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Genomic Analysis and Antibiotic Resistance Profiling of Tributyltin Degrading Bacteria, Pseudomonas Stutzeri

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KEYWORDS

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ABSTRACT

In the present study, an indigenous strain of Pseudomonas stutzeri exhibiting high tolerance to tributyltin (TBT) at a concentration of 1500 µg/L was isolated from the Cuddalore Old Town (OT) harbour, a prominent shipping zone in Tamil Nadu, India. Molecular identification techniques confirmed the taxonomic status of the isolate, which was further characterized for its growth kinetics under elevated TBT concentrations using various carbon sources. The isolate demonstrated significant potential for bioremediation, aligning with the growing emphasis on the use of native microbial communities as cost-effective and environmentally sustainable strategies for the remediation of organotin-contaminated marine environments. Genomic analysis revealed the presence of Antibiotic resistance gene, supporting its functional role in detoxification. Whole-genome sequencing and assembly using the Unicycler platform resulted in 22 contigs, with an estimated genome size of 4,192,731 base pairs and an average G+C content of 64.25%. The genome assembly exhibited an N50 value of 537,847 bp and an L50 count of 3, indicating high contiguity and assembly quality. These findings underscore the potential application of Pseudomonas stutzeri as a robust candidate for the bioremediation of TBT in marine ecosystems.

INTRODUCTION

Pseudomonas stutzeri is a non-fluorescent, denitrifying pseudomonad, widely distributed in soil and water. *Pseudomonas stutzeri* was first described by Burri and Stutzer in 1895 as *Bacillus denitrificans II* and renamed Bacterium stutzeri by Lehmann & Neumann in 1896. Thereafter, the taxonomic and nomenclatural status of this well-characterized species became confused, and was cleared up by van Niel & Allen only in 1952, when they retraced the history of the species, and redescribed it. *P. stutzeri* can be distinguished from other *Pseudomonas sp.* by many properties, of which the salient ones are: vigorous denitrifying ability; use of starch and maltose as carbon sources; and a characteristic and unusual colony structure; colonies of freshly isolated strains are wrinkled, tough and coherent, and have a light-brown colour which reflects the unusually high cytochrome content of the cells. Aerobic growth is possible in the presence of nitrate, nitrite, or N_2O producing copious amounts of nitrogen. Colony forms are varied and may produce pitting of the agar surface [24].

Work performed in recent years has clearly established firm bases for grouping the strains into a number of genomic variants (genomovars) that are phylogenetically closely related. Some strains have received particular attention because of specific metabolic properties such as denitrification, degradation of aromatic compounds, and nitrogen fixation. In such a way we identified and used this *Pseudomonas stutzeri* strain for biodegrading of Tributytltin (TBT).

Butyltin compounds have been widely used as stabilizers for chlorinated polymers, catalysts for a variety of chemical reactions, and biocides for boat paints [26]. Of particular importance to the environment is the high toxicity of tributyltin chloride (TBT). Present knowledge indicates that TBT is among the most toxic compounds known for aquatic ecosystems [12]. These compounds are released continuously into the water, are persistent and impact on organisms. The toxic effects of TBT compounds have been investigated since the early 1980s after researchers in France discovered that TBT released from antifouling paints caused abnormal and reduced growth in the pacific oyster [3]. In addition, various biological effects of TBT such as shell deformation in oysters and growth retardation in many other species have been well documented. However, the most harmful effect of TBT is the commonly observed effect of imposex, the development of male characteristics in female species, in the dog-whelk [2].

These facts forced the development of national legislations all over the world. The use of antifouling paints containing TBT has been banned on vessels under 25m in France since 1982, in England since 1987 and in the United States since 1989. More recently, the Marine Environmental Protection Committee (MEPC) of the International Maritime Organization (IMO) banned the use of TBT-based paint products on ships from 1 January 2003. By the beginning of 2008, the presence of such paints on ships had to be completely removed or covered [7].

Therefore, there is an urgent need to clean up TBT-polluted environments after the global banning due to the significant risks it poses to the human and aquatic organisms for its slow degradation rate. In selecting bioremediation agents, indigenous bacteria were documented to be of great potentials compared to non-indigenous. In this study, *Pseudomonas* isolated from contaminated surface sediment of Cuddalore coast, Tamilnadu. The isolate was able to resist TBT up to 1500 µg/L without addition of carbon source in Basal salt medium (BSM). Growth of *Pseudomonas* sp. was observed in BSM treated with TBT at concentration of 500µg/L and 1000 µg/L along with addition of glucose as carbon source. Growth of *Pseudomonas* sp. was observed in BSM with higher TBT concentration even with additional of carbon source. This study equally endorses the potentials of indigenous bacteria in bioremediation of TBT contamination and its Genomic analysis.

MATERIAL AND METHODS

Isolation of TBT tolerant Bacteria

In this method, sediment was collected from Cuddalore OT harbour, Tamilnadu. Sediment was appropriately diluted and spread plated onto Zobell Marine Agar (ZMA) plates supplemented with 100 mg L^{-1} TBT. The plates were incubated at room temperature (28 ± 2 °C) for 48 h. Individual predominant colonies were picked, purified and maintained. The cultures thus obtained were cultured on ZMA plates with different concentration of TBT (0 - 1500 µg/L). Then the tolerance capacity was noted and cultures were maintained on ZMA slants containing TBT (100 mg L^{-1}) at 4 °C. Purification of selected colonies of high TBT tolerant bacteria was followed by their identification.

Identification of TBT tolerant Bacteria

Pseudomonas stutzeri was identified from sediment samples collected from Cuddalore OT harbour area of Tamilnadu. The presence of bacteria was confirmed based on the macroscopic examination of colonies, microscopic examination and the different chemical tests which were performed as given below.

Gram staining

Gram staining was performed on the colony that had been isolated. A small number of bacteria from an active culture was spread onto a clean glass slide and gently heated. The slide was then covered with either gentian violet or crystal violet solution for one minute, rinsed with water, and treated with Gram's iodine as a mordant. Afterwards, it was decolorized using 95% ethyl alcohol and washed with water before being counter stained with safranin for 60–80 s and again rinsed with water. The cell was then examined under an electron microscope at a 100× resolution. Gram- negative cells was confirmed by appearance purple.

Carbohydrate fermentation test

Carbohydrate fermentation is a process that bacteria use to create energy. Most bacteria break down glucose into pyruvate through glycolysis. To test for carbohydrate fermentation, a special medium is used containing a single carbohydrate (such as glucose, lactose, sucrose, and maltose) along with colour indicators. One common indicator used is phenol red in NA broth. In this test, isolated colonies from a pure culture of the organism are inoculated into the broth and incubated at 35–37°C for 48 h. If the medium turns yellow, it means that carbohydrate fermentation reaction was successful.

Catalase test

A small amount of hydrogen peroxide (H_2O_2) was added to a bacterial broth and left to culture for 24 h in a clean bottle. After being mixed well, the presence of catalase positive bacteria was confirmed by the formation of bubbles within a minute. Conversely, the absence of bubbles indicated catalase negative bacteria.

Triple Sugar Iron (TSI)

The TSI agar recipe included glucose, a basic sugar, along with lactose and sucrose, which are both more complex sugars. Additionally, phenol red was added as a pH indicator. A sterile needle was used to touch a single colony on the agar. The agar slants were inoculated by stabbing through the centre of the medium to the bottom of the tube, then streaking over the top. The tubes were left to incubate at 35 °C in normal air for 18 to 24 h, with the caps slightly open. The findings are interpreted as follows.

Starch Hydrolysis

Starch agar, a mixture of 5 g peptic digest of animal tissue, 5 g sodium chloride, 1.5 g yeast extract, 1.5 g beef extract, and 2 g soluble starch is used. The agar content is 15 g. The pH of the medium (at 25 °C) is maintained at 7.4 ± 0.2, indicating a basic nutritive medium with starch. To test a specific organism, a single streak is made in a sterile manner to introduce the organism onto the centre of a designated plate. The inoculated plates are then placed in an incubator for 48 h at 37 °C. Following incubation, the plates are treated with iodine solution by saturating the surface for 30 s using a dropper. Any excess iodine is drained off. The clear zone around the bacterial growth line is then examined for analysis.

Fluorescent pigment production test

Pseudomonas stutzeri isolate was placed in test tubes with sterilized nutrient agar medium. The tubes were then incubated for five days and observed. A negative result was confirmed when a yellowish green fluorescent pigment was not visible under UV light (365 nm).

Nitrate reduction test

The bacterial suspension was inoculated into sterile nitrate broth tubes and incubated between 30–37 °C for a full day. Next, 6–8 drops of nitrite reagent A was added followed by 6–8 drops of nitrite reagent B and watched closely for a colour change to indicate a reaction. If no colour change occurred, zinc powder was introduced and allowed to stand for a few minutes for a red colour to show up.

Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. The quality of the DNA isolated was checked using agarose gel electrophoresis. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. Post DNA extraction, the library preparation [25] was carried out using the TruSeq DNA Nano kit from Illumina. The library was then processed and genomic sequencing was performed by Oneomics Private Limited, situated in Trichy, Tamil Nadu. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond and Suchard, 2010).

Genome assembly

Pseudomonas stutzeri was assembled using Unicycler. There were 22 contigs, an estimated genome length of 4,192,731 bp, and an average G+C content of 64.25%. The N50 length, which is defined as the shortest sequence length at 50% of the genome, is 537,847 bp. The L50 count, which is defined as the smallest number of contigs whose length sum produces N50, is 3.

Genome Annotation

The *Pseudomonas stutzeri* genome was annotated using RAST tool kit (RASTtk) and assigned a unique genome identifier of 286.10618. This genome is in the super kingdom bacteria and was annotated using genetic code 11.

Phylogenetic Analysis

The closest reference and representative genomes were identified by Mash/MinHash [19]. The global protein families (PGFams) [9] were selected from these genomes to determine the phylogenetic placement of this genome. The protein sequences from these families were aligned with MUSCLE (Edgar, 2004), and the nucleotides for each of those sequences were mapped to the protein alignment. The joint set of amino acid and nucleotide alignments were concatenated into a data matrix, and RaxML [23] was used to analyze this matrix, with fast bootstrapping [22] was used to generate the support values in the tree (Figure 3).

Growth of TPT resistant bacteria *Pseudomonas stutzeri*

Pseudomonas stutzeri was grown at 28 ± 2^0 C for 72 h in BSS medium supplemented with TBT and different carbon sources namely Sodium succinate (SS), Sodium citrate (SC), Glucose (Glu), Maltose (Mal), Sucrose (Suc) and Fructose (Fru) at the concentration of 0.4 %. Their growth rate was monitored by taking OD values in UV-Visible Spectroflorometer.

RESULTS

TBT tolerance of *Pseudomonas stutzeri*

In the study of TBT tolerance by different bacterial colonies, *Pseudomonas stutzeri* showed high tolerance capacity. It was selected for whole genome sequencing.

Fig1: ***Pseudomonas stutzeri on BSM agar medium supplement with 100*** μg/L of TBT

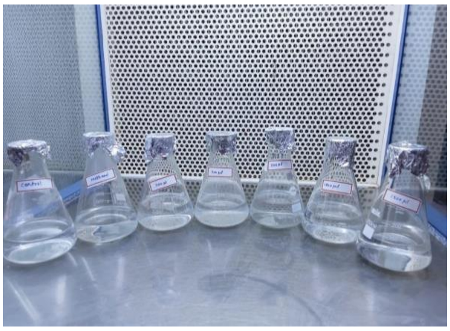

Fig 2: Growth of *Pseudomonas stutzeri* in BSS medium with different concentration of TBT

Genomic DNA Isolation from Bacteria *Pseudomonas stutzeri*

Table. 1Gemone details of *Pseudomonas stutzeri*

Genome Name	*Pseudomonas stutzeri*
Coarse consistency (%)	97.7
Fine consistency (%)	95.1
Completeness (%)	100
Contamination (%)	0.5
Contig count	22
DNA size (bp)	4,192,731
Contigs N50 (bp)	537,847
Contigs L50	3
Overpresent Roles	42
Underpresent Roles	27
Predicted Roles	1,799
Completeness Roles	1,092
Total Distinct Roles	2,755
Protein-Encoding Genes with Functional Assignment	2,712
Protein-Encoding Genes without Functional Assignment	1,301
% Protein-Encoding Feature Coverage	95.71
% Features that are Hypothetical	32.42
% Features that are in Local Protein Families	93.8

Genome annotation of *Pseudomonas stutzeri*

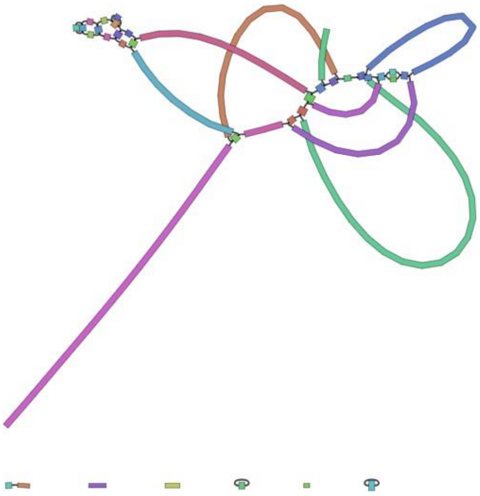

Fig.3 Genome assembly bandage plot of *Pseudomonas stutzeri*

The *Pseudomonas stutzeri* genome was annotated using RAST tool kit (RASTtk) and assigned a unique genome identifier of 286.10618. This genome is in the superkingdom Bacteria and was annotated using genetic code 11. The taxonomy of this genome is:

cellular organisms > Bacteria > Pseudomonadati > Pseudomonadota > Gammaproteobacteria > Pseudomonadales > Pseudomonadaceae > Pseudomonas > *Pseudomonas stutzeri*

This genome has 4,013 protein coding sequences (CDS), 77 transfer RNA (tRNA) genes, and 4 ribosomal RNA (rRNA) genes. The annotated features are summarized in Table 2.

The annotation included 930 hypothetical proteins and 3,083 proteins with functional assignments (Table 3). The proteins with functional assignments included 1,023 proteins with Enzyme Commission (EC) numbers, 862 with Gene Ontology (GO) assignments [4], and 747 proteins that were mapped to KEGG pathways [13]. PATRIC annotation includes two types of protein families [9], and this genome has 3,764 proteins that belong to the genus-specific protein families (PLFams) for, and 3,891 proteins that belong to the cross-genus protein families (PGFams).

Table 2 Genome annotation of *Pseudomonas stutzeri*

Annotated genome features	Reads
CDS(Coding Sequences)	4,013
Transfer Ribonucleia Acid	77
Ribosomal ribonucleic acid	0
Partial CDS(Coding Sequences)	0
Miscellaneous Ribonucleic Acid	4
Repeat Regions	0

Table.3 Protein feature of *Pseudomonas stutzeri*

Protein features	
Hypothetical proteins	930
Proteins with functional assignments	3,083
Proteins with EC number assignments	1,023
Proteins with GO assignments	862
Proteins with Pathway assignments	747
Proteins with PATRIC genus-specific family (PLfam) assignments	3,764
Proteins with PATRIC cross-genus family (PGfam) assignments	3,891

A circular graphical display of the distribution of the genome annotations is provided (Figure 1). This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. The colours of the CDS on the forward and reverse strand indicate the subsystem (fig4).

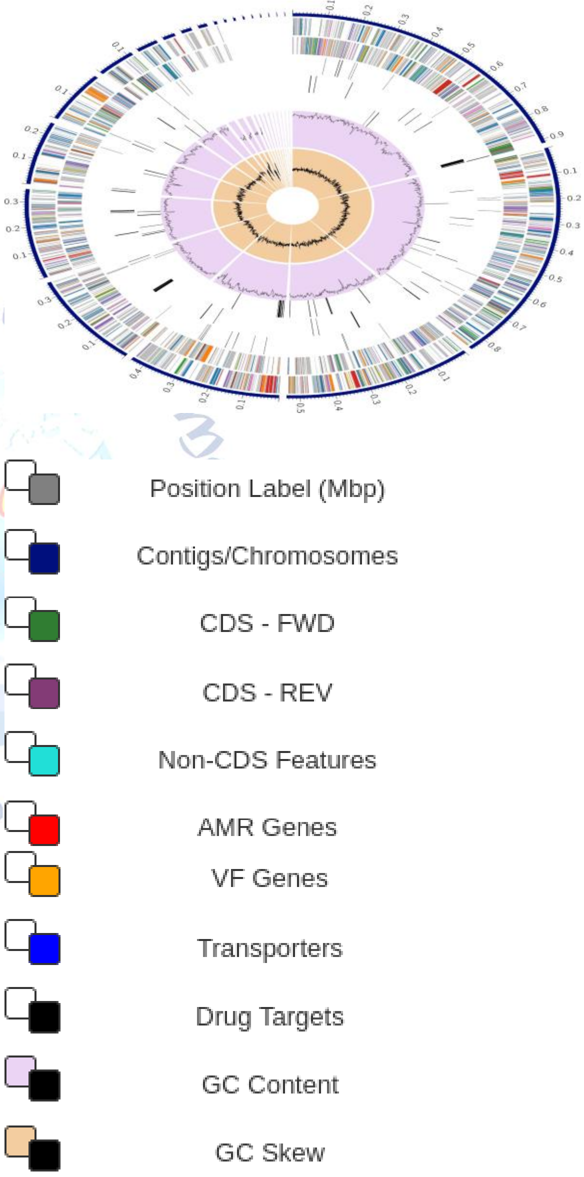

Fig. 4 Circular representation of the genome

Specialty Genes

Many of the genes annotated in have homology to known transporters [21], virulence factors [17][8], drug targets [27][14], and antibiotic resistance genes [18]. The number of genes and the specific source database where homology was found is provided (Table4.)

Table.4 Gene speciality of *Pseudomonas stutzeri*

Specialty Genes	Source	Genes
Antibiotic Resistance	CARD	4
Antibiotic Resistance	PATRIC	43
Drug Target	DrugBank	22
Drug Target	TTD	4
Transporter	TCDB	31
Virulence Factor	Victors	19
Virulence Factor	PATRIC_VF	1
Virulence Factor	VFDB	25

Subsystem Analysis

A subsystem is a set of proteins that together implement a specific biological process or structural complex [20] and the annotation includes an analysis of the subsystems unique to each genome. An overview of the subsystems for this genome is provided in Figure 5.

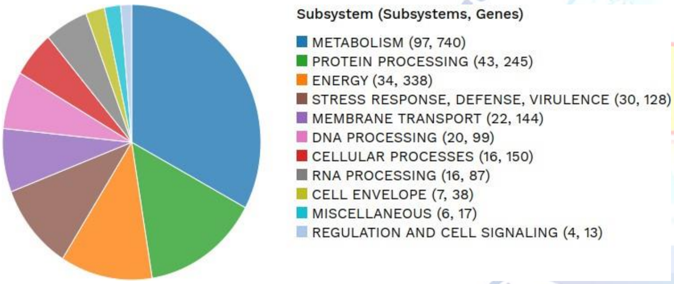

Fig. 5 Subsystem gene of *Pseudomonas stutzeri*

Antimicrobial Resistance Genes

The Genome Annotation Service uses a k-mer-based AMR genes detection method, which utilizes a curated collection of representative AMR gene sequence variants [25] and assigns to each AMR gene functional annotation, broad mechanism of antibiotic resistance, drug class, and, in some cases, specific antibiotic it confers resistance to. Please note, that the presence of AMR-related genes (even full-length) in a given genome does not directly imply an antibiotic-resistant phenotype. It is important to consider specific AMR mechanisms, especially the absence/presence of SNP mutations that convey resistance. A summary of the AMR genes annotated in this genome and the corresponding AMR mechanism is provided in Table 5.

Table.5 Antimicrobial resistance genes of *Pseudomonas stutzeri*

AMR Mechanism	Genes
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Antibiotic activation enzyme	KatG
Efflux pump conferring antibiotic resistance	MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexEF-OprN, MexEF-OprN system, MexJK-OprM/OpmH, TolC/OpmH
Gene conferring resistance via absence	gidB
Protein modulating permeability to antibiotic	OccD4/OpdT, OccD6/OprQ, OccK5/OpdH, OprD family, OprF
Regulator modulating expression of antibiotic resistance genes	OxyR

NCBI's manual curation of high-quality reference and representative genomes for phylogenetic analysis

The NCBI selects and arranges allusion and corresponding transcripts that they believe are of excellent purity and relevant to the investigation field. These genomes serve as the foundation for a variety of investigations. The usage of Mash/MinHash, a technique introduced by Ondov BD in 2016, is mentioned in the paragraph. This technique is used to identify genomes that are closely related to the reference and representative genomes that have been chosen. It is a technique for swiftly comparing and classifying genomes based on sequence similarity. The global protein PGFams (developed by Overbeek R in 2005) are picked from the genomes found using Mash/MinHash [8]. PGFams are most likely a collection of protein families that are utilized in this context to evaluate the evolutionary relationships and phylogenetic locations of genomes. The MUSCLE program, developed by Edgar RC in 2004, is used to align the protein sequences from the selected families. Alignment of protein sequences is a critical step in comparing sequences for evolutionary study. The aligned protein sequences' nucleotide sequences are mapped to the protein alignment. Identifying the genetic code, codon use, and other pertinent information for the nucleotide sequences may be part of this stage. The alignments of amino acids and nucleotides that arise are merged to form a single data matrix. This matrix is likely to contain information on the aligned sequences and their nucleotides, which can be utilized for further study. RaxML, a phylogenetic analysis tool.

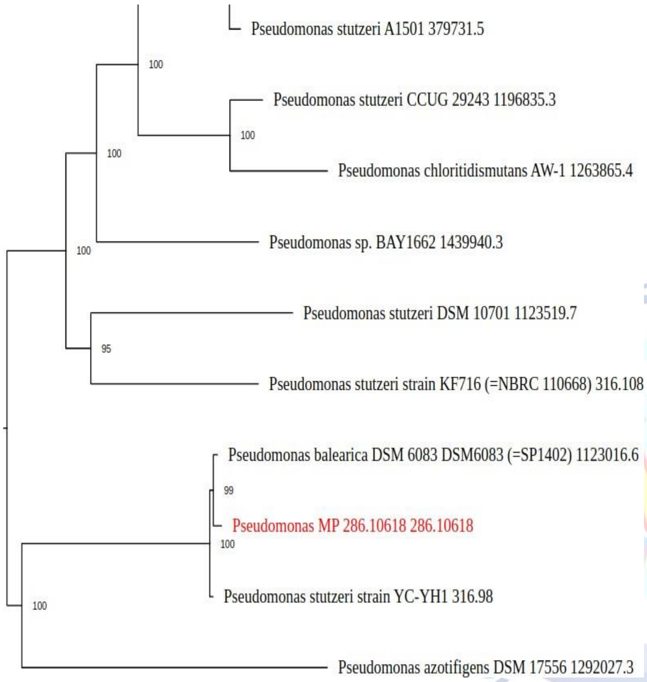

Fig. 6 Phylogenetic tree

Growth of TPT resistant *Pseudomonas stutzeri*

The growth rate of *Pseudomonas stutzeri* was studied using a UV-VIS spectrophotometer at 600 nm in BSM supplemented with 25 mg L^{-1} of TBT with different carbon sources, Sodium succinate (SS), Sodium citrate (SC), Glucose (Glu), Maltose (Mal), Sucrose (Suc) and Fructose (Fru).

Table. 6 Growth of P.stutzeri in different Carbon sources

Incubation period	TBT (25 mg L^{-1})					
0 hrs	SS(0.4 %)	SC(0.4 %)	SU(0.4 %)	Fru(0.4 %)	Mal(0.4 %)	Glu(0.4 %)
	0.012	0.084	0.087	0.094	0.1	0.01
72 hrs	0.31	0.76	0.76	0.8	0.452	0.32

DISCUSSION

Pseudomonas stutzeri is a widely distributed environmental bacterium found in soil, water, and marine sediments, known for its exceptional physiological and genetic adaptability that enables it to survive under diverse and sometimes extreme environmental conditions. It plays a crucial role in ecosystem functioning through its involvement in denitrification, where it converts nitrate into nitrogen gas, thereby contributing to the nitrogen cycle and helping to prevent nitrate accumulation in aquatic systems. In addition to its ecological importance, P. stutzeri is highly valued in bioremediation because of its ability to metabolize a broad range of organic compounds, including environmental pollutants such as hydrocarbons and chlorinated solvents, making it useful in the clean-up of contaminated sites. Its natural competence for DNA uptake also makes it a model organism for studying genetic transformation and microbial evolution. Similarly, research conducted in Malaysia by A. Abdussamad (2016) identified indigenous bacterial species such as Citrobacter sp., Klebsiella sp., and Acinetobacter sp., which demonstrated significant capacity to degrade the toxic organotin compound tributyltin (TBT) while tolerating high concentrations up to 1200 µg/L along with heavy metals, highlighting the vital role of naturally occurring microorganisms in managing industrial pollutants and supporting sustainable environmental restoration efforts.

CONCLUSION

In the percent study, we isolated an indigenous species of *Pseudomonas stutzeri* with high TBT tolerance (1500µ/L of concentration) from the Cuddalore OT harbour in Tamilnadu. Potential bacteria were identified by sequencing and its growth pattern in extreme concentrations of TBT in different carbon sources was studied as bioremediation by indigenous bacteria has been proposed as an economically efficient and effective method.

Conflict of interest statement

Authors declare that they do not have any conflict of interest.

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