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Biodegradation of chlorpyrifos pesticide by *Meiothermus silvanus* DSM 9946 isolated from agricultural runoff

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ABSTRACT

Indiscriminate usage of pesticides like chlorpyrifos can lead to extensive pollution and can seriously impact human life. After incubating water collected from agricultural runoff in the Kanchipuram district of Tamil Nadu state, it was observed that only one species could efficiently degrade chlorpyrifos. After performing the degradation studies, it was reported that the species could degrade chlorpyrifos completely within 5 d and could also reduce the chemical oxygen demand levels by 95.33%. The optimised experimental conditions were pH-8.5, input dosage-10 ppm and the temperature was maintained within 33°C–40°C. The species was characterised by performing 16s rRNA and has been identified as *Meiothermus silvanus* DSM 9946. As this bacteria belong to the *Thermus* genus it can withstand high temperature and it is chemotrophic indicating that it can acquire its nutrition through chemosynthesis by the oxidation of either organic or inorganic molecule. This specific feature might help in the oxidation/degradation of an organic molecule like pesticide, thus, reducing its toxicity. 2,2-dimethoxybutane, 2-heptanol, 6-amino-2-methyl, undecane compounds, 2,6-lutidine 3,5-dichloro-4-dodecylthiol, 2-nonenoic acid and carbromal were the metabolites detected in the degraded samples by gas chromatography–mass spectrometry.

Keywords: Pesticide; Metabolites; Chlorpyrifos; Pollution; Bioremediation; Xenobiotics

1. Introduction

Agriculture has revolutionised with the introduction of pesticides as it assures a constant amount of annual yields without much fluctuations [1,2]. Out of the chemical pesticides available in the market, organochlorine pesticides are highly notorious because of their recalcitrant nature [3,4]. This has led to the framing of rules forbidding the usage of most organochlorine pesticides like dichlorodiphenyltrichloroethane, lindane, etc [5,6]. Thus, in recent times there has been increased usage of organophosphorus pesticides like chlorpyrifos, malathion, parathion, methyl parathion, diazinon, phosmet, fenitrothion, terbufos, tetrachlorvinphos, azamethiphos, azinphos-methyl, etc [7–9]. The most commonly used amongst such pesticides are chlorpyrifos, malathion, parathion [10–12].

Chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is considered to be the fourth most sold pesticide in the world after endosulfan, monocrotophos and acephate [13]. The empirical formula of the pesticide is $C_9H_{11}C_{l3}NO_3PS$. The half-life varies between 60 and 120 d depending on its rate and time of application, method of application and external weather conditions [14]. The pesticide is stable from degradation under acidic and neutral conditions but as the pH increases the rate of hydrolysis increases [13]. Chlorpyrifos targets a diverse range of pests, mosquito, mites, ant which might affect the growth of the crops. It kills the target by inhibiting the enzyme acetylcholine.

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Chlorpyrifos is highly effective when it is been used in regulated quantities but in most cases, they are been used indiscriminately which leads to the problem. Chlorpyrifos has been detected in water bodies around the globe within the range of 2.86 n/L to 2 mg/L [15,16]. Activities like spray drifting, rinsing containers, crop handling, etc contaminate the environment with excessive quantities of chlorpyrifos [17–19]. Chlorpyrifos itself is not recalcitrant and has been termed moderately toxic. But the chemical promptly breaks down into chlorpyrifos oxon and further into 3,5,6-trichloro-2-pyridinol (TCP) [20]. The OH radicals present in the compound volatilises and converts into highly toxic chlorpyrifos oxon. TCP has been termed as highly recalcitrant by US EPA as its shelf life ranges from 65 to 360 d depending on circumstances.

The primary routes of entry of chlorpyrifos are by contact, ingestion and vapor [21]. Chlorpyrifos causes a wide range of health problems like cardiovascular, nephrological, respiratory, etc which can be highly fatal. A small quantity of chlorpyrifos might act as neurotoxic to foetuses/children [20]. Unregulated usage has also been reported to be responsible for the killing of non-target beneficial organisms. The decline of the population of such organisms negatively impacts the soil's fertility. Moreover, the unused amount of pesticide results in leaching which contaminates the groundwater and nearby water bodies. Thus, there is an urgent need to reduce the pollution load and decontaminate the aquatic and terrestrial ecosystems [22].

Scientists have put considerable effort into developing solutions for faster degradation of pesticides. Physical methods like ion exchange, adsorption, etc have been conventionally used but this process transfers the pollutants from one phase to another without completely destroying them [23]. Physicochemical methods like chemical treatment, volatilization, photodecomposition, and incineration have been widely used [24–26]. Among chemical treatment methods, advanced oxidation processes have gained popularity as it can be used extensively to treat wastewater treatment without leaving harmful chemicals [27–29].

Currently, another clean and sustainable method of reducing the contaminant load which is grasping a lot of attention in the scientific world is bioremediation [19,30,31]. A lot of studies have confirmed that certain strains of bacteria like Enterobacter, Pseudomonas, Klebsiella, Bacillus can efficiently degrade chlorpyrifos and its metabolite TCP over time. Metabolism of chlorpyrifos is of mainly two types – catabolism or co-metabolism/incidental metabolism [32]. In the case of catabolism, the xenobiotic acts as a nutrient for microbial growth and the organic compound get either completely or partially degraded. Co-metabolism on the other hand, is the incidental degradation of the xenobiotic that takes place during the execution of the routine metabolic pathway/activities of the microorganism [33]. The heterocyclic aromatic ring present in chlorpyrifos, that is, the pyridine ring can be efficiently cleaved and mineralised by microorganisms. The most important step in the degradation process is the further metabolism of TCP and TMP metabolites (3,5,6-trichloro-2-methoxypyridine) that are persistent in nature [32].

In this work, it is attempted to efficiently biodegrade chlorpyrifos and its persistent metabolites. Microorganism(s) can carry out the degradation process and it was further characterised and the intermediates metabolites formed after successful degradation were identified. Thus, the probable metabolic pathway is also mapped.

2. Materials and methods

2.1. Chemicals

20% chlorpyrifos of Arysta LifeSciences have been procured from an agricultural chemical shop in Chennai, India. The chemicals needed to prepare mineral salt media including nutrient broth and agar were purchased from SRL Chemicals (Chennai, India).

2.2. Enrichment and isolation of bacteria

Water samples were collected from the runoff streams located nearby agricultural areas in the Kanchipuram district of Tamil Nadu. The water was brought to the lab for further analysis. Basic water quality parameters like pH, total dissolved solids, COD (chemical oxygen demand) conductivity, and temperature were monitored.

The sample was serially diluted and it was cultured on sterilised Petri plates in triplicates with the help of pour plate method. The media for culture was mineral salt media (MSM) (dipotassium hydrogen phosphate 1 g/L, potassium dihydrogen phosphate 1 g/L, potassium nitrate 2 g/L, magnesium sulfate 0.2 g/L, calcium chloride 0.02 g/L, ferrous sulfate 0.01 g/L) and agar [34]. 10 ppm of 20% chlorpyrifos was also added to the media. The equation which was used for calculating the amount of chlorpyrifos to be added has been provided.

$$\text{Amount of chlorpyrifos to be added} = \frac{\text{Desired concentration of the pollutant}\left(\text{ppm}\right) \times \text{Total amount of solution to be prepared}\left(\text{L}\right)}{\text{Amount of active ingredient present}\left(\text{ml/L}\right)} \quad (1)$$

$$\text{Amount of active ingredient present} = \text{\% active ingredient present in the compound} \times 100 \quad (2)$$

The Petri plates were kept in an incubator and after 48 h colonies that could tolerate the stated level of pesticide were observed on the plates. From mixed colonies, individual colonies were selected and plated on freshly prepared plates supplied with agar and MSM. After 48 h one type of purified colony was observed and tagged as *CPs1*. It was further used for the experiments.

2.3. Identification of strains

The bacterial strain (*CPs1*) that could degrade chlorpyrifos considerably was chosen. It was subjected to several confirmatory tests including carbohydrate fermentation tests, oxidative fermentation test, indole production test, Voges–Proskauer test, methyl red test, citrate utilization test and urease test.

Further, the strain has been identified with the amplification of 16s rRNA. The DNA was isolated using

NucleoSpin® Tissue Kit (Macherey–Nagel) by following the user instructions. The quality of DNA was checked by agarose gel electrophoresis. The components used for Polymerase Chain Reaction (PCR) analysis are 2X Phire Master Mix, D/W, forward primer, reverse primer, DNA and their corresponding quantities are 5, 4, 0.25, 0.25, and 1 μL, respectively. The sequence of the forward primer used was CAGGCCTAACACATGCAAGTC while that of the reverse primer was GGGCGGWGTGTACAAGGC.

The PCR amplification was carried out in PCR thermal cycler (Model: GeneAmp 9700 PCR System, Applied Biosystems, USA) (Fig. S1). The removal of unwanted primers was done by using ExoSAP-IT (USB) which comprises two hydrolytic enzymes, that is, exonuclease I and Shrimp Alkaline Phosphatase (SAP) in a specialised buffer. The final sequencing reaction was done in a PCR thermal cycler (GeneAmp 9700 PCR System, Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufactures protocol. The components, that is, D/W-6.6 μL, 5X sequencing buffer-1.9 μL, forward primer-0.3 μL, reverse primer-0.3 μL, sequencing mix-0.2 μL, exosap treated PCR product-1 μL have been used for sequencing step. Sequence Scanner Software v1 was used to check the sequence quality. The sequence alignment and editing were done in Geneious Pro v5.1.

2.4. Procedure for parameter optimisation

Inoculated samples were subjected to certain parameter changes like pH, temperature, and initial dosage. The rate of degradation was observed when pH was varied within 4.5–9.5, a temperature within 25°C–65°C and initial dosage between 10–160 ppm. The derived optimised values at which maximum degradation was achieved were then fixed for further degradation studies.

2.5. Degradation of pesticides

Two 250 mL Erlenmeyer flasks were procured and freshly prepared autoclaved MSM broth was supplied into them. One of the flasks was inoculated with the bacterial culture and 10 ppm of chlorpyrifos was added to it. Another flask was supplied with MSM media and chlorpyrifos but with no microorganisms and thus, it served as control. pH, temperature, and initial dosage were fixed depending on the values derived from experimental studies. The flasks were then kept in an incubator for 5 d. Samples were withdrawn from the flasks and were further subjected to degradation studies.

2.6. Detection of wavelength for chlorpyrifos measurement

Standards containing chlorpyrifos from 10–50 ppm were measured in UV spectrophotometer and the detected wavelength was 290 nm. Alongside this, based on previous literature, 290 nm was the fixed wavelength for further measurement.

2.7. Analysis of the degraded samples

A part of the degraded product was removed in regular intervals of 24 h. The withdrawn samples were centrifuged, and the supernatant was kept for further analysis. The samples were subjected to a UV spectrophotometer (Company: LARK, Model: LI-UV-7000, optical system: double beam, wavelength range: 190–1,100 nm). Based on previous literature available, 290 nm was fixed to be the wavelength for chlorpyrifos measurement.

On the 5th day the samples were analysed with gas chromatography–mass spectrometry (GC-MS, PerkinElmer® Clarus® 680, Clarus 600 EI, United States) for detection of the metabolites present in the sample. The model of GC used in the analysis is the Clarus® 680. It consists of a fused silica column that is packed with Elite-5mS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 μm df). The components were separated utilising Helium as carrier gas at a rate flow of 1 mL/min. The predetermined temperature of injector was 260°C. The description of run conditions are: 60°C (2 min), followed by 300°C at the rate of 10°C/min, and 300°C, where it was kept for 6 min. The conditions for mass detector are transfer line temperature – 240°C, ion source temperature – 240°C, ionization mode electron impact – 70 eV, scan time – 0.2 s and scan interval – 0.1 s. The spectrums of components are compared against known ones which can be retrieved from GC-MS NIST (2008) library.

2.8. COD measurement

Selected strains were inoculated in an Erlenmeyer flask containing nutrient broth and 5 ppm chlorpyrifos. After 24 h of inoculation, the samples were subjected to centrifugation at 7,000 rpm for 10 min [35]. The pellets formed were resuspended thrice in distilled water. After washing, the pellets were added to an Erlenmeyer flask containing chlorpyrifos-contaminated agricultural wastewater. On the 2nd to 5th days after inoculation, samples were extracted and the reduction in COD was observed. The removal efficiency deduced from COD was calculated by the following equation:

$$\text{COD} = \frac{\left(C_1 - C_2\right)}{C_1} \tag{3}$$

where C_1 = initial COD levels and C_2 = COD level on 5th day.

3. Results and discussion

3.1. Characterization of bacterial strain

The purified stain that could tolerate high levels of chlorpyrifos was subjected to various tests and it was detected that the strain was coccus shaped, gram-negative, aerobic, oxidase-positive, and non-motile. The strain was found to be catalase negative and could degrade various sugars. It was further subjected to 16s rRNA and it was detected to be *Meiothermus silvanus* DSM 9946. The species belong to the genus *Meiothermus* and species *silvanus* is considered to be an organism that can sustain at lower temperatures or at less hot places [36]. It thrives in a slightly alkaline environment. According to previous literature, this type of bacterial species have been found in thermal water (temp.: 33°C, pH: 8.8) and geothermal spring (temp.: 56°C, pH: 8.9) of North Portugal

[37]. *M. silvanus* has been further detected in seawater near the coral colony of *Pocillopora meandrina* at Palmyra Atoll [38]. This reciprocates with the fact that this species of bacteria has been detected in the collected sample as the collection zone is in the vicinity of the coastline. Thus, the salinity is slightly higher (~4) because of salt intrusion. Also, the area suffers from impeded drainage due to the hard pan which can increase the salt concentration. Also, it must be mentioned that salinity varied throughout the area which might affect the distribution of the microorganism in the agricultural area. Since the sample was collected in May, the temperature and pH of the water of the sample were measured to be around 45°C and 8.3 which are suitable for the growth of *M. silvanus*.

3.2. Optimisation of varying parameters

Inoculated samples were kept in the incubator with varying pH, and temperature. It could be deduced that the optimal level of pH was between 7.5–8.5 for this particular microbial species. 93% degradation could be achieved at pH 8.5. Thus, microbial degradation is carried out best at a slightly alkaline range. As this kind of species can tolerate temperatures within 33°C–55°C, hence the rate of degradation was constant at around 96% throughout this range. When the temperature exceeded 65°C, the degradation started slowing down which could be attributed to the fact that the species might not be able to survive at very high temperatures. The effect of pH and temperature variation has been explained in Figs. 1 and 2, respectively.

The initial dosage of chlorpyrifos was also varied within the range of 10–160 ppm for studying the rate of degradation. Fig. 3 shows that till 40 ppm, there was complete degradation while from 70 ppm, the concentration slowly decreases. Thus, above 70 ppm, the proliferation of microbial species might be negatively affected which ultimately lowers the rate of degradation.

3.3. Degradation kinetics of chlorpyrifos

The inoculated and control samples were kept for 5 d at the following experimental conditions, that is, pH-8.5, temperature-33°C–40°C and initial dosage-10 ppm. The supernatant of control and inoculated cultures were extracted consecutively for 5 d and after centrifugation was subjected to measurement in UV spectroscopy at 290 nm. In case of inoculated culture, there was no trace of chlorpyrifos on the 5th day and the peak at 290 nm disappeared. The maximum degradation of control was 7.2% on the 5th day.

The relationship between the concentration of CP with time was studied with the help of first and zero-order kinetics. The inoculated sample follows first-order kinetics depicting that as time increases, the amount of chlorpyrifos reduces linearly while on the other hand, the control experiment when plotted follows zero-order kinetics. Figs. 4 and 5 explain the degradation kinetics. The coefficient of determination (R^2) and rate constant for an inoculated sample are 0.99 and 0.072 while those of control samples are 0.94 and 0.069, respectively. The algorithms followed for rate constant determination is:

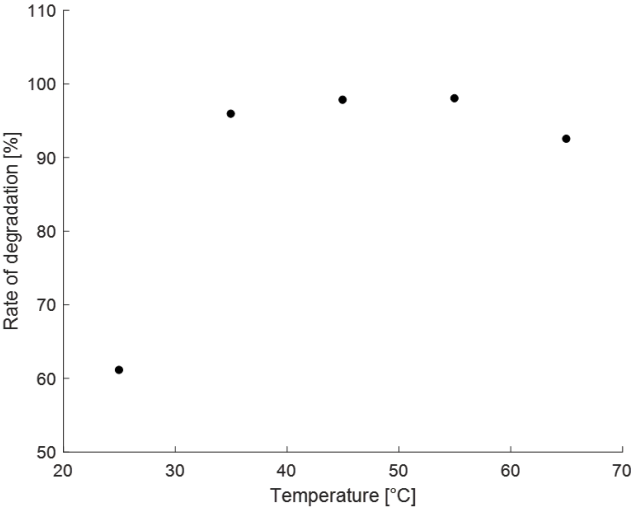


Fig. 2. Effect of varying temperature on the degradation rate.

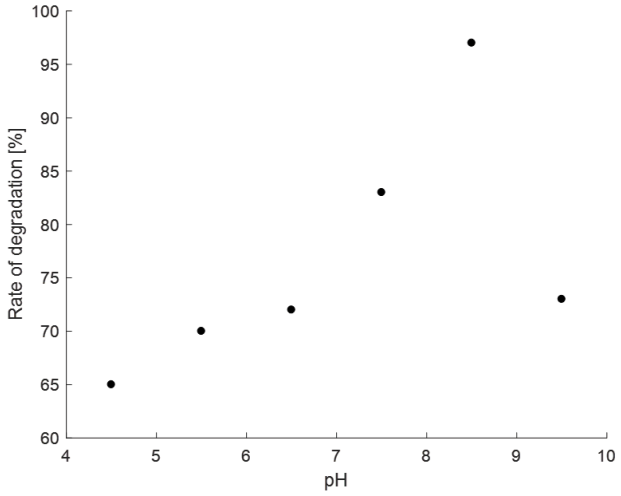


Fig. 1. Effect of varying pH on the degradation rate.

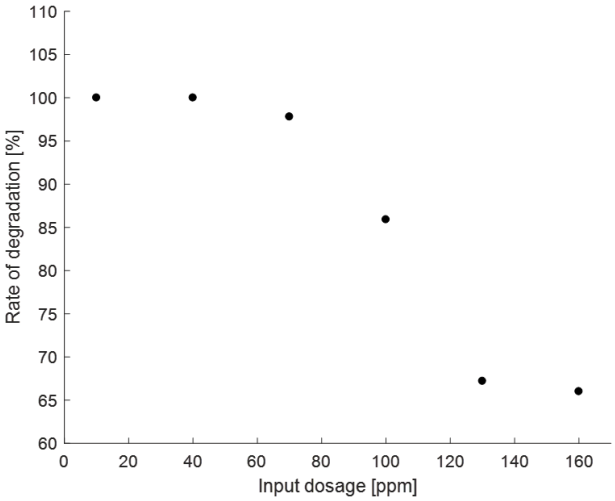


Fig. 3. Effect of varying input dosage on the degradation rate.

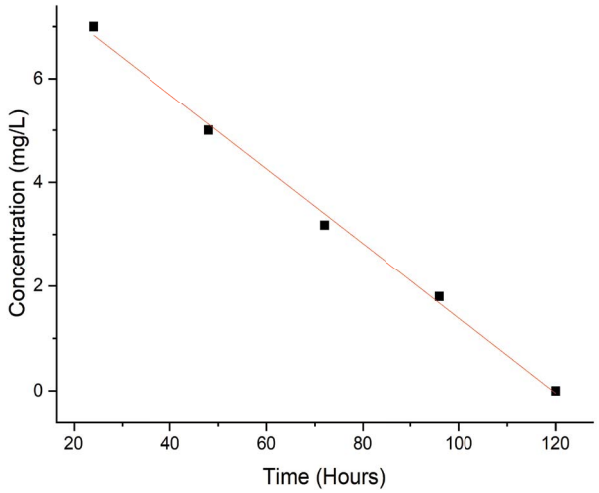


Fig. 4. Degradation kinetics of chlorpyrifos by *CPs1*.

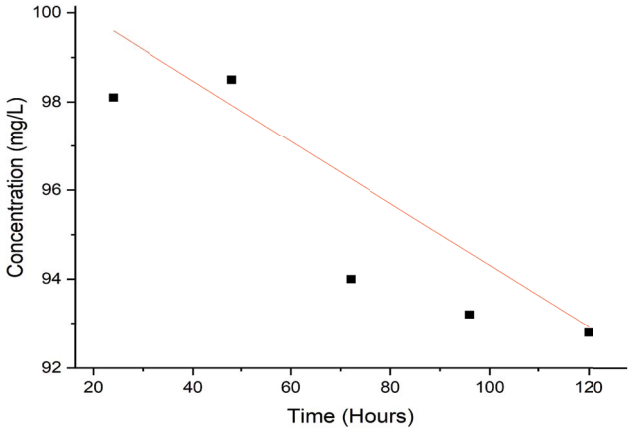


Fig. 5. Degradation kinetics of control sample.

$$\ln\left[A\right] = \ln\left[A\right]_0 - kt \tag{4}$$

where ln[*A*] = final concentration, ln[*A*]$_0$ = initial concentration, *k* = rate constant, and *t* = time.

The half-life of first-order model can be calculated by $t_{1/2} = 0.693/k$ and that of zero-order model can be calculated by $t_{1/2} = A_0/2k_0$.

where A_0 = initial concentration of CP, k_0 = rate constant for zero-order reaction.

It is evident from the calculated values that the half-life of chlorpyrifos in the uninoculated sample is quite high, that is, the chemical will take almost 62.83 h more to degrade without the help of microorganisms. Thus, the risk of accumulation post-application is extremely high. The inoculation of *M. silvanus* DSM 9946 stimulated the degradation process and half-life of the contaminant was reduced drastically. The degradation could not be attributed to physiochemical factors like photochemical degradation, oxidation etc as in the control, there was very limited degradation in the presence of abiotic controls. Moreover, literature studies have revealed that *M. silvanus* can thrive abundantly on substrates composed of phosphate. This can be linked to the fact that chlorpyrifos is a pesticide having phosphate group which can be a good source of nutrients for the microorganism [39]. Thus, *M. silvanus* DSM 9946 was found to be solely responsible for the complete degradation process.

3.4. Detection of metabolites

The degraded samples were subjected further to GC-MS to detect the metabolites formed. Several metabolites have been detected by GC-MS and a list of them has been provided in Table 1. The spectrum is shown in Fig. 6.

The highest area of the chromatogram was occupied by 2,2-dimethoxybutane followed by 2-heptanol, 6-amino-2-methyl and undecane compounds. The area covered by the major detected compounds has been shown in Table 2. 2,2-dimethoxybutane and nonenoic acid are two ingredients that are often added to agricultural chemicals mainly pesticides [40,41]. Nonenoic acid commercially called pelargonic acid, is added as an active ingredient as it also acts as herbicide. It is a naturally occurring fatty acid and is of food grade quality, thus, the toxicological effect is low [40]. Thus, these chemicals might have been added in commercial grade pesticides which ultimately have been detected in GC-MS. 2-heptanol, 6-amino-2-methyl is a tertiary alcohol which might be the result of the metabolism of chlorpyrifos pesticide. 2,6-lutidine 3,5-dichloro-4-dodecylthiol, a heterocyclic organic compound has been detected as an intermediate metabolite. 2,6-Lutidine is commercially termed as 2,6-dimethylpyridine [42]. The compounds that are dimethyl substituted products of the pyridine group have been termed as lutidine. Chlorpyrifos has a pyridine ring present in it and it can be stated that after metabolism, the diethyl group in chlorpyrifos might have been converted into dimethyl group. *M. silvanus* has been detected to possess transferases enzyme which in the past research has been involved in pesticide degradation. Abdel-Ghany et al. [43] detected the formation of volatile metabolites like tertiary dodecylthiol while chlorpyrifos was being metabolised with the fungal strain namely *Aspergillus flavus*. Undecane has been detected and it is a highly volatile compound from the alkane group. It has been previously reported that this compound is emitted from pesticides [44]. The proposed pathway of metabolism has been shown in Fig. 7.

3.5. Removal of COD

Two Erlenmeyer flasks were taken, out of which one served as control, that is, without any bacterial inoculation while the other one was inoculated with a microbial pellet of *CPs1*. Microbial pellet of *CPs1* has been extracted and added into the 2nd Erlenmeyer flask containing 5 ppm of pesticide. Both of the flasks were incubated for 5 d to detect the reduction in COD levels. The initial load of COD was around 150,00 mg·O_2/L on the 1st day for both flasks. For the control, the final COD on the 5th day was 13,800, that is, only 8% reduction. For the 2nd flask, the COD value came down to 700 mg·O_2/L. Thus, the COD reduction took place

Table 1
List of metabolites detected

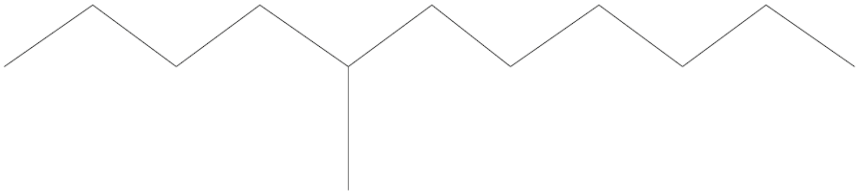
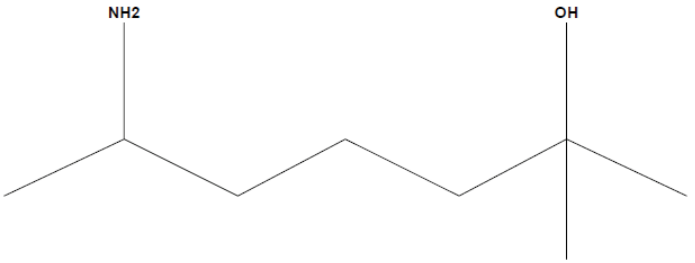
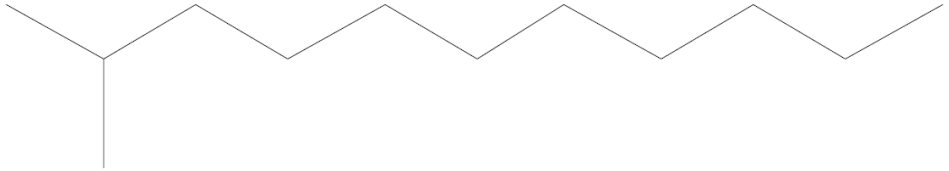
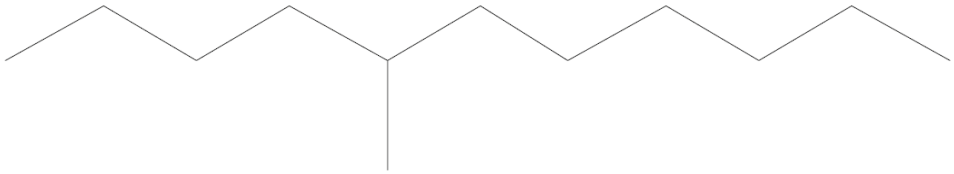
Detected compounds	Retention time	Empirical formula	Chemical structure
2,2-Dimethoxybutane	2.924	$C_6H_{14}O_2$	
2-Heptanol, 6-amino-2-methyl	4.029	$C_8H_{19}NO$	
Undecane, 2-methyl-	6.700	$C_{12}H_{26}$	
Undecane, 5-methyl-	8.471	$C_{12}H_{26}$	

Table 1 (Continued)

Table 1

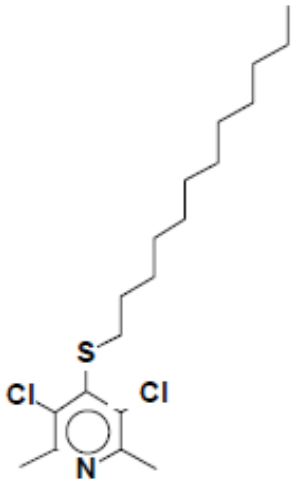
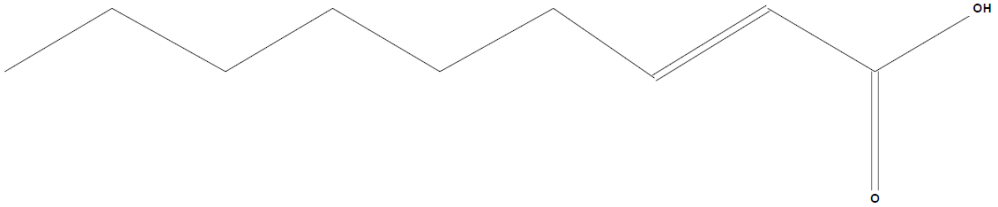
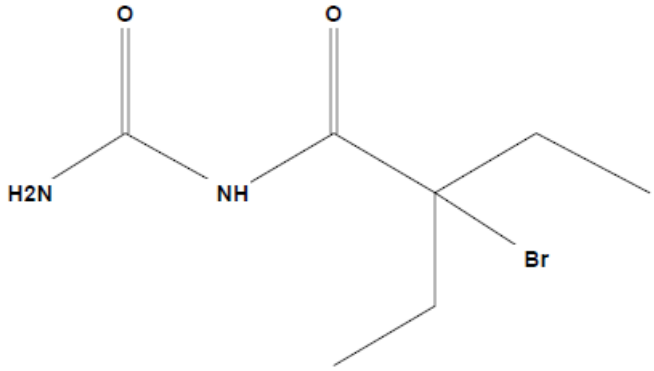
Detected compounds	Retention time	Empirical formula	Chemical structure
2,6-Lutidine 3,5-dichloro-4-dodecylthiol	27.728	$C_{19}H_{31}C_{12}NS$	
2-Nonenoic acid	31.650	$C_9H_{16}O_2$	
Carbromal	32.130	$C_7H_{13}BrN_2O_2$	

Table 2
Area covered by the detected compounds

Name of compound	Height of the compound	Area covered	Percentage of area
2,2-Dimethoxybutane	7,710,626	923,409.8	74.698
2-Heptanol, 6-amino-2-methyl	1,490,202	60,748.3	4.914
Undecane, 2-methyl-	3,911,305	205,488.9	16.623
Undecane, 5-methyl-	899,506	46,548.1	3.765

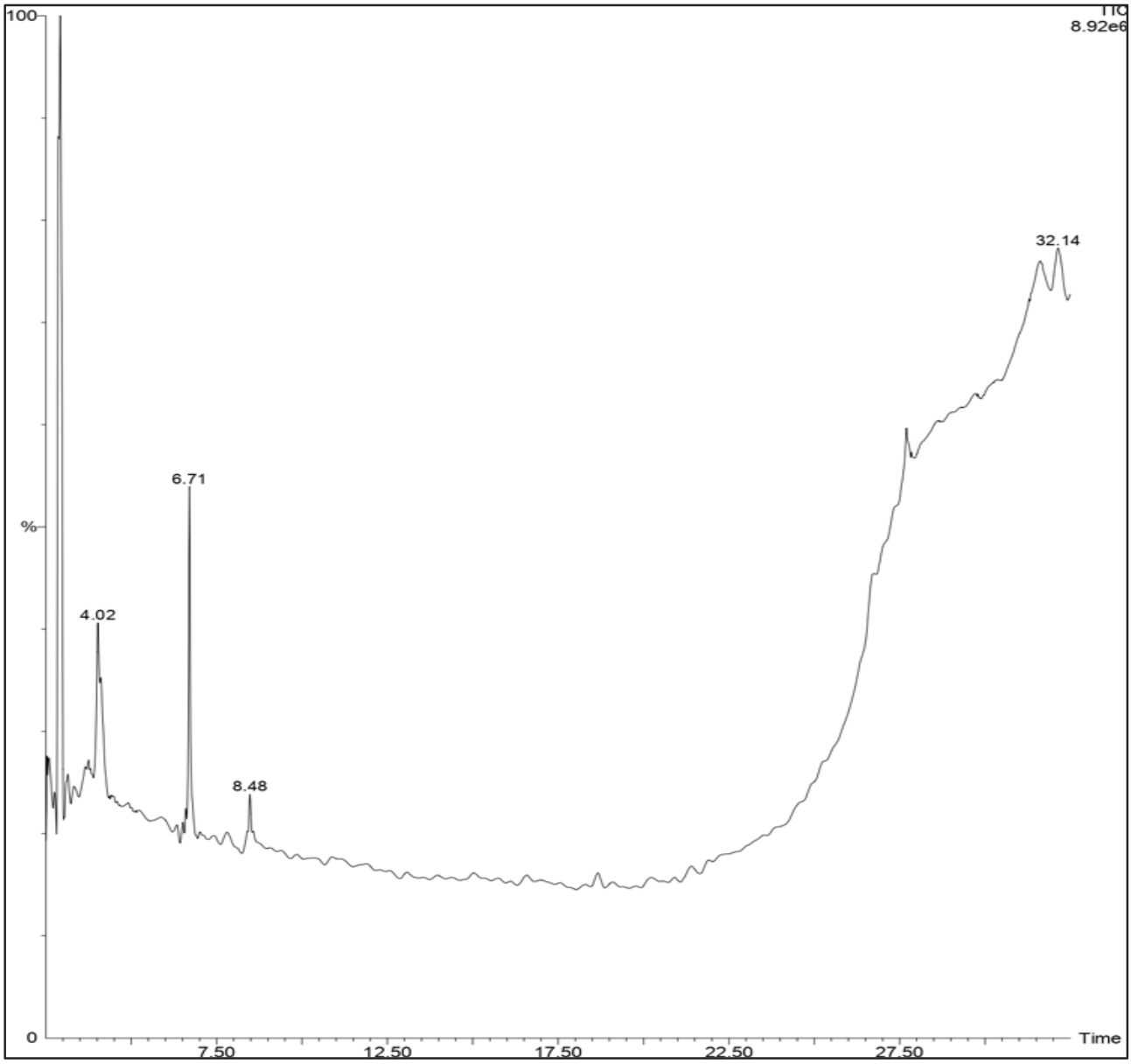


Fig. 6. Gas chromatography–mass spectrometry chromatogram.

by 95.33%. The gradual reduction in COD for the 2nd flask has been shown in Fig. 8. As can be seen that the chlorpyrifos content in the inoculated sample disappeared on the 5th day which was reflected in the COD reduction as well. The reduction is not 100%, the remaining 4.67% can be due to the metabolites formed during the process of degradation. Thus, it is evident that the strain *CPs1* is efficient in the reduction of the initial organic load.

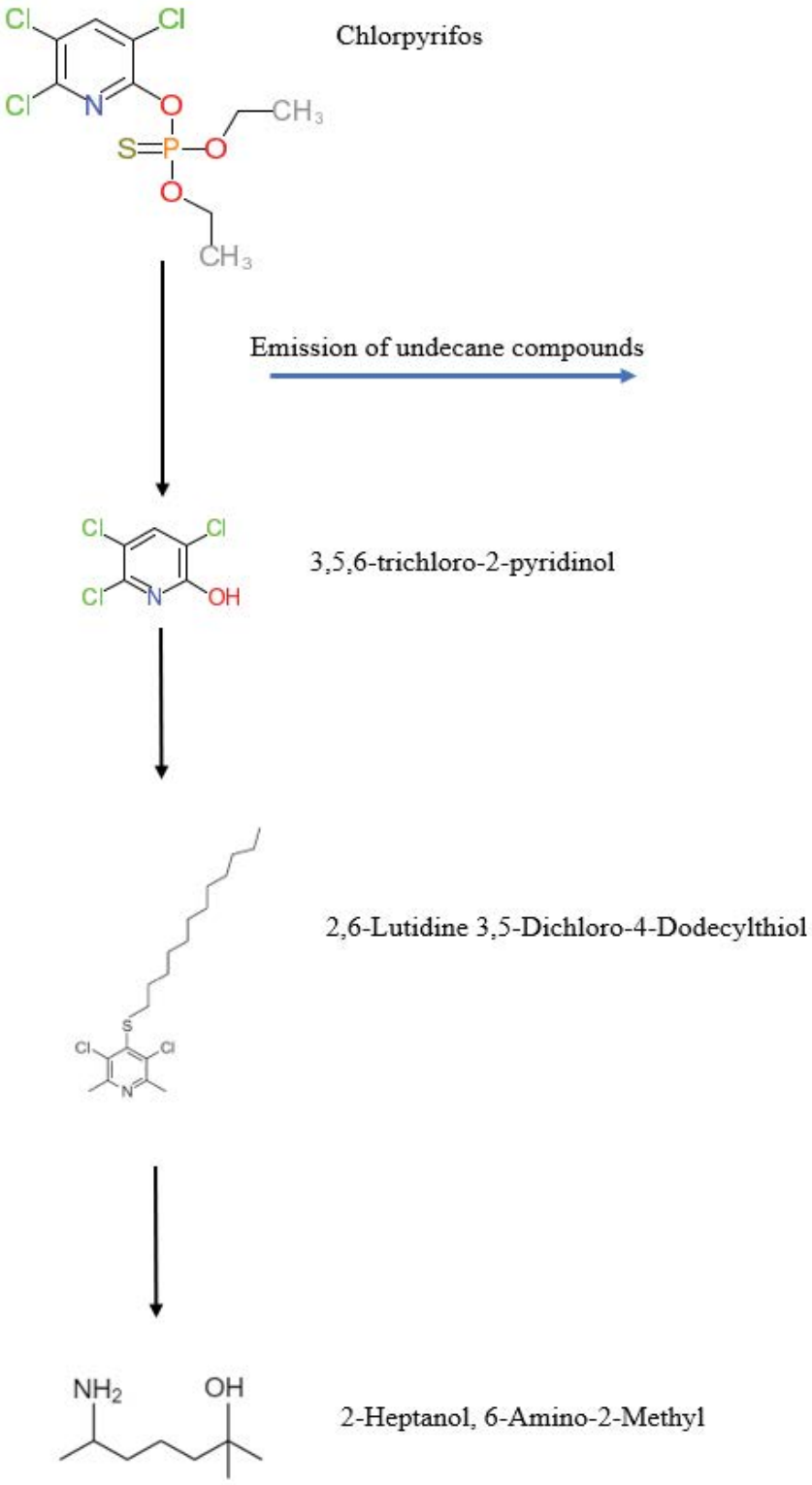


Fig. 7. Proposed metabolic pathway of degradation.

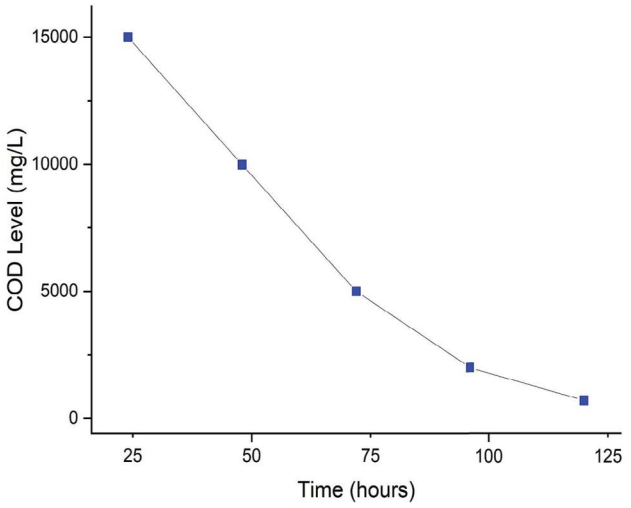


Fig. 8. Reduction in chemical oxygen demand levels with bacterial pellet.

4. Conclusion

In this paper, it could be concluded that *M. silvanus* DSM 9946 bacterial strain that was isolated from nearby runoff streams of agricultural areas in Kanchipuram district of Tamil Nadu state can efficiently degrade chlorpyrifos. The optimal temperature, pH, and initial concentration at which the species can efficiently degrade chlorpyrifos are 35°C–55°C, 8.5 and 10 ppm, respectively. As this bacterium belongs to the *Thermus* genus it can withstand high temperatures and it is chemotrophic in nature indicating that it can acquire its nutrition through chemosynthesis by the oxidation of either organic–inorganic molecules. This specific feature will help in the oxidation/degradation of the organic molecule, thus, reducing its toxicity. It has been observed through experimentation that the bacterial strain can completely degrade chlorpyrifos in 5 d and eventually can bring down the COD levels. The intermediate metabolites that have formed are comparatively less toxic and persistent than the parent compound. In the future, further research and experimentation need to be carried out to see how the metabolites formed can be rendered toxic-free. Also, it is important to observe how this strain can be feasibly utilised in treatment plants on a larger scale and whether it can degrade other xenobiotic compounds.

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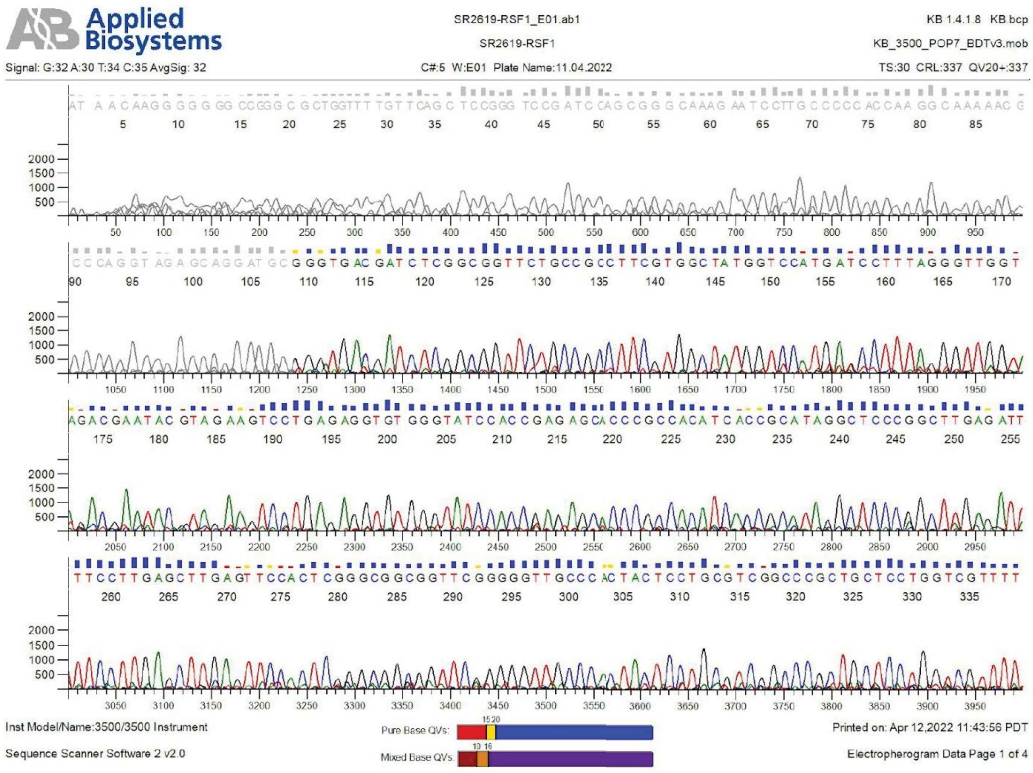
Supporting information



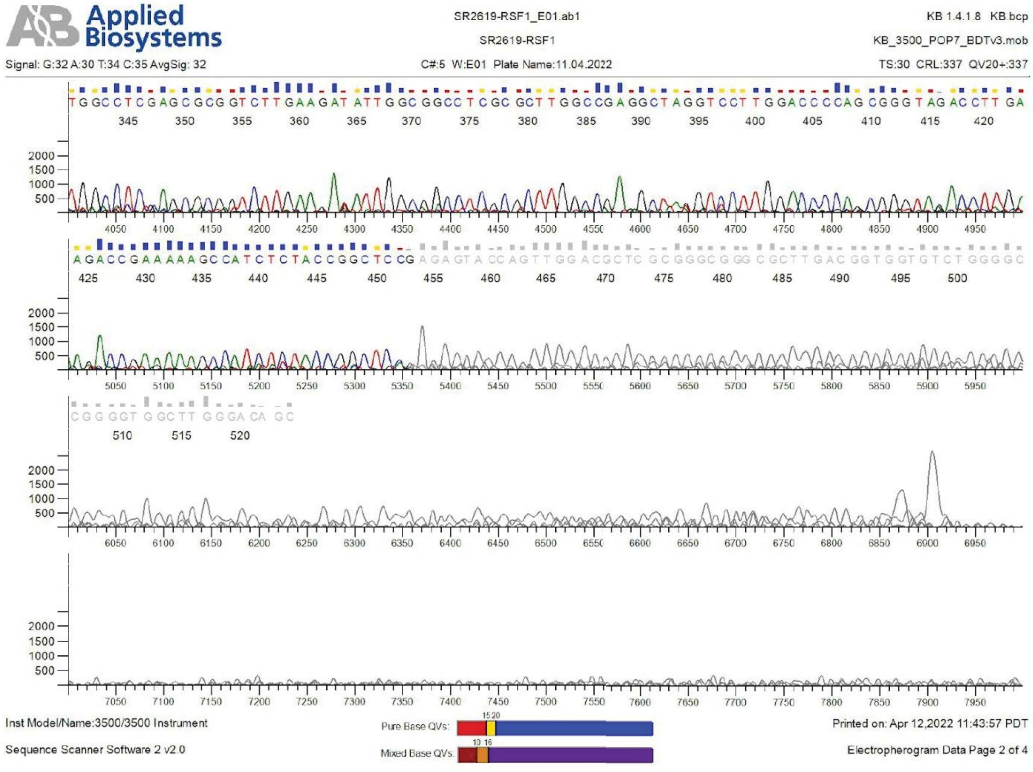
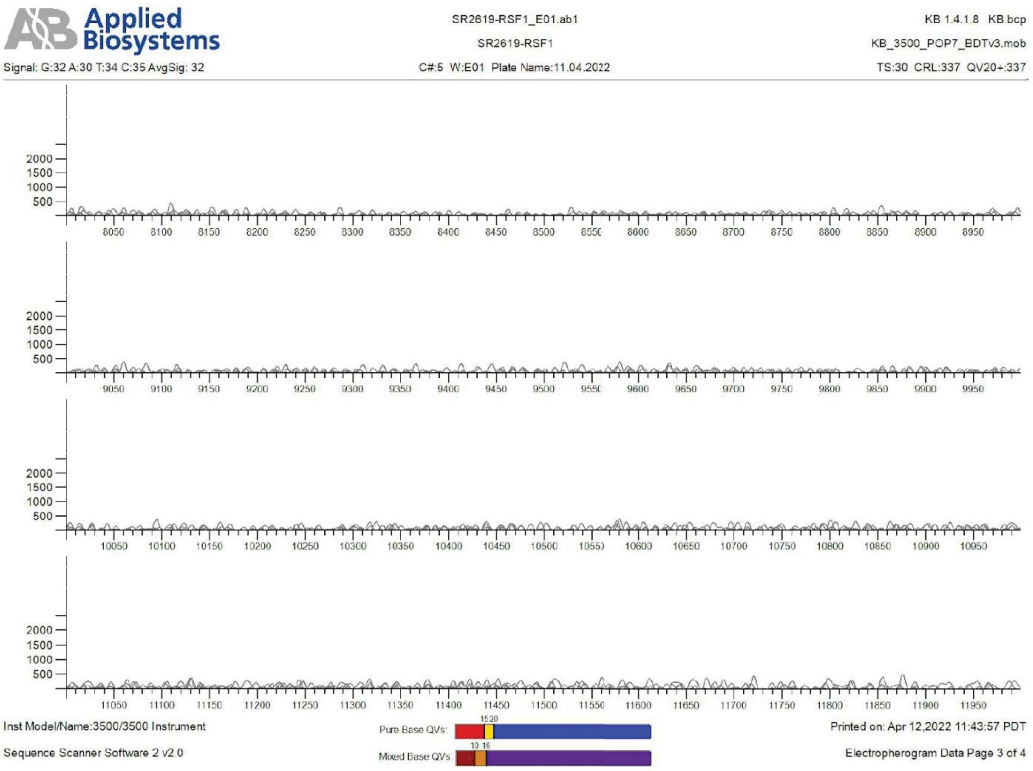


Fig. S1 (Continued)



S1. PCR sequencing results for characterisation of bacterial sample