The Present study

Cancer, medically known as malignant neoplasm, is a broad group of various diseases involving unregulated cell growth. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. Cancer can affect people of all ages and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). Prevention is undeniably the sensible maneuver towards the ultimate goal of cancer control. Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. Intervention with chemopreventive agents at the early stage in carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic drugs. However, most cancer chemotheraputants severely affect the host normal cells. Not surprisingly, the clinical success of these treatments has reached a plateau. A variety of bioactive compounds and their derivatives has been shown to inhibit carcinogenesis in a number of experimental systems involving initiation, promotion and progression. Hence, the use of natural products now has been contemplated of exceptional value in the control of cancer and its eradication program. It is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources including plants and marine organisms.

Researchers mainly focus on the agents that act by changing the biological properties of cancer cells so that they lose the ability to divide continuously. Hence treatment based on tumour differentiation and induction of apoptosis is beneficial to prevent the uncontrolled growth of tumour cells. Neovascularization or angiogenesis is an absolute requirement for tumour growth. Without angiogenesis the tumour ceases to grow even beyond a few millimeters in size The tumour growth and metastasis are dependent on the development of new blood vessels Inhibition of angiogenesis is now considered to be a promising approach for cancer therapy; efforts are being directed at overcoming tumour angiogenesis worldwide. In animal models, antiangiogenic compounds have proven very successful in inhibiting tumour development.

Pteridines, one of the larger families of bicyclic N-heterocycles, exert a wide range of biological functions in the living systems. Some naturally occurring pteridine derivatives carry out important metabolic transformations, play an important role in synthesis of aminoacids, nucleic acids, neurotransmitters, nitrogen monoxides and metabolism of purine and aromatic amino acid in human body. Based on its function these compounds have long been of interest in biological chemistry and medicinal chemistry. Some pteridine derivatives are practically used in the chemotherapy or diagnosis of various diseases. Pteridines play an essential role in growth processes and the metabolism of one carbon units as cofactors in enzyme catalysis and in biological coloration. Many synthetic pteridines proved useful in medicine as anticancer, antiviral, antibacterial and diuretic drugs. The reduced pterin, (6R)-L-*erythro*-5,6,7, 8-Tetrahydrobiopterin (BH_4) is an essential cofactor of three aromatic amino acid hydroxylases (phenylalanine, tyrosine, and tryptophan), lipid oxidase, three nitric oxide synthase (NOS) isoenzymes and cyanide monoxygenase. The NOS isoforms are known to be regulated at the transcriptional, translation and post-translational levels and BH_4 has come to light as an important factor that regulates the level and mode of NOS activity. NO is biologically synthesized by nitric oxide synthases (NOS). A broad spectrum of activities has been assigned to either the physiology or the patho-physiology of nitric oxide in tumor cells. Various direct and indirect mechanisms have been proposed for the anti-tumour properties of NO. NO displays a biphasic nature in cancer biology since it has both protumour and antitumour properties. The wide range of differing biological effects arising from exposure to NO is very much dependent upon many factors, such as formation and metabolism of NO, the type of NOS enzymes that are present, the interaction between NO utilizing processes, and crucially the concentration of NO that is present in the given system. Low level of NO has protumour activity whereas high output of NO results in antitumor activity. Conversely, depletion of BH_4, either pharmacologically or genetically was shown to impair NOS's activity; in each case repletion of BH_4 levels by treatment with either BH_4 itself, or the BH_4 precursor sepiapterin, was shown to restore NOS activity. Apart from the reduced pteridine, several other forms also posses anti-tumor property like folic acid, neopterin, 6-formylpterin, sepiapterin.

Pterin is a cofactor for cyanide oxygenase enzyme which metabolizes cyanide. Cyanide oxygenase is an enzyme involved in the oxygenolytic conversion of cyanide to carbon dioxide and ammonia. It has been recently reported that cyanide oxygenase is a pterin-dependent hydroxylase.

Based on the above stated facts, the present work has been focused on purification and characterization of pteridine derivative from cyanide degrading bacteria *Bacillus subtilis* and evaluation of its cytotoxic and antiangiogenic potentials in Dalton's Lymphoma Ascites (DLA) cell line induced ascites tumour model in Swiss albino mice.

Objectives

1. Isolation and identification of pterin producing microorganism among cyanate and cyanide degrading microorganisms from industrially contaminated soil and water samples.
2. Extraction, purification and characterization of pteridine derivative from the selected bacteria.
3. Evaluation of *in vivo* anticancer activity of purified pteridine using Daltons' Lymphoma Ascites tumour model in mice.

2.1. Introduction

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). In spite of its toxicity cyanide is found in a wide range of life forms including photosynthetic bacteria, algae, fungi and plants. Large amounts of cyanide are used in industries involved in the metal-plating, pharmaceuticals, synthetic fibers, plastics, coal gasification, coal coking, ore leaching, gold mining, and electroplating (Knowles and Bunch, 1986; White *et al.*, 1988; Yanase *et al.*, 2000). Cyanides can be removed from industrial wastes by biodegradation, physical and chemical methods (Patil and Paknikar, 2000; Ebbs, 2004). The biological treatment methods are feasible alternatives to physical and chemical methods, because a wide range of microorganisms are known to metabolize such chemicals (Castric, 1981; Fry and Meyers, 1981; Harris *et al.*, 1987).

The microbial degradation involves enzymatic pathways and generally degradation of cyanide is induced by the presence of cyanide in the media. Five general pathways for the biodegradation of cyanide are hydrolytic pathway, oxidative pathway, reductive pathway, substitution pathway and syntheses pathway (Adjei and Ohta, 1999; Sexton and Howlett, 2000; Yanase *et al.*, 2000; Kwon *et al.*, 2002). First three pathways are degradation pathways in which enzymes catalyze the conversion of cyanides into simple organic or inorganic molecules and it is further converted into ammonia, methane, CO_2, formic acid and carboxylic acid. Last two pathways are for the assimilation of cyanide in the microbe as nitrogen and carbon source (Urbanska *et al.*, 2002; Baxter and Cummings, 2006).

The oxidative pathway of cyanide conversion involves oxygenolytic conversion to carbon dioxide and ammonia. There are two types of oxidative pathway involving three different enzymes. The first oxidative pathway involves cyanide monooxygenase 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. Cyanases have been identified in numerous bacteria, fungi, plants and animals. Cyanide monooxygenase (CNO) 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). Cyanide monooxygenase is a pterin-dependent hydroxylase that means this enzyme requires pterin as a cofactor (Cabuk *et al.,* 2006). Its usually observed that cyanide-grown cells contain elevated levels of both cyanide mono-oxygenase and formate dehydrogenase (Kunz *et al.,* 1992). The second oxidative pathway utilizes cyanide dioxygenase to form ammonia and carbon dioxide directly (Ebbs, 2004).

Kunz *et al.* (2001) demonstrated that only reduced pterins among a number of compounds tested were able to complement the enzyme and thereby enhancing the CNO activity. He strongly suggested that these compounds are capable of serving as specific reductants for the enzyme. Other non-pterins such as ascorbate and glutathione showed no enhancing effect strongly suggesting that BH_4 and 6-tetrahydromethylpterin ($H_4$6MP) are capable of acting specifically as reductants for CNO. Utilization of $H_4$6MP finds analogy also with the properties of other pterin dependent hydroxylases for which this species has been shown to substitute almost universally for the naturally occurring BH_4 cofactor (Kaufmann, 1987; Kappock and Caradonna, 1996).

The detection of H_2monapterin and H_2biopterin in cell extracts of *Pseudomonas fluorescens* NCIMB 11764, each of which is capable of complementing proteins suggests that these compounds (H_4 monapterin and H_4biopterin, which can spontaneously oxidize to the more stable dihydro forms) can serve as natural cofactors for the enzyme. In addition to reduced monapterin and biopterin, H_2Neopterin was also found to support enzyme activity, implying that CNO has a rather broad specificity for pterin cofactors. There are distinct differences among CNO, AAH and NOS with regard to the oxidation state of pterin capable of supporting enzyme activity. The latter enzymes have a rather strict requirement for the fully reduced tetrahydropterins (in most cases BH_4 serves as the natural cofactor) (Marletta *et al.,* 1998; Presta *et al.,* 1998) whereas both the tetrahydro and dihydro forms were equally capable of enhancing the activity of CNO (Fernandez *et al.,* 2004).

Assuming that the cofactors 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 and actinomycetes from the soil and water samples for pteridine compound extraction.

2.2. Materials and methods

2.2.1. 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 laboratory within 48 h of collection and subsequent isolation of bacteria and actinomycetes were carried out.

2.2.2. Isolation of microorganisms

Microbial isolation from these contaminated soil and water samples were conducted by dilution plate method. Nutrient agar medium and starch casein agar medium (Himedia, Mumbai, India) were used for the isolation and enumeration of bacteria and actinomycetes respectively. The starch casein agar medium was supplemented with 10µg/mL amphotericin and 25µg/mL streptomycin (Himedia, Mumbai, India) to inhibit fungal and bacterial contamination respectively (Ravel *et al.,* 1998). Around 0.1mL of each dilution was spread plated on nutrient agar and starch casein agar plates in triplicates and incubated at room temperature for 24 h and 10 days respectively. After incubation period, the plates were examined for the presence of distinct bacterial and actinomycetes colonies. Pure colonies were obtained by quadrant streaking method and sub-cultured on respective medium and then preserved in 20% glycerol vials at -80^{o}C (Williams and Cross, 1971).

2.2.3. Selection of cyanate degrading microorganisms

Preliminary selection was carried out to exploit the ability of the microorganisms to utilize cyanate as nitrogen source. Selection of cyanate utilizing microorganisms was conducted in M9 minimal medium prepared without ammonium and citrate (Luque-Almagro *et al.,* 2005). The selection medium contains the following ingredients per litre of distilled water Na_2HPO_4-12.8 g, KH_2PO_4-3.0 g, NaCl-0.5 g, 1M $MgSO_4$-2.0 mL, 1M $CaCl_2$-0.1 mL, 50mM sodium

acetate. The pH of the medium was adjusted to 9.5 and autoclaved. Ten mL of 20% filter sterilized glucose was added. Various concentrations of membrane (0.2µm) sterilized sodium cyanate solution (10mM to 30mM) was added to the medium before plating and allowed to solidify (Maniatis *et al.*,1982). Each bacterial and actinomycetes isolate was streaked onto sodium cyanate plates and incubated for 5 and 20 days respectively at 37°C.

2.2.4. Selection of cyanide degrading microorganisms

Those microorganisms which were able to utilize and sustain at higher concentration of sodium cyanate were further tested for its ability to degrade cyanide. Cyanide fortified M9 minimal medium prepared as mentioned earlier (2.2.3) was used. Glucose (20%) and 2 mM sodium cyanide was used as sole source of carbon and nitrogen respectively. Glucose and sodium cyanide was membrane (0.2µm) sterilized and then added to autoclaved, cooled medium, plated and allowed to solidify. Bacteria and actinomycetes were streaked and incubated for 5 and 20 days respectively at 37 °C.

2.2.5. Growth of microorganisms and preparation of sample for pterin analysis

The bacteria and actinomycetes were grown in M9 minimal broth containing 10 mM glucose, 50 mM citrate and 2 mM sodium cyanide as carbon and nitrogen sources adjusted to pH 9.5 and incubated for 5 days and 15 days respectively at 37°C and 200 rpm. The cells were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were washed in 50 mM phosphate buffer, pH 7.0 and resuspended in the same buffer and stored at -20°C until use. A suspension of washed cells was diluted in phosphate buffer to produce a solution with an OD_{600} of 1.0. The cell suspension was sonicated in an ultrasonic water bath for 3 min and the cell debris was removed by centrifugation at 12,000 rpm for 10 min (Cho *et al.*, 1998; Lee *et al.*, 1999). Oxidations of samples were performed by mixing 200 µL of supernatant with equal volume of 0.2 N HCl and to this 15 µL of acidic iodine solution was added. The tubes were incubated in dark for 1h at room temperature. The tubes were then centrifuged at 10,000 rpm for 10 min at 4 °C to remove precipitated proteins. To the supernatant, 20 µL of 2% ascorbic acid was added to reduce excess iodine (Fukushima and Nixon, 1980). The samples were then filtered through 0.2 µm syringe filter and stored in dark for further analysis.

2.2.6. Selection of pterin producing organisms

2.2.6.1. Thin layer chromatography (TLC)

The TLC plates were coated with thin layer of silica gel G and activated at 110 ^{o}C for 2 h. Various solvent systems were used including (A) n-butanol: 5N glacial acetic acid (4:1) (Nemec *et al.*, 2003), (B) n-propyl alcohol: 29% NH_4OH in water (1:1), (C) isopropanol and 5% NH_3 (2:1) (Ikawa *et al.*, 1995). Plates were developed with the solvents under darkness and at room temperature. Presence of pterins were tentatively identified according to their Rf values and the colour of fluorescence under UV light at 254 nm (Pfleiderer, 1992) in comparison with pterin (Sigma, India) standard.

2.2.6.2. High Performance Thin Layer Chromatography (HPTLC)

Test samples (5µL) along with pterin standard were loaded on to the plate coated with silica gel 60 F_{254} using Hamilton syringe and run in CAMAG Linomat 5 instrument. The mobile phase used was n-butanol: 5N glacial acetic acid (4:1). The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG TLC SCANNER 3) and the image was captured at UV 254nm.

Based on the analysis for the presence of pterin, the strain VPW3 was chosen for further studies.

2.2.7. Identification and taxonomic positioning of 16s rRNA gene sequence of VPW3

The isolate VPW3 was identified to the genus level by observing their morphological and biochemical characteristics (Buchanan *et al.*, 1974). The bacteria was identified upto species level based on PCR amplification, 16s rRNA gene sequencing, BLAST analysis (Altschul *et al.*, 1990) and comparison with sequences in the Gen Bank nucleotide databases. The 16s rRNA gene was amplified using the universal primers, forward 5'-AGAGTTTGATCCTGGCTC AG-3' and reverse 5'- GGTTACCTTGTTACGACTT-3' (James, 2010). The Sequencing was outsourced at Xcelris Labs Ltd. (India). The 16S rRNA gene sequence was analyzed for the similarity and homology with the existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search available at http://www.ncbi.nlm.nih.gov/. The sequences were aligned using ClustalW program within BioEdit version 7.0.5.3 and phylogenetic tree was constructed by

neighbour joining method (Saitou and Nei, 1987) using Phylip software version 3.69. Treeview1.6.6 was used to visualize the phylogenetic tree and the bootstrap analyses of 1000 replicates were carried out (Hentschel *et al.*, 2001).

2.3. Results and Discussion

2.3.1. Isolation of microorganisms

Based on the colony morphology and their stability during subculturing, around 72 organisms have been isolated from soil samples collected from various regions of SIPCOT industrial area and nearby Vanappadi lake. Out of which 58 isolates were bacteria and 14 isolates were actinomycetes. The organisms were designated based on the area of sample collection (Table 2.1).

2.3.2. 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. 33 and 22% of the organisms tolerated 20 mM and 30 mM of sodium cyanate respectively (Table 2.2). Bacterial isolates VPW3 and MW1 alone were able to tolerate upto 35 mM of sodium cyanate. Among the selected 16 isolates, 11 bacteria designated as B2, B9, B13, B14, B19, MD2, MD4, MW1, VPW3, TCLW1, VP3 and 5 actinomycetes namely VA1, VA2, VA5, MDA1, MDA2 were able to tolerate upto 30mM of sodium cyanate. The selected strains were further subjected to secondary selection namely cyanide degradation. Among these, 12 isolates were able to utilize sodium cyanide as sole nitrogen source (Table 2.3).

The microorganisms involved in the biological treatment of cyanide and thiocyanate usually include a heterogeneous mixture of commonly found indigenous soil bacteria which have adapted due to continuously extended exposure to the treatment of these compounds (Mudder *et al.*, 2001). The current literature review states that the microorganisms able to assimilate cyanide can use it only as a nitrogen source, but not as the sole carbon source.

It has been reported that *P. fluorescens* NCIMB 11764 utilizes cyanide as a source of nitrogen (Harris and Knowles, 1983a, b; 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 of *Klebsiella pneumoniae*, *Moraxella*, *Serratia* and *Alcaligenes* species were isolated and identified as cyanide-degrading bacteria (Ingvorsen *et al.*, 1991; Kang and Kim, 1993) which utilized cyanide as a 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, have also been found to be capable of growing on cyanide as carbon and nitrogen source (Knowles, 1976).

Dorr and Knowles (1989) has reported the enzyme responsible for the conversion of cyanide into ammonia and carbon dioxide by cell free extracts of *P. fluorescens* NCIMB 11764 was found to be 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 xylooxidans* subsp. *denitrificans* (Ingvorsen *et al.*, 1991) and *B. pumilus* (Meyers *et al.*, 1991) were also found to convert cyanide to ammonia and formate.

2.3.3. Analysis of pterin

The cell extract of all the 12 isolates which 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 (A) n-butanol: 5N glacial acetic acid (4:1) was more suitable and showed maximum separation of compounds on TLC. Among the 12 isolates, only 10 strains showed blue colour fluorescence when observed under UV light at 254 nm. The blue fluorescence was intense in the cell extract of VPW3. There was 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 like tetrahydrobiopterin show no intense fluorescence. Normally in the cells, pterins occur in dihydro- or tetrahydro- form. Oxidation under the acidic condition converts both BH_4 and dihydrobiopterin into biopterin. On the contrary, under the alkaline condition, only dihydrobiopterin is oxidized into biopterin (Fukushima and Nixon, 1980)

The Rf value of fluorescent compound in the extract was almost similar to that of the standard pterin. The cell extract of the 10 isolates were subjected to HPTLC with the same solvent system. The spectrum of all the isolates along with the standard is shown in Fig.2.1. All the isolates showed a peak corresponding to the standard pterin with an Rf value of 0.79 (Table 2.4). 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 72 h which was less when compared with VA2 where the incubation period was found to be15 days and the yield was also comparatively lesser. Hence, depending on the fluorescence pattern, the incubation period, the yield of biomass, the peak height and peak area VPW3 has been selected for further study.

2.3.4. Morphological, physiological and biochemical characterization of the strain VPW3

The morphological, physiological and biochemical characteristics of the isolate VPW3 has been given in Table 2.5. The strain was Gram positive and strictly aerobic rods. It was capable of hydrolyzing starch, gelatin, casein and tween 80. It revealed that the organism produced the extracellular enzymes amylase, protease and lipase to metabolize the polymeric components of the nutrient mixture to monomeric forms for its growth. Apart from these, the strain showed positive result for catalase and urease. It utilized various carbon sources and produced acid. The test on triple sugar iron medium revealed that it was capable of fermenting sugar and produced acid and showed no growth in the anaerobic region and did not produce hydrogen sulphide gas. It reduced nitrate to nitrite. Assessing the physiological characteristics, the optimum temperature and pH for its growth was 35 ^{o}C and 7.5 respectively. But it was tolerant upto 55 ^{o}C and showed appreciable growth even in the slightly acidic pH. It was tolerant and showed good growth even at 10% NaCl concentration. Based on all of the above said characteristics the strain VPW3 has been tentatively identified as *Bacillus* sp.

2.3.5. Identification of the strain based on 16s rRNA sequencing

Sequencing of 16s rRNA resulted in an amplicon of 1417bp. For molecular identification BLAST search for the partial 16s rRNA sequence (1417bp) showed 99% of nucleotide similarity

with *Bacillus subtilis*. The phylogenetic tree was constructed based on neighbour joining method. The partial nucleotide sequence has been submitted in the NCBI GenBank nucleotide sequence database with the accession number JN989651.

2.4. Conclusion

Since industries in Ranipet are mostly engaged in leather, chemical, tool making and pharmaceuticals, this area has been chosen for our present study. Soil and water samples have been collected and a total of 72 bacteria and actinomycetes strains have been isolated. The microorganisms in this area would have acclimatized themselves to various heavy metals and cyanide complexes. Based on this, the organisms capable of utilizing cyanate and cyanide have been screened for our study. The microorganisms capable of degrading cyanide oxygenolytically possess cyanide monooxygenase enzyme, which require pterin cofactor obligately for the 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 organisms had the blue fluorescent compound in their cell extract. The cell extracts of 10 organisms has been subjected to HPTLC. Based on the incubation period, cell biomass yield, peak height and area VPW3 has been selected for further study. The organism has been identified as *Bacillus subtilis* based on morphological, physiological, biochemical characteristics and 16 rRNA gene sequencing. The purification and structural elucidation of the pteridine derivative present in this organism will be dealt in the next chapter.

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Table 2.1: 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

Table 2.2: Selection of cyanate degrading microorganisms

S.No.	Organisms	Control	10mM	20mM	30mM
Bacteria					
1	TCL1	+	+	+	-
2	TCL2	-	-	-	-
3	TCL3	-	-	+	-
4	TCL4	-	+	+	-
5	TCL5	-	+	-	-
6	TCL6	-	+	+	-
7	MD1	-		+	-
8	**MD2**	-		+	+
9	MD3	-	-	-	-
10	**MD4**	-	+	+	+
11	TCEW1	-	-	-	-
12	TCEW2	-	-	-	-
13	TCEW3	-	+	-	-
14	TCEW4	-	-	-	-
15	**TCLW1**	-	+	+	+
16	TCLW2	-	-	-	-
17	M1	-	-	-	-
18	M2	-	-	-	-
19	M3	-	-	-	-
20	M4	-	-	-	-
21	M5	-	+	+	-
22	M6	-	+	-	-
23	**MW1**	-	+	+	+
24	MW2	-	+	+	-
25	VPW1	-	-	-	-
26	VPW2	-	-	-	-
27	**VPW3**	-	+	+	+
28	VP1	-	+	-	-
29	VP2	-	-	-	-
30	**VP3**	-	+	+	+
31	VP4	-	-	-	-
32	VP5	-	+	-	-
33	CG1	+	-	-	-
34	CG2	-	-	-	-
35	CG3	-	-	-	-
36	CG4	-	-	-	-
37	CG5	-	-	-	-
38	CG6	-	-	-	-
39	CG7	-	-	-	-
40	B1	-	+	-	-
41	**B2**	-	+	+	+

Table 2 Contd.

42	B3	-	+	-	-
43	B4	-	-	-	-
44	B5	-	+	-	-
45	B6	-	+	-	-
46	B7	+	-	-	-
47	B8	-	-	-	-
48	**B9**	-	+	+	+
49	B10	-	-	-	-
50	B11	-	-	-	-
51	B12	-	-	-	-
52	**B13**	-	+	+	+
53	**B14**	-	+	+	+
54	B15	-	+	-	-
55	B16	-	-	-	-
56	B17	-	-	-	-
57	B18	-	-	-	-
58	**B19**	-	+	+	+
Actinomycetes					
1	**VA1**	-	+	+	+
2	**VA2**	-	+	+	+
3	VA3	-	-	-	-
4	VA4	-	-	-	-
5	**VA5**	-	+	+	+
6	VA6	-	-	-	-
7	VA7	-	+	+	-
8	**MDA1**	-	+	+	+
9	**MDA2**	-	+	+	+
10	MDA3	-	-	-	-
11	MWA1	-	-	-	-
12	VPWA1	+	-	-	-
13	VPWA2	-	-	-	-
14	VPWA3	-	-	-	-

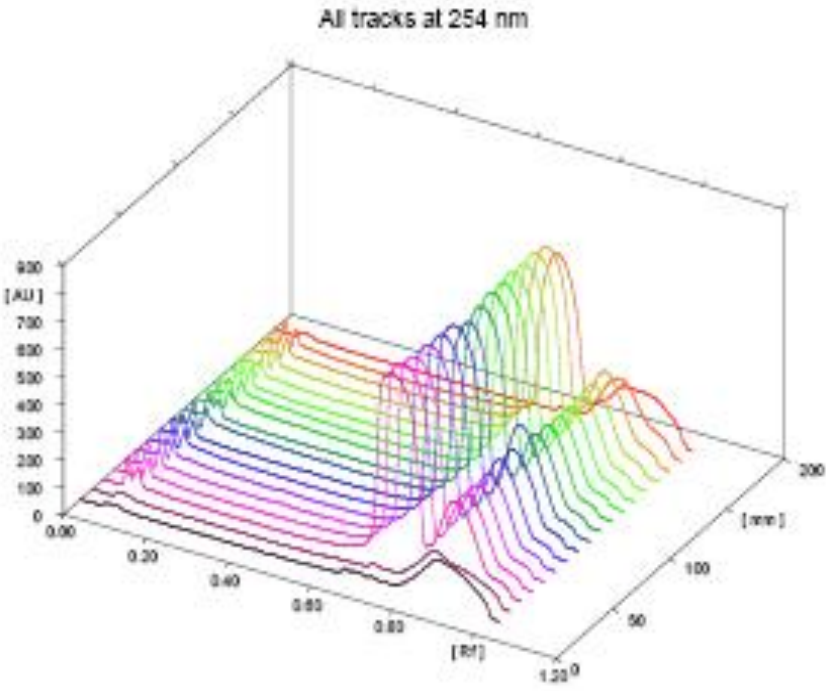
+ presence of growth; - no growth; Bold letters indicate selected strains for further study.

Table 2.3: Screening of cyanide degrading microorganisms

Organism	Growth at 2mM sodium cyanide concentration
Bacteria	
B2	**-**
B9	**+**
B13	**+**
B14	**+**
B19	**+**
MD2	**+**
MD4	**-**
MW1	**+**
VPW3	**+**
TCLW1	**-**
VP3	**-**
Actinomycetes	
VA1	**+**
VA2	**+**
VA5	**+**
MDA1	**+**
MDA2	**+**

+ presence of growth; - no growth

Fig. 2.1: HPTLC chromatogram of selected strains compared with standard pterin



Track No.	Applied position (mm)	Applied volume (µL)	Sample ID	Active
1	15	5	STD*	Yes
2	25	5	STD*	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*	Yes

*STD denotes standard pterin

Table 2.4: HPTLC analysis of pterin in selected strains

Track No.	Sample Code	Start Rf	Maximum Rf	End Rf	Maximum height	Area (%)
1	STD*	0.76	0.88	1.01	158.50	93.99
2	STD*	0.74	0.84	1.00	140.20	92.45
3	VPW3	0.79	0.86	0.97	292.50	29.82
4	MW1	0.79	0.86	0.99	281.90	29.09
5	B9	0.79	0.86	0.97	277.60	28.65
6	B14	0.79	0.86	1.00	285.70	29.70
7	B19	0.79	0.86	0.99	297.40	29.09
8	VA1	0.80	0.86	1.00	297.10	29.35
9	VA2	0.79	0.85	0.95	348.80	34.44
10	VA5	0.80	0.86	0.95	285.60	27.51
11	MDA1	0.80	0.86	0.99	280.80	26.93
12	MDA2	0.80	0.86	0.98	274.70	26.48
13	MW1	0.80	0.86	0.97	273.30	26.15
14	B9	0.80	0.86	0.94	276.20	26.16
15	B14	0.80	0.86	0.99	283.90	28.44
16	B19	0.80	0.86	1.01	294.20	30.17
17	VA1	0.79	0.87	1.01	235.30	31.75
18	STD*	0.73	0.88	1.00	164.4	95.00

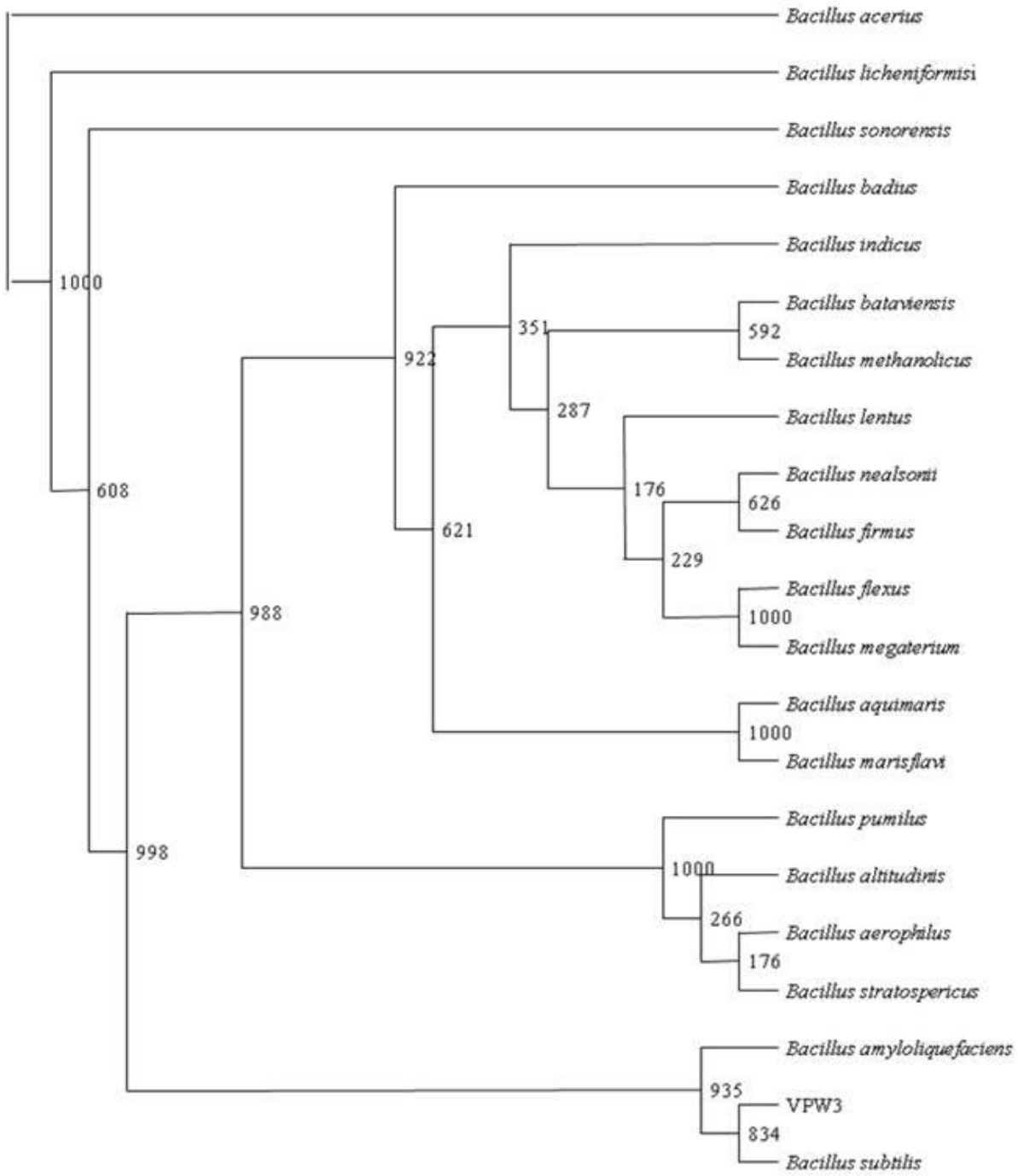
*STD denotes standard pterin

Table 2.5: Morphological, biochemical and physiological characterization of the selected Strain VPW3

Character	Response
Morphological	
Cell shape	Rod
Presence of spore	+
Physiological	
Growth under anaerobic conditions	-
Gram reaction	+
Range of temperature for growth	30 - 40˚C
Optimum temperature for growth	35˚C
Temperature tolerance	55˚C
Range of pH for growth	6.0 – 8.0
pH tolerance	5.0
NaCl tolerance	10%
Biochemical	
Catalase production	+
Oxidase production	-
Urease production	+
Hydrogen sulfide production	-
Nitrate reduction	+
Gelatin liquefaction	+
Methyl red test	V
Vogues proskauer test	+
Indole production	-
Citrate utilization	+
Hydrolysis of starch	+
Casein hydrolysis	+
Hydrolysis of Tween-80	+
TSI	A/-
Acid from carbohydrates	
Glucose	+
Mannitol	+
Fructose	+
Xylose	+

+ positive; - negative; V variable; A/- Acid production in slant/no growth in butt

Fig. 2.2: Phylogenetic tree analysis based on neighbor joining method for the selected strain VPW3



Bacillus subtilis 16S ribosomal RNA gene, partial sequence. GenBank: JN989651.1

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Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
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AUTHORS Nisshanthini,S.D. and Angayarkanni,J.
TITLE Isolation and characterization of pterin from bacteria
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1417)
AUTHORS Nisshanthini,S.D. and Angayarkanni,J.
TITLE Direct Submission
JOURNAL Submitted (03-NOV-2011) Microbial Biotechnology, Bharathiar University, Maruthamalai Main Road, Coimbatore, TamilNadu 641 046, India
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