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ISOLATION, SCREENING AND QUANTIFICATION OF EXOPOLYSACCHARIDE PRODUCE BY PLANT PATHOGENIC FUNGI

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ABSTRACT

Exopolysaccharides are polymers of carbohydrates secreted by some bacteria and fungi outside their cell walls. Exopolysaccharides extracted from plant pathogenic fungi have wide applications. Four species of fungi i.e. Penicillium sp., Aspergillus sp., Fusarium sp., Alternaria sp. and Candida sp. were isolated from different plants sources (chilli, tomato and mazie) and each species were tested for their polysaccharide producing ability. Polysaccharide production was recorded in incubating all four species in shaking flask, incubation condition of 28°C at 150 rpm for 14 days was found most suitable for exopolysaccharide secretion from each test species. Among all four species, Alternaria alternata produces maximum exopolysaccharide & minimum production was seen with Penicillium notaum. Total biomass (dry weight and wet weight) was also recorded. Maximum biomass was found to be in Fusarium equisetum. Total carbohydrate and protein produced was found to be highest in Alternaria alternata (approx 95% carbohydrate content). Fungal flora is affluent and thus can be used for the production of extracellular polysaccharides at a commercial level. The screening of such different natural sources for production of exopolysaccharides secreting fungi is promising as there is hope of isolation new productive microorganisms with properties superior to those of existing polymers. Isolation of exopolysaccharide secreting fungi from various fungi infected plants will be a valid alternative to plant and algal products considering that their properties are almost identical to those currently of used gums (Sutherland, 1956) and these exopolysaccharides activities can play a relevant role in biomedical and industrial applications, particularly in the field of antibacterial, emulsification, flocculation etc. for their intrinsic biocompatibility and potential low cost.

KEY WORDS

Exopolysaccharide, Natural gums, Plant pathogenic fungi, antibacterial, emulsification, flocculation

INTRODUCTION

At present, polysaccharides used at commercial level are almost entirely of plant and algal origin (Sutherland 1996). These high-molecular weight polymers, for example starch, alginate, arabic gum, carrageenan, agar and guar gum, are widely employed in the fermented food, pharmaceutical and cosmetic industries (Roller and Dea 1992; Wang and McNeil 1996; Manzi and Pizzoferrato 2000). However, microorganisms are known for their ability to synthesize exopolysaccharides (EPS), both homo- and hetero-polymers with different structural complexities (Seviour et al. 1992; Sutherland 1994). These microbial exopolysaccharides represent an attractive alternative to synthetic polymers and represent a valuable resource of biogenic and biodegradable polymers (Dave et al., 2016), suitable to replace petro based polymers in various technical applications (Moscovici, 2015; Meybod et al., 2015; Castillo et al., 2015; Schmid et al., 2016). Similarly, microbial exopolysaccharides are a better alternative to exopolysaccharides produced by plant or macro algae (Freitas et al., 2011; Moscovici, 2015; Meybod et al., 2015) as their production does not depend on geographical constraints and/or seasonal limitations. In some other cases, the microbial products have unusual molecular structures and peculiar conformations, thus

conferring unique and potentially interesting properties with potential industrial uses (Seviour et al. 1992; Sutherland 1996; Wang and McNeil 1996; Clementi 1997).The abilities of some of these microbial polysaccharides to lower cholesterol levels in human blood, to increase excretion of lipid and to stimulate the immunological system against antitumor activity and/or microbial diseases are some further potential fields of application (Bohn and BeMiller1995; Wang and McNeil 1996; Bao et al. 2001; Lee et al. 2002; Selbmann et al. 2002).

Additional advantage of microbial exopolymer production the manipulation of biosynthetic pathways to enhance productivity or to influence the chemical polysaccharide composition is comparatively easy in bacteria and fungi *(*Zhang and Cheung, 2011*).* However, their production is still relatively expensive and, at present, only few polysaccharides of microbial origin (e.g., curdlan, gellan and xanthan) are produced at commercial level (Sutherland 1996). This could be due to inadequate research: often, in fact, the physiology of production and optimization and scaling-up of EPS production processes either with new or already known selected strains have been rather neglected (Sutherland 1996). With the notable exceptions of Schizophillum commune (Rau et al. 1990; Bot et al. 2001), Sclerotium glucanicum (Wang and McNeil1996) and Aureobasium pullulans (Youssef et al. 1999), EPS production from plant pathogenic fungi has not been studied adequately yet. In 1992, Seviour *et al.* concluded their review work with the suggestion that further research work was still necessary and that undoubtedly, more EPS-producing fungi could be described. This suggestion appears still topical considering the number of recent papers dealing with screenings, selection and study of new plant pathogenic fungi able to produce EPS (Park et al. 2001; Sinha et al. 2001; Kim et al. 2002; Lee et al. 2002; Reis et al. 2002).

Rational screening could be based on physiological characteristics apparently common to several EPS producers (Seviour et al. 1992) such as the production of sclerotia or phytopathogenicity. So far, however, systematic studies on a possible correlation between these characteristics and the ability to produce EPS have not been reported. The aim of the present study was to explore the potential of several plant pathogenic fungal species for EPS production. Also, the polysaccharides produced by these fungi might be of superior properties to those of the existing polymer because of the different physiological properties as well as environmental effects.

MATERIALS AND METHODS

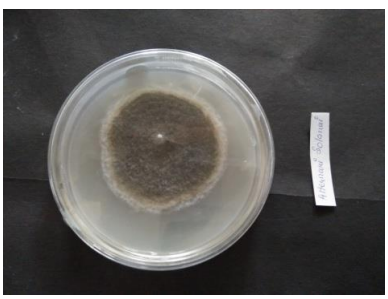
1. Screening of sample for isolation of fungi: -

Isolation of fungi from various infected crop and vegetable plants (Chilli, tomato, maize) sources by serial dilution method (Prescott et al., 1993) or directly plated on different types of media (PDA/CDA/YEPG agar/SDM etc.) for isolation of fungi. The plates were incubated at 29 ^{0}C for 6-7 days. Each isolated fungi was studied by colony characteristics and microscopic observations to obtain pure culture. (*Fig, 1*)

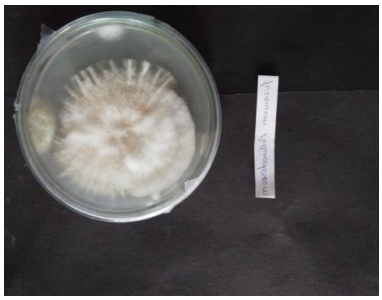
2. Screening of isolated cultures for the extracellular polymer production: -

The isolated pure cultures were screened for exopolysaccharides production using Shake flask method. The cultures were inoculated in 150 ml of enriched medium (Glucose- 40; Yeast extract- 1.0; Peptone-0.5; KH_2PO_4 -0.5; $MgSO_4.7H_2O$-0.5) and incubated on a rotary shaker (*Fig,2*) at 29°C and 150 rpm for 2 weeks. After 14 days of incubation (*Fig,3*), the culture will be centrifuged at 4000 rpm for 15 min. (Shamy and Nehad, 2010) and the supernatant was collected for EPS isolation and settled fungal cells were used for biomass calculation. To the clear supernatant, chilled 95% ethanol was added in ratio of 1:3 and was kept overnight at 4^{0}c. The precipitated or floated exopolysaccharides were collected next day after decanting ethanol and dissolved in small volume of deionized water and partially purified by dialysis (pretreated dialysis bags as mentioned below were used) against deionized water at 4 ^{0}C for 2 days by changing water after 24 hrs. (*fig,* 4) (Decho, 1990 and Decho and Lepez, 1993). The dialyzed exopolysaccharide suspension were then freeze using Lyophilizer or dried at 60˚c (Kumaran et al., 2014; Al-Manhel, 2017) for exopolysaccharides recovery (*Fig,* 5). The lyophilized or heat dried exopolysaccharide stored at -20 ^{0}C for subsequent analysis.

Fig: 1 Isolated fungi



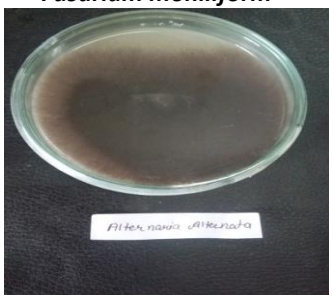
Alternaria Solanai



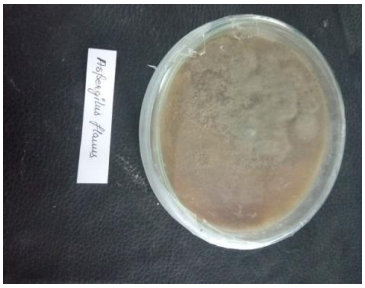
Fusarium pallirodosium

Fusarium moniliform

Fusarium equisetum

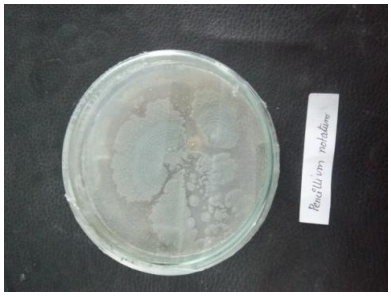


Aspergilus flavus



Alternaria Alternata

Candida Albicans



Pencillium notum

Fusarium oxysporium

Fig: 2 Shaking Incubator

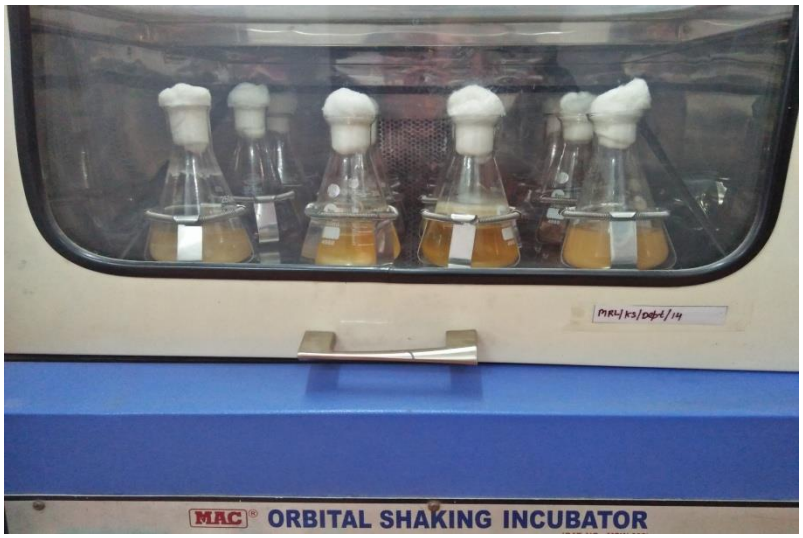


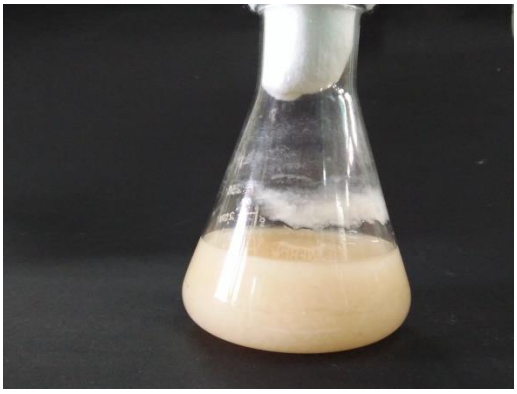
Fig.3 After 14 Days incubation culture

Alternaria Alternata

Alternaria Solanai

Aspergillus flauvs

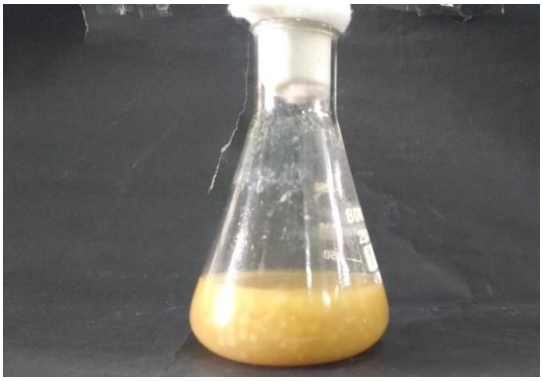
Candida albicans



Fusarium equisitum

Fusarium Moniliform

Fusarium oxysporium



Penicillium notaum

Fusarium Palirodosium

Fig: 4 Dialysis EPS

Fig: 5 Dried Pure Exopolysaccharide

3. Pretreatment of Dialysis membrane: -

The dialysis tube (Himedia MW cut off 13,000 Da) was cut to the desired length and soaked in deionized water for 15 min. the tube was boiled in large quantity of 10mM sodium hydrogen carbonate for 30 min. The membrane was then transferred to 10mM EDTA solution and soaked for 30 min. It was then heated in boiling deionized water for 30 min. and cooled by soaking the membrane in deionized water and stored in de ionized water containing 0.05% sodium azide at 4 ^{o}C till further use. The membrane was washed with deionized water before use.

4. Isolation and quantification of exopolysaccharides: -

Total protein and total carbohydrate content in the isolated polymer were estimated by Lowry's method (1951) and phenol sulphuric-acid method (Dubois et al., 1956) respectively.

Phenol sulphuric acid method for estimation total carbohydrate content: -

The carbohydrate content in the procured samples was determined by phenol sulphuric acid method (Dubosis et.al 1956). 1 mg/ml of sample was prepared in deionized water and was mixed with 1 ml of 5% phenol followed by addition of 5 ml 96% sulphuric acid and the tubes were mixed thoroughly. The tubes were gently shake and then placed in water bath maintained at 25-30^{o}c for 20 min. One blank tube as a control was also maintained for each isolate which contained the entire assay reagent with deionized water instead of sample under study. The total sugar content in the sample was calculated using the standard curve of glucose measured at 490 nm.

% Carbohydrate in sample = $\frac{Absorbance\ of \frac{1mg}{ml} test\ sample}{Concentration\ of\ sample}$ **X 100**

Lowry's Method for estimation total protein content: -

Total soluble protein content present in he samples was estimated by FolinLowry method (Lowry et al., 1951). The assay is based on the biuret reaction of proteins with cupric sulfate at alkaline conditions and the Folin-Ciocalteau phosphomolybdotungstate reduction to heteropolymolybdenum blue which is measured at 660 nm. The reaction is primarily due to the presence of the amino acids tyrosine and tryptophan, and to a lesser extent cystine, cysteine, and histidine.

Reagents

Reagent A – 2 % Na_2CO_3 in 0.1 N NaOH

Reagent B – 0.5 % $CuSO_4$ in 1 % Potassium sodium tartarate

Reagent C – Reagent A and Reagent B in the ratio of 50:1

Regent D – 1N Folin-Ciocalteau reagent

Procedure:

To 1 ml sample, 5 ml reagent C was added and mixed well. The mixture was incubated at room temperature for 10 min. To this added 0.5 ml reagent D, mixed and kept at dark for 20 min. The resulting color was measured at 660 nm.

RESULTS AND DISCUSSION

The results were recorded that 9 fungal isolated obtained from the fruit, vegetable and crop plants originating from Udaipur (Raj.) (*fig*1). After further experiment was carried out with 9 isolates of fungi on shaker incubator at 150 rpm at 28ºc for 14 days and grown in enriched medium (Glucose- 40; Yeast extract-1.0; Peptone-0.5; KH_2PO_4 -0.5; $MgSO_4.7H_2O$- 0.5). After 14 days of incubation isolated fungi have the potential

to produce exopolysaccharide except one fungi i.e. *Penicillium notaum.* After 14 days Accroding to the measurement the best yield of total mycelium (wet and dry) was produced by *Fusarium pallirodosiumis is* 26.49gm/150ml and 3.88gm/150ml and lowest amount of total mycelium produced by fungi *Fusarium oxysporium* is 13.67gm/ 150ml wet mycelium and 3.79 gm/150ml dry mycelium as shown in table (1).

Table 1: Total mycelium (Dry and Wet)

Name of fungus	Wet Mycelium (gm) R1	R2	R3	Average	SD	SE	Dry Mycelium (gm)R1	R2	R3	Average	SD	SE
Fusrium moniliform	14.91	13.64	17	15.18	2.81	0.98	0.87	0.82	0.91	0.86	0.05	0.03
Candida	21.84	21.5	21.12	21.48	2.42	0.20	0.68	0.78	0.73	0.73	0.045	0.029
Fusarium oxysporium	11.07	9.84	10.47	10.46	2.66	0.35	0.65	0.51	0.57	0.57	0.040	0.041
Alternaria solanai	8.36	12.67	9.64	10.33	2.5	1.25	2.3	2.26	2.32	2.29	0.040	0.018
Aspergilus flavus	16.62	18.48	17.38	17.48	17.49	2.08	0.71	0.78	0.66	0.71	0.5	0.032
Fusarium Equisitum	51.48	47.12	52.47	50.35	3.18	1.64	1.47	1.41	1.36	1.41	0.036	0.032
Alternaria Alternata	17.88	22.24	18.79	19.63	1.98	1.32	1.98	1.87	1.93	1.92	0.055	0.032
Fusarium pallirodosium	22.93	24.22	22.83	23.83	1.04	0.44	0.71	0.79	0.65	0.71	0.040	0.014
Penicillum notaum	13.84	14.62	15.58	14.68	2.40	0.50	0.28	0.31	0.36	0.32	0.062	0.023

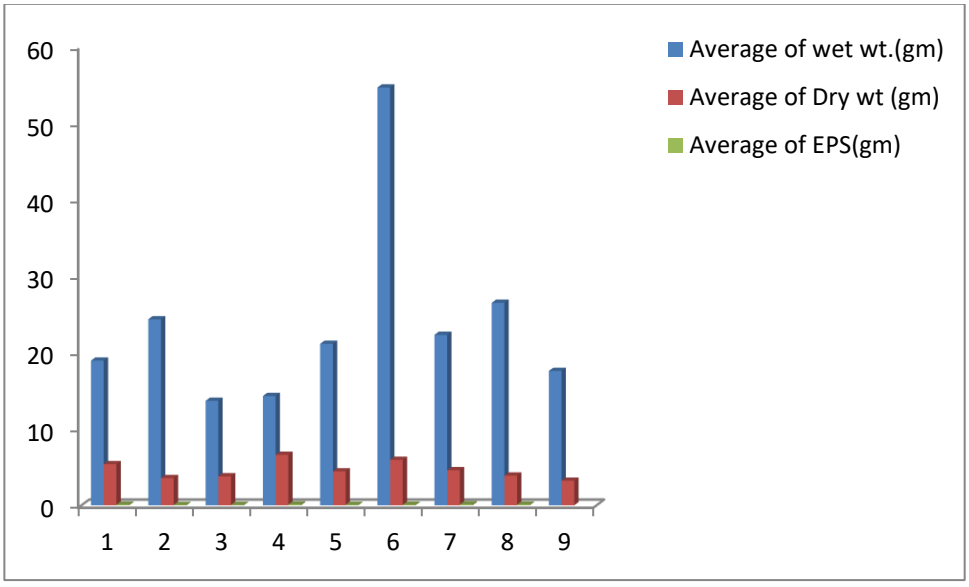
Majority of the polysaccharides used currently in industries, medical and agriculture fields are of plant, algae and bacterial origin (Dilna et al., 2015). The exopolysaccharides isolated from these fungi had different biological behaviour (Yang et al., 2008). The exopolysaccharide produced by plant pathogenic fungi from medicinal plants have been reported to possess various biological activities like antioxidant, antiproliferative activities etc. (Orlandelli et al., 2016; Zhang et al., 2017) Extracellular polysaccharides (EPSs) produced by various white rot fungi shows beneficial biological activities including anti-tumour, immuno-stimulating, and hypoglycaemic activities etc. (Shiao et al.,1994; Sutherland 2002; White et al., 2002; Cheung et al., 2003).

Based on the results of measurements of dry weight exopolysaccharide (gm / 150 ml) as well as quantification assay results as shown in (Table 2), which investigated that the fungal isolate *Alternaria alternate* produced best yield exopolysaccharide is 0.117gm/ 150 ml and produced wet and dry mycelium is 22.30 gm and 1.23 gm per 150 ml respectively and carbohydrate and protein is 95 % and 4% respectively.

Table 2: Recovery of Exopolysaccharide

Name of fungus	Biopolymer + bottle (R1)	Bottle	EPS1 (gm)	EPS1 (mg)	Biopolymer + bottle (R2)	Bottle	EPS2 (gm)	EPS2 (mg)	Biopolymer + bottle (R3)	Bottle	EPS 3 (gm)	EPS 3 (mg)	Average of EPS (gm)	SE
Fusrium moniliform	19.4	19.32	0.08	80	19.98	19.88	0.1	100	19.67	19.58	0.09	90	0.09	0.006
Candida albicans	18.03	17.99	0.04	40	17.65	17.59	0.06	60	18.35	18.3	0.05	50	0.05	0.006
Fusarium oxysporium	19.2	19.15	0.05	50	19.37	19.31	0.06	70	19.18	19.13	0.05	50	0.053	0.003
Alternaria solanai	9.36	9.24	0.12	120	9.45	9.34	0.11	110	9.39	9.28	0.11	110	0.113	0.003
Aspergilus flavus	9.42	9.36	0.06	60	9.47	9.41	0.06	60	9.55	9.48	0.07	70	0.063	0.003
Fusarium Equisitum	9.52	9.44	0.08	80	9.43	9.36	0.07	70	9.5	8.99	0.06	60	0.07	0.006
Alternaria Alternata	9.08	8.97	0.11	110	9.14	9.02	0.12	120	9.01	8.89	0.12	120	0.117	0.003
Fusarium pallirodosium	9.34	9.24	0.1	100	9.38	9.29	0.09	90	9.42	9.32	0.1	100	0.097	0.003
Penicillum notaum	Not significant													

Fig.2 Graph of Total biomass and Exopolysaccharide



1. *Fusarium monilifirmae*
2. *Candida albicans*
3. *Fusarium oxysporium*
4. *Alternaria solanai*
5. *Aspergilus flavus*
6. *Fusarium equisetum*
7. *Alternaria alternate*
8. *Fusarium pallirodosium*
9. *Penicillum notaum*

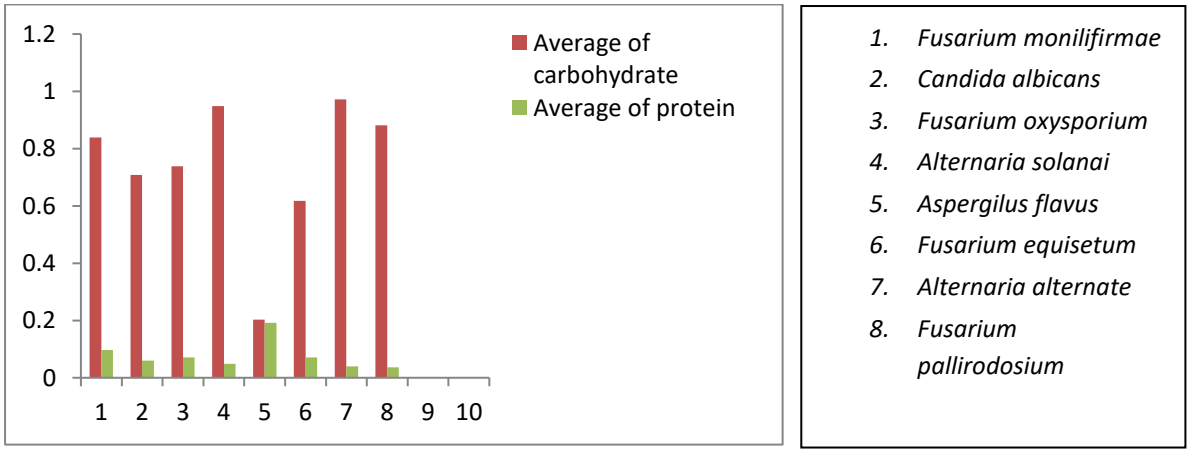
Table 3: Carbohydrate estimation

Name of fungus	Absorbance of sample				SD	SE	Carbohydrate conc. mg/ml	% of Carbohydrate
	R1	R2	R3	Average				
Fusrium moniliform	0.836	0.849	0.831	0.838667	0.009292	0.005	0.83	83
Candida	0.704	0.711	0.709	0.708	0.003606	0.002	0.72	72
Fusarium oxysporium	0.731	0.744	0.739	0.738	0.006557	0.004	0.79	79
Alertnaria solanai	0.942	0.954	0.949	0.948333	0.006028	0.003	0.92	92
Aspergilus flavus	0.202	0.211	0.196	0.203	0.00755	0.004	0.24	24
Fusarium Equisitum	0.623	0.611	0.619	0.617667	0.00611	0.004	0.61	61
Alternaria Alternata	0.972	0.968	0.975	0.971667	0.003512	0.005	0.95	95
Fusarium pallirodosium	0.881	0.878	0.884	0.881	0.003	0.002	0.94	94

Table 4: Protein estimation

Name of fungus	Absorbance of Sample						Protein conc. mg/ml	% of Protein
	R1	R2	R3	Average	SD	SE		
Fusrium moniliform	0.096	0.103	0.091	0.096667	0.006028	0.003	0.11	11
Candida albicans	0.056	0.063	0.061	0.06	0.003606	0.002	0.07	7
Fusarium oxysporium	0.074	0.068	0.071	0.071	0.003	0.002	0.09	9
Alertnaria solanai	0.051	0.045	0.049	0.048333	0.003055	0.002	0.06	6
Aspergilus flavus	0.196	0.191	0.189	0.192	0.003606	0.002	0.23	23
Fusarium Equisitum	0.072	0.065	0.077	0.071333	0.006028	0.003	0.09	9
Alternaria Alternata	0.045	0.039	0.034	0.039333	0.005508	0.003	0.04	4
Fusarium pallirodosium	0.035	0.043	0.031	0.036333	0.00611	0.004	0.04	4

Fig. 3 Graph of carbohydrate and protein



CONCLUSION

The present study suggested that all the fungal isolates produced exopolysaccharides. Among the isolates the *Alternaria alternata* produced significant quantity of exopolysaccharides compared to other isolates. Further their exopolysaccharide may find application in various such as emulsification, flocculation, antibacterial etc. industrial sectors.

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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