

EVALUATION AND OPTIMIZATION OF ANTIOXIDANT POTENTIALITY OF *XYLARIA FEEJEENSIS*  
HMJAU22039

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Received: 27 June 2016, Revised and Accepted: 04 July 2016

## ABSTRACT

**Objective:** Antioxidants neutralize free radicals generated in the human body and prevent them from causing damage. The present study is aimed to evaluate and optimize the antioxidant activity of *Xylaria feejeensis* HMJAU22039 an endophyte isolated from *Tectona grandis*.

**Methods:** The antioxidant potential was measured by 1-1diphenyl-2-picryl-hydrazil (DPPH) radical scavenging assay and nitric oxide (NO) scavenging assay. Total phenolic content (TPC) of the samples was measured by Folin-Ciocalteu reagent based assay, and the values were obtained from the regression equation:  $y = 0.006141x + 0.157733$ .

**Results:** Potato dextrose yeast extract broth medium was selected as the basal medium as it reported a high antioxidant activity. The basal medium was optimized with 2% dextrose, 0.2% yeast extract, and 200 g/L potato infusion. Dextrose and yeast extract were selected as carbon and nitrogen sources, respectively, as they reported high antioxidant activity. The antioxidant activity and phenolic content are highest at static condition (18.14 mg/g). Incubation temperature of 30°C and pH of the initial medium at 6 were found to be optimum conditions for high antioxidant activity. An incubation period of 20 days reported the highest antioxidant activity and phenolic content. Methanol extract recorded high antioxidant activity with a DPPH (73.86%) followed by ethyl acetate and chloroform fractions.

**Conclusion:** The study highlights the importance of different physicochemical parameters in the production of secondary metabolites having antioxidant properties. The results reveal a significant positive correlation between DPPH radical scavenging assay, NO scavenging assay, and TPC.

**Keywords:** 1-1diphenyl-2-picryl-hydrazil, Total phenolic content, Endophytic fungus, *Tectona grandis*.

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## INTRODUCTION

Natural products are naturally derived bioactive metabolites and byproducts from microorganisms, plants, or animals. These products have been exploited for human use for many years and plants have been the main source of the compounds used for medicine. Besides plants, microorganisms also constitute a major source of natural products with preferable bioactive properties. Researchers are in quest of new bioactive metabolites from new sources. Endophytic fungi appear to be one such interesting source of research. As a consequence of their contribution to the host plant, endophytes may produce a surplus of substances that may have potential use in modern medicine, agriculture, and industry. Bioactive compounds produced by endophytes have been promising prospective utility in safety and human health concerns. Endophytes provide a wide range of bioactive secondary metabolites with a unique structure, including alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, terpenoids, steroids, tetralones, xanthenes, and others [1]. These bioactive secondary metabolites have a wide range of application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents [2-4].

Antioxidants also called as free radical scavengers are chemicals that interact and neutralize free radicals, thus preventing them from causing damage to the organism. Free radicals are produced constantly in the human body during normal physiological processes and cause various degenerative processes such as aging, inflammation, cardiovascular diseases, atherosclerosis, diabetes, cancer, cataracts, Alzheimer's disease, and neurodegenerative disorders [5]. Antioxidants can be either used as dietary supplement or as a drug. Food industries use synthetic antioxidants such as butylated hydroxyanisole, tert-butylhydroquinone, and butylated hydroxyl toluene as food additives to prevent lipid peroxidation. Synthetic antioxidants are found to be carcinogenic and are reported to involve other toxic side effects thus compelling the

search for natural antioxidants. As a result, attention has been drawn on the characterization of the antioxidant properties of products from several natural resources and isolation and identification of those important constituents. Plants and mushrooms are major sources of natural antioxidants. In recent times, fungi have emerged as the new sources of antioxidants in the form of their secondary metabolites [6]. Discovery of pestacin and isopestacin as antioxidant compounds from *Pestalotiopsis microspora* an endophyte in *Terminalia morobensis* led to the exploration of the antioxidant potential of this less explored group of fungi. Graphis lactone A was isolated from *Cephalosporium* sp. IFB-E001, an endophytic in *Trachelospermum jasminoides*. The compound was confirmed to have stronger antioxidant activity *in vitro* as compared to butylated hydroxytoluene and ascorbic acid [7].

## METHODS

## Isolation and identification

*Xylaria feejeensis* HMJAU22039 was isolated from *Tectona grandis* and grown on potato dextrose agar (PDA) medium substituted with chloramphenicol. After 1 week of growth, the culture was transferred to PDA slants and stored at 4°C. Molecular identification of the strain was confirmed by NFCCI, Pune.

## Antioxidant activity assays

*Free radical scavenging activity measured by 1-1diphenyl-2-picryl-hydrazil*

The free radical scavenging activity of all the extracts was measured by DPPH radical scavenging assay [8]. An aliquot of 1 ml of 0.1 mM DPPH solution in methanol and 0.5 ml of extract were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 minutes, and absorbance was measured at 517 nm. The DPPH radical scavenging effect was calculated according to the

following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A0 is the absorbance of the control and A1 is the absorbance of the sample.

#### Determination of nitric oxide scavenging activity

An equal amount (6 ml) of sodium nitroprusside (5 mM) solution was mixed with 6 ml of extract and incubated at 27°C for 2½ hrs. Afterward, 0.5 ml of the reaction mixture was mixed with an equal amount of Griess reagent, and absorbance was taken at 546 nm [9]. The nitric oxide (NO) scavenging effect was calculated according to the following equation:

$$\text{NO scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance of the sample.

#### Determination of total phenolic content

Total phenol content was estimated using Folin-Ciocalteu (FC) reagent based assay using gallic acid as standard [10]. The extract was dissolved in methanol (1 mg/mL), and 500 µL of (50%) FC reagent was added followed by the addition of 1.5 mL of 20% of Na<sub>2</sub>CO<sub>3</sub>. The final volume was made up to 5 mL by adding distilled water. The mixture was kept at room temperature for 30 minutes, and the absorbance was recorded at 765 nm. This procedure was also repeated to aliquots of 10-100 µg/mL methanolic gallic acid solutions which were used as a standard for the calibration curve. Total phenolic value of the samples was obtained from the regression equation:  $y = 0.006141x + 0.157733$  with R<sup>2</sup> = 0.9975 and expressed as mg/g gallic acid equivalent.

#### Medium optimization

##### Standardization of basal medium

Standardization of basal medium for optimum antioxidant activity consists of (g/L) Potato dextrose broth containing potato (200.0 g) and dextrose (20.0 g); Czapek-Dox Broth containing NaNO<sub>3</sub> (3.0 g), K<sub>2</sub>HPO<sub>4</sub> (1.0g), MgSO<sub>4</sub> (0.5 g), KCl (0.5g), FeSO<sub>4</sub> (0.01 g), and sucrose (30.0g); Sabouraud dextrose broth containing peptone (10 g/L) and glucose (40 g/L); potato dextrose yeast extract broth (PDYEB) containing potato (200.0 g), dextrose (20.0 g), and yeast extract (2.0 g); Malt extract broth (ME) containing ME (20.0 g), peptone (1.0 g), and glucose (20.0 g) media were used. The culture was kept in static condition and an initial pH of 7 for all cultures. After 14 days of incubation at 26 ± 2°C, the culture filtrate was extracted 3 times with ethyl acetate. The antioxidant activity was measured by DPPH, NO scavenging assay, and the total phenolic content (TPC) was recorded.

##### Effect of carbon source

Various carbon sources such as dextrose, sucrose, glucose, lactose, and maltose were amended separately into the basal medium (PDYEB) at a concentration of 2%. *X. feejeensis* HMJAU22039 was inoculated to each of the media and incubated at 26 ± 2°C in dark for 14 days in static condition, and their respective antioxidant activity was recorded.

##### Effect of nitrogen source

Various nitrogen sources such as sodium nitrate, casein, yeast extract, peptone, and ME were amended into the basal medium (PDYEB) at a concentration of nitrogen source 0.2%. *X. feejeensis* HMJAU22039 was inoculated to the respective medium and incubated at 26 ± 2°C in dark for 14 days in static condition, and their respective antioxidant activity was recorded.

##### Effect of shaking

*X. feejeensis* was inoculated into PDYEB basal medium and was grown in static and shaking conditions. In shaking condition, the broth culture was kept on an orbital shaker at 120 rpm and 240 rpm for 1 week. After 14 days, the antioxidant activity and phenolic content were recorded.

##### Effect of temperature

The strain HMJAU22039 was inoculated into PDYEB basal medium and was grown in various ranges of temperature from 20 to 40°C at a difference of 5°C for 14 days in dark under static conditions, and the antioxidant activity and TPC were recorded.

##### Effect of pH

Initial pH was adjusted from 5 to 9 at a difference of one to PDYEB medium and was incubated for 14 days in dark at 26 ± 2°C under static condition, and their antioxidant activity and TPC were recorded.

##### Determination of incubation period

The strain HMJAU22039 was inoculated into PDYEB medium and incubated up to 25 days in static condition in dark at 26 ± 2°C. Their antioxidant activity and TPC were measured at an interval of 5 days from the initial day of inoculation till the 25<sup>th</sup> day.

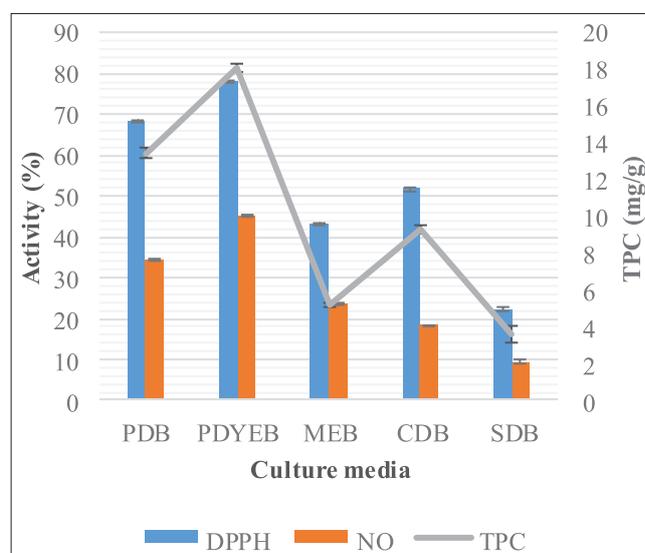
##### Effect of solvent

The fungal strain was inoculated into 5 flasks containing PDYEB basal medium. The temperature was maintained at 26 ± 2°C in dark. After 14 days of incubation, the culture was passed through four layers of cheesecloth to remove solids and extracted with ethyl acetate, chloroform, diethyl ether, and toluene. The fungal mat was crushed, soaked in methanol for 24 h, and filtered. The antioxidant activity and the TPC of all the solvent extracts were recorded.

## RESULTS

### Standardization of basal medium

Although PDA medium was used for the isolation of *X. feejeensis* HMJAU22039, further standardization of medium showed that PDYEB was a better medium for antioxidant activity (Fig. 1). Comparative study of antioxidant activity measured by DPPH, NO scavenging activity,



**Fig. 1: Effect of different media on antioxidant potential of *Xylaria feejeensis*. DPPH: DPPH radical scavenging activity; NO: Nitric oxide scavenging activity; TPC: Total phenolic content. All values are reported as mean±SD (n=3). DPPH: 1-1diphenyl-2-picrylhydrazil, NO: Nitric oxide, SD: Standard deviation**

and TPC indicated a significantly higher antioxidant activity and TPC (18.1 mg/g) in PDYEB by *X. feejeensis* compared to others. Hence, PDYEB medium was used to optimize different environmental parameters for antioxidant activity and phenol production.

#### Effect of carbon source

Table 1 shows the effect of carbon source on antioxidant activity and phenol content. A significantly higher value of DPPH radical scavenging (69.34%), NO scavenging activity (38.89%), and total phenol content (14.31 mg/g) were produced when dextrose was the carbon source. It was followed by glucose with DPPH, NO scavenging activity, and TPC at 61.38%, 36.41%, and 11.42 mg/g, respectively. Sucrose, maltose, and lactose showed moderate to low amount of antioxidant activity and phenol content.

#### Effect of nitrogen source

Table 2 shows the effect of nitrogen source on antioxidant activity and phenol content. A significantly higher DPPH (70.39%), NO scavenging activity (41.63%), and total phenol content (16.81 mg/g) were produced when yeast extract was the carbon source by *X. feejeensis*. Although sodium nitrate as nitrogen source gave good antioxidant activity, it is very less compared to that of yeast extract. ME, casein, and peptone showed very less antioxidant activity.

#### Effect of shaking

Table 3 shows the effect of shaking on antioxidant activity and phenolic content. A higher DPPH (71.34%), NO scavenging activity (43.17%), and total phenol content (17.14 mg/g) were produced when the culture was kept at static condition by *X. feejeensis* HMJAU22039. The antioxidant activity was lowered when the culture was kept at 120 rpm shaking condition and was significantly lowered at 240 rpm shaking condition.

#### Effect of temperature

Maximum antioxidant activity of DPPH, NO scavenging, and TPC 72.41%, 44.14%, and 14.11 mg/g, respectively, was recorded at 30°C (Fig. 2). Low antioxidant activity and phenol content were found at very low and high temperatures 20°C and 40°C, respectively. An increasing antioxidant activity was recorded at a temperature between 20°C and 30°C after which a decrease in activity and phenol content was observed.

#### Effect of pH

Medium with initial pH 6 was found to be optimal for DPPH (68.12%), NO scavenging activity (39.03%), and total phenol content (16.61 mg/g) (Fig. 3). pH 5 and pH 7 also reported good antioxidant activity. Very little antioxidant activity was observed at pH 9.

#### Effect of incubation period

The incubation period at 20 days was observed to be optimum for maximum antioxidant activity, with 84.32%, 52.14%, and 24.42 mg/g for DPPH, NO scavenging, and TPC, respectively (Fig. 4). Maximum antioxidant activity was reported after the fungus reached its stationary phase after 20 days of incubation, the antioxidant activity was slightly lowered on the 25<sup>th</sup> day of the incubation period.

#### Effect of solvent

Maximum antioxidant activity of DPPH, NO scavenging, and TPC at 73.86%, 44.97%, and 18.36 mg/g, respectively, by *X. feejeensis* was reported by methanolic extract of the culture followed by ethyl acetate and chloroform extract (Table 4). Diethyl ether and toluene reported low antioxidant activity.

## DISCUSSION

Recent research showed that a number of potent antioxidants had been isolated from endophytic fungi [11-13]. Like many other metabolites

**Table 1: Effect of carbon source on antioxidant potential of *Xylaria feejeensis***

C source	DPPH	NO scavenging	TPC
Glucose	61.38±0.25	36.41±0.43	11.42±0.35
Dextrose	69.34±0.31	38.89±0.38	14.31±0.14
Maltose	33.12±0.56	18.13±0.33	4.37±0.59
Sucrose	59.64±0.12	28.64±0.52	8.83±0.28
Lactose	34.39±0.18	20.14±0.15	2.14±0.1

All values are reported as mean±SD (n=3). DPPH: DPPH radical scavenging activity, NO: NO scavenging activity, TPC: Total phenolic content, SD: Standard deviation, DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide

**Table 2: Effect of nitrogen source on antioxidant potential of *Xylaria feejeensis***

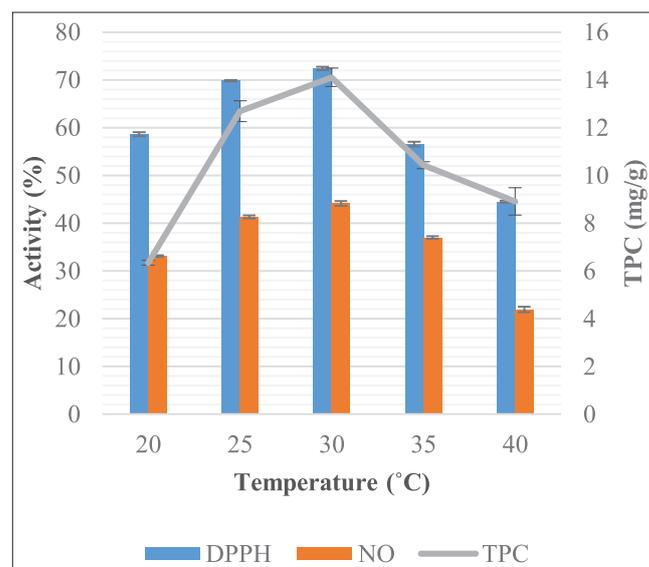
N source	DPPH	NO scavenging	TPC
Yeast extract	70.39±0.23	41.63±0.27	16.81±0.5
Malt extract	34.33±0.45	17.41±0.13	4.73±0.16
Peptone	38.19±0.44	19.29±0.48	5.97±0.22
Sodium nitrate	49.14±0.3	21.45±0.46	9.36±0.39
Casein	42.47±0.51	26.39±0.57	7.19±0.34

All values are reported as mean±SD (n=3). DPPH: DPPH radical scavenging activity, NO: NO scavenging activity, TPC: Total phenolic content, SD: Standard deviation, DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide

**Table 3: Effect of shaking on antioxidant potential of *Xylaria feejeensis***

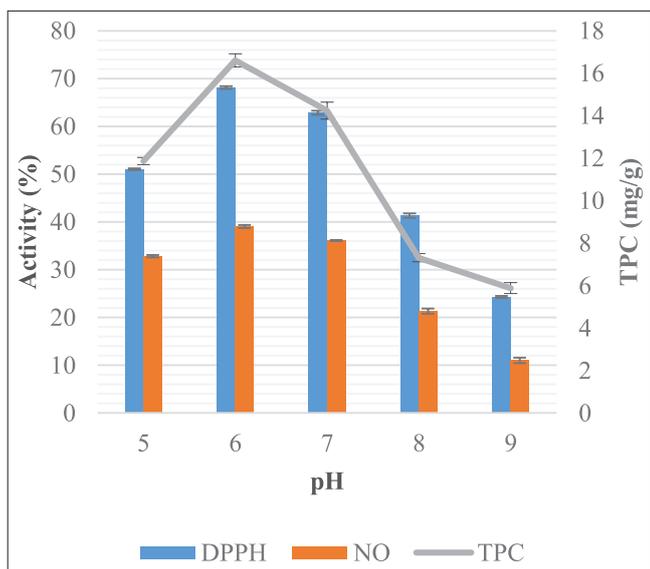
Condition	DPPH	NO scavenging	TPC
Static	71.34±0.5	43.17±0.2	17.14±0.18
Shaking 120 rpm	64.81±0.42	40.23±0.37	15.71±0.24
Shaking 240 rpm	53.91±0.45	33.76±0.16	11.32±0.31

All values are reported as mean±SD (n=3). DPPH: DPPH radical scavenging activity, NO: NO scavenging activity, TPC: Total phenolic content, SD: Standard deviation, DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide

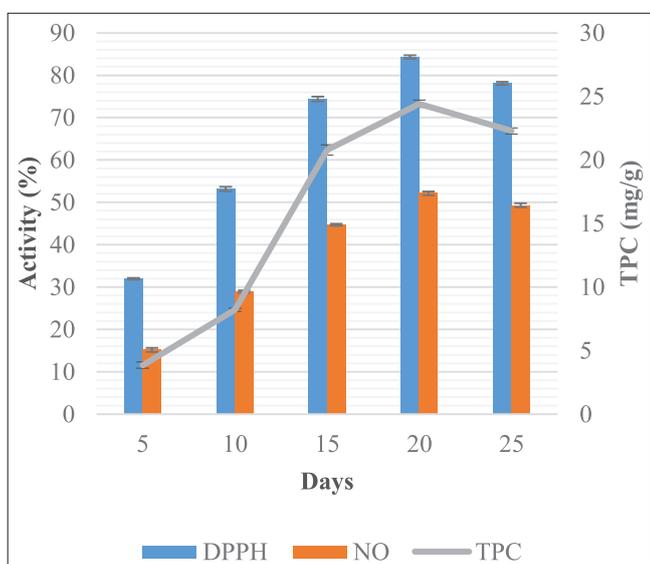


**Fig. 2: Effect of incubation temperature on antioxidant potential of *Xylaria feejeensis*. DPPH: DPPH radical scavenging activity; NO: NO scavenging activity; TPC: Total phenolic content. All values are reported as mean±SD (n=3). DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide, SD: Standard deviation**

productions of antioxidant compounds such as phenols and flavonoids depend on media components and their conditions. Variation of



**Fig. 3: Effect of pH on antioxidant potential of *Xylaria feejeensis*. DPPH: DPPH radical scavenging activity; NO: NO scavenging activity; TPC: Total phenolic content. All values are reported as mean±SD (n=3). DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide, SD: Standard deviation**



**Fig. 4: Effect of incubation period on antioxidant potential of *Xylaria feejeensis*. DPPH: DPPH radical scavenging activity; NO: NO scavenging activity; TPC: Total phenolic content. All values are reported as mean±SD (n=3). DPPH: 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide, SD: Standard deviation**

**Table 4: Effect of extraction with different solvents on antioxidant potential of *Xylaria feejeensis***

Solvent	DPPH	NO scavenging	TPC
Ethyl acetate	69.24±0.54	38.43±0.55	15.02±0.32
Chloroform	60.23±0.41	31.14±0.37	13.11±0.36
Diethyl ether	39.07±0.2	15.84±0.24	7.89±0.11
Methanol	73.86±0.49	44.97±0.26	18.36±0.4
Toluene	21.76±0.19	9.33±0.58	6.29±0.17

All values are reported as mean±SD (n=3). DPPH: DPPH radical scavenging activity, NO: NO scavenging activity, TPC: Total phenolic content, SD: Standard deviation, DPPH 1-1diphenyl-2-picryl-hydrazil, NO: Nitric oxide

physicochemical and nutritional parameters such as the type of carbon and nitrogen sources, pH, shaking, temperature, incubation period, and inoculum size of the fungal strain can greatly influence secondary metabolite biosynthesis [14]. Therefore, optimization of these parameters is a vital step for the enhancement of bioactive metabolites. The present study demonstrated the basic composition of potato dextrose yeast extract medium to be the best for the effective production of antioxidant activity of *X. feejeensis*. Dextrose and yeast extract were found to be promising carbon and nitrogen sources for obtaining good antioxidant activity by *X. feejeensis*. Many researchers found dextrose as an optimal carbon source for growth and metabolite synthesis [15]. Yeast extract as a competitive nitrogen source for optimum growth and metabolite production of another *Xylaria* species has been reported [16]. Potato infusion has been maintained at a constant level of 200 g/L throughout the experimental design as components present in the infusion are required by the fungi for a variety of regulatory functions and biosynthesis of various secondary metabolites. Static culture of *X. feejeensis* was found to be more suitable for antioxidant activity compared to shake flask culture. This reinforces earlier observation of different researchers who have used static conditions to optimize antioxidant activity [17]. It might be caused by the low amount of phenols produced under shaking conditions, which are important for antioxidant activity. This study also demonstrated that 20 days of incubation period was optimum for antioxidant activity, and the subsequent decline in bioactivity could be due to the depletion of nutrients available for the fungi. This reduction might also be caused by the degradation of secondary metabolites (phenols) produced by fungi as demonstrated by the reduction in the TPC. Earlier studies on fast growing species such as *Aspergillus*, *Penicillium*, and *Chaetomium* showed 10-15 days to be the optimum incubation period for antioxidant activity [18]. The increase in the optimum incubation period for antioxidant activity of *Xylaria* might be due to the relatively slow growth rate of the strain. A temperature of 30°C was found to be optimum for antioxidant activity which is also supported by earlier studies that report temperatures between 25°C and 30°C as optimum for antioxidant activity [19]. It is assumed that low temperature ceases the metabolic activity of the fungus, and high temperature kills the cell of the fungus. Very less activity was detected at pH extreme and was optimally best between the pH 6 and 7. The present results corroborate the previous studies done on *Saccharomyces cerevisiae* where very less antioxidant activity was reported at pH extremes [20]. The pH of a culture is associated with permeability of the cell wall and membrane which might affect ion uptake and loss to the nutrient medium [21]. Delayed metabolite production due to delayed mycelial growth may be the reason for reduced antioxidant activity in extreme pH conditions.

## CONCLUSION

The study highlights the importance of different media and physicochemical parameters in the production of secondary metabolites having antioxidant properties by *X. feejeensis* HMJAU22039. The results reveal a significant positive correlation between the DPPH radical scavenging activity, NO scavenging activity, and TPC.

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