Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 6 Issue 6 June 2023

Research Article

Screening and Identification of Novel Laccase Producing Entomopathogenic Fungus *Beauveria pseudobassiana* PHF4 and Optimization of Production Conditions of Enzyme

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DOI:10.31080/ASMI.2023.06.1259

Received: May 08, 2023

Published: May 19, 2023

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Abstract

In the present study, twenty-nine positive fungal isolates for production of laccase were obtained from decaying wood and soil samples from Shimla district of Himachal Pradesh, India. The positive isolates were confirmed by brown halo formation owing to oxidation of guaiacol by laccase enzyme. Out of the 29 isolates, the isolate PHF4, which produced the maximum laccase activity, was recognized as *Beauveria pseudobassiana*. Laccase displayed good activity in production medium comprising peptone (1.0%, w/v), malt extract (0.5%, w/v), glucose (2.0%, w/v) and $CuSO_4$ (0.005%, w/v). Optimization of inoculum size, incubation time, carbon source, carbon source concentration, nitrogen source, nitrogen source concentration, copper sulphate concentration, temperature and pH for laccase production was performed. The optimal inoculum size and incubation time for the laccase production were found to be 6 discs (1 cm in diameter) and 8 days respectively. Glucose (2.0%, w/v) and yeast extract (2.0%, w/v) were the most appropriate carbon and nitrogen source respectively for the production of enzyme. The presence of 0.003% (w/v) copper sulphate enhanced the laccase production. A temperature of 25°C and pH 6.0 were found to be optimum for laccase production. Under the optimum conditions, the maximum activity of enzyme was 21.47 U/ml which was 2.9 times higher than the unoptimized conditions. This is the first record of *Beauveria pseudobassiana* being isolated and optimized for the production of laccase.

Keywords: Laccase; Optimization; *Beauveria pseudobassiana;* Guaiacol; Yeast Extract

Introduction

Laccases (EC 1.10.3.2) are member of copper-containing oxidases which catalyze the breakdown of a broad diversity of inorganic and organic compounds, for example lignin, aromatic amines, phenolic substrates, phosphates, ketones and ascorbates [1,2]. To carry out reaction, laccases only need molecular oxygen and not hydrogen peroxide [3]. Laccases are able to convert substituted phenolic compounds into simpler ones by one-electron oxidation. This process is supplemented by reduction of oxygen to water [4].

In a Japanese lacquer tree called *Rhus vernicifera*, laccase was found for the first time [5]. Main sources of laccase enzyme are fungi but it is also secreted by bacteria, insects and higher plants. Gut of animals and plant tissues are sources of laccase-producing microbes [6]. Laccases are generally extracellular in nature, but bacterial laccases are generally periplasmic or intracellular [7-9]. Main sources of fungal laccases are: *Neurospora, Aspergillus, Trametes, Agaricus* and *Pleurotous*. Few laccases from marine fungi like *Diaporthe phaseolorum* and *Cerrena unicolor* have the ability to

decolorize textile dyes [10]. Crude extract from the fungus *Trametes pubescens* is utilized in the decomposition of chlorophenols and from *Pichia pastoris* is used for dye degradation such as indigo carmine [11].

Due to high catalytic efficiency of laccases, they are exploited for industrial applications in several sectors of biotechnology such as in the food sector for stabilization of wine and beer, in paper industry for delignification of pulp and for treatment of effluents, in pharmaceutical industry for synthesis of pharmaceutically important compounds like actinocin, which has anticancer property, in cosmetic industry in permanent hair dyes, as biosensors for identification of toxic compounds, in degradation of xenobiotic compounds like pesticides and polychlorinated hydrocarbons, detoxification of dyes, for the generation of energy in biofuel cells without producing greenhouse gases [12,13].

Due to numerous biotechnological applications, researchers have made attempt to isolate laccase producing microorganisms. In a study, laccase emitting fungus *Nectriella pironii* was isolated from soil [14]. In another study, laccase producing fungus *Galactomyces geotrichum* was also isolated from soil [9]. Cultivation conditions and medium composition are the key aspects affecting laccase production [15]. Culture conditions such as temperature, pH and type of nitrogen and carbon sources impacted the expression of laccase in fungi [16]. Copper sulphate can be added to the medium as inducer to boost laccase production from the chosen fungus. The concentrations of nitrogen and carbon sources are the most adaptable limiting factors for the synthesis of laccase [17].

The present work is focused on screening of laccase producing fungi and isolation of *Beauveria pseudobassiana* PHF4. The optimization of various culture conditions for production of laccase was performed.

Materials and Methods

Chemicals

All the chemicals used in the present investigation such as glucose, peptone, malt extract, copper sulphate, yeast extract, sodium chloride, sucrose, maltose, starch, lactose, ammonium chloride, tryptone etc. were of high analytical grade and procured from Hi-Media and Sigma, Aldrich (U.S.A).

Screening for laccase producing microorganism

In sterile sample collection bags, 25 samples of soil and 15 samples of decayed wood were gathered from various locations of Shimla district of Himachal Pradesh, India. To isolate laccase producing microorganisms, the decaying wood fungal samples were directly inoculated whereas samples taken from soil were serially diluted and streaked on potato dextrose agar (PDA) plates having guaiacol (0.02% v/v), chloramphenicol (0.01% w/v) and were incubated for 6-7 days for the initial screening [18]. This method assists in the selection of laccase-producing microorganisms by the formation of brown coloured halos zones around the growth of colony [19]. The positive isolates were further sub cultured and the activity of laccase was checked with the help of guaiacol assay method. The isolate PHF4 created a distinguished size of brown halo zone formation around the colony and so it was further selected for identification.

Identification of selected fungal isolate

The fungal isolate with high laccase production was identified by 18S rRNA sequencing carried out by Biologia Research India Pvt, Ltd. (Delhi, India). For multiple nucleotide sequence analysis, sequences of closely linked strains were downloaded from the National Center for Biotechnology Information (NCBI) service BLAST and aligned by using MEGA 11 software. The Neighbor-joining method was used to create the phylogenetic tree and a bootstrap consensus tree was estimated from 1000 replicates [20].

Extracellular laccase activity

Guaiacol assay method was used to calculate the activity of laccase [21]. The reaction mixture was prepared using 3 ml of sodium acetate buffer (10 mM), 1 ml of guaiacol (2 mM) and 1 ml of fungal supernatant. Additionally, a blank reaction was also setup comprising sodium acetate buffer (5 ml). The reaction tubes were kept at 30°C for 15 min. The mixture developed reddish brown colour as a result of laccase enzyme oxidizing the guaiacol. The oxidized substrate was read at 450 nm with the help of LABINDIA analytical UV/VIS spectrophotometer.

Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min.

Optimization of various parameters for extracellular laccase production by selected fungal isolate

Optimization of medium for laccase production

The chosen fungal isolate PHF4 was cultured in ten diverse production media (M1-M10) having pH 5.5 and was incubated at 25°C. The media reported by different researchers are mentioned in table 1.

Medium	Components (g/l)	Reference(s)
M1	Glucose (10.0), Peptone (3.0), $FeSO_4$ (0.0005), KH_2PO_4 (0.001), $MnSO_4$ (0.005), $ZnSO_4$ (0.4), $MgSO_4$ (0.005), K_2HPO_4 (0.0005),	[21]
M2	Peptone (10.0), Malt extract (5.0), $CuSO_4.5H_2O$ (0.005), Glucose (20.0)	[22]
M3	Yeast extract (2.5), Starch (20.0), H_3PO_4 (1.0), $FeSO_4$ (0.01), Na_2HPO_4 (0.05), $CaCl_2$ (0.01), $MgSO_4$ (0.5), $CuSO_4$ (0.002), $ZnSO_4$ (0.001), $MnSO_4$ (0.001)	[23]
M4	Sodium Chloride (5.0), $CaCl_2$ (1.0), Peptone (15.0), Tween 80 (1.0)	[24]
M5	Peptone (0.25), Yeast extract (0.25), Olive oil (12.0), Glucose (12.5), $MgSO_4.7H_2O$ (0.5), KCl (0.5), KH_2PO_4 (2.0)	[25]
M6	Sucrose (30.0), $FeSO_4$ (0.01), $MgSO_4$ (0.5), K_2HPO_4 (1.0), KCl (0.5)	[18]
M7	Glucose (1.0), Bagasse (10), Meat peptone (5.0), $CuSO_4$ (0.002)	[9]
M8	Glucose (10.0), $CuSO_4.5H_2O$ (2.0), K_2HPO_4 (0.4), $(NH_4)_2SO_4$ (3.3), KH_2PO_4 (0.6), $ZnSO_4.7H_2O$ (0.007), $MnSO_4.H_2O$ (0.5), $FeSO_4$ (0.05)	[26]
M9	Yeast extract (6.0), Maltose (30.0), Peptone (2.0), KH_2PO_4 (0.5), $MnSO_4$ (0.2), $MgSO_4$ (0.5), K_2HPO_4 (0.2),	[27]
M10	Peptone (5.0), Glucose (20.0), K_2HPO_4 (0.5), Yeast extract (0.5), $FeSO_4.7H_2O$ (0.001), $MgSO_4.7H_2O$ (1.0)	[28]

Table 1: Composition of different media screened for extracellular laccase production from fungal isolate PHF4.

Optimization of inoculum size and incubation time

To observe the impact of inoculum size, it was varied from 2 to 7 discs (1 cm in diameter) in the medium which is used for production of laccase. To optimize the time of incubation, the *Beauveria pseudobassiana* was grown for time period varying from 1 to 10 days. The production medium was assessed for optical density at 660 nm after every 24 hrs for 10 days.

Optimization of carbon source and its concentration

To select best carbon source, various sugars for example glucose, maltose, lactose, galactose, sucrose, fructose, xylose and starch

were added to the medium at 1% (w/v) concentration. A control without carbon source was also run. The carbon source which led to highest production of laccase was supplemented to the medium at different concentrations of 1%, 2%, 3%, 4%, 5% and 6% (w/v)

Optimization of nitrogen source and its concentration

Diverse sources of nitrogen including ammonium chloride, sodium nitrate, tryptone, beef extract, yeast extract, urea, peptone, ammonium nitrate and ammonium sulphate at concentration of 1% (w/v) were checked for their impact on production of laccase. A control was run without the source of nitrogen. To analyze the influence of the selected nitrogen source, it was supplemented in the production medium at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% (w/v)) and enzyme activity was evaluated.

Effect of concentration of copper sulphate on production of laccase

Various concentrations of copper sulphate (0.001%, 0.003%, 0.005%, 0.007%, 0.009% and 0.011% (w/v)) were used to check the impact on laccase production and laccase activity was assayed.

Optimization of temperature and pH

To investigate consequences of change of temperature on production media, fungal isolate was grown at various temperatures such as 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. For optimization of pH, production medium was prepared at various values of pH such as 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The medium was inoculated with selected fungal isolate and activity of laccase was calculated.

Results and Discussion

Screening for laccase producing fungal isolates

Isolates were analyzed for the production of laccase. Their capability of oxidation of guaiacol caused brown halo zones in the vicinity of fungal mycelium on the PDA plates having guaiacol. This confirmed the laccase production by the organism. 29 fungal isolates were found to give extracellular laccase activity. The isolate PHF4 was observed to have good brown halo zone around colony and highest laccase activity of 5.45 U/ml (Figure 1a).

(a)

(b)

Figure 1: (a) PDA plate showing growth of fungal isolate PHF4 (b) Phylogenetic tree of *Beauveria pseudobassiana* PHF4 using neighbor-joining method.

Identification of the fungal isolate PHF4

The fungal isolate PHF4 was characterized by using 18S rRNA gene sequencing and was found to be *Beauveria pseudobassiana* (NCBI Accession No.: ON916161.1). MEGA 11 software was used to examine the 18S rRNA sequences of various strains for evolutionary relationships and phylogenetic variation. The obtained sequence was further analyzed and the results showed 99.69% homology with *Beauveria pseudobassiana* strain YFCC (Figure 1b).

Optimization of various parameters for extracellular production of laccase by *Beauveria pseudobassiana* PHF4

Optimization of medium

The production medium M2 containing glucose (2.0%, w/v), malt extract (0.5%, w/v), peptone (1.0%, w/v) and $CuSO_4$ (0.005%, w/v) showed the highest laccase activity of 7.37 U/ml among the various production media used (Table 2). Peptone and malt extract enhanced the production of laccase. This might be due to the fact that the complex nitrogen sources are usually preferred more because they are broken down to create nucleic acids, proteins and amino acids which may provide all essential nutrients to the microbial system for the production of enzyme [15]. Researchers have reported the rise in the synthesis of laccase by fungi when copper or various aromatic compounds are added to the medium [29]. In a previous study, culture medium composed of glucose, peptone, ammonium tartarate, yeast extract, KCl, KH_2PO_4, $MgSO_4$ and $CuSO_4$ gave maximum laccase production from *Penicillium chrysogenum* [24]. For *Neofusicoccum, Ulocladium, Lophiostoma, Pringsheimia, Hormonem* and *Dothiorella* the highest laccase production was observed using medium containing $C_4H_{12}N_2O_6$, yeast extract, $MgSO_4 \cdot 7H_2O$ and KCl [17].

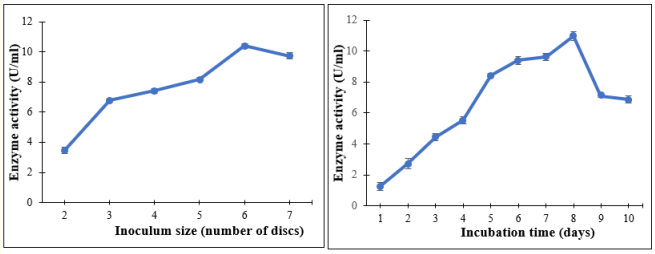
Name of medium	Enzyme activity (U/ml)
M1	3.52 ± 0.16
M2	7.37 ± 0.11
M3	5.29 ± 0.14
M4	2.47 ± 0.27
M5	0.54 ± 0.08
M6	1.66 ± 0.14
M7	0.81 ± 0.10
M8	1.09 ± 0.17
M9	2.84 ± 0.20
M10	6.22 ± 0.15

Table 2: Effect of various media on production of laccase from *Beauveria pseudobassiana* PHF4.

Effect of inoculum size and incubation time

The maximum activity of laccase (10.38 ± 0.13 U/ml) was achieved by the inoculum size of 6 discs (1 cm in diameter) (Figure 2a). The size of inoculum is a major factor for enzyme production, as with the size, the active growing cells increase in number. This could be due to the synthesis of appropriate amount of mycelium which causes the right amount of production of laccase. It has been reported that with the increase in the size of inoculum, the activity of laccase enzyme decreased which could be due to decreased synthesis of enzyme as the nutrients were primarily got consumed in the growth of fungus [30]. Earlier, the highest activity of laccase in *Aspergillus ochareaceous* was observed with inoculum size of 3 discs (1.5 cm in diameter) [31].

Optimal incubation time for maximum laccase production (10.98 ± 0.28 U/ml) was found to be 8 days (Figure 2b). After 8 days, enzyme production decreased which can be related to depletion of minerals and nutrients that would have resulted into adding up of toxic compounds in the medium and decrease in the laccase production [32]. The highest production of laccase in *Penicillium chrysogenum* was observed on 5th day [24]. In previous studies, the highest enzyme production in *T. harzianum* and *P. purpurogenum* was obtained on sixth day [33]. In *G. geotrichum,* the optimal time for production of laccase was found to be 14 days [9].



(a) (b)

Figure 2: (a) Effect of inoculum size on the production of laccase from *Beauveria pseudobassiana* PHF4 (b) Effect of incubation time on laccase production from *Beauveria pseudobassiana* PHF4.

Optimization of carbon source

Maximum production of extracellular laccase (11.54 ± 0.13 U/ml) was attained with the medium enriched with glucose (1%, w/v) as carbon source (Figure 3a). Least effective carbon source for laccase production was sucrose with activity of 0.68 ± 0.34 U/ml. Other researchers have reported that when sucrose was present in excess amount, it led to the reduction in the laccase production by blocking induction and permitted only constitutive production

[34]. Glucose has been observed as ideal source of carbon for production of laccase by *P. ostreatus* and *T. versicolor* [35]. A 3-fold increase in the production of laccase by *Coriolus versicolor* MTCC 138 was observed when glucose was used instead of fructose [36] whereas, highest laccase activity from *Trametes versicolor* was observed in presence of maltose as carbon source [37].

Various concentrations of glucose were used in the production medium and 2% (w/v) was found to give the maximum laccase production of 13.66 ± 0.27 U/ml (Figure 3b). It was observed that the addition of glucose in the production medium beyond particular concentration inhibited the laccase synthesis in the fungus. Rise in glucose concentration leading to reduction in production of laccase is possibly attributed to operon recessive effect due to catabolic repression [38]. The glucose concentration of 0.8% (w/v) was found best for production of laccase by *T. versicolor* and *P. ostreatus* [35]. In another study, glucose concentration of 1% (w/v) was observed to be best for laccase production by *A. alternata* [39].

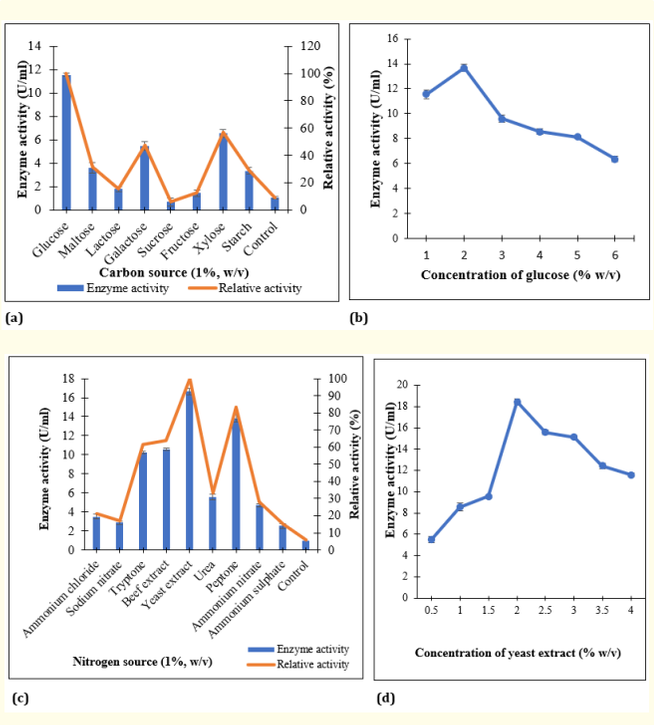


Figure 3: (a) Effect of different carbon sources on production of laccase from *Beauveria pseudobassiana* PHF4 (b) Effect of different concentrations of glucose on production of laccase from *Beauveria pseudobassiana* PHF4 (c) Effect of different nitrogen sources on laccase production from *Beauveria pseudobassiana* PHF4 (d) Effect of different concentrations of yeast extract on production of laccase from *Beauveria pseudobassiana* PHF4.

Optimization of nitrogen source

Nitrogen source is crucial for synthesis of enzymes in microbes. It acts as a source for many types of amino acids and cell growth factors which are essential for cell metabolism and synthesis of enzyme [40]. When yeast extract was added as nitrogen source, the maximum activity of laccase (16.64 ± 0.3 U/ml) was recorded (Figure 3c). Organic nitrogen sources intensify the production of majority of the fungal laccases. Nitrogen is reported as limiting factor for the ideal laccase production in some fungi such as *Botryosphaeria* sp. and *Pycnoporus cinnabarinus* [41]. Many workers have reported maximum laccase production using soya meal as nitrogen source [35,42].

In the current investigation, the highest laccase production of 18.45 ± 0.24 U/ml was attained with 2% (w/v) yeast extract concentration (Figure 3d). At higher concentration of yeast extract, reduction in the activity of laccase can be owing to inability of the fungus to hydrolyze yeast extract. In a previous study, when soya bean (0.6%) was used as only nitrogen source in the production, there was 31-fold rise in the activity of laccase when compared with unoptimized medium [42]. The production of laccase from *Ganoderma* sp. has been increased by the addition of yeast extract [43]. Yeast extract is an undefined growth supplement which is made up of nucleotides, peptides, amino acids and additional constituents of yeast cells. Malt extract and beef extract were also reported to enhance the activity of laccase [31].

Effect of copper sulphate on production of laccase

In this study, the maximum laccase production (20.2 ± 0.11 U/ml) by *Beauveria pseudobassiana* PHF4 was noted when 0.003% (w/v) concentration of copper sulphate was added to the medium (Table 3). According to reports, many fungal species, including *M. roridum, A. flavus* and *Peniophora* sp. are strongly induced to produce laccase when copper is present in the production medium. In addition to controlling the expression of the laccase gene, copper also influences the stability and activity of the enzyme positively by inhibiting the activity of extracellular proteases [44]. However, laccase production does not always benefit from increase in copper concentration. High concentrations of copper ion act as a potent inhibitor of fungal growth. In a study, laccase production from *Penicillium chrysogenum* was considerably improved at 0.002% (w/v) of copper sulphate concentration [24]. In *Dichomitus squalens* and *Trametes versicolor,* the highest laccase activity was noted with copper sulphate concentration of 0.005% (w/v) [39]. A copper sulphate concentration of 30 μM was found best for production of laccase from *G. lucidum* [45].

Concentration of copper sulphate % (w/v)	Enzyme activity U/ml
0.001	9.26 ± 0.24
0.003	20.20 ± 0.11
0.005	17.49 ± 0.30
0.007	12.46 ± 0.25
0.009	9.24 ± 0.19
0.011	7.43 ± 0.19

Table 3: Effect of concentration of copper sulphate on the production of laccase from *Beauveria pseudobassiana* PHF4.

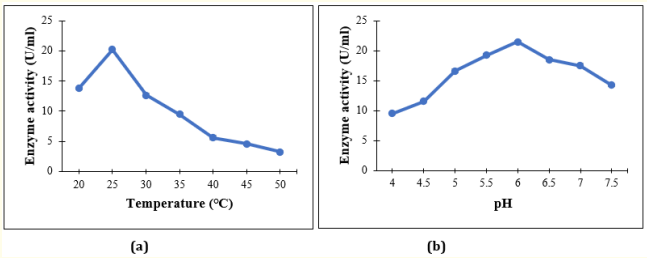


Figure 4: Effect of (a) temperature and (b) pH on the production of laccase from *Beauveria pseudobassiana* PHF4.

Effect of temperature and pH

In the present study, highest laccase production (20.25 ± 0.16 U/ml) (Figure 4a) was observed at 25°C. Temperature is the key factor that strongly influences the activity of fungal enzymes [16]. As the temperature rose further, the laccase activity dropped. Higher temperatures have been suggested to have caused deactivation of enzyme, which reduced the laccase activity [46]. For ideal laccase production, majority of the fungi have been grown at temperature between 25°-30°C [47]. The maximum activity of laccase in *Hormonema* sp. was noted at 80°C. The ideal temperature for laccases ranges typically between 30-60°C, but higher temperature (70°C) have been observed for *G. lucidum* [17]. *A. alternata* showed best laccase activity at 30°C [39].

The production medium having pH 6.0 exhibited highest laccase activity of 21.47 ± 0.18 U/ml (Figure 4b). This may be due to the organism's need for a pH that is slightly acidic for both laccase production and its metabolic processes. These could also be because this pH upholds the crucial three-dimensional structure essential for high laccase activity. pH of medium is crucial for absorption of nutrients, growth of organism and stimulation of production of enzymes via various pathways of signaling. The highest laccase activity for *B. subtilis* was found at pH 6.5 [48]. Another study found that *T. harzianum* needs a pH of 5.0 for growth and production of laccase [33]. Recently, the highest laccase production has been found at pH 5.0 from *Ganoderma leucocontextum* [43].

Conclusion

The present study shows that *Beauveria pseudobassiana* PHF4 has the ability to produce extracellular laccase. The production of laccase is positively influenced by a number of production parameters, including inoculum size, incubation time, carbon source, carbon source concentration, nitrogen source, nitrogen source concentration, copper sulphate concentration, temperature and pH. The activity of laccase obtained under optimized conditions was 21.47 U/ml, which was 2.9 times higher than the unoptimized conditions. The laccase enzyme obtained from *Beauveria pseudobassiana* PHF4 can be used in synthesis of pharmaceutically important compounds and in degradation of xenobiotic compounds like pesticides and polychlorinated hydrocarbons.

Fundings

Financial assistance from University Grant Commission (UGC), Grant number [UGC-Ref. No.:2922/(CSIR-UGC NET JUNE 2019)] to Ms. Divya Sharma and from Council of Scientific and Industrial Research (CSIR), New Delhi, Grant number [File No. 09/237(0170)/2018-EMR-I] to Ms. Manpreet Kaur is thankfully acknowledged.

Compliance with Ethical Standards

This article does not contain any studies with human participants performed by any of the authors.

Ethical Declarations

The article contains no data of research with animal objects.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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