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MYCELIAL BIOMASS, EXO & ENDO POLYSACCHARIDES IN WILD STRAIN OF *SPARASSIS CRISPA*

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ABSTRACT

Submerged culture conditions were studied for the production of Exo & Endo polysaccharide (EPS, IPS) from *Sparassis crispa.* Maximum mycelial biomass was observed at 30 ^{0}C and pH 6.5. The best culture media for mycelial growth was MEB with 7.08± 0.15g/L biomass production at 10 days of incubation period. However 7 days was found to be sufficient for obtaining good amount of biomass as its rate of increase was high with a very low increase thereafter. Under optimized culture conditions, the maximum EPS concentration obtained was 3.25±0.55g/L at 100rpm while the IPS produced was 2.58±0.6g/L.

Keywords: *S. crispa,* submerged culture, mycelial biomass, optimization, Exopolysaccharides, Endopolysaccharides.

INTRODUCTION

Mushroom are macroscopic, epigeous, fleshy or leathery, umbrella like frting bodies, bearing their sporangium either on gills or lining the tubes, and are made up of mycelium formed in the substratum (Sharma and Jaitly, 2017). Consumption of mushroom has increased undoubtedly due to their pleasing aroma, flavor, and remarkable nutritional value in a number of countries. In 21st century extensive production and consumption of mushroom worldwide is termed “Non-green Revolution" (O’Neil *et al.,* 2013). The study of mushroom for their edibility index revealed that a fresh fruiting body contains 0.9% mannitol, 0.28% reducing sugar, 0.59% glycogen and 0.91% hemicelluloses and sugars like raffinose, sucrose, glucose, fructose and xylose are dominant carbohydrates (Mc Connel and Esselen, 1947). In different studies most of the pharmaceutically valuable polysaccharides were found to be originated from mushroom (Tabata *et al.*, 1981; Yang *et al.*, 2000) with interesting biological functions, including antimicrobial, and immune-stimulating activity (Ganeshpurkar *et al.*, 2010). A number of reports on pharmaceutical polysaccharides from fungi like *Ganoderma lucidum*, *Cordyceps* spp., *Lentinus edodes Pteridium aquilinum* are available (Yang and Liau, 1998; Kim *et al.*, 2001). Traditionally, edible and medicinal mushrooms have been produced in solid culture using composts or lingo-cellulosic wastes, such as straw or wood, a process that usually takes several months to produce fruiting bodies. Submerged culture gives rise potential advantages of higher mycelial production in a compact space and shorter time with low chances of contamination. Moreover, produced biomass shall be used as food or feed as protein supplement and source of polysaccharides (Jong and Birgmingham, 1993).

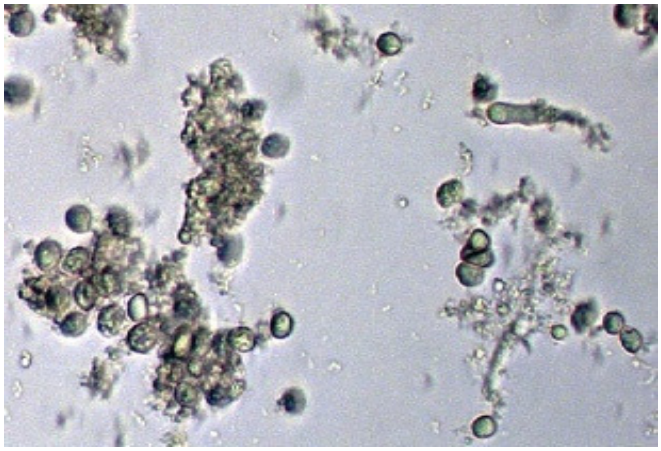
Sparassis crispa, also known as cauliflower mushroom which means “a blossom”. It is an edible and medicinal mushroom growing in the temperate regions of Europe and North America (Chandrasekaran *et al.,* 2011) and a cultivable species in Asian countries. *S. crispa* has been an integral part of human diet due to its potential of reducing the risk of colon cancer (Nowacka *et al.,* 2021). Despite its popularity, exopolysaccharide production from wild strains of *S. crispa* have not been exactly defined and studied to date. Therefore present study has been undertaken.

MATERIALS AND METHODS

Micro-organism

MUSN-80 (Fig. 1.) was a culture collection which had been isolated from the fruiting bodies of *S. crispa*. originally collected from foothills of Uttarakhand in India. The pure culture was isolated on potato dextrose agar (PDA) and stored at 4ºC till further analysis.

(a)



(b)

Fig. 1.a, b : Identified *S.crispa* MUSN-80 in natural habitat during collection and spore structure

Inoculum preparation:

S. *crispa* fresh seed was prepared on PDA medium 3 days prior to experiments.

Fig. 2 : Seed culture of *S. crispa from slant*

Effect of culture medium:

Five different culture broths were analyzed for maximum growth including 2 Synthetic *i.e.* YPB and Czapek-Dox and three natural media *i.e.*, Potato extract, Malt extract and Oatmeal extract were analyzed. 5 mm of plug of the inoculum from seed plate transferred into a 250 mL Erlenmeyer flask containing a 50 mL of sterilized culture media (each). The flasks were incubated at 25°C on a rotatory shaker incubator at 4 different rotations 100, 150, 200, 250 rpm for 10 days. Each treatment was replicated three times. The mycelial biomass produced in each treatment has been harvested by filtration which was oven dried at 50^{0}C until constant weight and represented as dry mycelial weight (DCW)g/L.

Effect of incubation temperature

The effect of incubation temperature on *S. crispa* biomass production was determined using optimized medium, incubation period and rotation rate. The optimized medium was dispensed into 250 ml. conical flasks (50 ml. per flask). Initial pH was maintained at neutral *i.e.* 7. Each flask was inoculated aseptically with 5mm plug inoculum of freshly cultured mycelium. Plates were incubated at 15, 20, 25, 30, 35, 40 and 45°C for 10 days on rotatory a shaker incubator and biomass produced was determined.

Effect of pH value:

At the optimized conditions i.e. medium, incubation time and temperature, initial pH value was taken 4.5, 5.0, 6.0, 6.5, 7, 7.5, 8, and 8.5.with 0.2M Phosphate Buffer. 50mL of each treatment was dispensed into 250 ml. Erlenmeyer flasks and replicated thricely. After sterilization they were inoculated (5mm plug seed culture) and incubated at 25 °C on a shaker incubator (100 rpm) for 10 days and biomass produced was determined.

Estimation of exopolysaccharides and endopolysachharides at optimized conditions:

Samples collected at regular interval of 24 hrs for 10 days from experimental shake flasks at optimized conditions were filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, UK). Filterate was then centrifuged at 5000*rpm* for 10 min. The resulting supernatant was mixed with four volumes of absolute ethanol, stirred vigorously, and then left overnight at 4^{0}C. The precipitated EPS was centrifuged at 5000 rpm for 10 min, and the supernatant was discarded. The precipitate of pure EPS was dried and weighted

Estimation of endopolysaccharide at optimized conditions

Samples collected at regular interval of 24 hrs for 10 days from experimental shake at optimized conditions were filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, UK). Filtered mycelium was air dried and centrifuged at 5000*rpm* for 10 min. Supernatant was discarded and pellets were mixed with four volumes of absolute ethanol, stirred vigorously, and then left overnight at 4^{0}C in order to precipitate the IPSs. The precipitate of pure IPS was dried and weighted.

RESULTS AND DISCUSSION

The fruiting body of *MUSN-80 (Fig 1a.)* was obtained from the Udham Singh Nagar of Foothill region of Uttarakhand India. The morphological and microscopic analysis of basidiospores structure *(Figure 1b)* revealed and validate the mushroom to be as *Sparassis crispa*. It is the first report from this region to the best of knowledge.

Effect of culture Medium on mycelial biomass

The mycelial growth of five different culture media was observed in the range 7.08 ± 0.15~ 0.859 ± 0.06g/L DCW at 10 days after inoculation (1). Two of these nutrient media MEB and OMB are favorable for the vegetative growth of *S.*

crispa. However, mycelial pellets density was observed to be very thin in CZB, moderately thin in YEB, and moderately compact in PDB. The result (considering Table 1.) on the 10th day showed that S. crispa had maximum dry mycelial weight 7.08g/L in MEB media followed by OMB with 5.42g/L at maximum incubation time i.e. 10th day. The least growth was recorded on Czapek-Dox broth *i.e.* 1.9 g/L DCW. However there was very less increase in the biomass after 7th day of incubation. Incubation period of 7 days is sufficient for obtaining good amount of biomass. The results explain the tendency and composition of natural culture media for the luxuriant growth of mycelia biomass over the synthetic media. Natural media has been reported to be highly supportive for the mycelial growth of mushroom by several authors (Sharma and Jaitly, 2017; Ngadin *et al.*, 2019). The agitation rate of 100rpm was found to be most suitable for the maximum growth of S. crispa pellets (Fig. 3). That may be due to the high agitation rates of flask may disturb cells to grow and assimilate the nutrients present in the substrate medium.

Table 1 : Analysis of culture media and incubation time on mycelial biomass of *S. crispa*

I.P. (days)	DCW (g/L)				
	MM-1	MM-2	MM-3	MM-4	MM-5
3	0.859 ± 0.06	1.05 ± 0.12	1.93 ± 0.12	2.67 ± 0.38	2.95 ± 0.07
5	0.97 ± 0.04	1.47 ± 0.31	2.88 ± 0.10	3.89 ± 0.19	4.17 ± 0.33
7	1.36 ± 0.21	1.91 ± 0.04	4.05 ± 0.18	5.12 ± 0.44	6.21 ± 0.76
10	1.9 ± 0.03	2.17 ± 0.13	4.41 ± 0.16	5.42 ± 0.40	7.08 ± 0.15

**MM-1: CZB; MM-2: YEB; MM-3: PDB; MM-4: OMB; MM5- MEB; DCW:Dry cell weight*

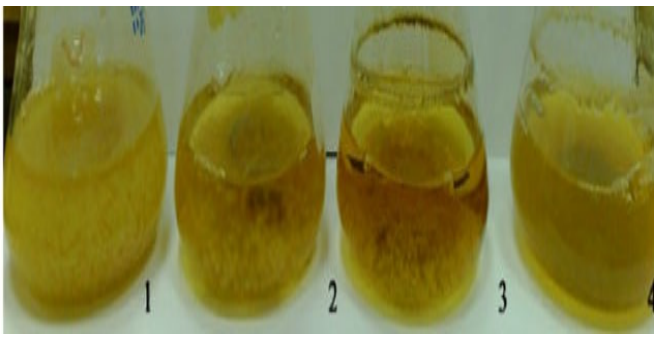


Fig. 3 : Analysis of agitation rates for the biomass of *S. crispa*

**1= 100rpm, 2= 150 rpm, 3= 200rpm, 4= 250rpm*

Effect of temperature on mycelial biomass:

After optimization of media the temperature optimization was conducted with optimized culture broth *i.e.* MEB. The maximum Mycelial biomass 6.18g/L was obtained at 30^{0}C which was a slight more than the biomass at 25^{0}C i.e. 5.84g/L. The mycelium growth was about negligible above 35^{0}C indicating that high temperatures like 40^{0}C, 45^{0}C restricts the mushroom mycelium to grew. While warm environment with a temperature ranging between 25^{0}C-30^{0}C support the mycelium to bloom completely. Cheong, JC *et al.* (2008) reported 23˚C as the optimum temperature for mycelial growth of *S. crispa*, which is nearby optimum temperature 25˚C obtained in this study. Majority of mushroom such as *Volvariella volvacea* (Moonmoon *et al.,* 2008) and *Macrolepiota procera* (Shim *et al.*, 2005) do not have mycelial growth at temperatures beyond 30˚C which is similar to our findings. Valuable ezymes which catalyse fungal metabolic processes denatures beyond 35˚C, it might be the main reason for not having any growth beyond the stated temperature (Kibar and Peksen, 2011).

Table 2 : Analysis of Temperature on mycelial growth of *S. crispa*

I.P. (days)	TEMPERATURE (^{0}C)					
	20	25	30	35	40	45
3	0.75 ± 0.07	1.30 ± 0.60	1.24 ± 0.47	0.65 ± 0.06	0.24 ± 0.11	0.18 ± 0.06
5	1.41 ± 0.17	4.27 ± 0.50	3.50 ± 0.74	1.23 ± 0.67	0.66 ± 0.08	0.62 ± 0.07
7	3.30 ± 0.05	7.04 ± 0.46	5.77 ± 0.25	2.40 ± 0.42	1.55 ± 0.38	1.25 ± 0.11
10	5.17 ± 0.33	8.45 ± 0.50	7.34 ± 0.34	3.14 ± 0.08	1.92 ± 0.05	1.37 ± 0.16

Effect of pH on mycelial biomass:

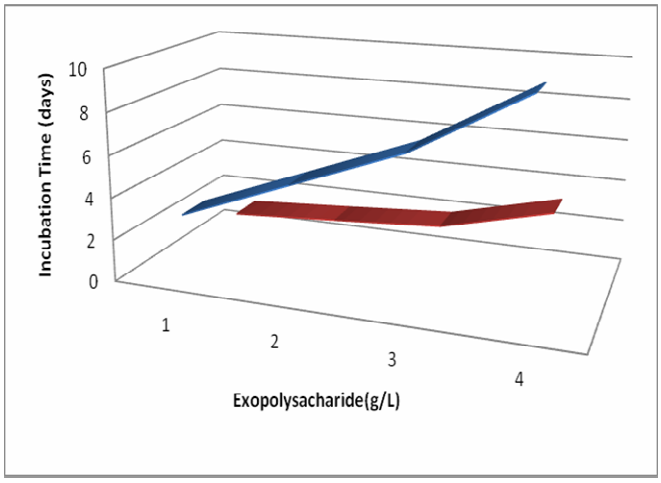
This study shows that slightly acidic to neutral initial media pH has been most favorable for mycelial growth of *S. crispa* (Figure 3). The MEB medium with initial pH of 6.5 resulted in highest mycelial growth among the pH levels tested (pH 5 - 9). However, mycelial growth measurements within the initial pH range 6.5 – 7.5 were statistically in a close range (Table 3). This study confirms the finding by Cheong *et al.* (2008) in terms of the most favorable initial pH of media.

Table 2 : Analysis of Temperature on mycelial growth of *S. crispa*

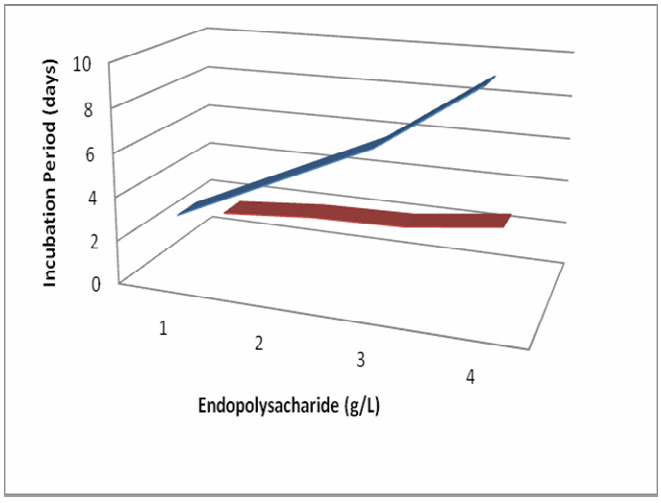
I.P.	pH								
	5	5.5	6	6.5	7	7.5	8	8.5	9
33	0.17±0.20	0.24±0.11	0.50±0.03	0.84±0.18	0.77±0.19	0.56±0.03	0.30±0.09	0.19±0.08	0.17±0.05
55	0.95±0.51	1.26±0.29	1.64±0.40	3.92±0.39	3.16±0.11	2.36±0.52	1.47±0.08	1.30±0.24	0.759±0.30
77	1.84±0.55	2.43±0.48	3.90±0.82	7.50±0.25	5.90±0.58	5.24±0.59	2.51±0.29	2.10±0.11	1.84±0.12
110	2.05±0.34	4.30±0.05	6.80±0.22	9.58±0.13	7.79±0.65	6.23±0.16	4.09±0.59	3.56±0.21	2.16±0.73

EPS and IPS produced:

During the EPS and IPS experiments, the fermentation conditions of temperature, initial pH, agitation rate, and growth period were fixed at 30 °C, 6.5, 100rpm, and 10days, respectively. The EPS and IPS production was calculated and the results depicted (figure 5). In the present study maximum EPS concentration (3.24 ± 0.5g/L) was obtained in culture grown at optimal conditions, whereas maximum IPS concentration (2.76g/L) was obtained. It has also been reported that many kinds of mushroom have more acidic pH optima for mycelial growth and EPS accumulation during their submerged cultures. It has been reported that all kinds of mushroom have more acidic pH optima for mycelial biomass and EPS accumulation during the submerged cultures (Kim SW et al., 2003; Shu C.H. and Lung MY, 2004)



(a)



(b)

Fig. 5a, b : Time profiles of EPS and IPS production respectively in shake flask cultures under optimized conditions

CONCLUSION

To date, many investigators have studied the production of mushroom polysaccharide by submerged cultures. Nevertheless, relatively few authors have used shake flask methodology for medium optimization. The designed shake flask culture method was proved to be a useful optimization technique for determining submerged culture condition of *S. crispa*. The optimization strategy established in this study maybe worth attempting with other mushroom fermentation processes for enhanced production of mushroom polysaccharides particularly those with pharmaceutical potential due to less required materials and times. Our results are very helpful in the production of bioactive polysaccharides from *S, crispa.*

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