

Isolation, Identification And Characterisation Of Dye-Adapted Bacteria From Textile Effluents Mixed With Sewage Released Into The River Amaravathy, Karur, Tamilnadu, India.

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Abstract: In Karur, the textile factories discharge millions of litres of untreated effluents into the drains that eventually empty into river, Amaravathy. The release of coloured compound into water bodies is undesirable not only because of their impact on photosynthesis of aquatic plants but also due to the carcinogenic nature of these dyes and their breakdown products. In this work, bacteria capable of decolorising Textile effluents mixed with sewage (TES) were isolated. Based on the results of these various biochemical tests, the isolates were identified. The identification was conformed by 16s rRNA sequencing. They were identified as *Bacillus cereus* AK1968 and *Pseudomonas* sp. AKDYE14. The sequences were deposited in GENBANK. The accession numbers were JN689235 and JN674167 respectively.

Key words: Karur, Amaravathy, Textile Effluents mixed with Sewage (TES), 16srRNA sequencing, *Bacillus cereus*, *Pseudomonas* sp., GENBANK

I. Introduction

1.1 Amaravathy river basin at the down stream of Karur Town is severely polluted due to discharge of partially treated effluent by the textile bleaching and dyeing units. At present 487 bleaching and dyeing units are in operation. These bleaching and dyeing units are located on either side of Amaravathy river within 2 Km from the river. By average one unit generates 30 kilo litres per day (KLD) of trade effluent. These units have provided either individual effluent treatment plant (IETP) or joined in common effluent treatment plant (CETP). After treatment the effluent is discharged into river Amaravathy. The total dissolve solids (TDS) in the river discharge is in the range of 5000 – 10000 mg/L and the chloride is in the range of 2000 – 4000 mg/L. Daily 14600 KLD of partially treated effluent is discharged into the river. The effluent reaching the river is in dark brown in colour. During summer period there is no water flow in river, only effluent flow can be noticed. In monsoon period the colour in river water can be noticed up to Kattali where the river confluences with river Cauvery [1].

1.2 Dyes are usually aromatic and heterocyclic compounds and are often recalcitrant, some of them being toxic and even carcinogenic [2]. Many microorganisms are capable of degrading azo dyes, including bacteria, fungi, and yeast [3]. The efforts of isolating dye-degrading bacteria started in the 1970s, when *Bacillus subtilis* was first isolated in 1977, followed by *Aeromonas hydrophila* in 1978 and *Bacillus cereus* in 1980. After that, several other dye-degraders such as *Pseudomonas* and *Aeromonas* strains were reported [4] (Banat *et al.*, 1996). Recently, bacterial cultures capable of degrading dyes were listed by Banat *et al*[4]. Pearce *et al.* [5] and Forgacs *et al.* [6]. Numerous studies have demonstrated the ability of bacteria in monoculture to degrade dyes anaerobically and aerobically [4]. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. A highly alkali thermostable *Bacillus* sp. SF isolated from the wastewater drain of a textile finishing company was found to be capable of degrading an azo dye [7]. An azo dye-reducing, endospore forming bacterium was isolated from textile industry wastewater that can decolorize the azo dye Remazol Black B [8].

1.3 Recently, several researchers have identified single bacterial strains that have very high efficacy for removal of azo dyes. In contrast to mixed cultures, the use of a pure culture has several advantages. These include predictable performance and detailed knowledge on the degradation pathways with improved assurance that catabolism of the dyes will lead to nontoxic end products under a given set of environmental conditions. Another advantage is that the bacterial strains and their activity can be monitored using culture-based or molecular methods to quantify population densities of the bacteria over time. Knowledge of the population density can be extrapolated to quantitative analysis of the kinetics of azo-dye decolorisation and mineralization [9]. Microorganisms have developed enzyme system for the decolorisation and mineralization of azo dyes under certain environmental conditions [9, 10, 11]. Preliminary characterization of the bacterial isolate

regarding its morphological, biochemical characters and decolourisation activity as well as the molecular identity gives useful information with regard to the further application of strain for various purposes [12]. So, present study was designed to isolate the dye adapted and efficient dye decolourising bacterial strains from their native habitat, the textile effluents mixed with sewage (TES) identify and characterise them.

II. Materials And Methods

2.1 Sample collection

The untreated textile effluents along with sewage were released into the river Amaravathy by small-scale dyeing industries in Karur. Amaravathy river basin is located between north latitude $10^{\circ}06'51''$ N and $11^{\circ}02'10''$ N and longitudes $77^{\circ}03'24''$ E and $78^{\circ}13'06''$ E in Tamilnadu, India. Samples were collected in sterile plastic bottles from a place where these **textile effluents and sewage (TES)** confluence with the river Amaravathy. Samples were transported to the laboratory in an ice-packed chest box within 1 hr of collection.

2.2 Isolation of bacteria from TES and Screening of bacterial isolates

One ml of freshly collected TES was serially diluted (10^{-1} – 10^{-3}) in physiological saline. A loopful of each dilution was inoculated, by streaking onto triplicate set of agar plate containing the basal medium. **The basal medium** contained (gL⁻¹): NaCl - 0.3, MgSO₄·7H₂O - 0.42, KCl - 0.29, K₂HPO₄ - 1.27, KH₂PO₄ - 0.85, NaNO₃ . 0.42 and deionised water 1,000 ml. Agar No. 1 was added at 1.5% (w/v) to prepare solid medium. The plates were incubated at 37°C for 24 – 48hr. Morphologically different colonies B1, B2, B3, B4, and B5 developed were picked separately and inoculated separately into 10ml liquid broth and incubated at 37°C for 24hr. **Liquid Broth Medium** contained (g/L): Tryptone 10.0, Yeast 1.0, Agar 0.7, NaCl 10.0 & de-ionized water 1000ml. Both the media used were at pH 7.0. Sterilization was done by autoclaving at 121°C at 15psi for 15min.

TriPLICATE set of 100ml Erlenmeyer flasks each containing 25ml of TES were inoculated with 1ml of a 24hr liquid broth culture B1, B2, B3, B4, and B5 separately. Two sets of controls were also set up: positive control consisted of duplicate flasks inoculated with 1ml of heat-treated cells and negative controls consisted of uninoculated flasks. The flasks were incubated at 37°C and observed daily for decolourisation of medium (evidence of degradation) and increase in turbidity (evidence of growth). The cultures showing maximum turbidity and decolourisation were selected on the basis of spectrophotometer readings, identified and used for further studies. Each agar plate (mixed with sterilized TES) was inoculated with a loopful of the culture. Incubation was done at 37°C for 24 – 48hr. Colonies was picked and purified by repeated subculture and Gram stained. Colonies which were distinctly seen were used for various identification tests. Various tests (motility, presence/absence of spore, urease, citrate utilization, hydrogen sulphide production, MR, VP, catalase, oxidase, indole, starch hydrolysis and fermentation of various sugars) were carried out according to the methods of [13] Cruickshank *et al.* [14] Garrity *et al.* and [15] Holts *et al.*

2.3 16s rRNA sequencing

Identification of the isolated strain that achieved the best decolourisation was performed by 16S rDNA sequence analysis. 16S rRNA gene/DNA was obtained according to [16] Pitcher *et al.* using TE buffer, Lysozyme, Proteinase K, isopropanol and ethanol. The 16SrRNA gene was amplified by PCR using the specific Primer sequences, the forward primer, 16sF- AGAGTTGATCMGGCTCAG and the reverse primer, 16sR-AAGGAGGTGWTCCARCC and the DYEnamic ET Dye Terminator Cycle Sequencing Kit for the automated Mega Base 500 system (GE Healthcare) [17]. The sequences were aligned using the CLUSTAL X program and analyzed with the MEGA software 2001 [18, 19]. Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA, using Kimura's DNA substitution model [20]. The phylogenetic reconstruction was done using the Neighbour Joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software [21].

III. Results

Five different colonies B1, B2, B3, B4 and B5 were found to be growing in the medium with TES and they were isolated. On the basis of spectrophotometer readings it was found that the isolates B3 and B4 showed maximum hydrolysis whereas B1, B2 and B5 isolates showed only slight hydrolysis in liquid broth (Table 1), thus they were not selected for further study of the dye degradation. The desired bacterial strains with strong decolourising ability on TES, B3 and B4, were isolated from TES and identified. The results of various biochemical characters were given in the Table 2. The colony of bacterial isolate B3 was circular, flat, and smooth. The bacteria of the isolate B3 was a gram positive rod shaped, non-motile and facultative anaerobic spore forming bacteria. The optimum growth temperatures ranged from 30 to 50°C. They could grow at pH values of between 4.3 and 9.3. The organism produced heat resistant spores and these might germinate if cooling was too slow. They could hydrolyse casein, gelatin and starch, could reduce nitrate, and could produce

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acid from maltose, mannitol, glucose, fructose and galactose. They showed negative result for indole test, methyl red test, voges-proskauer test, and citrate catalase test. The bacteria of B4 colonies were rod shaped, gram-negative, aerobic, non-spore forming bacteria. B4 species also typically showed a positive result to the catalase test, oxidase test, the absence of gas formation from glucose, indole negative, methyl red negative, Voges-Proskauer test negative, and citrate positive. The bacterial strains B3 and B4 were tentatively identified as *Bacillus cereus* (Plates 1 to 4) and *Pseudomonas* sp. (Plates 5 & 6) to respectively by referring to Bergey's Manual of Determinative Bacteriology [15]. Based on the phenotypic and biochemical characteristics and genetic and phylogenetic analysis, the new strains were identified and christened as *B.cereus* AK1968 and *Pseudomonas* sp. AKDYE 14. The 16S rDNA sequences of both of these bacteria were deposited in GenBank. The accession numbers were JN689235 and JN674167 respectively.

16s rDNA sequence of *Bacillus cereus* AK1968

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>
CCGTCAATGGCGGGGCTGCCTAATTACATGCAAGTCAGCGAATGGATAAGAGCCTGCTTATGAAGTTAGCGCGGACGGGTGAGTAAC
ACGTGGGTAACCTGCCATAAGACTGGGAAACTCGGGAAACCGGGCTAATACCGATAACATTGAAACCGCATGGITCGAAATTGAAAGG
CGGCTTCGGCTGCACTTATGGATGGACCCGCGTCGATTAGCTAGTGGTGAGGTAAACGGCTACCAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATGCCAACACTGGGACTGAGACACGCCAACACTCTACCGGAGGCAGCAGTGGAAATCTCCGAATGGACGAAAGTCTGACCGGA
GCAACCGCGTGAAGTGAAGGCTTCCGGCTGAAAACACTCTGGTAGGGAAAGAACAAAGTGTAGTTGAATAAGCTGGCACCTTGACCGTA
CTAACACCAGAACGCCAGGCTAACTACGTGCCAGCAGCGCTAATACGTAGGGCAAGCGTTATCCGAATTATTGGGCTAAAGCGCGCG
CAGGTGGTTCTAAGTCTGATGTGAAGGCCACGGCTAACCGTGGAGGGTCACTGGAAACTGGGAGACTTGAGGCAGAAAGAGGAAAGTGG
ATTCCATGTGAGCGTGAATGCGTAGAGATATGGAGGAACACCGATGGCAAGGCAGCTTCTGCTGTAACGTGACACTGAGGCGCGAAA
GCGTGGGAGGAAACAGATTAGATACCCCTGGTAGTCCACGCCAACAGTGGAGTGAAGTGTAGGGTTAGGGGCTTCCGCCCCTTAGTGTGAAAGT
TTAACCGCATTAAGCAGCTCCGCCATGGGGAGTACGGCCGAGGCTGAAATCAAAGGAATTGACGGGGGCCACCAGCGGTGGAACATTGG
TTTAATTGAAAGCCACGGGAGAACCTTACCCGGTTTGAACTCTTGGAAACCCCTGGGATTGGGTTTCCCTTGGGAGCAAGGTACCGGTG
GGGCCAGGGTGTGCAAGTGTGTTGAGAAGGGTGGGAAAGTCTCGCAAAAGGGGAAACCTTGTGATTGGTGCCTCCCTTAAGGTGGC
AATTAAAGGGGATGCGGGGAAACCGGAGGAAGGTGGGAAAGAAGTCAAATTCTCTCCCTTGTGACCTGGGGTACCCAACACTGGTCAAT
GGGAGGTACAAGAGGTCAAGACCCGGAGGGGGGGTAATTCTCAAACCGTTCCGTTGGGTTGGGTGCAAAATTGCTACCTGAAG
GTGGAATTGTTGTTATTGCGGGTCAGCTGCCGCGTGAATAAGTTCCGGGCCTGGACACCCGCCCTCACCCCCGGGAGTTGTACCCC
CGAAGTCGGTGGGGAACCTTTGGAGCCAGCGCTCTTGTGACTACGATTTGG
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Sequence Analysis with BLAST : Gb|Q329658.1; *Bacillus cereus* 16S ribosomal RNA gene, partial sequence Length=1451; Score = 2167 bits (1173) ; Strand=Plus/Plus .

16s rDNA Sequence of *Pseudomonas* sp. AKDYE14

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>
AAAATTAGGGGAACCGCCCATAGAGTAACAAGTCCCCCGAAGGTTAGACTAGCTACTTCTGGAGCAACCCACTCCATGGTGACGGCGG
TGTGTACAAGGCCCGGAACGTATTACCGTGAECTCTGATTACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCC
GGACTACGATCGTTTATGGGATTAGCTCACCTCGGGCTTGTACCGACCTTGTACCGACATTGAGCACGTGTGAGCCTGGCTAAGGACAAG
GCCATGATGACTTACGTCATCCCCACCTCCCGGTTGTACCGCAGTCCTCTAGACTGCCACCCGAGGTGCTGTAACTAAGGACAAG
GGTTGGCTCGTTACGGACTTAACCAACATCTCACGACACGAGCTACGACAGCCATGCAGCACCTGTGTGAGTCCCGAAGGCACCAATC
CATCTGAAAGTCTCAGCATGTCAGGCCAGGTAAAGTCTCGGTTGCTGAAATTAAACACATGCTCCACCGCTTGTGCGGGGCCCCGT
CAATTCAATTGAAATTAAACCTCGGGCCGTACTCCCCCGGGCTCAACTTAAAGGGTTAGCCGGCGACTAAAATCTCAAGGGTCCCCA
ACGGGCTATTGACATCGTTTCCGGCTGGGACTACCAGGGTATCTAAATCCGTTTGCCCCCACGTTTGACCCCTCAGTGGTGGTATC
AACTCCAAGGGTCCCCCTCGCAAACCTGGGGTGTCTCTTTATCTACACCATTAGCGGCTCCTCAGGAATATTCCAGCCCCCCCCCT
ACCCGCTATTCTAGITCCCCCGTGTGGATAGCCGGTCCCCAGGGTTAAACCCCGGGGGGGTATTCACTCCGACCTTATACAACCC
CCCTTACACC CGGTGTTATTACCCCATAAATTCCAAGATAAAAGACACTGTGCTACCCGCTCCTATATCTGCCCTCG
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Sequence Analysis with BLAST : Dbj|AB045280.1; *Pseudomonas* sp.

HMD H5 for 16S rRNA, partial sequence Length=1488

Score = 1232 bits (667) ; Strand=Plus/Minus

Table 1 Ranking of bacterial isolates B1, B2, B3, B4 & B5 by their Dye decolourising ability based on visual identification.

S.No.	Purified colonies	Ranking of decolourisation in Liquid Broth Medium	Ranking of decolourisation in Basal Medium
1.	B1	-----	-----
2.	B2	++	+
3.	B3	+++	+++
4.	B4	+++	+++
5.	B5	-----	-----

Key: ----no decolourisation + slight decolourisation ++ moderate decolourisation +++ intense decolourisation

Table: 2. Morphological and biochemical characteristics of B3 and B4 colonies isolated from the Textile Effluents mixed with Sewage (TES).

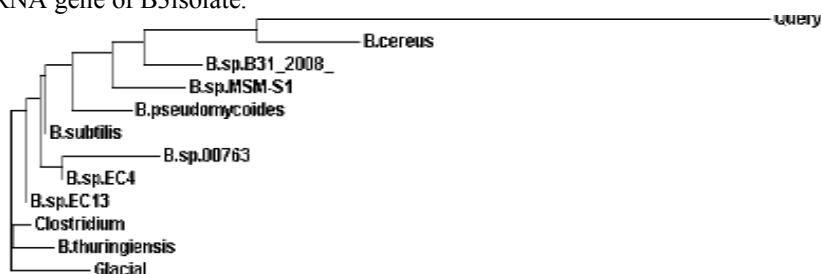
S.No.	Biochemical Test	Characteristics of	
		B3 isolate	B4 isolate
1.	Gram staining	+	-
2.	Morphology	Rods	Rods
3.	Motility	-	+
4.	Spore	+	-

5.	Spore position	Terminal	-
6.	Spore shape	Ellipsoidal	-
7.	Indole	-	-
8.	Methyl Red	-	-
9.	Voges-Proskauer	-	-
10.	Citrate test	-	+
11.	Catalase test	-	+
12.	Nitrate Reduction test	+	+
13.	Oxidase test	-	+
14.	Hydrolysis of:		
i)	Casein	+	-
ii)	Gelatin	+	-
iii)	Starch	+	-
iv)	Urea	-	-
15.	Acid from:		
i)	D-Maltose	+	+
ii)	Mannitol	+	-
iii)	D-Glucose	+	+
iv)	D-Fructose	+	+
v)	Galactose	+	+

+ = positive; - = Negative

IV. Discussion

16S ribosomal RNA gene sequence of B3 isolate was showing common ancestry with *Bacillus cereus* 16S rRNA gene with highest similarity. The other neighboring species which were showing high similarity with the 16S rRNA gene of *Bacillus* sp. B31(2008), *Bacillus* sp. MSM-S1, *Bacillus pseudomycoides* strain N14, *Bacillus subtilis* strain BSF01, *Bacillus* sp. 00763 and *Bacillus* sp. EC4. 16S rRNA gene of *Clostridium* sp. W52, *Bacillus thuringiensis* strain CMST-MSU-APL 2, Glacial ice bacterium G50-TS3 are distinctly showing similarity to the 16S rRNA gene of B3 isolate.



Dendrogram 1. showing the relationship of *Bacillus cereus* with other bacteria

16S rRNA gene sequence of B4 isolate was showing close evolutionary relationship with *Pseudomonas* sp. **HMD H5** 16sRNA gene with highest similarity and sharing common ancestry with the 16S rRNA gene of *Pseudomonas* sp. V7 and *Pseudomonas* sp. BWDY-42. 16S ribosomal RNA gene of *Pseudomonas* sp. K50 and *Pseudomonas* sp. Y2-1-1, *Pseudomonas oleovorans* strain DT4 were showing nearest similarity with the 16S rRNA gene of B4 isolate. 16S rRNA gene of *Pseudomonas* sp. SMCC B0259 and *Pseudomonas mendocina* strain NaF-C-1 are far distantly similar with 16S rRNA gene of B4 isolate.



Dendrogram 2. showing the relationship of *Pseudomonas* sp. with other species

B. cereus was reported to decolorize cibacron red P4B by 81% using the combination of ammonium nitrate and sucrose and to decolorizes cibacron black PSG by 75% using yeast extract and lactose. The preliminary decolorisation studies carried out with bacteria species isolated from the dye effluents, indicated that *B. cereus* performed best when compared to *Mycobacterium acidophilus* and *Streptococcus faecalis* [22].

Pseudomonas sp.(99%) which has been proved to have decolorising ability against both azo and triphenylmethane dyes [23] . Based on the phenotypic characteristics and phylogenetic analysis, strain was identified as *Pseudomonas* sp.

Heterotrophic bacteria, such as *Escherichia coli*, *Bacillus* sp. *Pseudomonos* sp., *Staphylococcus* sp., etc., are usually dominant in the activated sludge system, degrading and eventually mineralizing organic compounds to carbon dioxide and water [24, 25].

A study discovered effluent adapted strains of *Acinetobacter*, *Bacillus* and *Legionella* with potentials for colour removal and strains of *Acinetobacter*, *Bacillus* and *Pseudomonas* with potential use for COD removal. The municipal landfill site soils yielded strains of *Bacillus* sp. with potentials for use in colour and COD removal. This may be due to the significant exposure of these organisms to a myriad of chemicals and materials some of which contain dyes which are deposited in the landfill which may cause a release of dyes to the soil [25] strain of bacterium *Bacillus subtilis* with strong decolorising ability was isolated from Textile effluent to decolorize the textile Azo dye Crystal violet (100 mg/L) within 24hr in aerobic and static conditions [26]. [27] Khadijah *et al* reported 1540 bacterial isolates and screened for their ability to degrade selected azo dyes.

Interestingly, the bacterial species used in carrying out the decolorization of Azo dye Crystal violet was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus subtilis* showed decolorising activity through a degradation mechanism rather than adsorption. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors[26].

A wild-type isolate *Pseudomonas luteola* was able to decolorize reactive black B while *P. aeruginosa* NBAR12 was capable of decolorising 12 different dyes [28]. *Pseudomonas aeruginosa* was also reported to decolorize certain azo dyes. Many *Pseudomonas* strains were also found to be capable of denitrification [4]. A total of 24 isolates were obtained; eighteen organisms belonging to the genera, *Bacillus*, *Acinetobacter*, *Legionella*, *Staphylococcus* and *Pseudomonas* were isolated from the textile effluents (effluent-adapted bacteria) while six isolates belonging to the genus *Bacillus* were isolated from the landfill site (effluent non-adapted isolates) [26]. As observed by [3] Chen *et al.*, screening on solid media after adaptation and enrichment gave effective dye decolorising isolates; bacteria colonies with decolorisation zone and fungi showing growth and decolorisation on solid culture which were further picked out and enriched in the liquid media to develop the deferent decolorising consortium. Adapted local microbial isolates from textile wastewater contaminated sites can effectively be used in the aerobic treatment of these effluents before discharging into the environment [29].

Reports indicate that though several microorganisms may seem to have a potential for dye degradation, very few strains can withstand the conditions of dyeing effluents, thus the effluent-adapted strains may be better candidates for potential bioremediative uses [30]. The bacterial isolates *Bacillus cereus* and *Pseudomonas* sp. originated from the dye contaminated textile wastewater of local industry can easily adapt to the prevailing local environment. Microbial decolorisation and degradation is an environment friendly and cost-competitive alternative to chemical decomposition processes. Moreover decolorisation and degradation can also detoxify the effluent effectively without leaving any residues [31]. Microbial degradation of azo dyes by extra cellular enzymes in *Pseudomonas* sp., and *Pseudomonas luteola* and *Mycobacterium avium* has already been reported [32]. Individual and synergistic effects of *B.cereus* and *Pseudomonas* sp. have been proved to be successful in degradation of dye house effluent in dye contaminated ecosystems[33].

V. Conclusion

The use of microorganisms like bacteria and fungi for the removal of synthetic dyes from industrial effluent offers considerable advantages. The process is relatively inexpensive, it is simple method and running costs are low, the end products of complete mineralization are not toxic. Therefore, the bacterial strains identified in this work *B.cereus*AK1968 and *Pseudomonas* sp.AKDYE14 can be used to develop an effective biological treatment system for the wastewaters contaminated with azo dyes.

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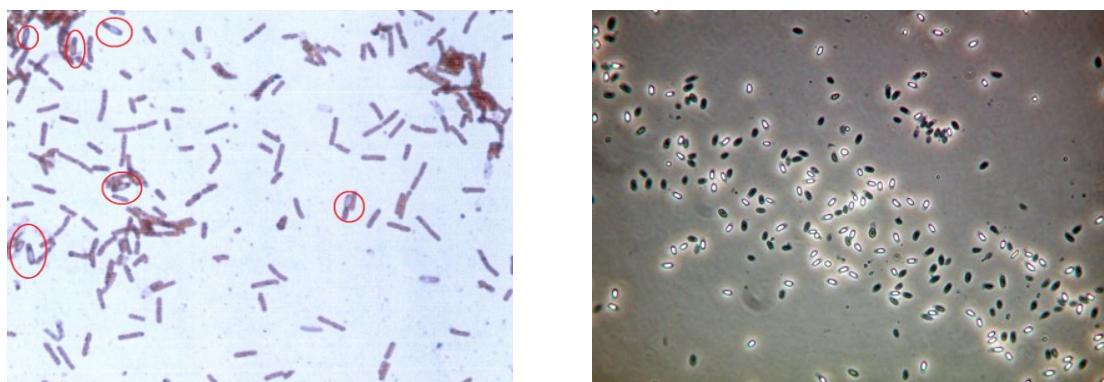


Plate 1 & 2 Microscopic view of *Bacillus cereus* AK1968 and its spores

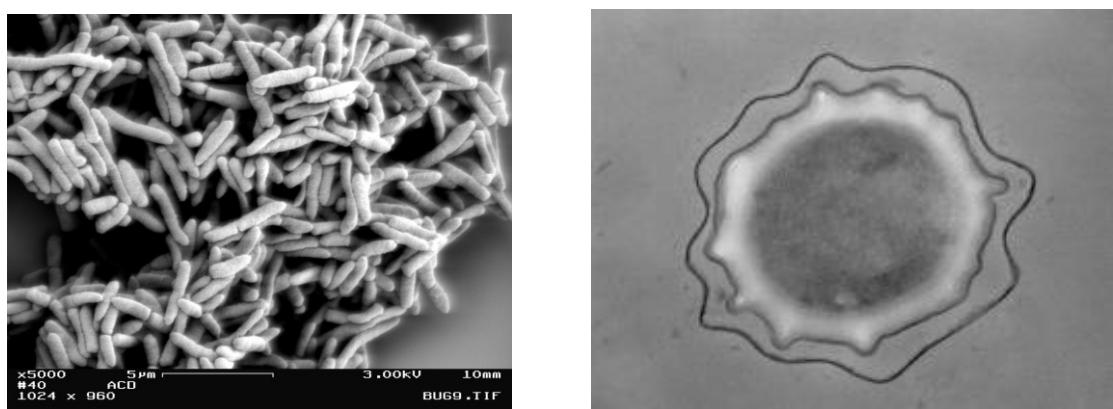


Plate 3 & 4 Electron miocrograph of *Bacillus cereus* AK1968 and its spore

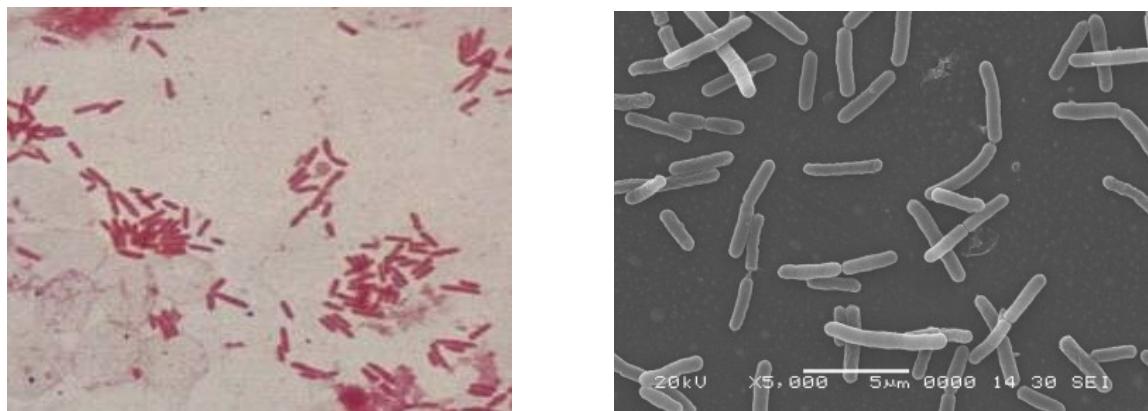


Plate 5&6 Microscopic view and Electron micrograph of *Pseudomonas* sp. AKDYE14