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Optimized culture conditions for enhanced recovery of exopolysaccharide from *Pseudolagarobasidium acaciicola*: a novel fungus isolated from the fruit body of *Russula nigricans*, a wild edible mushroom of Odisha, India

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Fungal exopolysaccharides (EPS) are becoming important due to their multifarious applications with different structural forms and easy recovery. The objective of this study was to optimize submerged culture condition of a new fungal isolate *Pseudolagarobasidium acaciicola* obtained from fruit body of an edible mushroom, *Russula nigricans.* The study analyses the optimization of different parameters for enhanced production of EPS by one factor-at-a-time (OFAT) method. The influence of incubation period, initial pH value, temperature, mode of culture (static shake), culture vessel, carbon and nitrogen sources, and enhancers was studied. OFAT method revealed pH 6 with 7 days incubation statically and in dark in 150 ml Erlenmeyer flask, chemical factors like sabouraud dextrose HiVeg broth medium, xylose, yeast extract, tryptophan, K_2HPO_4, $CaCl_2$ and vitamin C as good conditions and components for maximum biomass and EPS production. Optimized medium developed in this study was a combination of the individually screened nutrient component, estimated the maximum EPS (1002.3 ± 189.72 mg/l) which was later expelled to 1468.1 ± 227.86 mg/l after addition of olive oil and Tween 80 at a concentration 250 : 50 µl v/v, which was much higher, and reported first time from this fungus (it means that early when medium was formulated with different chemical components we got the optimized medium giving 1002.3 mg/l of EPS but when addition of oils was performed we got more amount, i.e. 1468.1 mg/l). EPS production in a new medium might facilitate its industrial-scale production and use as a bioactive product for the welfare of mankind.

Keywords: Exopolysaccharide, optimization, *Pseudolagarobasidium acaciicola*, submerged culture.

MANY microbes produce bioactive polysaccharides which are high-molecular-weight polymers composed of long chains of monosaccharide units linked with glycosidic bonds and release the constituent monosaccharides/oligosaccharides on hydrolysis[1–3]. Polysaccharides are diversified due to their structure, properties, and

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functions and are useful in the food, pharmaceutical, pollution management and cosmetic industries[4–6]. Several fungi and bacteria have been explored for exopolysaccharide (EPS) production. Microfungi belonging to *Alternaria*, *Aspergillus*, *Candida*, *Fomes*, *Fusarium*, *Mucor* and *Penicillium* are widely reported along with some macrofungi[7–13].

In general, the biosynthesis of polysaccharides, and their production depend upon the microbe, nutritional and cultural conditions. Submerged cultures have been proved to be effective, as they require less space and time, have least chance of contamination and are a cost-effective method to obtain EPS[14–18]. It has been reported that the food industry needs 70,000 tonnes/year polysaccharides as thickening agents, stabilizers and textures[19–22]. Hence, the search for a new source with enhanced potential of polysaccharide production is imperative.

Several studies have been carried out to improve the extracellular metabolite production by considering the cultural and nutritional conditions in solid state and submerged fermentation[23–28]. A combination of several nutritional and environmental factors using one-factor-at-a-time (OFAT) method, response surface methodology, orthogonal matrix method, Plackett–Burman design and central composite design are the most useful experimental and statistical designs to get more enhanced EPS yield[29].

Carbon sources are important ingredients of media used for metabolic activity[10,30], which also affect EPS production. It reveals the species specific presence towards carbon utilization and synthesis of microbial cells[31–35]. Nitrogen sources like yeast extract were found to be one of the most important sources resulting in enhanced EPS production by fungi. Peptone was also found suitable for maximum EPS productivity by *Calocybe indica*, *Hirsutella* sp. and *Xylaria nigripes*[36,37]. The influence of phosphate on EPS production using K_2HPO_4 and KH_2PO_4, ionic salts, amino acids, fatty acids, oil and surfactants have been studied[38–45]. The role of pH, incubation period and specific temperature has also been evaluated by several researchers[46–49].

To the best of our knowledge, no published records are available for EPS production by *Pseudolagarobasidium acaciicola*. Nevertheless, optimization of media has been reported. Hence, in the present study, different experimental set-ups were used to standardize the medium components for enhanced production of EPS. To analyse the interactive behaviour of different media components and their contribution towards EPS production was also one of the objectives of this study.

P. acaciicola was isolated from wild edible mushroom, *Russula nigricans* grown in Odisha, India, was obtained from the culture collection of Plant Pathology and Microbiology Division, Regional Plant Resource Centre, Bhubaneswar. The fungal culture was maintained on sabouraud dextrose hiveg agar (SDA) medium slants and subcultured twice in a month. Slants were incubated at 25° ± 2°C for 7 days in static condition (EYELA LTI-700) and then stored at 4°C. Then the culture of *P. acaciicola* was transferred to sabouraud dextrose HiVeg broth (SDB) (culture medium consisting of dextrose 20 g/l, peptone 10 g/l) by punching of 6 mm (4 nos) plate culture disc and transferring to 50 ml media sterilized in 250 ml Erlenmeyer flasks, and incubated at 25°–30°C ± 2°C for 7 days[50].

In pure form, genomic DNA was extracted and ITS region of rDNA was successfully amplified by using fungal universal primers ITS4 and ITS5. PCR was set-up with ABI-BigDye ® Terminator cycle sequencing kit for sequencing. Raw sequence obtained from ABI 3100 automated DNA sequencer was edited manually for inconsistency. The sequenced data were aligned with publicly available sequences and analysed to obtain the identity.

The extraction and precipitation of EPS was carried out according to Shih *et al.*[50] and Ahmed *et al.*[51], the culture filtrate was collected through Whatman no. 1 filter paper. Isopropanol was added to the culture filtrate (1 : 1 v/v) with shaking and kept overnight at 4°C. The precipitated EPS was recovered by centrifuging at 5500 rpm for up to 30 min (Eppendorf centrifuge 5430R) and lyophilized (MINI LYODEL, DELVAC). Estimation was done by phenol sulphuric acid method[52] using a UV spectrophometer specord 50 (Analytikjena).

Seed culture of *P. acaciicola* was inoculated in liquid SDB medium 50 ml, pH 6.0 ± 0.2, in 250 ml Erlenmeyer flask and incubated at different incubation periods (3, 5, 7, 9, 12, 15 and 20 days) at 25°–30°C ± 2°C.

Seed culture was grown in liquid broth of SDB medium at different pH values (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), with inoculum size and flask volume mentioned same as above, and kept in a incubator for 7 days at 25°–30°C ± 2°C.

The seed culture was inoculated as above and incubated at various temperatures (25°C, 30°C, 35°C and 40°C) keeping other factors constant (pH 6.0, incubation period 7 days, 250 ml Erlenmeyer flask having 50 ml medium).

Seed culture was inoculated in different volumes of Erlenmeyer flask (100, 150, 250, 500 and 1000 ml capacity) with other factors maintained constant as above and cultured for 7 days at 25°–30°C ± 2°C.

Erlenmeyer flask of 150 ml capacity with 50 ml of SDB broth medium was kept in a incubator for 7 days at 25°–30°C ± 2°C at static as well as shaking conditions at 50, 75 and 100 rpm in light and dark conditions (12 h intervals) separately. All experimental conditions were maintained constant.

SDB broth medium was used at a concentration of 3%, 4%, 5%, 6% and 7% as well as diluted to 1.5%, 0.75%, 0.375% and 0.187% to analyse its influence on EPS production by *P. acaciicola*. All other parameters for the experiment were kept constant.

Six types of enhancers were added to the basal medium: (a) Amino acids – Glycine, asparagine, tryptophan, phenylalanine and glutamine at 1 g/l concentration[42]. (b) Plant oils – Different concentrations of olive oil and peppermint oil (0.5%, 1.0%, 2.0% and 4.0%)[45,53,54]. (c) Ionic salts – $FeSO_4$, $CaCl_2{\cdot}2H_2O$, $ZnSO_4$ and $MgSO_4$ at a concentration of 0.5% (refs 40, 42). (d) Vitamins – Including M, C, B1 and B6 at a concentration of 1 g/l (refs 42, 55). (e) Phosphate – Different sources of phosphate, viz. KH_2PO_4, K_2HPO_4, Na_2HPO_4 and Ca_3PO_4 at a concentration of 0.5, 1.0, 2.0 and 4 g/l (refs 38, 40, 42). (f) Surfactants – Tween 20 and Tween 80 at a concentration of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (ref. 54).

Precipitation efficiency of different solvents for EPS was determined using ethanol, isopropanol and acetone individually and/or in different combinations and ratios.

An experiment was designed to determine the effect of carbon and nitrogen supplements in two types of SDB medium: (a) laboratory composed medium and (b) commercial basal medium.

Different carbon sources (2%) such as glucose, galactose, lactose, fructose, xylose, sucrose, starch, maltose and mannitol were independently supplemented to the medium containing 1% peptone (medium composition was kept same as SDB broth). Different nitrogen sources (1% w/v), including yeast extract, peptone, Chile saltpetre ($NaNO_3$), calcium nitrate, ammonium chloride, ammonium nitrate, urea, ammonium sulphate and potassium nitrate were introduced separately in the medium containing 2% w/v dextrose in order to examine the effect on EPS production. All other cultural and environmental conditions were maintained the same.

Supplementation of the above-mentioned carbon sources was done in the primary SDB medium at the same composition, pH maintained at 6.0 and incubated at 25°–30°C ± 2°C for 7 days.

Supplementation of the above-mentioned nitrogen sources was done in the basal SDB medium at the same concentration, pH maintained at 6.0 and left for 7 days for incubation at 25°–30°C ± 2°C.

Factorial design was planned to determine the C : N ratio using xylose and peptone (same as SDB medium) pH 6.0 ± 0.2, in 150 ml Erlenmeyer flask containing 50 ml medium. Culture broth was incubated at 30° ± 2°C for 7 days (Box 1).

To determine the impact of oil, optimized medium was supplemented with different ratios of olive oil (O) and Tween 80 (T) (Box 2).

Yield of EPS by *P. acaciicola* was studied periodically. As shown in Figure 1, culture filtrate collected at different incubation periods exhibited highest yield at 7 days (128.57 ± 5.35 mg/l) which decreased after 9 days of incubation. Gradual enhancement in mycelia biomass yield was observed during the experiment; maximum biomass yielded (5.43 ± 0.37 g/l) was at 20 days of incubation.

The result obtained in the present study exhibits the suitability of pH 6 for better production of EPS by the fungus (Figure 2). At lower and higher values of pH, EPS productivity was found to decrease. The ideal temperature for EPS synthesis by *P. acaciicola* was recorded to be 30°C, producing 98.1 ± 24.34 mg/l of EPS. Reduction in EPS production was clearly observed at high temperatures (Figure 3). A good mycelia biomass could be observed in the fungal culture grown at 25°–35°C; however, there was a decline in the growth of fungus yielding 0.12 ± 0.09 g/l of dry biomass at higher temperatures.

Table 1 shows the influence of culture conditions on cellular development and EPS yield. According to the table, static with dark condition supports good EPS yield and

Box 1.

Experimental run	Factor A (%)	Factor B (%)
1	0.5	0.5
2	1.0	
3	1.5	
4	2.0	
5	2.5	
6	3.0	
7	0.5	1.0
8	1.0	
9	1.5	
10	2.0	
11	2.5	
12	3.0	
13	0.5	1.5
14	1.0	
15	1.5	
16	2.0	
17	2.5	
18	3.0	

Factor A, Xylose; Factor B, Peptone.

Box 2.

Experimental run	Factor (A)	Factor (B)
1	–	–
2	+	–
3	–	+
4	1	4
5	1	5
6	1	6
7	4	1
8	5	1
9	6	1

*Factor A, Tween 80; Factor B, Olive oil, 1, 50 µl/50 ml optimized medium; 4, 200 µl/50 ml optimized medium; 5, 250 µl/50 ml optimized medium and 6, 300 µl/50 ml optimized medium.

Table 1. Effect of culture conditions on mycelia growth and exopolysaccharide (EPS) production*

Culture conditions	Dark		Light	
	Mycelial biomass (g/l)	EPS (mg/l)	Mycelial biomass (g/l)	EPS (mg/l)
Static shake (rpm)	1.94 ± 0.07	127.53 ± 4.8	1.77 ± 0.14	24.07 ± 5.97**
50	2.72 ± 0.54	71.17 ± 5.36	1.87 ± 0.13	93.2 ± 18.36
75	1.48 ± 0.41	102.13 ± 26.03	1.63 ± 1.53	72.9 ± 17.11
100	0.99 ± 0.15	31.07 ± 10.66	0.73 ± 0.16	7.6 ± 1.39

*Fermentation was carried out in Erlenmeyer flask (150 ml) with 50 ml SDB at pH 6.0.
**Values are mean ± SD of triplicate experiments.

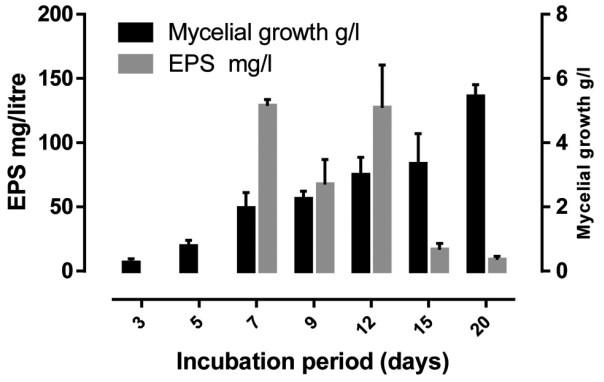

Figure 1. Effect of incubation period (days) on mycelia growth and exopolysaccharide (EPS) production.

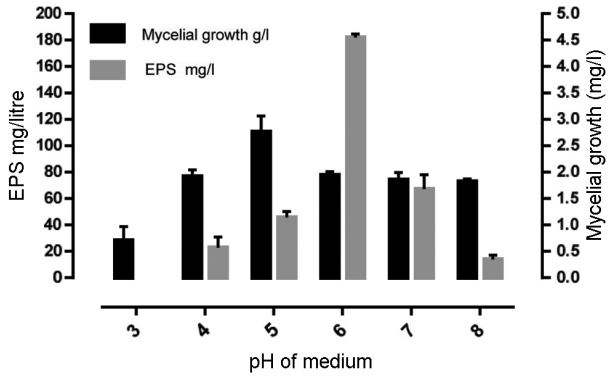

Figure 2. Effect of pH of the medium on mycelia growth and EPS production.

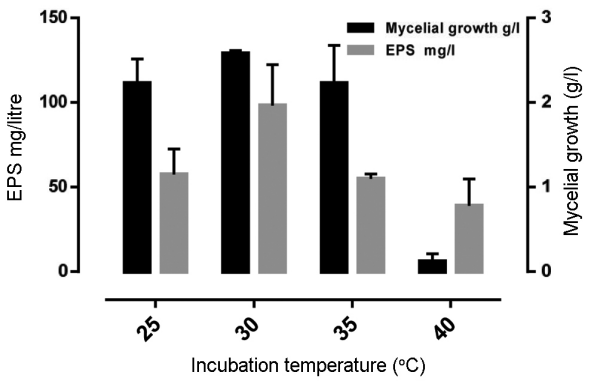

Figure 3. Effect of incubation temperature on mycelia growth and EPS production.

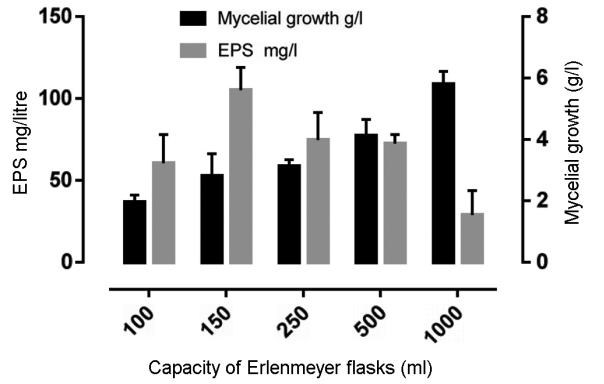

Figure 4. Effect of Erlenmeyer flask volume (capacity) on mycelia growth and EPS production.

biomass, 127.53 ± 4.8 mg/l and 1.94 ± 0.07 g/l respectively, compared to shaking and dark condition at 75 rpm (102.13 ± 26.03 mg/l EPS and 1.48 ± 0.41 g/l respectively).

The influence of surface area was evaluated and found to be more in 150 ml flask (Figure 4). Least was observed in Erlenmeyer flask of 1000 ml capacity with the amount of polysaccharide reduced to one fourth comparatively instead yield more biomass of 5.8 ± 0.42 g/l. The basic concentration of commercial basal media SDB impacted on EPS production extracellularly. No EPS was noticed at lower SDB concentration. In the present study, 3% of SDB was found to be optimum.

The impact of enhancers was also studied and it was observed that independent factors exhibited effect on EPS production (Table 2). Addition of $MgSO_4$ and $CaCl_2$, induced the fungus for EPS production, whereas $ZnSO_4$ and $FeSO_4$ did not help in EPS yield by this fungus. Similarly, vitamins B6 and C impacted better on EPS production compared to vitamins B1 and M. Correspondingly higher concentration of K_2HPO_4 affected EPS production, whereas it was inhibited in case of other phosphate sources (Figure 5). Data obtained on mycelial growth of the organism (Figure 6) depict the positive role of phosphorus in terms of phosphate, irrespective of the source. All

Table 2. Effect of minerals, vitamins and amino acids on mycelia growth and EPS yield*

Chemical components	Mycelia growth (g/l)	EPS (mg/l)
Mineral (0.5%)		
Calcium chloride	1.93 ± 0.52	28.93 ± 3.97**
Magnesium sulphate	3.16 ± 0.6	27.73 ± 3.61
Ferrous sulphate	0	0
Zinc sulphate	0	0
Vitamins (1 g/l)		
M	2.63 ± 0.25	17.13 ± 8.16
C	1.95 ± 0.26	16.47 ± 2.58
B6	3.18 ± 0.2	20.73 ± 2.70
B1	2.95 ± 0.22	0
Amino acid (1 g/l)		
Phenylalanine	2.77 ± 0.37	0
Asparagine	3.23 ± 0.33	0
Glutamine	2.94 ± 0.21	84.47 ± 15.51
Tryptophan	1.76 ± 0.37	105.13 ± 12.14
Glycine	2.87 ± 0.22	0

*Fermentation was carried out in Erlenmeyer flask (150 ml) with 50 ml medium at pH 6.0.
**Values are mean ± SD of triplicate experiments.

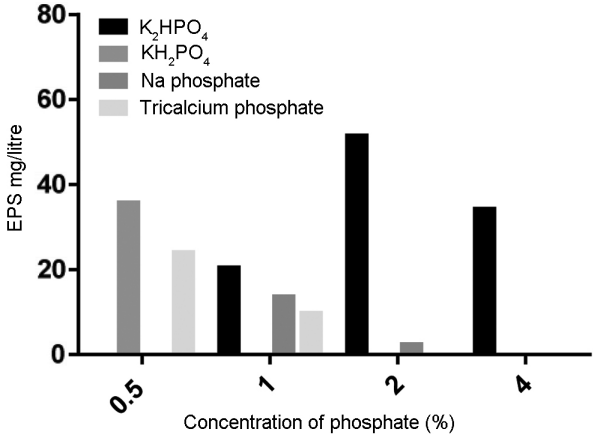

Figure 5. Effect of phosphate source (%) on EPS production.

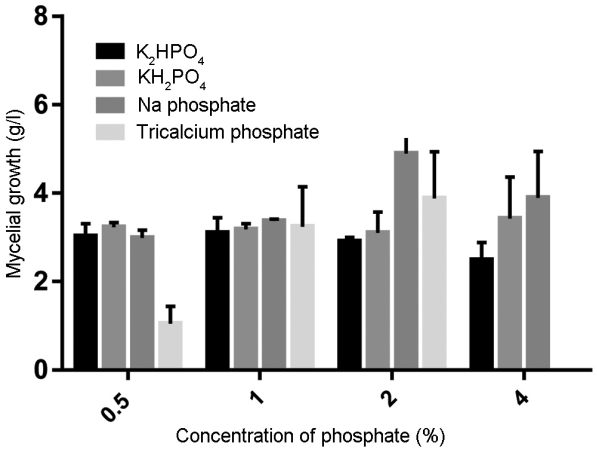

Figure 6. Effect of phosphate source (%) on mycelia growth.

total $CaCl_2$, vitamin C and K_2HPO_4 contributed more to EPS production yielding 28.93 ± 3.97, 34.33 ± 6.24 and 51.47 ± 18.95 mg/l respectively, which was found greater according to the basal medium, i.e. 22.3 ± 4.9 mg/l (normal SDB medium).

In the present study addition of olive oil (Figure 7) and Tween 80 (Figure 8) resulted in more EPS synthesis whereas Tween 20 showed no effect respectively. The fungus developed good mycelial biomass in the presence of Tween 80 and/or olive oil. Precipitation efficiency and maximum recovery of EPS by solvent extraction was possible considering isopropanol, ethanol and acetone independently with different ratios and at different temperatures. Isopropanol was the most suitable at ratio 1 : 2 v/v for extraction and precipitation of EPS by this organism followed by ethanol and acetone.

An experiment was designed (matched paired set-up) for determination of the most suitable carbon and nitrogen supplements with basal medium and laboratory composed medium. Tables 3 and 4 show that supplementation of carbon and nitrogen sources in laboratory composed medium did not have much effect compared to SDB medium for mycelia growth as well as EPS production.

However, supplementation of more carbon sources (glucose and xylose) had more impact on EPS production. Other carbon sources in both cases contributed less. Supplementation of nitrogen sources other than basic medium components did not enhance EPS production by this fungal culture (Table 4). It was noticed that xylose and peptone in the ratio 2 : 1 w/v enhanced EPS production.

Individual experiments exhibited difference in experimental components. We planned to combine the source of primary components and make a new medium in comparison with laboratory composed medium. It was formulated with the SDB medium (30 g/l), xylose (2%), yeast extract (1%), L-tryptophan (1 g/l), olive oil (1.0%),

Table 3. Effect of carbon sources on mycelia growth and EPS production*

Carbon sources (20 g/l)	Laboratory composed medium		Commercial synthetic medium	
	Mycelial biomass (g/l)	EPS (mg/l)	Mycelial biomass (g/l)	EPS (mg/l)
Glucose	0.77 ± 0.06	38.23 ± 26.73	2.62 ± 0.42	63.23 ± 5.85**
Lactose	0.63 ± 0.27	14.23 ± 3.23	3.69 ± 0.39	14.13 ± 2.8
Mannitol	0.73 ± 0.17	12.57 ± 4.33	2.01 ± 0.56	12.5 ± 0.87
Xylose	0.79 ± 0.11	44.13 ± 8.64	1.95 ± 0.44	10.4 ± 1.39
Galactose	0.59 ± 0.04	24 ± 10.82	2.55 ± 1.11	6.67 ± 2.66
Maltose	1.22 ± 0.18	12.63 ± 4.9	4.5 ± 0.43	0
Starch	1.81 ± 0.24	2.07 ± 1.25	2.17 ± 0.56	0
Sucrose	0.64 ± 0.31	0	1.86 ± 0.3	0
Fructose	4.07 ± 0.3	0	4.07 ± 0.30	0
No carbon	0.24 ± 0.16	1 ± 0.2	2.23 ± 0.45	22.3 ± 4.9

*Fermentation was carried out in Erlenmeyer flask (150 ml) with 50 ml SDB at pH 6.0.
**Values are mean ± SD of triplicate experiments.

Table 4. Effect of nitrogen sources on mycelia growth and EPS production*

Nitrogen sources (10 g/l)	Laboratory composed medium		Commercial synthetic medium	
	Mycelial biomass (g/l)	EPS (mg/l)	Mycelial biomass (g/l)	EPS (mg/l)
Peptone	0.81 ± 0.1	11.97 ± 1.34	4.92 ± 0.84	2.7 ± 1.13**
Yeast extract	4 ± 0.86	23.07 ± 9.51	4.44 ± 0.33	24.8 ± 2.5
Calcium nitrate	0.12 ± 0.08	0	4.12 ± 0.89	4.27 ± 1.8
Potassium nitrate	0.09 ± 0.01	0	0	0
Ammonium sulphate	0	0	1.47 ± 0.78	0
Ammonium chloride	0	0	1.47 ± 0.78	0
Sodium nitrate	0	0	2.83 ± 0.64	0
Urea	0	0	0	0
No nitrogen source	0.21 ± 0.04	3.03 ± 0.49	2.23 ± 0.45	22.3 ± 4.9

*Fermentation was carried out in Erlenmeyer flask (150 ml) with 50 ml medium at pH 6.0.
**Values are mean ± SD of triplicate experiments.

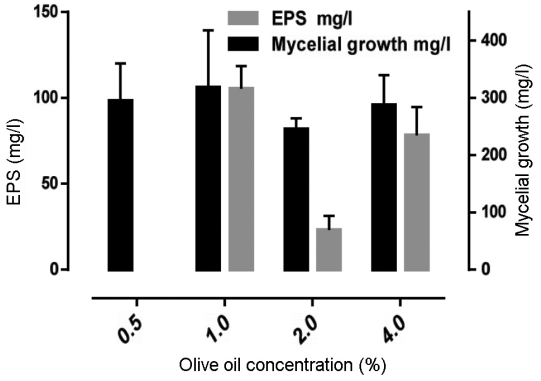

Figure 7. Effect of olive oil (%) on mycelia growth.

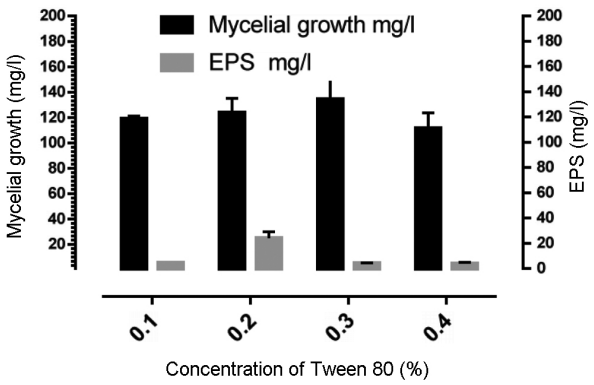

Figure 8. Effect of Tween 80 (%) on mycelia growth.

Tween 80 (0.2%), vitamin C (1.0 g/l), K_2HPO_4 (2 g/l), $CaCl_2$ (0.5%), pH 6.0 ± 0.2, temperature 30° ± 2°C with incubation period of 7 days, inoculum size 24 mm in 50 ml of medium (150 ml conical vessel) in static condition and dark and repeated measure difference was followed to examine the role of enhancers, especially olive oil. In normal SDB basal medium experimented condition, the fungus produced EPS of 22.3 ± 4.9 mg/l with pH 6.0, 7 days of incubation period, static dark condition, whereas in optimized medium it was 1002.3 ± 189.72 mg/l. We also considered the addition of oil in different combinations with and without Tween 80. Independently, it did not show prominent results. However, combination of Tween 80 and olive oil at a concentration ratio of 50 : 250 µl induced 1468.1 mg/l of EPS (Figure 9). The growth performance of the fungus under these experimental conditions is displayed in Figure 10, which shows growth-promoting activity of the enhancer. In the

optimization process of different factors added singly to the SDB medium with control, EPS precipitation was nil. Formulation of optimized medium was based on data obtained with reference to those in the basal SDB medium (22.3 ± 4.9 mg/l).

Several fungi have been reported as important for EPS production with strong biological activities against many dreadful diseases[56–58]. Hence attention is given to the new source. We have considered the *P. accacicola* since no work is evident in this context[59]; also it is novel fungus showing other bioactive metabolites production like laccase enzyme and cytotoxic sesquiterpenes[59–61] and isolated from *R. nigricans*, a wild edible mushroom from Odisha.

Different cultures, including *Alternaria alternata*[10], *Schizophyllum commune*[35], *Pleurotus pulmonaris* and *Trametes versicolor*[62,63] show 9, 14 and 7 days respectively, of highest EPS yield.

The present study also corroborates with the above, demonstrating the requirement of 7 days of incubation for better production of EPS by this fungus at significant level ($P < 0.05$). The influence of pH and temperature has also been examined in the present study and corroborated with the observations of Patil *et al.*[64], who studied the role of pH and incubation temperature on the production of bacterial metabolites. Most of the fungi preferred a wide range of optimum pH values like 3.0 (*Mucor rauxii*[3], *Alternaria alternate*[10]), 5.0–6.0 (*Schizophyllum commune*[35], *Hirsutella* sp.[36]), 6.0–7.0 (*Stemphilium* sp.[32], *Ganoderma* sp., *Agaricus blazie*, *L. squarrosulus*[51] etc.). Our study showed good production (181.93 ± 2.70 mg/l) EPS at pH 6.0 ± 0.2.

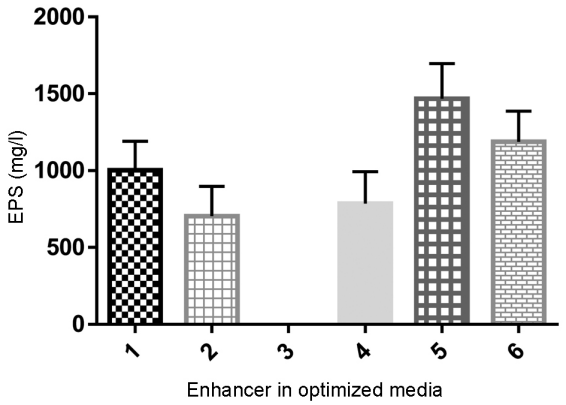

Figure 9. Effect of Tween 80 and olive oil (ratio) on EPS production – in optimized medium. 1, Control (optimized medium without oils); 2, Optimized medium with olive oil; 3, Optimized medium with Tween 80; 4, 50 : 200 µl v/v (T : O); 5, 50 : 250 µl v/v (T : O); 6, 50 : 300 µl v/v (T : O).

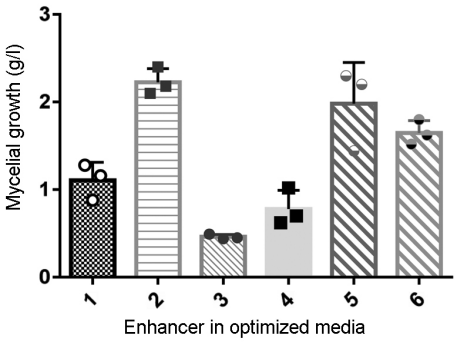

Figure 10. Effect of Tween 80 and olive oil (ratio) on mycelia growth – in optimized medium. 1, Control (optimized medium without oils); 2, Optimized medium with olive oil; 3, Optimized medium with Tween 80; 4, 50 : 200 µl v/v (T : O); 5, 50 : 250 µl v/v (T : O); 6, 50 : 300 µl v/v (T : O).

Fungal cultures, generally prefer temperature range from 15°C to 30°C for biomass and EPS production. *A. blazie* showed highest EPS production (1.268 g/l) in medium of starch and yeast extract at pH 6.8 and temperature 20°C (ref. 33). *P. flabellatus* yield 540 mg/l of EPS in yam (yam dextrose broth medium) at 30°C, while *Pleurotus ostreatus* yielded 2700 mg/l of EPS at 25°C (ref. 42). All produced EPS in optimized medium. In this study, we observed 30°C to give best yield (98.1 ± 24.34 mg/l of EPS), without addition of other enhancers.

Reduction of cellular biomass and EPS yield was found at higher incubation temperature (above 30°C), as recorded by Li *et al.*[36] and Zhang *et al.*[65] in *Ganoderma lucidum*.

Maximum EPS was produced under dark condition by *P. acaciicola.* However, many reports elucidated the importance of shaking condition for enhanced production of EPS[66–68]. Work on *Coriolus versicolor* by Ahmed *et al.*[2] showed highest yield of EPS at shaking condition 700 mg/l in yeast malt extract medium at 5–7 days of incubation period. In our study, shaking at 75 rpm versus static condition had no significant impact on mycelia development and EPS production ($P > 0.05$). To the best of our knowledge, the effect of culture vessel on EPS yield under liquid culture condition of *P. acaciicola* has not been studied earlier.

The use of surfactants like Tween 80 has shown a remarkable effect on mycelia growth and EPS production[69]. Its stimulating effects on EPS production by *S. commune* and *Botryosphaeria rhodina* have also been demonstrated[70]. Li *et al.*[71] studied a new fungal source, *Bionectria ochroleuca* providing optimized medium having glucose yeast extract, $MgSO_4$ and Tween 80 yielding 2.65 ± 0.16 g/l of EPS. In the present study positive effect of Tween 80 was observed, while negative effect of Tween 20 on EPS production was found negligible and hence not measured. Though the nutrient function of Tween 80 as an enhancer has not been confirmed in many other fungi, we planned a separate experiment with optimized media by adding Tween 80 and olive oil in different ratios[44,72]. Individually Tween 80 did not perform well, but in combination with olive oil, viz. 50 : 250 µl v/v concentrations; gave better results. These were analysed and found to be remarkably different at $P < 0.05$ level.

Our findings also confirm those of Li *et al.*[73], who reported higher yield of EPS in optimized medium with

potato extract 20%, peptone 0.5%, K_2HPO_4 0.2%, $MgSO_4$ 0.05% and sucrose 2.5%, in case of *Hirsutelle* species (2.27 g/l of EPS) at 4 days of incubation. Carbon and nitrogen were found to play a key role in cellular biomass development and metabolite production in *Pleurotus* sp.[74] and *Tricholoma matstaka*[75]. Similarly, higher K_2HPO_4 concentration affected EPS production, whereas it was inhibited in case of other phosphate sources[37,40,76]. EPS yield in the presence of K_2HPO and KH_2PO_4 did not show significant difference ($P < 0.05$), though mycelia biomass significantly differed in both cases. This indicates the importance of both types of potassium phosphates in EPS production.

Positive impact of ionic salts like $MgSO_4$ and $CaCl_2$ was observed. The stimulatory effect of $MgSO_4$ in *C. versicolor* and *Stemphilium* sp. is well reported[32,77]. Similar phenomenon is evidenced in the present study for vitamin C, whereas other vitamins like B6 and M did not support much growth and EPS production[42,78].

Two types of basal media, i.e. laboratory composed and commercial have been used to observe the impact of supplementation of carbon sources. Supplementation of more glucose and xylose in SDB medium had more impact on EPS production[35,79]. The mycelia biomass varies in different carbon sources added to SDB medium, it enhanced EPS production significantly ($P < 0.05$). This may be because of negative regulatory operation due to carbon–carbon effect. Utilization of both carbon components in the basal medium for better EPS production corroborated with the results of Adebayo-Tayo *et al.*[79] and Joshi *et al.*[35], who reported xylose to be the best for EPS production by *Schizophyllum* (4.26 g/l of EPS), *Maramius* sp. and *Fomes* sp. On the other hand, yeast extract acted as a good nitrogen source for EPS yield. It has been reported that production of microbial metabolites is influenced by the presence of yeast extract, tryptophan, etc. either solely or in combination[80,81], which corroborates with our findings. Hence, variation in different important media components and/or cultural conditions will have considerable influence on the amount of microbial metabolites, especially EPS.

In our preliminary experiments, the OFAT[35] method was used to examine the effect of environmental factors and medium constituents on EPS production[51]. Results obtained indicate that the crucial medium components significantly affecting the EPS production are xylose, yeast extract, tryptophan, vitamin C, $CaCl_2$ and K_2HPO_4, and olive oil in combination with Tween 80. The individual components exhibited their own candidature and contributed towards EPS production. The whole scenario changed when we combined the individual factors and made a new optimized medium for enhanced EPS production, in which *P. acaciicola* produced five times higher amount of EPS and production also increased up to 7 times when olive oil and Tween 80 were added. The possible cause is the initiation of EPS biosynthesis in fungal cells due to mutualistic interaction of these components and their contribution towards enhanced metabolism of EPS. Variation in cellular growth and EPS yield was noticed in each set of experiments; this may be due to seasonal variation. It has been observed that biomass yield is not directly related to EPS production, which was similar to the results of Ahmed *et al.*[2] on *C. versicolor*. According to them[2] yield of EPS is not associated with exponential development and is not a result of primary metabolism, but secondary metabolites. Hence, the data recorded in the present study and formulation of an optimized protocol for enhanced production of EPS under submerged culture condition by *P. acaciicola* may help exploit its potential at a mega scale and in drug discovery programmes.

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