 2007; Reyes *et al.*, 2007)**. The most effective antibacterial agent was the methanolic extract, which was followed by the culture filtrate, whereas other extracts failed to prove any capacity to stop the development of test organisms **(Tripathi and Tiwary, 2013)**. **Tripathi and Tiwary (2013)** documented that *S. aureus* and *B. subtilis* were more sensitive to methanolic extract and culture filtrate of *S. commune* and *P. aeruginosa* showed no zone of inhibition, our finding showed resemblance with this study. **Joshi et al. (2013)** found that yeast extract and xylose were the most efficient sources of carbon and nitrogen for the synthesis of mycelial biomass and EPSs, which showed similarity with our finding except mycelial biomass which was effective on glucose medium. The most crucial ingredient for fungi is nitrogen, which is used in the production of both primary and secondary metabolites **(Joshi *et al.*, 2013)** and yeast extract is a suitable nitrogen source which has been already documented by many researchers **(Park *et al.*, 2001; Lin, 2011; Lee *et al.*, 2004)**.

CONCLUSION

Present research revealed that *S. commune* showed higher antibacterial response against Gram-positive (especially *S. aureus*) bacteria as opposed to Gram-negative one. Studied mushroom also showed the high level of FRS, reducing power and chelating effect of ferrous ions activities which also contained high amount of total phenol but lowest amount of flavonoid. In addition, the optimization strategy of carbon and nitrogen sources recognized this investigation could be worthwhile to try with some other wild mushrooms as well. The basic outcomes observed in this work are valuable for the further implication of the cultivation method for making of EPSs on a large scale. Hence, more studies should be performed to increase the production of polysaccharides with a variety of biological properties that have the potential to be developed into purposeful foods. To find out the main functional components more research work is essential for the isolation, characterization and extraction of the primary ingredients in diverse wild edible, poisonous and medicinal mushrooms.

Acknowledgments: The Head of the Department of Botany at Tripura University is acknowledged by the writers for offering a variety of facilities. The authors are also grateful of the Forest Department of Tripura, Government of Tripura, India for allowing us to conduct fieldwork in forest regions. The Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India is acknowledged by the first author for the financial support provided through a project (Sanctioned order no. BT/463/NE/TBP/2013).

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