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Original Research Article

Isolation of Pyridine Degrading Bacteria from Soils Contaminated with Petrochemical Industry Effluents in Purba Medinipur

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ABSTRACT

Pyridine and its derivatives are of major concern as environmental pollutants due to their toxic nature. Bioremediation is considered to be an alternative process to clean up the environment polluted with heterocyclic pollutants including pyridine and its derivatives. Our primeval objectives were first to isolate the pyridine degrading bacteria from the petrochemical industry effluent enriched soil sample collected from effluent discharge site in Purba Medinipur, West Bengal and further to explore morphological, biochemical, molecular characteristics and the optimum conditions under which these strains can most efficiently breakdown pyridine. In this study, one new bacterial strain, *Shewanella algae* (designated as MM) capable of utilizing pyridine as a sole carbon source was isolated from the contaminated site and we have explored different research approaches alongwith 16S rDNA gene analysis method for unveiling this intended purpose. Furthermore, it can be used effectively for the treatment of pyridine bearing wastewater and as a possible inoculum in a biofilter treating pyridine-laden gas.

Key Words: Bioremediation, growth rate, pyridine, *Shewanella algae*

INTRODUCTION

A vast amount of aromatic compounds as pollutants are being discharged into the environment by different industries, following the wide range of implications of aromatic compounds among the top chemicals utilized. Amongst this aromatic heterocyclic compounds, pyridine and its derivatives are of foremost concern as environmental pollutants following their recalcitrant, toxic and teratogenic property. It is a heterocyclic volatile aromatic compound regarded as a weak organic base, colorless liquid with penetrating, empyreumatic odor, a threshold odor concentration of 0.1 ppm (58.6 mg l^{-1}) (Pandey *et al.* 2007; Mohan *et al.* 2003; Mohan *et al.* 2004). Pyridine is used as a solvent in paint and rubber industries, as an intermediate in manufacturing insecticides and herbicides for agricultural purposes and in research for successful extraction of plant hormones. On the other hand, it is also employed in the alcohol denaturation process and to formulate diverse products for instance medicines, vitamins, food flavorings, dyes, adhesives and in waterproofing of fabrics (Mohan *et al.* 2005; Lataye *et al.* 2006).

Pyridine biodegradation by some soil microorganisms was first documented in 1914 (Kost and Modyanova, 1978). USEPA has documented pyridine as a hazardous substance in its list of priority pollutants (Lataye *et al.* 2006; Padoley *et al.* 2006). Other severe physiological impacts are its mild toxicity for inhalation; its skin and severe eye irritant property and also on exposure it can result into gastrointestinal upset, liver and kidney damage, headache, dizziness, insomnia, nausea, anorexia, frequent urination, dermatitis and carcinogenesis. The standard set by Occupational Safety and Health Administration (OSHA) as well as American Conference of Governmental Industrial Hygienists (ACGIH) was that the exposure limit is 5 ppm average over a 10 h work shift (Agency for Toxic Substances and Disease Registry U.S., 1992). Accordingly, scientists have aimed to generate effective, economically reasonable practice for pyridine clean-up from the environment.

Different physico-chemical methods for the management of pyridine pollution have been devised. These consist of adsorption process (Lataye *et al.* 2006; Mohan *et al.* 2005; Mohan *et al.* 2004; Sabah *et al.* 2002; Kumar *et al.* 1995; Zhu *et al.* 1988), sorption process in zeolites (Bludau *et al.* 1998), biodegradation alone (Pandey *et al.* 2007; Padoley *et al.* 2006; Mohan *et al.* 2003; Uma *et al.* 2002; Li *et al.* 2001; Fetzner,

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1998; Uma *et al.* 1998; Uma *et al.* 1997; Rhee *et al.* 1997; Rhee *et al.* 1996) and ozonation with biodegradation (Stern *et al.* 1997). Nowadays, biological treatment or bioremediation is a potent and well-accepted approach for successful removal of pyridine. A number of microorganisms have been reported till date and are capable of degrading pyridine and its derivatives, e.g., aerobic microorganisms like *Rhodococcus* sp. Chr-9 (Jiquan *et al.* 2012), *Rhodococcus* sp. (Sun *et al.* 2011; Dindar *et al.* 2013), *Lysinibacillus cresolivorans* (Yao *et al.* 2011), *Pseudomonas pseudoalcaligenes-KPN* (Pandey *et al.* 2007), *Pseudomonas sp.* (Mohan *et al.* 2003), *Bacillus consortia* (Uma *et al.* 2002), *Bacillus coagulans* and *Bacillus sp.* (Uma *et al.* 1997; Uma *et al.* 1997), *Nocardioides sp.* (Rhee *et al.* 1997), *Pimelobacter* (Rhee *et al.* 1996), *Nocardia* (Pandey *et al.* 1991), *Micrococcus sp.* (Sims *et al.* 1986), *Corynebacterium sp.* and *Brevibacterium sp.* (Shukla *et al.* 1974; Shukla *et al.* 1975). Several *Achromobacter sp.* are found to be capable of transforming monohydroxylated pyridine. 2 and 3 monohydroxylated pyridine were converted to 2,5-dihydroxypyridine and 4-hydroxypyridine was converted to 3,4-dihydroxypyridine. On the other hand, *Arthrobactor* strains were also capable of degrading hydroxylated pyridine. *Bacillus sp.* is capable of transforming the carboxy pyridine (2-carboxypyridine). The transformation starts with a hydroxylation at position 6 of the heterocyclic aromatic ring, leading to 6-hydroxypicolinic acid. Pyruvic acid is the end product of this metabolism. The metabolism of hydroxylated and carboxylated pyridine, starts with a hydroxylation (Kaiser *et al.* 1996). Pyridine is also found to be degradable by a few Actinomycetes strains. Another introspection revealed that the strain PDB9^T (most closely related to the type strain of *Rhodococcus sp,* sharing 99% 16s rDNA similarity) found to be capable of degrading pyridine. From the results of several pyridine degradation tests, strain PDB9^T was shown to be very useful microorganism capable of degrading high concentrations of pyridine (approx 3500 mg l^{-1}) and was assigned as a new species, viz., *R. pyridinovorans* (Yoon *et al.* 2000). Interestingly, *Pseudomonas putida* MK1 with varying concentrations of phenol were synergistically utilized to observe its effect in the pyridine degradation. The main objective of this work was to demonstrate that calcium alginate immobilization of microbial cells can efficiently increase the tolerance of the microorganism to phenol and in turn results in increased degradation of pyridine. This study was an imperative landmark in the treatment of wastewater in the presence of compound toxicity (Kim *et al.* 2006). A novel bacterial strain, *Shinella zoogloeoides* BC026, which utilizes pyridine, has been isolated from the activated sludge of a coking wastewater treatment plant and is capable of degrading upto 1,806 mg l^{-1} of pyridine in 45.5 h (Bai *et al.* 2009). Various information of pyridine treatment in suspended aerobic bioreactor systems for liquid effluent are existing (Padoley *et al.* 2006; Uma *et al.* 2002; Uma *et al.* 1998; Pandey *et al.* 1997; Pandey *et al.* 1991) and Pandey *et al.* 2007 has reported biotreatment of waste gas containing pyridine using biofilter systems. Till date several works are in progress to isolate new and efficient microbial species to degrade pyridine. The objective of the present study was to isolate and characterize bacterial strains capable of degrading high strength of pyridine and to identify the optimum conditions under which this strain can most efficiently breakdown pyridine.

MATERIALS AND METHODS

Chemicals and growth medium

The isolated strain MM was grown on basal salts medium (BSM) prepared with deionized water (Milli-Q, Millipore) with pyridine as the only carbon source. The BSM was filter sterilized aseptically (0.2µm filter, Pall Corporation, Pall India Pvt. Ltd., India). All the chemical components used were AR grade with more than 99% purity and purchased from local chemical manufacturer (S.D. Fine Pvt. Ltd., India; Merck, India). Bacteriological grade chemicals were purchased from Himedia (Mumbai, India).

Table 1. Composition of the Basal Salt Medium (BSM).

Component	Concentration (g L^{-1})
KH_2PO_4	0.91
$Na_2HPO_4 \cdot 2H_2O$	2.39
KNO_3	2.96
$(NH_4)_2SO_4$	1.97
$MgSO_4 \cdot 7H_2O$	0.5
$CaCl_2 \cdot 2H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.02
$MnSO_4 \cdot 7H_2O$	0.0008
$ZnSO_4 \cdot 7H_2O$	0.0004
$Na_2MoO_4 \cdot 2H_2O$	0.001
$CoCl_2 \cdot 6H_2O$	0.0004

Soil collection and determination of pH

Soil sample was collected from 1 ft depth of the selected petrochemical industrial effluent site. Next, 5 gm dry soil was dissolved in 25 ml 0.5 mM $CaCl_2$ (pH-7.0) solution and then followed by measurement of its pH (Reddy, 2007).

Isolation of strains

Soil sample obtained from petrochemical industrial effluent site, was suspended and vortexed with Millipore water and then allowed to stand for 10 min. One milliliter of the supernatant was serially diluted up to 10^{-10} in sterile phosphate buffer (pH 7.0). Spreading of serially diluted sample was then aseptically done on basal salts medium agar 2% (w/v) plates with pyridine as a sole carbon and energy source in comparison with plate devoid of pyridine. The plates were then incubated at 30±1°C and colonies developed were observed in detail. Single colonies were obtained by repeated sub culturing. Following this process, one isolate (MM) was found to have profuse growth and maintained by periodical transfer onto LB agar slant with pyridine and was stored at 4 °C for further study. Additionally, glycerol stock of the culture was prepared and stored at – 80 °C.

Strain identification

The morphological, physiological and biochemical tests of the isolate MM are listed in Table 2. This bacterial strain was taxonomically identified under the genera *Shewanella* based on the tests (Table 2) as well as Buchanan and Gibbons, Bergey's Manual (Bergey's Manual of Determinative Bacteriology, 1974). This bacterial strain was further confirmed as *Shewanella algae* (designated as MM) by 16S rDNA sequence analysis.

Table 2. Biochemical and morphological characteristics.

Biochemical Characteristics	
Gram straining	Negative
Spore stain	Positive
Klingler iron agar slant	Butt-yellow, Slant-red
Catalase	Positive
Oxidase	Positive
Indole	Negative
Methyl red	Positive
Voges-Proskauer	Positive
Citrate	Negative
H_2S production	Positive
Glucose	Positive
Fructose	Positive
Maltose	Negative
Sucrose	Negative
Lactose	Negative
Starch	Negative
Arabinose	Negative
Urease	Positive
Gelatinase	Positive
Ornithine decarboxylase	Positive
DNase	Positive
Morphology under microscope	
Cell type (shape)	Rods
Color	Yellowish white
Size	0.5–0.6×1.6–2.8μm
Surface	Smooth
Arrangement	Coherent cluster
Density	Opaque
Elevation	Convex
Motility	Positive

Genomic DNA isolation and sequencing of 16S rDNA gene (rDNA)

Genomic DNA was isolated using standard bacterial procedures (Goldberg *et al.* 1984). Then 16S rDNA gene was amplified by PCR amplification using following primers: 63f (5'-AGGCCTAACACATGCAAGTC-3'), 1387r (5'-GGGCGGAGTGTACAAGGC-3') (Marchesi *et al.* 1998). The PCR mixture (50 μL) comprised of 25 pmol of each primer, 200μM of each of deoxynucleoside triphosphate, PCR buffer (Promega, Madison, WI, USA), 0.5U of Taq DNA polymerase (Promega, Madison, WI, USA) and 10 ng of DNA per μL. The PCR was performed in a thermalcycler (Eppendorf, Germany) with an initial denaturation step at 94°C for 2 min, 28 amplification cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final polymerization for 15 min (Na *et al.* 2005). PCR product (~1.5 kb) was visualized on 1.2% agarose gel and further excised followed by cloning in a pGEM-T vector (Promega, Madison, WI, USA). Both strands of the 16S sequences were sequenced by the dideoxynucleotide method (Sanger *et al.* 1977). Nucleotide sequence similarities were compared using BLAST (National Center for Biotechnology Information databases). The phylogenetic tree was constructed by the maximum likelihood method by using MEGA4.

Plasmid identification Test

The plasmid identification test was performed to find out whether the microbial isolate MM was carrying any natural plasmids or not. Purification of plasmid DNA using alkaline lysis is based on differential denaturation of chromosomal & plasmid DNA in order to separate the two (Reddy 2007).

Scanning electron micrograph of the isolated strain

Scanning electron micrographs of the MM strain was carried out by using a scanning electron microscope (SEM) (Model LEO435VP, LEO Electron Microscopy Ltd., England). For SEM, the microorganisms were fixed with 2% glutaraldehyde aqueous solution for 1 h at less than 20 °C, washed with phosphate buffer (pH 7.0) and then dehydrated with ethyl alcohol, dried and finally provided with a metal coating of gold.

Antibiotic Sensitivity Assay

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of aerobic and facultative anaerobic bacteria to various antimicrobial compounds. Here we tested four compounds, viz., Chloramphenicol (C), Streptomycin (S), Ampicillin (A), Fluconazole (F). If the agar plate has been inoculated with a suspension of the isolate to be tested prior to the placing of disks on the agar surface, simultaneous growth of the bacteria and diffusion of the antimicrobial compounds occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The point at which critical mass is reached is demonstrated by a sharply marginated circle of bacterial growth around the disk (Reddy 2007).

Acclimatization of cultures and biodegradation studies

The inoculum for the entire experiments was arranged by inoculating the isolated strain MM in the LB medium containing 500 mg l^{-1} of pyridine and incubating at 30 °C, 180 rpm until the bacteria grew into the logarithmic phase. Harvesting of the bacterial cells has been carried out by centrifuging at 3,000 x g for 5 min. The cells were washed for several times with the BSM. The bacterial pellet was then resuspended by vortex, and diluted with the BSM to an optical density at 600 nm (OD600) of 1–2 (Model UV Pharmaspec-1700 Shimadzu, Japan). Finally, the bacterial suspension was used as inoculum in the biodegradation experiment.

The biodegradation experiment was carried out by implementing a series of 250 ml Erlenmeyer flasks as batch reactors. Each flask contained 100 ml of the BSM with a specific concentration of pyridine (in the range of 400–3,000 mg l^{-1}) and same initial amount of the inoculum. The flask without the bacterial inoculum was used as a negative control under the same condition. All flasks were shaken at 30°C, 180 rpm, and sampled periodically. OD_{600} values were measured against time by using a spectrophotometer (Model UV Pharmaspec-1700 Shimadzu, Japan).

RESULTS AND DISCUSSION

Biochemical and morphological characterization

As depicted in Table 2, the strain (MM) was a rod-shaped bacterium with dimensions of 0.5–0.6 × 1.6–2.8 μm under the SEM (Figure 1). The results of physiological tests indicated that it was a gram-negative, aerobic, motile strain, and resistant to Fluconazole (Table 3, Figure 3). Absence of natural plasmid indicates that the pyridine degrading gene is not episomal in nature (Figure 2).

Table 3. Antibiotic sensitivity test.

Ampicilin	Sensitive
Streptomycin	Sensitive
Chloramphenicol	Sensitive
Fluconazole	Resistant

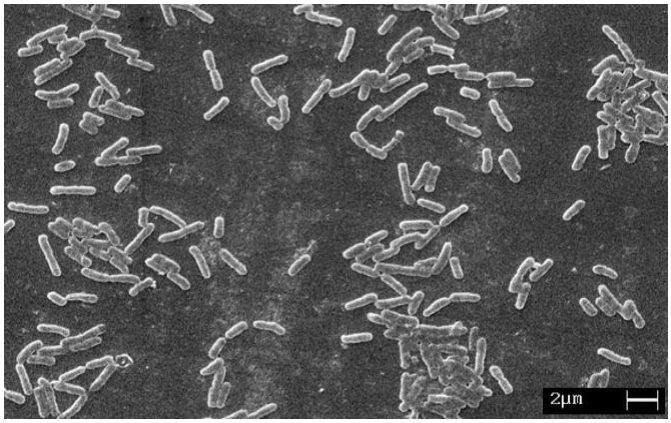

Figure 1. Scanning electron micrograph of profusely grown pyridine degrading isolate.

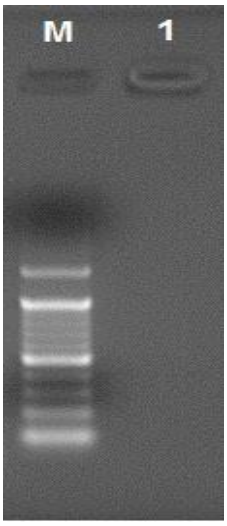

Figure 2. Plasmid identification of isolated strain ran on 1.0% agarose gel (M: 100 bp Molecular Marker, Lane 1 is showing no visible bands in comparison to molecular marker).

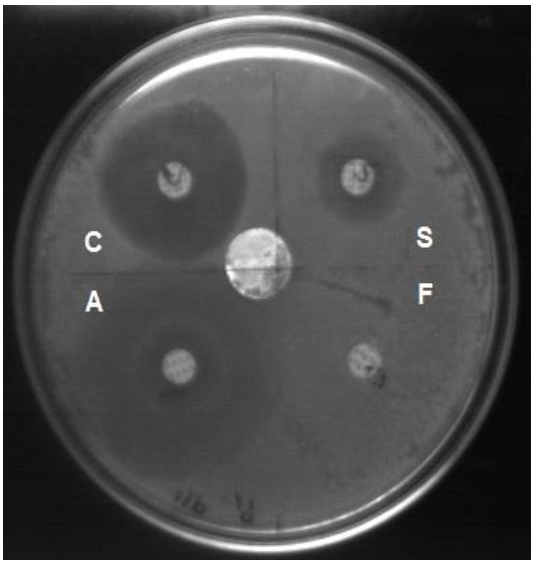

Figure 3. Antibiotic sensitivity assay (C: Chloramphenicol, S: Streptomycin, A: Ampicillin, F: Fluconazole).

Identification of the strain MM

Figure 4 illustrates that the corresponding gene fragment (~1.5 kb) was amplified from the genomic DNA. The PCR product generated in this approach with Taq polymerase enzyme usually comprises 'A' overhangs. Following this, these products were ligated in pGEM-T vector using TA cloning method. After ligation reaction the positive clones (pGEM-T vector with desired insert) were selected by Blue-White screening, and positive clones were isolated. The isolated vector with insert having the desired size of 4.5 kb was sequenced in forward and reverse direction. The sequence has been analyzed with existing 16S rDNA sequences in the GenBank database and upon subsequent phylogenetic analysis by MEGA4, the strain (designated as MM) was found to have 100% similarity to *S. algae* (Gen Bank Accession No. SAU91544.1) (Figure 5). In this way, the morphological and physiological characterization data were supported and above strain was confirmed as *S. algae.*

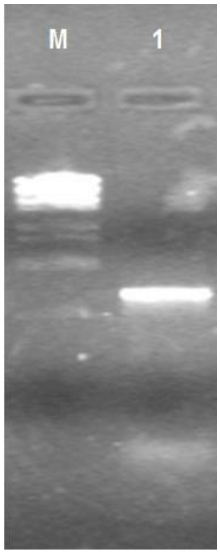

Figure 4. Lane 1 is showing the PCR product (~1.5 kb) of isolated pyridine degrading strain with Lambda DNA/BstEII digest as marker (M) ran on 1.2% agarose gel.

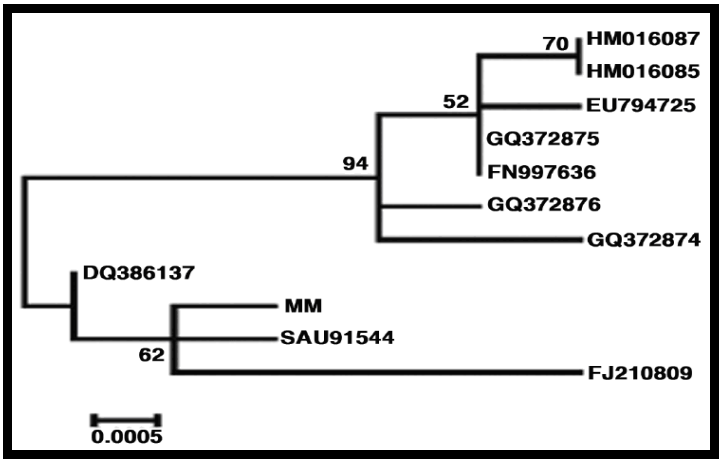

Figure 5. Phylogenetic Tree by Maximum Likelihood Method.

Effects of pH and temperature on the biodegradation of pyridine

To find the optimal conditions for the cell growth, further biodegradation experiments under different pH (3.0–11.0) (Figure 6) and temperatures (20–40°C) (Figure 7) were accomplished. The strain MM could grow better in the pH range of 5.0–9.0 and temperature range of 25–35°C with an optimum at pH 7.0 and 35°C.

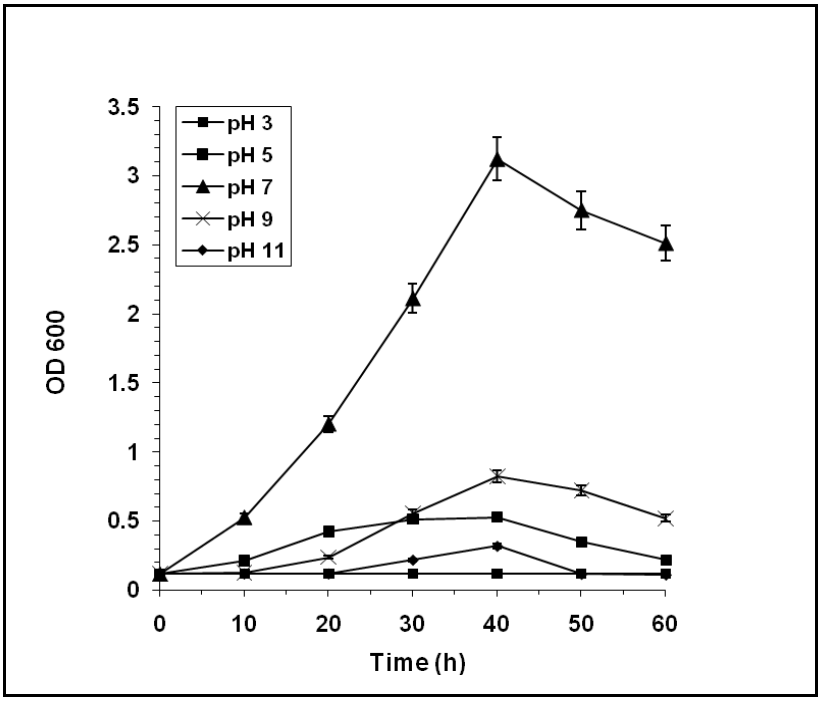

Figure 6. Effect of pH on cell growth of isolated pyridine degrading strain in BSM (the values represent the Mean ± S.E.M. of 3 independent experiments performed).

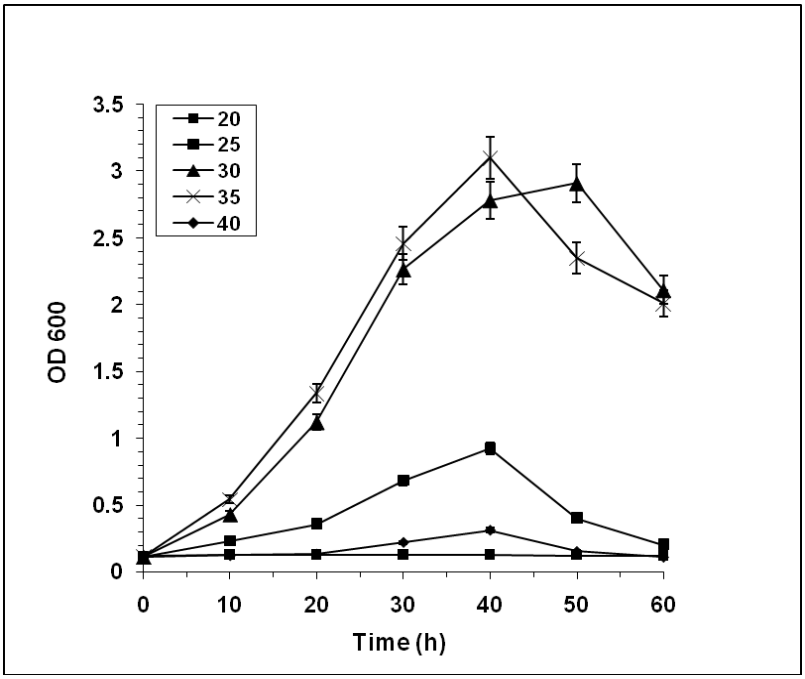

Figure 7. Effect of Temperature on cell growth of isolated pyridine degrading strain in BSM (the values represent the Mean ± S.E.M. of 3 independent experiments performed).

Biodegradation of pyridine

For the biodegradation experiment with different initial pyridine concentrations (in the range of 400–3,000 mg l^{-1}), Figure 8 shows the growth of the MM strain. Pyridine of 393, 922, 1,919, and 2,614 mg l^{-1} were degraded by the strain MM as OD increases with time (h) gradually. By utilizing pyridine, MM strain grew in a well-fashioned manner and stabilized at OD 600 1.418, 1.821, 3.64 and 2.604 corresponding to the initial pyridine concentration from low to high (Figure 8).

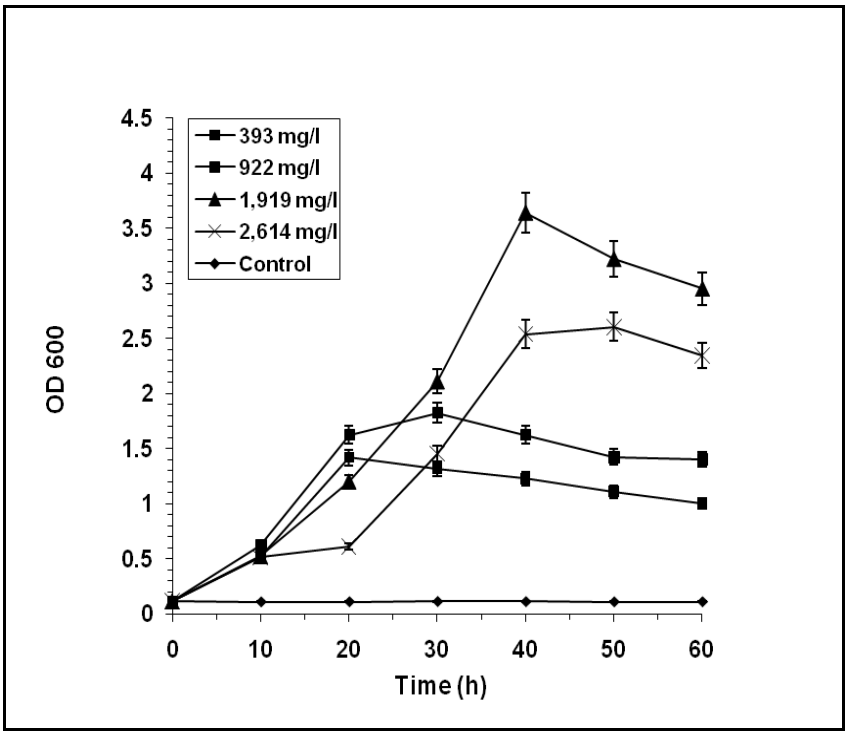

Figure 8. Pyridine degradation and cell growth of the isolated pyridine degrading strain with different initial concentrations in BSM (393 mg l^{-1}, 922 mg l^{-1}, 1,919 mg l^{-1}, 2,614 mg l^{-1}, and sterile control) (the values represent the Mean ± S.E.M. of 3 independent experiments performed).

CONCLUSIONS

The rationales of this study was to isolate the pyridine degrading bacteria from the soil contaminated with petrochemical industry effluents at Purba Medinipur (W.B.) and to investigate its physiological characteristics. The pure stain was isolated and identified as *S. algae*. This experimental results show that the isolated stain, *S. algae* efficiently degrades different initial concentrations in BSM (393 mg l^{-1}, 922 mg l^{-1}, 1,919 mg l^{-1}, 2,614 mg l^{-1}) implying *S. algae* used pyridine as sole carbon and energy source. The strain could grow better in the pH range of 5.0–9.0 with an optimum at pH 7.0. Isolate *S. algae* can degrade high strength pyridine and compared to the other organisms reported in the literature, the strain *S. algae* was capable of degrading 2,614 mg l^{-1} of pyridine effectively. The study indicated that the information presented herein laid the foundation for further treatment of pyridine bearing wastewater and the possible use of an inoculum of *S. algae* in a biofilter treating pyridine-laden gas.

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