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Spectral characterization of a pteridine derivative from cyanide-utilizing bacterium *Bacillus subtilis* - JN989651

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Soil and water samples were collected from various regions of SIPCOT and nearby Vanappadi Lake, Ranipet, Tamilnadu, India. Based on their colony morphology and their stability during subculturing, 72 bacteria were isolated, of which 14 isolates were actinomycetes. Preliminary selection was carried out to exploit the ability of the microorganisms to utilize sodium cyanate as nitrogen source. Those organisms that were able to utilize cyanate were subjected to secondary screening viz., utilization of sodium cyanide as the nitrogen source. The oxygenolytic cleavage of cyanide is dependent on cyanide monooxygenase which obligately requires pterin cofactor for its activity. Based on this, the organisms capable of utilizing sodium cyanide were tested for the presence of pterin. Thin layer chromatography (TLC) of the cell extracts using n-butanol: 5 N glacial acetic acid (4:1) revealed that 10 out of 12 organisms that were able to utilize cyanide had the pterin-related blue fluorescent compound in the cell extract. The cell extracts of these 10 organisms were subjected to high performance thin layer chromatography (HPTLC) for further confirmation using a pterin standard. Based on the incubation period, cell biomass yield, peak height and area, strain VPW3 was selected and was identified as *Bacillus subtilis*. The Rf value of the cell extract was 0.73 which was consistent with the 0.74 Rf value of the pterin standard when scanned at 254 nm. The compound was extracted and purified by preparative High Performance Liquid Chromatography (HPLC). Characterization of the compound was performed by ultraviolet spectrum, fluorescence spectrum, Electrospray Ionization-Mass Spectrometry (ESI-MS), and Nuclear Magnetic Resonance spectroscopy (NMR). The compound is proposed to be 6-propionyl pterin (2-amino-6-propionyl-3*H*-pteridin-4-one).

***Keywords*:** cyanide monooxygenase, *Bacillus subtilis*, HPLC, ESI-MS, NMR, 6-propionyl pterin

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Introduction

Pteridines are heterocyclic nitrogen compounds that are metabolically important as cofactors of enzymes associated with growth and differentiation (Chapman, 1969) viz., aromatic amino acid (phenylalanine, tyrosine, and tryptophan) hydroxylases (AAH), nitric oxide synthases (NOS) and cyanide monooxygenase (CNO). The redox potential of some pteridines indicates that they play a key role in cellular electron transport (Rembold, 1975). Pterin compounds are broadly classified into 2 major classes, 'conjugated' and 'unconjugated'. The classification is based on the complexity of the side chains. Folic acid and methanopterin belong to the conjugated type, which has a linkage of p-aminobenzoic acid to pterin. Biopterin, molybdopterin and pterin-containing glycosides belong to the unconjugated type since they bear less complex side chains at the 6-position of the pterin (Cho *et al*., 1998).

Pterin derivatives were found to be produced in abundance by certain prokaryotes, especially cyanobacteria (Forrest *et al*., 1958; Hatfield *et al*., 1961; Forrest and Van Baalen, 1970; Matsunaga *et al*., 1993). A high concentration of pterins (2-amino 4-hydroxy pteridines) was first reported in *Anacystis nidulans* (Forrest *et al*., 1957). *Synechococcus* sp. and *Spirulina platensis* are the other cyanobacteria that produce pterin derivatives. Anaerobic photosynthetic bacteria *Chlorobium tepidum* (Cha *et al*., 1995), *Chlorobium limicola* (Cho *et al*., 1998), a chemoautotrophic archaebacterium *Sulfolobus solfataricus* (Lin and White, 1988), and a methanogenic bacterium *Methanoculleus thermophilus* were found to contain pterin glycosides. The functions of parent biopterin have been studied in detail but the physiological roles of other forms remain obscure.

Cyanide is a potent poison that arises in the environment by natural and anthropogenic means. Cyanide is highly toxic to living organisms since it inactivates the respiration system by tightly binding to cytochrome C oxidase (Solomonson and Spehar, 1981; Chena and Liu, 1999; Yanase *et al*., 2000). Microbial degradation of cyanide involves enzymatic pathways and generally these are induced by the presence of cyanide in the medium. The oxidative pathway of cyanide conversion involves oxygenolytic conversion to carbon dioxide and ammonia. The oxidative pathway involves cyanide mono-

oxygenase and cyanase. Cyanide monooxygenase (Raybuck, 1992; Ebbs, 2004) converts cyanide to cyanate. The cyanate is then catalyzed by cyanase resulting in the conversion of cyanate to ammonia and carbon dioxide. Cyanide monooxygenase is located in the cytosolic fraction of cells induced with cyanide and requires both reduced pyridine nucleotide (NADH) and a source of reduced pterin as a cofactor (Kunz *et al*., 2001; Fernandez *et al*., 2004). Assuming that the cofactor production also increases with increased production of metabolic enzymes, an attempt has been made in the present study to isolate cyanate- and cyanide-utilizing bacteria, including actinomycetes, from the soil and water samples for extraction of pteridine compound(s).

The separation of pteridines has been accomplished by a variety of chromatographic techniques and in particular, High-Performance Liquid Chromatography (HPLC), which has become the method of choice to separate and quantify pterin derivatives, mainly because of its speed, reproducibility and cost (Kaneko *et al*., 2001). For concentration of samples prior to HPLC analysis, Dowex resin columns (Fukushima and Nixon, 1980; Ziegler, 1985) and solid-phase cartridges with (Werner *et al*., 1987) or without (Slazyk and Spierto, 1990; Rippin, 1992) direct insertion of cartridges into the HPLC-eluent stream, have been used. To remove protein from the same, precipitation by acids (Fukushima and Nixon, 1980; Braiutigam and Dreesen, 1982), ultrafiltration (Powers *et al*., 1988; Candito *et al*., 1993) and deproteinization on a first column using column switching (Huber and Lamprecht, 1995) have been employed (Werner *et al*., 1996).

Unfortunately, the reduced pterins (tetrahydrobiopterin-BH_4) show no intense fluorescence, because the rings of the molecule are not in the fully oxidized, aromatic state. To overcome the difficulty in measuring BH_4 using fluorescence detection, the differential oxidation method was introduced by Fukushima and Nixon (1980). By this differential oxidation method, oxidation under the acidic condition converts both BH_4 and dihydrobiopterin into biopterin; whereas, under the alkaline condition, only dihydrobiopterin is oxidized into biopterin.

Though pterins have immense potential in the field of medicine, the synthesis or purification of substantial amounts of these pigments from a microbial source have so far not been reported. With this information in mind, our present work aimed at screening for the presence of pterins among certain bacteria with cyanide degrading ability isolated from an industrial area. Extraction and purification of a pterin compound by an HPLC technique, was followed by characterization of the purified compound based on ultraviolet/visible absorption spectrometry, infrared spectroscopy, excitation/emission properties, electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance spectroscopy (NMR) studies.

Materials and Methods

Location and sample collection

Soil and water samples were collected aseptically in sterile containers from ten different places in the SIPCOT industrial region, Ranipet (12° 55′ 39″ N, 79° 19′ 48.72″ E) and nearby Vanappadi Lake (12°57′52″N, 79°19′2″E), Tamilnadu, India. The samples were brought to the laboratory within 48 h of collection and subsequently the isolation of bacteria (including actinomycetes) was carried out.

Isolation and screening of microorganisms for the presence of pterin

Microbial isolation from the contaminated soil and water samples was performed by serial dilution and spread plates. Nutrient agar medium and starch casein agar medium (Himedia) were used for the isolation and enumeration of bacteria and actinomycetes respectively. The screening of the isolates was based on their ability to utilize sodium cyanate as sole source of carbon and nitrogen when added to M9 minimal medium at various concentrations (10 mM, 20 mM, and 30 mM). Organisms that were selected were further screened for their ability to utilize sodium cyanide at 2 mM concentration. The presence of pterin in the bacterial isolates was confirmed by Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) techniques.

Identification and taxonomic positioning of 16S rRNA gene sequence of VPW3

Based on the screening results, the isolate VPW3 was selected for further studies. The isolate VPW3 was identified to the genus level by observing its morphological and biochemical characteristics (Buchanan *et al*., 1974). The bacteria was identified to the species level based on the 16S rRNA gene nucleotide sequence obtained by PCR amplification of the 16S rRNA gene, followed by DNA sequencing, BLAST analysis (Altschul *et al*., 1990) and comparison with sequences in the GenBank nucleotide databases. The 16S rRNA gene was amplified using the universal primers, forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-GGTTACCTTGTTACGACTT-3′ (James, 2010). The DNA sequencing was outsourced to Xcelris Labs Ltd. The 16S rRNA sequence was analyzed for its similarity and homology with the existing sequences available in the data bank of the National Center for Biotechnology Information (NCBI) using the BLAST search software available at http://www.ncbi.nlm.nih.gov/. The sequences were aligned using the ClustalW program within BioEdit version 7.0.5.3 and a phylogenetic tree was constructed by the Neighbor-joining method (Saitou and Nei, 1987) using PHYLIP software version 3.69. Treeview1.6.6 was used to visualize the phylogenetic tree and a bootstrap analysis using 1,000 replicates was carried out (Hentschel *et al*., 2001).

Culture conditions

The bacterium VPW3 was grown in specialized M9 minimal medium. The production medium contains the following ingredients (per L of distilled water): Na_2HPO_4 12.8 g, KH_2PO_4 3.0 g, NaCl 0.5 g, 1 M $MgSO_4$ 2.0 ml, 1 M $CaCl_2$ 0.1 ml, 50 mM sodium acetate. The pH of the medium was adjusted to 9.5 and it was then autoclaved. 10 ml of 20% filter-sterilized glucose and membrane (0.2 μm)-sterilized 2 mM sodium cyanide was added after cooling the medium to room temperature (Maniatis *et al*., 1982). The seed culture

was prepared by inoculating the bacterium in nutrient agar broth for 24 h at 37°C prior to fermentation. The seed culture at 1% concentration and OD_{600} adjusted to 1.0 was used as inoculum for fermentation. The broth was incubated at 37°C for a period of 5 days. The cultured cells were harvested by centrifugation at 8,000 × *g* and suspended in 10 mM phosphate buffer, pH 7.0, and stored at -20°C until further use (Almagro *et al.*, 2005).

Extraction of pteridine derivatives

The cells were sonicated in an ultrasonic water bath and centrifuged at 10,000 × *g*. To the supernatant, 0.3 M perchloric acid was added for deproteinization (Klein *et al.*, 1991). Activated charcoal was then added directly, with intermittent swirling, and incubated for 5 min. The process was carried out in the dark. The mixture was filtered through a Whatman No.1 paper filter and the filtrate was discarded. The bed was then washed with 100 ml of water and the fluorescent substances eluted with ethanol containing 1% NH_3. The eluate was concentrated *in vacuo* (Ikawa *et al.*, 1995).

Oxidation of sample

The resulting eluate (1.0 ml) was mixed with an equal volume of 0.2 N NaOH. The oxidation of the sample was initiated by the addition of 100 µl of acidic iodine solution [0.9% (w/v) iodine and 1.8% (w/v) potassium iodide in 0.1 N HCl]. The tubes were incubated in the dark at room temperature for 1 h. The tubes were centrifuged at 10,000 × *g* for 10 min at room temperature to remove the precipitated proteins. To the supernatant, 100 µl of 2% (w/v) ascorbic acid was added to reduce the excess iodine. The sample was filtered through a 0.2 µm syringe filter and stored at -20°C until further use (Fukushima and Nixon, 1980).

Purification of pteridine derivative

Conventional purification: The eluate was initially purified by silica (60–120 mesh) column chromatography using a chloroform: methanol step gradient (100:0 to 0:100). A volume of 250 ml was collected per step and analyzed for the presence of blue fluorescent compound by TLC. The fractions containing the blue fluorescent compound were pooled and concentrated *in vacuo*.

Purification by preparative HPLC: The concentrated sample was subjected to preparative HPLC equipped with an octadecylsilane column (Deltapak C18) equipped with a UV detector. The column was eluted isocratically with 2% aqueous methanol at a flow rate of 20 ml/min. The UV-absorbing peak at 254 nm with a retention time of 4.2 min was collected and concentrated. The purity was determined by analytical HPLC using a NovaPak C18 column eluted with 5% aqueous methanol. The purified pterin was concentrated by rotary evaporator and stored at -20°C for further study (Cho *et al.*, 1998; Fernandez *et al.*, 2004).

Characterization of pure compound

Ultraviolet/Visible absorption spectrometry: Ultraviolet/Visible absorption spectra for the purified compound along with standards, pterin and biopterin dissolved in 10 mM phosphate buffer, pH 6.0, were recorded in the range of 200–700 nm on a Jasco V-630 spectrophotometer.

Fluorescence spectrometry: The excitation/emission properties of the sample were recorded using a Jasco FP 6600 spectrofluorometer. The compound was excited at 360 nm and its emission was observed in the range of 400–600 nm.

Infrared spectrometry: A pinch of KBr pellet was oven dried and transferred into a mortar. The purified sample at about 0.1–2% was added, mixed and ground to a fine powder. The mid-range IR spectra, 4000–400 cm^{-1} was recorded on a Nicolet Avatar Model FT-IR spectrophotometer.

Electrospray ionization-mass spectrometry (ESI-MS): The electrospray mass spectra were recorded on a THERMO-Finnigan LCQ Advantage Max ion-trap mass spectrometer. The sample (dissolved in methanol) was introduced into the ESI source through a Finnigan Surveyor autosampler. The mobile phase, methanol:water (9:1), was pumped at the rate of 250 µl/min. Ion-spray voltage was set at 5.3 KV and capillary voltage at 34 V. The MS scan ran up to 2.5 min and the print-outs of the spectra were an average of more than 10 scans. The chromatogram is created by plotting the total ion current (TIC) in a series of mass spectra recorded as a function of retention time.

Nuclear magnetic resonance spectrometry (NMR): The ^1H of the sample was recorded using a Bruker Avance III, at 500 MHz with DMSO–d_6 as the solvent.

Table 1. Isolation of bacteria and actinomycetes from industrially contaminated soil and water samples

Industries	Bacteria	Actinomycetes
Soil samples		
Chemical industries	B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, B14, B15, B16, B17, B18, B19	–
Pharmaceutical industries	MD1, MD2, MD3, MD4, M1, M2, M3, M4, M5, M6	MDA1, MDA2, MDA3
Fabrication industries	CG1, CG2, CG3, CG4, CG5, CG6, CG7	–
Leather industries	TCL1, TCL2, TCL3, TCL4, TCL5, TCL6	–
Vanapadi lake	VP1, VP2, VP3, VP4, VP5	VA1, VA2, VA3, VA4, VA5, VA5, VA7
Water samples		
Pharmaceutical industries	MW1, MW2	MWA1
Fabrication industries	TCEW1, TCEW2, TCEW3, TCEW4, TCLW1, TCLW2	–
Leather industries	–	–
Vanapadi lake	VPW1, VPW2, VPW3	VPWA1, VPWA2, VPWA3

Results and Discussion

Isolation of bacteria

Based on the colony morphology and stability during subculturing, 72 organisms were isolated from soil samples collected from various regions of the SIPCOT industrial area and nearby Vanappadi lake. Of the 72 bacterial isolates, 14 were actinomycetes. The organisms were designated based on the area of sample collection (Table 1).

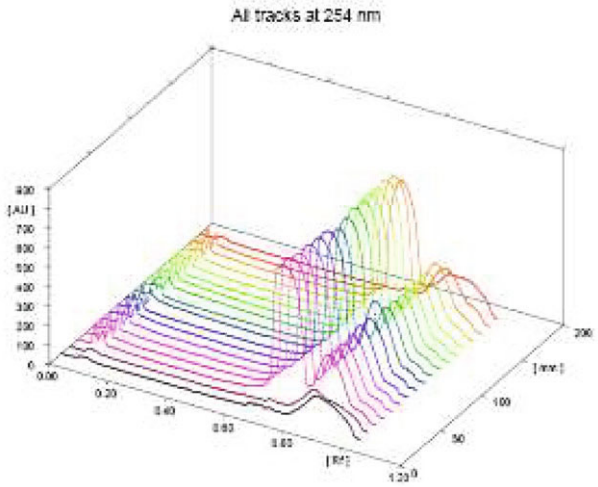
Screening of microorganisms for the presence of pterin

Selection of microorganisms based on the ability to degrade cyanate and cyanide: Out of 72 isolates, 46% of the organisms were able to utilize 10 mM sodium cyanate as nitrogen source. Of these isolates, 33% and 22% tolerated 20 mM and 30 mM of sodium cyanate respectively. Only isolates VPW3 and MW1 were able to tolerate up to 35 mM of sodium cyanate. Among the 16 selected isolates, 11 bacterial isolates designated as B2, B9, B13, B14, B19, MD2, MD4, MW1, VPW3, TCLW1, VP3, and 5 actinomycete isolates namely VA1, VA2, VA5, MDA1, MDA2 were able to tolerate up to 30 mM of sodium cyanate. The selected strains were then tested for cyanide degradation. Among these, 12 isolates were able to utilize sodium cyanide as sole nitrogen source.

The microorganisms involved in the biological treatment of cyanide and thiocyanate usually include a heterogeneous mixture of commonly found indigenous soil bacteria that have adapted due to chronic exposure to these compounds (Mudder *et al*., 2001). It has been reported that *P. fluorescens* NCIMB 11764 utilizes cyanide as a source of nitrogen (Harris and Knowles, 1983a, 1983b; Rollinson *et al*., 1987). Furuki *et al.* (1972) have reported a bacterium utilizing 40– 50 µg/ml of cyanide as a nitrogen source with an additional source of carbon. On the other hand, the species *Klebsiella pneumoniae, Moraxella*, *Serratia*, and *Alcaligenes* species were isolated and identified as cyanide-degrading bacteria (Ingvorsen *et al*., 1991; Kang and Kim, 1993) that utilized cyanide as a sole source of carbon and nitrogen. A *Bacillus pumilus* strain (Skowronski and Strobel, 1969) was also found to grow on cyanide as the sole source of carbon and nitrogen. Two facultative autotrophs, both actinomycetes of the genus *Nocardia*, and in another case a Gram-positive filamentous organism, probably again an actinomycete, converting cyanide to ammonia, were found to be capable of growing on cyanide as a sole source of carbon and nitrogen (Knowles, 1976).

Dorr and Knowles (1989) have reported that the enzyme responsible for the conversion of cyanide into ammonia and carbon dioxide in cell-free extracts of *P. fluorescens* NCIMB 11764 was cyanide monooxygenase, with the formation of cyanate as a metabolic intermediate. White *et al.* (1988) reported the conversion of cyanide to formate and ammonia by a *Pseudomonas* sp. isolated from industrial waste water. In addition, *Alcaligenes xylosoxidans* subspecies *denitrificans* (Ingvorsen *et al*., 1991) and *B. pumilus* (Meyers *et al*., 1991) were also found to convert cyanide to ammonia and formate. The reason behind choosing cyanide degrading bacteria was to increase the production of the pteridine derivative that acts as a cofactor for cyanide degrading enzymes. Our aim is to utilize the purified pteridine derivative for anticancer therapy.

Analysis of pterin : The cell extract of all the 12 isolates that were able to utilize sodium cyanide were oxidized and analyzed for the presence of pterin by TLC and HPTLC methods and compared with the standards. Among the various solvent systems used n-butanol: 5 N glacial acetic acid (4:1) was the most suitable and showed the maximum separation of compounds. Among the 12 isolates, only 10 strains showed blue fluorescence when observed under UV light at 254 nm. The blue fluorescence was intense in the cell extract of VPW3. There was an absence of fluorescence in the cell extract of B13 and MD2. One of the properties of pteridines is intense fluorescence, which is observed only when both the rings are in the fully oxidized and aromatic state. The reduced pterins, such as tetrahydrobiopterin, show no intense fluorescence. Normally in the cells, pterins occur in the di-



Track No.	Applied position (mm)	Applied volume (µl)	Sample ID	Active
1	15	5	STD[a]	Yes
2	25	5	STD[a]	Yes
3	35	5	VA2	Yes
4	45	5	MW1	Yes
5	55	5	B9	Yes
6	65	5	B14	Yes
7	75	5	B19	Yes
8	85	5	VA1	Yes
9	95	5	VPW3	Yes
10	105	5	VA5	Yes
11	115	5	MDA1	Yes
12	125	5	MDA2	Yes
13	135	5	MW1	Yes
14	145	5	B9	Yes
15	155	5	B14	Yes
16	165	5	B19	Yes
17	175	5	VA1	Yes
18	185	5	STD[a]	Yes

[a] STD denotes standard pterin

Fig. 1. HPTLC chromatogram of selected strains compared with standard pterin.

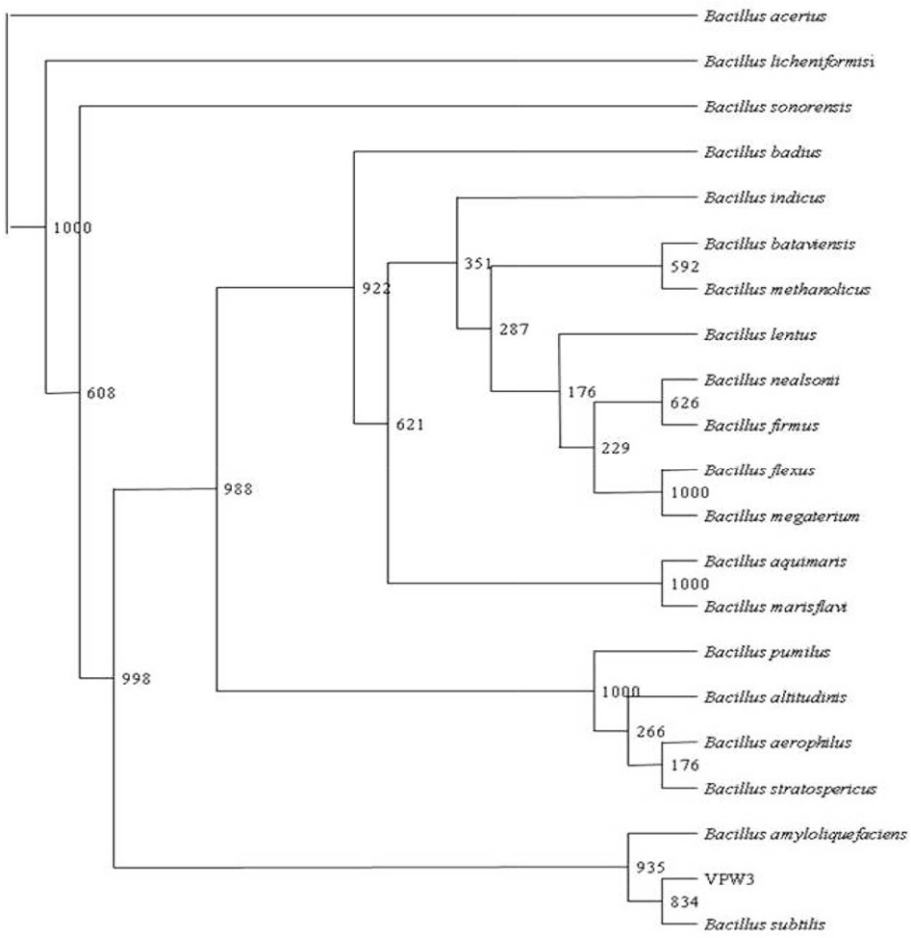


Fig. 2. **Phylogenetic tree analysis based on neighbor joining method for the selected strain VPW3.**

hydro- or tetrahydro-form. Oxidation under acidic conditions converts both BH_4 and dihydrobiopterin into biopterin. In contrast, under alkaline conditions, only dihydrobiopterin is oxidized into biopterin (Fukushima and Nixon, 1980).

The Rf value of the fluorescent compound in the extract was similar to that of the pterin standard. The cell extracts of the 10 isolates were subjected to HPTLC with the same solvent system. The spectrum of all the isolates along with the standard are shown in Fig. 1. All the isolates showed a peak corresponding to the pterin standard, with an Rf value of 0.79. The strains VA2, VPW3, and B14 showed a maximum peak area of 34.44, 29.82, and 29.7 respectively. The biomass yield was high in the case of VPW3 and the incubation time was also less, whereas in VA2 the incubation period was 15–20 days and the yield was also comparatively less. Hence, based on the fluorescence pattern, the incubation period, the biomass yield, the peak height and the peak area, VPW3 was selected for further study.

Identification and taxonomic positioning of the 16S rRNA gene sequence of VPW3

Based on morphological, physiological and biochemical characteristics, the isolate VPW3 was tentatively identified as *Bacillus* sp.. PCR amplification of the 16S rRNA gene resulted in a sequence fragment of 1,417 bp. For molecular identification, a BLAST search using the partial 16S rRNA sequence (1,417 bp) showed 99% nucleotide similarity with *Bacillus subtilis*. A phylogenetic tree was constructed based on the Neighbor-joining method (Fig. 2). The partial rRNA nucleotide sequence has been submitted in the NCBI GenBank nucleotide sequence database with the accession number JN989651.

Purification of pterin derivatives

The sample was loaded onto a conventional column and eluted using a chloroform-methanol solvent gradient. The fractions containing the blue fluorescent compound were pooled and concentrated *in vacuo*. The concentrated sample was subjected to further purification in preparative HPLC, eluted isocratically with 2% aqueous methanol. The RT value of the pterin standard and biopterin were 4.4 min and 4.9 min respectively (Fig. 3) when scanned at 254 nm. Similarly, the sample had a retention time of 4.2 min, which is similar to that of the pterin standard. The sample corresponding to this retention time was collected and dried *in vacuo*. The purity of the compound was confirmed by analytical HPLC (Fig. 4).

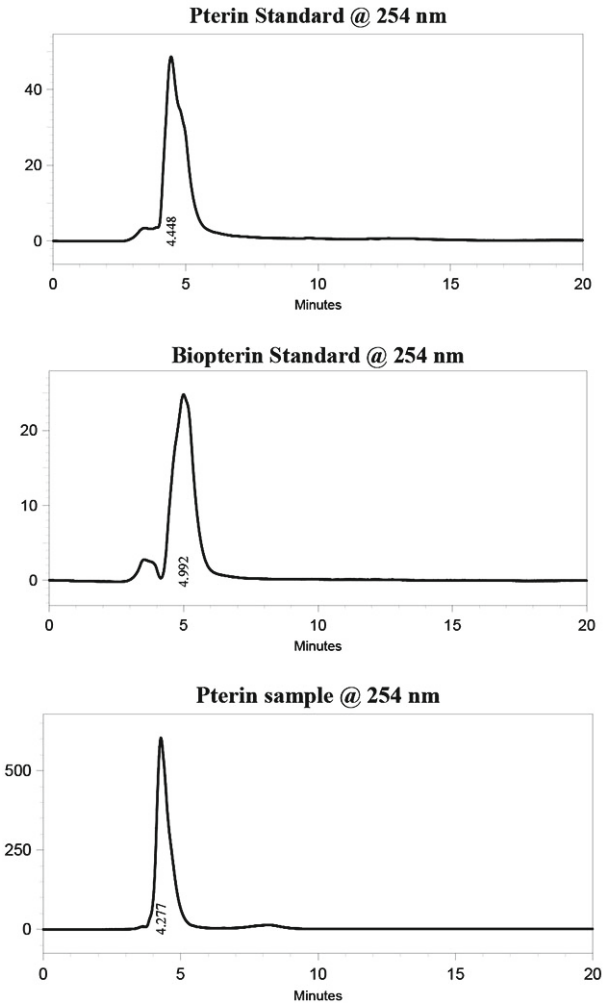


Fig. 3. **HPLC Profile for standard pterin, biopterin and sample.**

Characterization of the purified compound

UV-Vis absorption spectrometry: The UV-Vis spectrum of the sample showed two peaks at 254 nm and 360 nm, which is very similar to the spectra of the standards, pterin, and biopterin (Fig. 5). The pterin compound in the cell extract of *P. fluorescence* in Na_2HPO_4-KH_2PO_4 buffer pH 7.0 showed

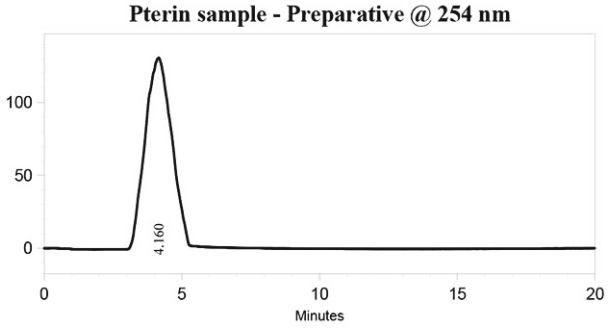


Fig. 4. **Preparative HPLC profile of the sample.**

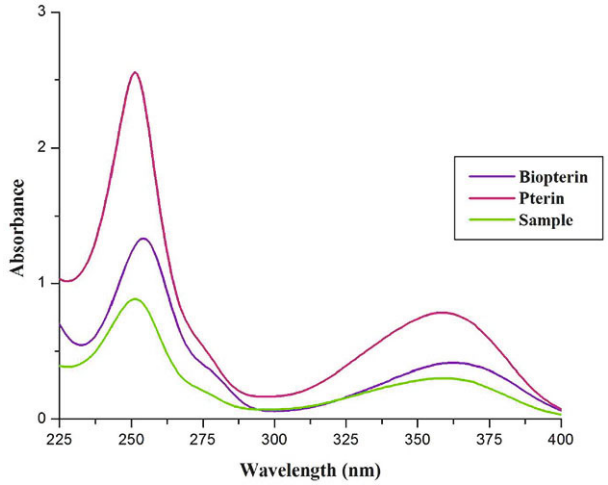


Fig. 5. **Ultraviolet/Visible absorption spectra of standards and sample.**

UV absorption maxima at 254 nm and 350 nm (Fernandez *et al.*, 2004). This is in agreement with our result. The UV-Vis spectra of tepidopterin showed absorption maxima at 276 and 350 nm at neutral pH (Cho *et al.*, 1998). The UV-Vis spectrum of purified cyanopterin in water generated four apparent peaks at 192, 236, 276, and 345 nm (Lee *et al.*, 1999).

Fluorescence spectrometry: The compound was excited at 360 nm, which elicited emission at 450 nm (Fig. 6). The excitation at higher wavelength gave maximum selectivity, excitation at 355 nm and emission at 450 nm were used to simultaneously analyze three pteridines (Hibiya *et al.*, 1995). Tepidopterin has a typical fluorescence spectrum of a pterin compound, which emits maximally at 450 nm when excited at 360 nm (Cho *et al.*, 1998). Klein *et al.* (1991) have reported the fluorescence properties of some of the naturally occurring pteridines. Accordingly, D-Neopterin, D-, and L-biopterin, and pterin, when excited at 363, 362, 358 nm, had their emission maxima at 450, 445, 445 nm, respectively.

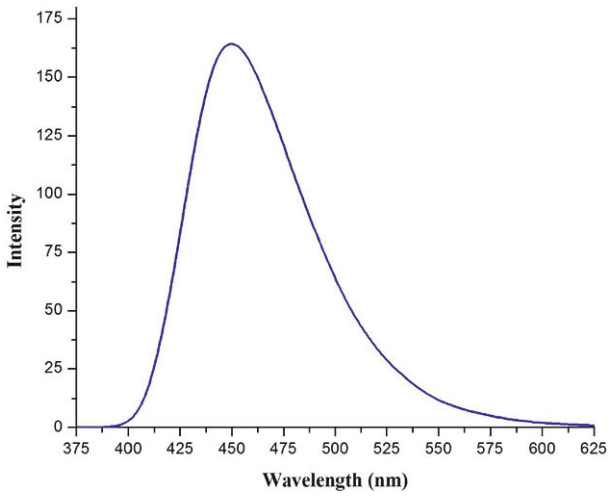


Fig. 6. **Fluorescence emission spectra of the sample.**

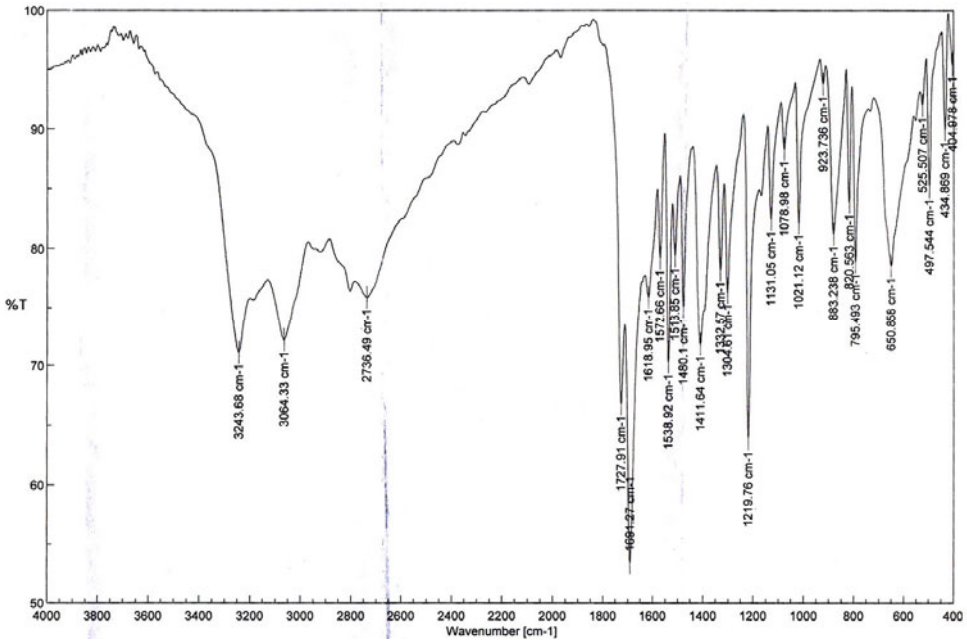


Fig. 7. **Infrared spectra of the sample.**

Infrared spectrometry: A medium sharp peak at 3243 cm^{-1} may be due to N-H stretching, which indicates the presence of an -NH_2 group. The peaks at the region of 1400–1550 cm^{-1} (Fig. 7) indicate the presence of a C=C of an aromatic system in the compound. Two sets of bands in the region around 1600 cm^{-1} and another around 1500 cm^{-1} were consistent with an aromatic compound. Well-defined and characteristic peaks in the region of 3200–2700 cm^{-1} are normally carbon- and hydrogen-containing species and were assigned to various forms of C-H stretching. The peaks at 1729 and 1691 cm^{-1} indicated the presence of a C=O group (Coates, 2000). So, overall, the IR spectrum of the compound suggested that the compound may be an aromatic heterocyclic compound containing amino and carbonyl groups.

ESI-MS spectrometry: The ESI-MS spectrum indicated the mass of the compound to be 219 (Fig. 8).

NMR spectrometry: The ^{1}H NMR spectrum in DMSO-d_6 showed resonances for nine protons (Fig. 9): δ 1.41(3H, t, -CH_3); δ 3.57 (2H, q, -CH_2); δ 5.83 (1H, s, -NH); δ 9.24 (2H, br. s, -NH_2); δ 9.73 (1H, s, -H). The peaks were similar

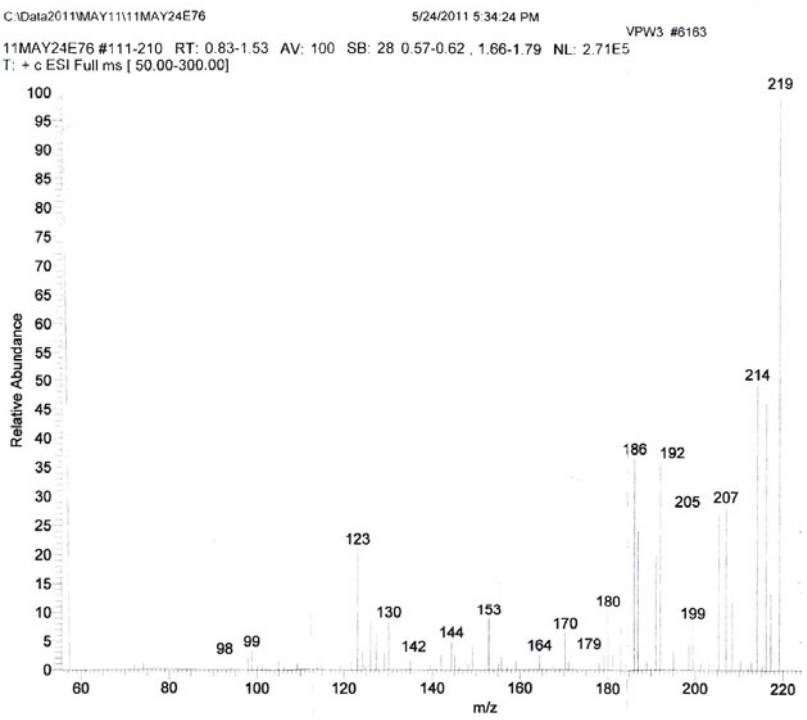


Fig. 8. **ESI-MS spectra of the sample.**

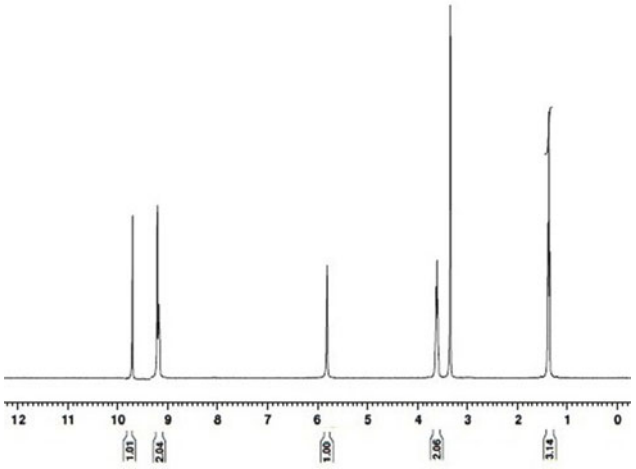
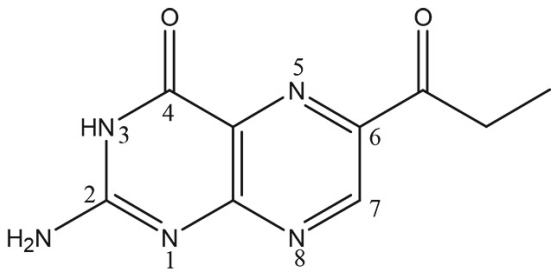


Fig. 9. ^{1}H NMR of the sample in DMSO.

to a previously reported pterin derivative, 6-propionyl pterin (Baur *et al*., 1988). The UV-Vis, IR, ESI-MS, and the ^{1}H NMR spectra of the of the compound were also in accordance with the compound, 6-propionyl pterin (Fig. 10).

The Drosophila mutant "sepia" lacks the enzyme pyrimidodiazepine synthase, leading to an overproduction of yellow pigments identified as sepiapterin (Forrest and Mitchell, 1954; Nawa, 1960), deoxysepiapterin (formerly called isosepiapterin) (Viscontini and Mohlmann, 1959) and sepiapterin C (Sugiura *et al*., 1973). Their chemical structures are very similar and have been elucidated as 6-L-Lactyl, 6-propionyl, and 6-acetyl-7,8-dihydropterins (Sugiura *et al*., 1973). Another natural source of deoxysepiapterin has been found in a blue-green alga, *Anacystis nidulans* (Forrest and Mitchell, 1954) and its formation is also observed on oxidation of 5, 6, 7, 8-tetrahydrobiopterin glucoside (MacLean *et al*., 1966). Sepiapterin and deoxysepiapterin are formed on air oxidation from 5,6,7,8 tetrahydrobiopterin in phosphate buffer at pH 4.0 (Schircks *et al*., 1978; Pfleiderer, 1979) or by an acid-catalyzed dehydration of 7,8-dihydrobiopterin (Katoh and Akino, 1966). MnO_2 or Br_2 oxidation of the extract led to the formation of a blue fluorescent oxidation product 6-(L-lactyl-pterin) from sepiapterin (Forrest and Mitchell, 1954) and blue fluorescent compound 6-propionyl pterin from isosepiapterin (Forrest *et al*., 1959). In concordance with earlier reports the 6-propionyl pterin purified in the present study might have been the oxidation product of 5,6,7,8-tetrahydrobiopterin which is the cofactor of cyanide monooxygenase. Kobayashi and Forrest (1970) reported the presence of a new pteridine derivative in *Bacillus subtilis*, the neopterin residue of neopterinyl glucuronic acid appears to be derived without loss of carbon atoms, from guanosine triphosphate.



2-Amino-6-propionyl-3*H*-pteridin-4-one

Fig. 10. **Proposed structure of the compound (6-propionylpterin).**

Conclusion

Since industries in Ranipet are mostly engaged in leather, chemicals, tool making and pharmaceuticals, this area was chosen for our present study. Soil and water samples were collected and a total of 72 bacterial strains, including actinomycetes, were isolated. The microorganisms in this area would have had to become acclimatized or adapted to various heavy metals and cyanide complexes. Based on this, organisms capable of utilizing cyanate and cyanide were screened for our study. The microorganisms capable of degrading cyanide oxygenolytically possess the enzyme cyanide monooxygenase, which obligately requires pterin cofactor for enzyme activity. In this regard, the selected organisms have been subjected to further screening to determine the presence of pterin. Out of 12 organisms capable of utilizing cyanide, 10 microbes had the blue fluorescent compound in their cell extract. The cell extracts of these 10 organisms were subjected to HPTLC. Based on the incubation period, cell biomass yield, peak height and area, isolate VPW3 was selected for further study. The organism has been identified as *B. subtilis* based on morphological, physiological, and biochemical characteristics and its 16S rRNA gene sequence. The pterin cofactor was purified and it has blue fluorescence, showing UV absorption maxima at 254 and 360 nm, with maximum blue fluorescence at 450 nm when excited at 360 nm, the mass of the compound was found to be 219. Also the ^{1}H NMR spectra of the compound was consistent with earlier findings and hence the compound is proposed to be 6-propionyl pterin.

The reduced as well as the oxidized form of pterin exerts multiple functions. The importance of the compound 6-propionyl pterin in the treatment of cancer remains to be explored.

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