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Research Article
Antioxidant Potential of *Penicillium expansum* and Purification of its Functional Compound

[1]Priyanka Chandra and [2]Daljit Singh Arora

[1]Crop Protection Division, ICAR-Indian Institute of Wheat and Barley Research, 132001 Karnal, India
[2]Laboratory of Microbial Technology, Department of Microbiology, Guru Nanak Dev University, 143005 Amritsar, India

Abstract

Background: Fungi are the good source of various biological active secondary metabolites. **Materials and Methods:** The antioxidant potential of *Penicillium expansum* isolated from soil of Punjab, India was studied and a three-step optimization strategy which includes, one-factor-at-a-time classical method and different statistical approaches (Plackett-Burman design and response surface methodology) were applied to enhance the antioxidant potential. Antioxidant activity was assayed by different procedures and compared with total phenolic content. **Results:** Primarily, different carbon and nitrogen sources were screened by classical methods, which revealed sucrose as carbon source is most suitable for antioxidant activity. Sodium nitrate, yeast extract and peptone were good sources of nitrogen but sodium nitrate was the best among them. Significance of the components of Czapek dox's medium with respect to antioxidant activity was evaluated with Plackett-Burman design, which supported sucrose and $NaNO_3$ to be the most significant. In second step, sucrose and $NaNO_3$ along with temperature were taken as three variables for response surface methodology to study their interaction. Response surface analysis showed significant enhancement in the antioxidant potential of *Penicillium expansum*. A compound was purified from the ethyl acetate extract which demonstrated potent antioxidant activity. **Conclusion:** The present study demonstrated potential of soil fungi to have antioxidant activity similar to plants and mushrooms thus further highlighting their significance as new sources of natural antioxidants and thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. The fungi may provide easier set up for production and purification of natural antioxidants as compared to higher plants.

Key words: Antioxidant activity, *Penicillium expansum*, Box-Behnken design, fungi, Plackett-Burman design, response surface methodology

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Corresponding Author: Priyanka Chandra, Crop Protection Division, ICAR-Indian Institute of Wheat and Barley Research, 132001 Karnal, India

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Reactive Oxygen Species (ROS), such as superoxide anion radical, hydroxyl radical and H_2O_2 can cause oxidative damage of DNA, proteins, lipid and small cellular molecules. Increasing evidences has suggested that many human diseases, such as cancer, cardiovascular disease and neurodegenerative disorders are the results of the oxidative damage by reactive oxygen species. The antioxidants are known to play an important role in protection against disorders caused by oxidative damage. Antioxidants can delay or inhibit the initiation or propagation of oxidative chain reaction and thus prevent or repair damage done to the body's cells by oxygen[1].

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroxyquinone (TBHQ) are usually used as food additives by the food industry to prevent lipid peroxidation. However, their applications have been limited because they exhibit toxicity and carcinogenic potential, show lower efficiency than natural antioxidants and require high manufacturing costs. Thus, there is a need to identify natural and possibly more economic and effective antioxidants[2].

In recent years, great interest in finding antioxidants from natural sources has been considered. Numerous crude extracts and pure natural compounds from plants, mushrooms and fungi were reported to have antioxidant and radical-scavenging activities[3].

Natural antioxidants are also in high demand for application as nutraceuticals as well as food additives because of consumer's preferences[4]. Natural products from microbial origin have played and still playing an invaluable role in drug discovery[5]. Fungi have proved to be capable of biosynthesizing secondary metabolites bearing conspicuous structural diversity, which could be further enlarged by structural modifications. Mushrooms (fruiting body) and plants have been known for production of antioxidant compounds but more recently many other filamentous fungi have attracted the attention of scientific community because of their ability to produce wide range of secondary metabolites, many of which are known to possess antioxidant activity. Hence, the present study was planned to explore the antioxidant potential of fungi and *Penicillium expansum* isolated from soil was selected for the optimization of the activity, screening of functional compound and its purification.

MATERIALS AND METHODS

The fungal culture was isolated from the soil of sugarcane field, Harike Punjab, India and identified on the basis of standard protocols and the identity was confirmed by National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India. To study the antioxidant potential, the fungus was grown on 50 mL Czapek dox's broth (sucrose 3%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05%, $FeSO_4$ 0.001%, pH 7.0). The medium was inoculated with two discs (8 mm) of fungal mycelia obtained from 6-7 days grown culture on yeast extract glucose agar plates. After incubation under stationary conditions at 25°C for 10 days, the culture broth was filtered through Whatman filter paper No. 1 and the filtrate so obtained was analyzed for antioxidant potential by different assay procedures and extracellular total phenolic content was estimated by Folin-Ciocalteau (FC) method.

Chemicals: All the chemicals and media/components were purchased from Hi-media Pvt., Ltd., Mumbai, India. Precoated silica gel 60 F254 plates were used for thin layer chromatography (Merck, Dermstadt, Germany). All the solvents used in the study were of analytical grade. For analytical techniques, HPLC grade and NMR grade solvents were used.

Optimization of physiochemical parameters: Different physio-chemical and nutritional parameters were optimized to enhance their antioxidant potential. To select the basal medium for the growth of fungus leading to best antioxidant activity, the different media viz., Czapek Dox's Medium (CDM), 2% Malt Extract Medium (MEM), Potato Dextrose Medium (PDM) and yeast extract glucose medium (YEM) were screened. Then, to monitor the time profile for antioxidant potential of fungus with respect to incubation period, the activity was monitored every 5th day upto 30 days of growth under static conditions. To see the effect of shaking on the antioxidant activity of the fungus was grown on Czapek dox's broth under shaking at different rpm 100, 150, 200 and 250 and compared with antioxidant potential of the fungus grown under static conditions. Further, the activity was checked in the culture broths obtained from the fungus grown on Czapek dox's broth medium at different pH values (2-11). To check the effect of inoculum size on the antioxidant potential, 1-5 discs were inoculated in different flasks containing 50 mL of Czapek dox's broth medium. The diameter of all the discs was 8 mm. And to see the effect of age of inoculum on antioxidant potential, fungus was grown in different petri plates and from 3rd day onwards till 10th day, two fungal discs from each plate were further inoculated in the flasks containing 50 mL broth of Czapek dox's medium. The flasks were incubated for 10 days and the antioxidant potential of the fungal isolates was estimated.

Medium optimization using one-factor-at-a-time classical method

Screening of different carbon and nitrogen sources: To find out the best carbon source, sucrose in the Czapek dox's medium was replaced with same concentration of one of the sugars (glucose, maltose, lactose, starch and glycerol) and to work out the best nitrogen source, sodium nitrate ($NaNO_3$) in Czapek dox's medium was substituted with one or the other inorganic nitrogen source [potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), ammonium chloride ($(NH_4)_2Cl$), ammonium sulphate ($(NH_4)_2\ SO_4$), ammonium dihydrogen sulphate ($(NH_4)H_2SO_4$)] or nitrogen rich organic supplement (yeast extract, peptone, malt extract, urea, casein and soyabean meal).

Medium optimization using statistical methods

Plackett-Burman experimental design: The screening of most significant parameters affecting antioxidant potential was studied by the Plackett-Burman design. Five factors, which are components of Czapek dox's medium (sucrose, $NaNO_3$, K_2HPO_4, KCl and $MgSO_4$) were examined. Total 14 tests were designed including 12 combinations and 2 repetitions at central point which contain different concentration of each factor and the effect of each factor was determined by the difference between the average of the positive and negative responses. The significance level of effect of each factor was determined by student's t test. The most common mean of assessing significant value is the p-value which was also evaluated for each factor.

Optimization by response surface methodology using box-behnken design: On the basis of results obtained from screening of different carbon and nitrogen sources through one-factor-at-a-time classical method and different components by Plackett-Burman design, sucrose and $NaNO_3$ were found to be the best for antioxidant activity. Sucrose as carbon source, $NaNO_3$ as nitrogen source and temperature were taken as independent variables for the optimization by RSM using Box-Behnken design of experiments. Each variable was studied at three levels (-1, 0, +1), for sucrose these were 1, 3 and 5% and $NaNO_3$: 0.05, 0.2 and 0.35%, temperature: 15, 25 and 35°C.

The experimental design included 17 flasks with five replicates having all the three variables at their central coded values. Different assays such as: DPPH assay, reducing power, ferrous ion and nitric oxide ion scavenging activity, FRAP assay and their total phenolic contents were taken as a responses $G_{(1-6)}$. The mathematical relationship of response G (for each parameter) and independent variable X (X_1 sucrose, X_2 $NaNO_3$ and X_3 temperature) was calculated by the following quadratic model equation:

$$G_{(1-6)} = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$$

where, G is the predicted response, β_0 is intercept, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are squared coefficients and β_{12}, β_{13} and β_{23} are interaction coefficients. The MINITAB statistical software was used to obtain optimal working conditions and generate response surface graphs. Statistical analysis of experimental data was also performed using this software.

The optimized values of factors were validated by repeating the experiment in triplicate flasks. The best medium and optimized conditions obtained were used for further study.

Extraction with different organic solvents: To work out the best organic solvent for extraction of bioactive component, the culture broth was treated with different solvents viz., petroleum ether, chloroform, ethyl acetate and butanol. Solvent extracted components were then evaporated to dryness in vaccuo and the resulting solids were reconstituted in DMSO which was then checked for their antioxidant potential by various assays.

Thin layer chromatographic analysis and screening of functional compound in the ethyl acetate fungal extract using dot blot assay: Concentrated ethyl acetate extract (10 µL) was loaded onto TLC plates and dried at 80°C for 10 min. which were then developed in the solvent system of chloroform and ethyl acetate (1:1). Chromatograms so developed were observed under UV light 254 and 365 nm and also observed in iodine chamber. Various separated spots were noted as their Rf values. The developed TLC plates were sprayed with the ethanolic solution of 0.4 mM DPPH[6]. All detected active antioxidants constituents were noted according to their Rf values.

Extraction and purification of active components from ethyl acetate extract of *Penicillium expansum*: Purification was carried out by column chromatography using silica gel. The collected fractions were subjected to thin layer chromatography and antioxidant activity testing using dot blot and different assay procedures.

HPLC analysis: High-pressure liquid chromatography analyses were performed using a Dionex P680 HPLC. Acetonitrile (75% aqueous solution) was used as mobile phase at a flow rate of 0.3 mL min^{-1} and injection volume was 20 mL at a column temperature 25°C. The detections were monitored at different wavelengths (lmax) i.e., 225, 250, 275 and 300 nm.

Chromatographic ferric chloride-potassium ferricyanide sprays analysis: Thin layer chromatography of purified compound was developed in chloroform and ethyl acetate (1:1) and equal volumes of aqueous 1% solutions of each salt (ferric chloride and potassium ferricyanide) were mixed together and sprayed on the TLC plates. Phenols give an immediate blue color[7].

UV spectrum analyses: The UV spectra were recorded using Shimadzu UV mini 1240 UV-visible spectrophotometer in the UV region (200-600 nm) and ethyl acetate were taken as control.

NMR (^{1}H and ^{13}C) spectroscopy and infrared (IR) spectrum analyses: The ^{1}H and ^{13}C NMR spectra of the purified compound were recorded on JEOL-FT NMR AL 300 MHz spectrometer (Bruker, Germany). Infrared (IR) spectrum was taken in the infrared region (400-4000 cm^{-1}) using varian 660-IR fourier-transform IR spectrophotometer.

Antioxidant potential of purified compound and estimation of half maximal effective concentration (EC_{50}) value: Antioxidant potential of purified compound was evaluated by all the various assay procedures (DPPH assay, reducing power, ferrous ion scavenging activity, FRAP assay and nitric oxide ion scavenging activity) and EC_{50} value was also calculated. The EC_{50} represents the amount of sample (mg extract mL^{-1}) necessary to scavenge free radicals by 50%. The EC_{50} value is also the effective concentration at which the absorbance for reducing power is 0.5. Such EC_{50} value was calculated from the graph plotting inhibition percentage against extract concentration[8].

Thermostability of antioxidant bioactivity: To check the temperature sensitivity of the purified compounds for antioxidant activity, the compounds were subjected to 100°C for 2 h and were then assayed for the residual antioxidant activity.

Antioxidant activity

Assay procedures for antioxidant activity and determination of Total Phenolic Content (TPC): Different assay procedures for antioxidant activity was used as described earlier[9]. The total phenolic content was determined colorimetrically using the Folin-Ciocalteau (FC) method according to Arora and Chandra[9].

Toxicity tests: Toxicity tests were carried out as described earlier[9].

RESULTS

Effect of different physiochemical parameters on antioxidant potential and TPC: Antioxidant activity of *Penicillium expansum* was best expression in the CDM followed by YEM then in MEM while the activity was least in PDM, hence Czapek dox's medium was used for the further studies. To see the effect of growth period on antioxidant potential and TPC, the activity of *Penicillium expansum* was recorded every 5th day upto 30 days of growth. The antioxidant potential measured by different assay procedures was best expressed on 10th day. To see the effect of shaking, the fungus was grown at different RPM and was compared with the activity of static culture and result was found to be highest at static conditions. The antioxidant potential of *Penicillium expansum* was highest at pH 7. Almost similar results were found at pH 5, 6, 7. Comparatively, low activity was observed at pH 4, 8 and 9 while no activity was found at pH 2, 3, 10, 11 and 12. Two discs were found to demonstrate maximum antioxidant potential and it remained stable after the increase in the number of disc. Similar profile was observed for inoculum size for all the assay procedures. The TPC was also found highest in the extracellular culture broth of the fungus inoculated with two discs and it also remained stable even after increasing the number of discs. *Penicillium expansum* demonstrated highest antioxidant activity, by using the 7 days grown inoculum and activity remained stable by further increase of inoculum age. Similar results were found for TPC, which was also highest with the inoculum of age of 7 days.

Optimization of medium by classical method using one-factor-at a-time

Effect of carbon sources on antioxidant potential and TPC: The antioxidant potential of *Penicillium expansum* was best expressed in sucrose and DPPH, ferrous, NO ion scavenging

Table 1: Effect of carbon sources on antioxidant potential of *Penicillium expansum*

Activity (%)	Dextrose	Maltose	Lactose	Starch	Sucrose	Glycerol
DPPH scavenging activity	70.30±0.01	60.80±0.01	58.30±0.51	55.20±0.12	75.20±0.45	50.40±0.11
Reducing power	0.51±0.7	0.50±0.34	0.43±0.14	0.32±0.2	0.56±0.01	0.32±0.08
Fe^{2+} scavenging activity	50.20±0.45	45.30±0.12	40.30±0.32	37.40±0.02	54.20±0.01	30.10±0.1
FRAP activity	46.20±0.21	42.40±0.1	40.30±0.05	36.30±0.02	50.20±0.02	30.20±0.48
NO scavenging activity						
30 min	24.30±0.3	20.20±0.03	20.10±0.006	18.70±0.4	30.10±0.003	15.40±0.04
60 min	38.30±0.02	24.30±0.01	25.30±0.4	22.10±0.01	36.20±0.1	21.20±0.1
90 min	40.30±0.01	30.20±0.01	30.20±0.9	28.50±0.8	40.40±0.002	25.40±0.001
120 min	45.20±0.05	37.50±0.07	35.40±0.02	32.30±0.01	46.10±0.1	30.20±0.5
180 min	48.20±0.2	42.50±0.01	42.60±0.03	40.90±0.7	50.50±0.01	35.50±0.02
TPC (mg mL^{-1})	6.50±0.01	5.10±0.12	4.80±0.02	4.20±0.01	7.10±0.10	3.20±0.04
Biomass (mg)	331.00±0.2	245.00±0.1	155.00±0.01	285.00±0.07	185.00±0.8	102.00±0.1

Table 2: Effect of nitrogen sources on antioxidant potential of *Penicillium expansum*

Nitrogen sources	DPPH scavenging activity	Reducing power	Fe^{2+} scavenging activity	FRAP activity	NO scavenging activity	TPC (mg mL^{-1})	Biomass (mg)
Nitrogen rich organic supplements							
Yeast extract	72.1±0.1	0.50±0.2	50.2±0.02	45.6±0.4	45.8±0.2	6.2±0.2	332±0.02
Peptone	72.1±0.3	0.50±0.1	50.2±0.002	45.5±0.04	45.9±0.1	6.2±0.004	320±0.01
Malt extract	61.2±0.04	0.32±0.02	42.4±0.001	38.4±0.1	37.5±0.3	4.8±0.6	278±0.01
Casein	65.4±0.003	0.40±0.003	45.2±0.4	40.3±0.3	40.2±0.04	5.3±0.4	257±0.5
Soybean meal	60.1±0.01	0.30±0.67	42.3±0.3	38.7±0.2	38.9±0.2	4.5±0.4	184±0.9
Urea	19.2±0.03	-	-	-	-	1.2±0.3	108±0.2
Inorganic nitrogen sources							
KNO_3	45.2±0.4	0.25±0.2	28.9±0.3	22.2±0.4	20.1±0.1	3.2±0.9	180±0.01
$(NH_4)_2SO_4$	48.9±0.001	0.29±0.04	30.2±0.4	25.6±0.5	25.3±0.3	3.8±0.08	185±0.1
$(NH_4)H_2SO_4$	42.3±0.002	0.21±0.004	26.4±0.001	23.3±0.2	22.2±0.002	3.2±0.2	160±0.01
NH_4NO_3	40.1±0.03	0.20±0.033	23.3±0.003	20.2±0.1	18.3±0.01	2.2±0.1	158±0.06
$NaNO_3$	75.3±0.03	0.56±0.1	54.2±0.4	50.2±0.2	50.5±0.1	7.1±0.3	185±0.3
$(NH_4)_2Cl$	52.3±0.006	0.30±0.4	36.4±0.5	32.3±0.1	32.1±0.1	4.2±0.2	162±0.1

and FRAP activity were 75.2, 54.2, 50.5 and 50.2%, respectively and reducing ability was 0.56. Dextrose was followed by sucrose and the DPPH, ferrous, NO ion scavenging and FRAP activity were 70.3, 50.2, 48.2 and 46.2%, respectively while 0.51 of reducing power was found. The TPC follow the same profile, sucrose (7.1 mg mL^{-1}) supported the maximum TPC followed by dextrose (6.5 mg mL^{-1}), maltose (5.1 mg mL^{-1}), lactose (4.8 mg mL^{-1}), starch (4.2 mg mL^{-1}) and glycerol (3.2 mg mL^{-1}). The biomass was found highest in dextrose followed by starch, maltose, sucrose, lactose and glycerol (Table 1).

Effect of nitrogen sources on antioxidant potential: Sodium nitrate was the best nitrogen source to support maximum antioxidant potential of *Penicillium expansum* and DPPH, ferrous, NO scavenging ion and FRAP activity were 75.3, 54.2, 50.5 and 50.2%, respectively while reducing ability was 0.56. Sodium nitrate was followed by peptone and yeast extract while urea supported the poorest activity. The TPC was also highest (7.1 mg mL^{-1}) in sodium nitrate while yeast extract and peptone showed similar TPC (6.2 mg mL^{-1}). The biomass was found to be highest in yeast extract followed by peptone (Table 2).

Statistical optimization of the medium

Plackett-Burman design for selection of significant components: A Plackett-Burman experiment was designed to evaluate the influence of five different components (sucrose, $NaNO_3$, K_2HPO_4, KCl and $MgSO_4$) of the Czapek dox's medium in order to obtain better antioxidant activity. Antioxidant potential of all the fungal isolates along with their biomass and extra-cellularly produced total phenolic content were estimated in all the 14 runs of different combinations of the media components. Invariably high activity was found among run 5, 13 and 14. The results were subjected to regression analysis and the analysis of variance (ANOVA) which revealed that of the five variables only sucrose and $NaNO_3$ have statistically significant affect on antioxidant potential for all the fungi with $p \leq 0.005$ and $p \leq 0.05$, respectively. The antioxidant potential in run order 5 was highest with 78.5, 58.9 and 55.0% of DPPH, ferrous and NO ion scavenging activity. The FRAP activity of 57.4% was recorded and the reduction power was 1.1. Run order 14 showed the scavenging effect of 75.8, 55.0 and 50.9% on DPPH, ferrous and NO ion, respectively, however, the reducing power was 0.78 with 51.8% of FRAP activity. Run order 13 showed 75.6, 54.0, 50.5 and 50.9% of DPPH, ferrous, NO ion scavenging and

Table 3: Results of Plackett-Burman design experiment for antioxidant potential of *Penicillium expansum*

Run	DPPH scavenging activity	Reducing power	Fe^{2+} scavenging activity	FRAP activity	NO scavenging activity	TPC (mg mL^{-1})	Biomass (mg)
1	70.1	0.71	45.0	42.4	50.1	7.20	186.0
2	78.5	0.98	50.2	49.8	55.8	10.10	192.0
3	33.2	0.08	17.7	15.5	20.6	2.20	110.0
4	50.8	0.28	38.1	35.5	40.0	4.40	192.0
5	78.5	1.10	58.9	57.4	55.0	10.90	214.0
6	72.3	0.42	50.1	48.7	54.8	5.60	185.0
7	35.4	0.10	13.6	10.6	15.5	0.70	164.0
8	45.8	0.22	35.0	35.0	36.1	1.20	120.0
9	20.2	0.10	13.0	12.8	13.8	0.98	102.0
10	60.2	0.58	48.1	46.9	47.9	4.10	118.0
11	62.0	0.32	40.0	41.2	40.2	6.70	120.0
12	0.0	0.00	0.0	0.0	0.0	0.00	0.0
13	75.6	0.70	54.0	50.9	50.5	7.50	234.0
14	75.8	0.78	55.0	51.8	50.9	7.80	230.0

Table 4: Results of Box-Behnken design experiment for antioxidant potential of *Penicillium expansum*

Run	DPPH scavenging activity	Reducing power	Fe^{2+} scavenging activity	FRAP activity	NO scavenging activity	TPC (mg mL^{-1})
1	70.30	0.60	50.3	48.6	48.0	6.6
2	74.30	0.77	50.1	50.2	50.8	7.6
3	70.50	0.61	50.3	48.9	48.8	6.6
4	35.10	0.17	28.2	25.8	26.1	1.2
5	65.30	0.32	48.1	44.2	45.8	4.2
6	68.80	0.57	52.3	50.3	48.3	6.0
7	70.10	0.63	54.5	54.8	55.3	7.2
8	79.20	1.50	70.2	69.6	65.3	12.2
9	67.30	0.60	48.3	45.2	46.8	6.3
10	65.80	0.40	45.0	46.2	45.2	4.2
11	78.20	1.40	60.2	58.2	65.3	13.9
12	75.60	1.17	52.3	50.2	51.2	11.2
13	76.60	0.80	51.8	51.4	50.3	7.9
14	75.20	0.79	50.2	50.2	50.8	7.8
15	76.60	0.80	51.2	51.2	50.6	8.0
16	75.20	0.90	51.0	50.6	51.8	7.6
17	75.80	0.80	55.0	51.8	50.9	7.8

FRAP activity, respectively and reducing power was 0.70. The TPC was found highest in run order 5 (10.90 mg mL^{-1}) followed by 14 (7.80 mg mL^{-1}) and 13 (7.50 mg mL^{-1}). The biomass was observed highest in run order 13 followed by 14 and 5 (Table 3).

Box-Behnken design for optimization of the medium composition for antioxidant activity: The results for Box-Behnken design of *Penicillium expansum* are described in Table 4. The final predictive equations for each response: DPPH free radical scavenging activity (G_1), reducing power (G_2), ferrous ion scavenging activity (G_3), FRAP activity (G_4) and nitric oxide ion scavenging activity (G_5) and total phenolic content (G_6) obtained were as follows:

$$G_1 = 75.76-2.35X_1-5.387X_2+4.48X_3-7.042X_1^2-6.168X_2^2+ 2.13X_3^2-9.85X_1X_2+1.4X_1X_3-0.27X_2X_3$$

$$G_2 = 0.87+0.105X_1-0.13X_2+0.34X_3-0.22X_1^2-0.103X_2^2+ 0.11X_3^2-0.15X_1X_2+0.15X_1X_3-0.022X_2X_3$$

$$G_3 = 51.8-0.3X_1-4.13X_2+5.43X_3-1.14X_1^2-5.97X_2^2+ 5.58X_3^2-5.47X_1X_2-2.8X_1X_3-1.15X_2X_3$$

$$G_4 = 51.0-0.075X_1-3.887X_2+5.86X_3-1.42X_1^2-6.20X_2^2+ 5.15X_3^2-6.17X_1X_2-2.17X_1X_3-2.2X_2X_3$$

$$G_5 = 50.58-0.85X_1-4.9X_2+6.4X_3-2.7X_1^2-4.44X_2^2+ 5.9X_3^2-6.3X_1X_2+1.9X_1X_3-3.1X_2X_3$$

$$G_6 = 51.0-0.075X_1-3.88X_2+5.86X_3-1.42X_1^2-6.20X_2^2+ 5.15X_3^2-6.1X_1X_2-2.1X_1X_3-2.2X_2X_3$$

The interactive effect (X_1X_2) was found significant for DPPH free radical scavenging activity ($p<0.05$). Linear effect (X_2) was most significant with $p<0.005$ on reducing power, FRAP activity, ferrous ion and nitric oxide ion scavenging activity while X_1^2 effect significantly at $p \leq 0.05$ only on reducing power and nitric oxide ion scavenging activity. Sucrose at a concentration of 4.5 and 0.05% of $NaNO_3$ and 35°C were

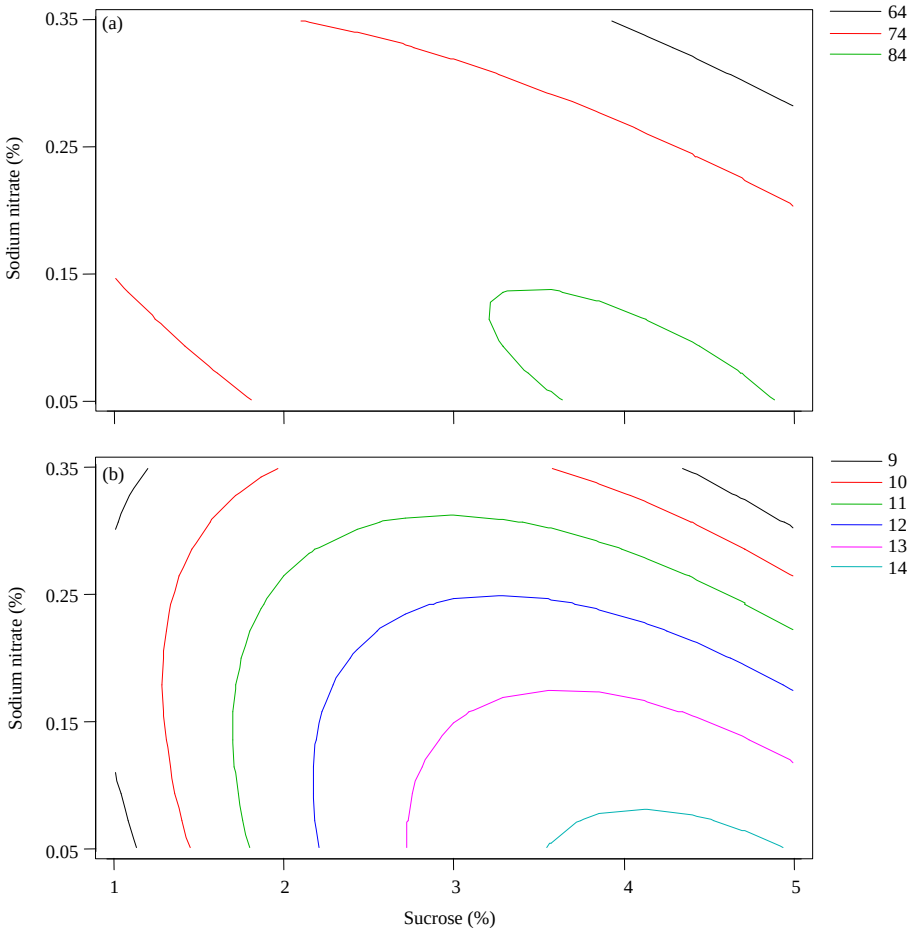

Fig. 1(a-b): Contour graph showing effect of different variables on antioxidant potential of *Penicillium expansum* (hold value: Temperature at 35°C) (a) 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity (Percentage activity) and (b) Total phenolic content (mg mL^{-1})

found to be most suitable for the antioxidant potential of *Penicillium expansum* (Fig. 1a). At these conditions, DPPH free radical, nitric oxide ion, ferrous ion scavenging and FRAP activity was 84, 70, 68 and 65%, respectively and reducing power was 1.5. Linear effect (X_3) was significant with $p \leq 0.005$ while interactive effect (X_1^2) showed significance for total phenolic content at $p \leq 0.05$. The response surface graphs showed the highest amount of TPC (14 mg mL^{-1}) obtained at 4.5% of sucrose and 0.05% of $NaNO_3$ at 35°C (Fig. 1b).

The optimized conditions obtained from analysis were 4.5% sucrose, 0.05% $NaNO_3$ and incubation temperature of 35°C. The verification experiment using the optimum medium composition was carried out in triplicates. *Penicillium expansum* showed 85.1, 70.6 and 70.1% scavenging effect for DPPH radical, ferrous ion and nitric oxide ion, respectively. The TPC obtained was 12.5 mg mL^{-1} and reducing power showed absorbance of 1.5 with 65.2% for FRAP activity.

Extraction of bioactive component/s with different organic solvents: The results revealed that ethyl acetate to be the best solvent to elute the components responsible for antioxidant potential that was followed by chloroform and butanol extract. However, extracts obtained from petroleum ether did not show any activity.

Thin layer chromatographic analysis and screening of functional compound in ethyl acetate extract: Concentrated ethyl acetate extract of *P. expansum* was subjected to TLC analysis. The TLC resolved into 2 bands with Rf value of 0.15 and 0.53 among which only one band (Rf value: 0.53) showed positive antioxidant activity.

Purification of the active compound: The fractions obtained from the column chromatography were subjected to TLC and the fractions having single bands with Rf value of 0.53 were pooled and considered as purified compound.

Chromatographic spray analyses, UV spectra and HPLC profile: The TLC of purified compound was sprayed with ferric chloride-potassium ferricyanide which gave positive reaction and indicated that this compound belong to phenolic group. To confirm the purity of the compound, HPLC analysis was carried out and single peak was observed for the compound with retention time of 6.12 min. The UV spectrum demonstrated maximum absorbance at 213 nm.

NMR (^{1}H and ^{13}C) spectroscopy and infrared (IR) spectrum analyses: The ^{1}H NMR of the compound showed peaks, corresponds to aromatic groups and presence of -OH group has also been indicated. The ^{13}C NMR of compound also showed the presence aromatic region. The IR spectrum indicates the presence of hydroxyl groups in this compound. The bands corresponding to carboxyl (C=O), carbonyl (C-O) and C-C bonds groups were observed. The IR spectrum also confirmed the presence of methyl ($-CH_3$) or methylene ($-CH_2$) group.

Antioxidant activity of the purified compound: The purified compound obtained was analyzed for its antioxidant potential by various assay procedures and the result showed compound to be a potent antioxidant. The maximum DPPH radical, ferrous and NO ion scavenging activity at 1 mg mL^{-1} was 87.2, 74.2 and 81.3%, respectively. The reducing power of 1.6 was observed while the ferric ion reduction capacity was 74.6%. The EC_{50} value of compound was 12 µg which was similar for all the assay procedures.

Thermostability of the isolated compound: The purified compound was found to be relatively thermostable as at 100°C it suffered a slight loss in its antioxidant activity. After 1 h, the activity of purified compound decreased only by 5%, while it suffered a maximum loss of 10% after 2 h.

Toxicity testing: The purified compound showed no mutagenicity as no bacterial colony was observed on agar plates containing fungal extracts, while more than 1000 colonies were observed on positive control (sodium azide) containing plate. Similarly, results obtained from MTT assay revealed that the purified compound was non cytotoxic.

DISCUSSION

The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging[10]. Antioxidants can deactivate radicals by two major mechanisms, Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET). The HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation and SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals and radicals[11]. Hence, various assay procedures were used for the estimation of antioxidant potential. The results obtained from various assay procedures prove the potent broad spectrum antioxidant activity of *P. expansum*.

A total antioxidant activity assay using one chemical reaction seems to be rather unrealistic, yet there are numerous published methods claiming to measure total antioxidant activity *in vitro*[12]. Thus, to validate the reliability of different methods, the correlation between the different assays is necessary and thus efforts have been made to establish correlation between different assays. All were found to be positively correlated but r^2 value was variable. These variations in correlation coefficient among different antioxidant assays indicate that a single assay is not sufficient to evaluate the total antioxidant activity.

Previous studies indicate phenolic compounds to be the major antioxidants of medicinal plants, mushrooms, essential oils, spices, fruits and vegetables[13]. The interest in the phenolic compounds has increased tremendously due to their prominent free radical scavenging activity, attributed to their redox properties, which allow them to act as reducing agents or hydrogen atom donor[14]. Hence, TPC in extracellular filtrate was estimated so as to work out its correlation with antioxidant activity. Plotting total phenolic content versus DPPH, ferrous ion scavenging activity, reducing power, nitric oxide ion scavenging activity and FRAP assay yielded a positive correlation. The study reflected that total phenolic contents were moderately to highly associated (r^2 = 0.225-0.845) with antioxidant properties of the tested fungus.

The analysis of the results for antioxidant potential of *P. expansum* assayed demonstrated static culture conditions to be more suitable as compared to shake flask. This supports the earlier contention of various researchers who have used static conditions[15]. The optimum period of incubation for antioxidant potential of *P. expansum* was found to be 10 days, it supports the concept for the production of secondary metabolites during late phase of the cycle and subsequent decline in antioxidant activity

after 10th day could be due to the exhaustion of nutrients available for the fungus to produce such bioactive compounds. Gogoi *et al.*[16] also reported highest bioactivity from *Fusarium* sp., at 9-10th days. The present results corroborate the previous studies in which there was no bioactivity at pH extremes[17].

Optimization of fermentation medium is very important for maximizing the yield of many secondary metabolites[18]. The testing of different carbon sources revealed sucrose to be the most promising for obtaining the best antioxidant activity by fungal isolate which is in consonance with earlier studies[19]. During the screening of nitrogen sources for their effect on antioxidant activity, sodium nitrate was found to be the best among organic and inorganic nitrogen sources followed by peptone and yeast extract. Several studies proved sodium nitrate to the best nitrogen source for the production of antioxidant metabolites as well as many other bioactive metabolites[20].

Most of the recent optimization efforts have relied on statistical experimental design and response surface analysis[21]. Plackett-Burman design was applied for screening of medium constituents effective for antioxidant potential and its analysis showed sucrose and $NaNO_3$ to be significant. The variables screened by Plackett-Burman design were subjected to statistical and mathematical optimization tool response surface methodology for optimization of concentration of sucrose and sodium nitrate. This empirical technique enables to evaluate the interaction between independent variables and to predict the response in an effective experimental design[22].

The analysis of variance of the quadratic regression model suggested that the model is very significant. The R^2 value (multiple correlation coefficient) closer to 1 denotes better correlation between the experimental and predicted responses[22] and the results demonstrated high R^2 value. The response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and combined effects of each independent variable upon the response and the optimized conditions obtained from them helped in the enhancement of the activity which was further verified. After the application of RSM, the results of *Penicillium expansum* showed significant increase in the antioxidant activity and DPPH radical, ferrous ion and nitric oxide ion scavenging activity was enhanced by 1.13, 1.3 and 1.2 folds, respectively while reducing potential and ferric reduction rate was enhanced by 3.0 and 1.3 folds. The production of TPC was enhanced by 1.7 folds.

The TLC of the purified compound on silica gel plates were developed in chloroform and ethyl acetate (1:1, v/v) and sprayed with the mixture of ferric chloride-potassium ferricyanide which gave positive reaction. This result indicated the phenolic nature of the compound which is in consonance with earlier studies[7]. Phenolic compounds are aromatic in nature and show intense absorption in the UV region of the spectrum[23]. The HPLC equipped with a photo diode array detector, which establishes the light absorbance spectrum from visible and UV wavelengths of each detected compound and in the present study the absorption for purified compound was detected between 225-300 nm. This also gives the indication of presence of phenolic compounds. The obtained results can be further supported by the UV spectra, as the purified compound showed the maximum absorption at 213 nm. This study further verifies the presence of phenolic compounds and is in consonance with earlier study which demonstrated absorption maxima of phenolic derivatives between 210-300 nm[24].

The characterization of the compound revealed the presence of basic structure of phenolic compounds as it possesses the aromatic rings with hydroxyl and oxo group. The IR study shows the presence of the hydroxyl groups in the purified compound which are responsible for their high antioxidant activity. As described by Bors *et al.*[25] there are three structural features that are important determinants for the antioxidant potential of phenolic compounds, (a) Dihydroxy structure in the aromatic ring, (b) The 2, 3-double bond in conjunction with the 4-oxo group (which allows conjunction between the rings or electron delocalization and (c) The presence of OH group in aromatic ring and it is the most significant determinant of electron-donating activity. Presence of all these groups has been proved in the purified compound by using various analytical techniques (IR and NMR). Hence, the study confirms the presence of phenolic compound as active components in the fungus.

The novelty of present study has been proven as most of the literature is available on antioxidant activity of plants and mushrooms though some of the fungi are known to produce antioxidant activity. To the best of our knowledge apparently this is the first systematic report on antioxidant activity of *Penicillium expansum* estimated by different assay procedures and its optimization by statistical methods. The present study get further credence as the purified compound did not show any mutagenicity or cytotoxicity.

CONCLUSION

The present study demonstrated potential of soil fungi to have antioxidant activity similar to plants and mushrooms thus further highlighting their significance as new sources of natural antioxidants and thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. The fungi may provide easier set up for production and purification of natural antioxidants as compared to higher plants. The obtained results suggest *Penicillium expansum* produce potent antioxidants with non-mutagenic and non-cytotoxic properties and their further investigations *in vivo* can be useful for their wider use. Furthermore, easier downstream processing of the fungal compounds as compared to phytochemicals offers a ray of hope for further development of chemotherapeutic agents as antioxidants are used as protective measure in various diseases.

SIGNIFICANT STATEMENT

The results obtained from the present study prove the potent broad spectrum antioxidant activity of *P. expansum*. Natural products from microbial origin have played and still playing an invaluable role in drug discovery.

Potential of soil fungi to have antioxidant activity similar to plants and mushrooms thus further highlighting their significance as new sources of natural antioxidants and thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. The fungi may provide easier set up for production and purification of natural antioxidants as compared to higher plants.

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