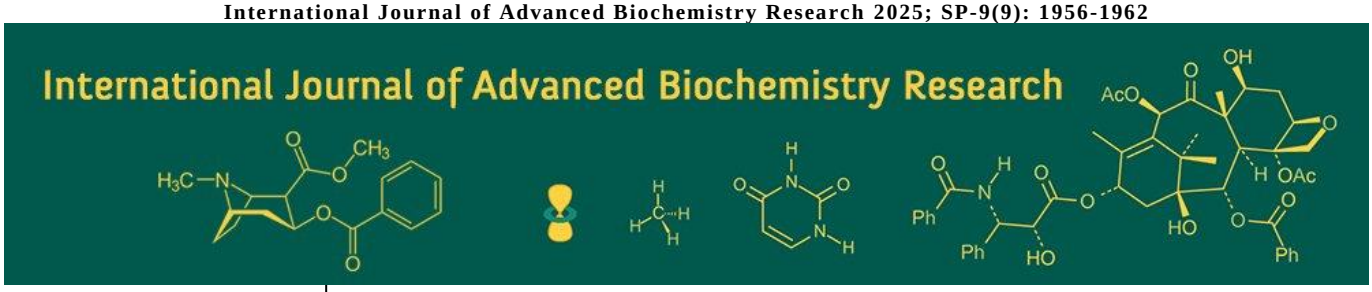

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Screening and identification of pyroxasulfone degrading bacteria

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Abstract
A laboratory experiment was carried out at the Food Quality Testing Laboratory, N.M. College of Agriculture, Navsari Agricultural University, Navsari during of year 2023-24. In this study 26 bacterial culture previously isolated from pyroxasulfone applied soil at food quality testing laboratory were used for determination of degradation potential of isolated microbes, they were separately inoculated in BHM broth supplemented with pyroxasulfone as sole carbon source and incubated at room temperature at different time interval. Pyroxasulfone was extracted using acetonitrile and analyzed by GC-MS/MS. The maximum degradation of pyroxasulfone on BHM medium was recorded in HP 1 culture on 5th,10th and 15th day after inoculation which was followed by culture HP 7. Among 26 bacterial cultures HP 1 and HP 7 showed that the highest degradation of pyroxasulfone as compared to others. These two cultures *viz*., HP 1 and HP 7 were identified by 16S rRNA gene sequencing. Based on sequence homology, the culture HP 1 and HP 7 were identified as *Streptomyces* sp. HP 1 and *Staphylococcus* sp. HP 7.

Keywords: Pyroxasulfone, 16S rRNA, Gas chromatography, Degradation

1. Introduction
Herbicide play an important role in agricultural production, and their use is currently essential for ensuring and enhancing crop yields. (Wang *et al.*, 2023) [3]. Herbicides remain the most effective, efficient and economical way to control weeds; and its market continues to grow even with the plethora of generic products. With the development of herbicide-tolerant crops, use of herbicides is increasing around the world that has resulted in severe contamination of the environment. The strategies are now being developed to clean these substances in an economical and eco-friendly manner (Singh and Singh, 2016) [21].
Microbial biodegradation is environment-friendly treatment technique for detoxification of persistent organic pollutants, compared with conventional methods. Some bio- technological applications such as biodegradation of organic pollutants could be given as examples of biodegradation. Bacteria and fungi are both responsible for biodegradation of polyaromatic hydrocarbons (PAHs) and other petroleum hydrocarbon. Microbial populations have high biodegradation capacity for organic material (Kanat, 2018) [8].
Pyroxasulfone (PYS), a pre-emergence herbicide registered for weed control in maize, wheat and soybean in India. Efficacy of any pesticide depends upon various environmental factors e.g. soil properties, rainfall and its physicochemical properties. The DT50 of pyroxasulfone in soil ranged from a low of 15.4 d to a high of 53.3 d, and loss was more rapid under warm, moist conditions. These results indicate that pyroxasulfone would last long enough to provide residual weed control, but would not persist excessively to injure rotational crops (Mueller, 2017) [16] the information regarding the impact of biotic factors particularly microbial degradation of PYS is not available.

2. Materials and Methods
2.1 Reagents and Chemicals
Technical- grade pyroxsulfone (99.73% pure) was procured from M/S Chromatopak Analytical Instrumentation (India) Pvt. Ltd., Jawahar Nagar, Goregaon West Mumbai- 400 062, MS grade Dichloroethane (99.8% pure), Sodium sulphate, Magnesium sulphate, Sodium chloride, MS grdae Acetonitrile, acetone (99.9% pure), Nutrient agar media, Bushnell and Haas agar media.

2.2 Screening of Pyroxasulfone Degrading Bacteria by Enrichment Culture

2.2.1 Medium for screening

Bushnell and Haas agar (BHM) media with following composition was used: $MgSo_4$ 0.2; $CaCl_2$ 0.02; KH_2PO_4 1.0; $FeCl_2$ 0.05; NH_4NO_3 1.0; Agar 15.0.

The medium for screening was composed of BHM media and pyroxasulfone. BHM media was autoclaved and isolated microbes were separately inoculated in BHM broth then supplemented with 10 mg/L pyroxasulfone were incubated at room temperature. Samples were analyzed after 0(after 2 hrs.) 5, 10, and 15 days interval for growth and biodegradation rate. Growth rate was measured at 600 nm on spectrophotometer. Pyroxasulfone was extracted using acetonitrile and analyzed by GC-MS/MS as described in screening procedure.

2.2.2 Screening procedure

The screening procedure of Sharma, 2013 was followed with some modifications. The bacteria was used to previously isolated from pyroxasulfone applied soil at Food Quality Testing Laboratory. All isolated microbes were screened for their efficacy to degrade pyroxasulfone. For determination of degradation potential of isolated microbes, they were separately inoculated in BHM broth supplemented with pyroxasulfone as sole carbon source and were be incubated at room temperature.

For screening procedure taken 50 ml BHM media containing pyroxasulfone in separating funnel. Added NaCl and added 15 ml dicholomethane (DCM) in separating funnel and Kept a flask with funnel under the nozzle of separating funnel and added Sodium sulphate or MgSO4 on the cotton plug placed inside the funnel. Mixed the separating funnel properly and released the gas intermittently after shaking allowed the funnel to settle down and extract the DCM layer and also repeated the above step 2, 3 times. Taken 4 ml aliquot in test tube and evaporate it to dryness with TurboVap at 40 ℃. Finally made up the volume to 2 ml using acetonitrile and filtered it in to glass vial and dilute it on 100 µl: 900 µl acetonitrile (1 ml) and quantified on GC-MS/MS.

2.3 Screening of Biosurfactant Production using Blue Agar and Blood Agar

2.3.1 Blue agar

The potential of isolation microorganisms to produce biosurfactants, which improve pyroxasulfone's bioavailability was examined. To identify the synthesis of biosurfactants Cetyl Tri Methyl Ammonium Bromide (CTAB) was added to blue agar plates. Culture was spot inoculated and incubated to observe the formation of blue halos around the colonies.

2.3.2 Blood agar

To see hemolysis, cultures were injected in spots on blood agar plates (nutrient agar containing 5% human blood) and allowed to incubate at room temperature. A clear zone surrounding a colony was indicated of hemolytic activity.

2.4 Identification of Potential Isolate/s by 16S rRNA

Potential isolate was identified by 16S rRNA sequence homology. Sequencing service was taken from SLS Research Pvt. Ltd, Surat. Sequence homology was searched from available database using nucleotide BLAST tool and isolated organism was identified and gene sequence was submitted for accession number.

3. Results and Discussion

3.1 Isolation of Bacterial Cultures

Twenty six (26) bacterial cultures used in the present study were previously isolated at Food Quality Testing Laboratory. These cultures were for screening of pyroxasulfone degrading isolates to check the degradation of pyroxasulfone.

3.2 Screening of Pyroxasulfone Degrading Isolates using GC-MS/MS

The breakdown of pyroxasulfone by different 26 bacterial isolates was studied in BHM broth. Results were shown in Table 1. All of these species were assessed for total pyroxasulfone degradation under similar environmental condition at four interval times (0, 5, 10 and 15 days). The results obtained in the study reveals that there was no any degradation of pyroxasulfone was recorded on 0 day. However pyroxasulfone degradation was recorded in the later sampling intervals (5, 10, 15 DAI) in 26 bacterial strains along with control which was devoid of bacterial inoculation. The maximum degradation of pyroxasulfone was recorded in HP 1 culture on 5th, 10th and 15th DAI which was followed by culture HP 7. The maximum pyroxasulfone degradation HP 1 and HP 7 were the two bacterial species that degraded pyroxasulfone highest in BHM medium as compared to other culture. Although, a consistent pyroxasulfone degradation was also recorded in other cultures along with control on 5^{th}, 10^{th} and 15^{th} DAI. On 15^{th} DAI, the maximum degradation of pyroxasulfone was recorded in HP 1 culture (67.74%) followed by HP 7 (62.10%), HP 19 (58.90%). Other cultures have also exhibited pyroxasulfone degradation capability which was found to be in the range of 42.05 to 50.82%. However, these cultures were not considered for further study as control treatment exhibited 39.31% degradation on 15 DAI.

Table 1: Degradation percentage and growth at 600nm of bacterial culture HP 1-HP 26 of different time interval

Culture	Days after Inoculation							
	0 (after 2hrs)		5		10		15	
	Growth (600nm)	Degradation (%)	Growth (600nm)	Degradation (%)	Growth (600nm)	Degradation (%)	Growth (600nm)	Degradation (%)
Control	0.00	0.00	0.00	8.55	0.00	24.28	0.00	39.31
HP 1	0.00	0.00	0.552	19.49	0.785	45.61	0.962	67.74
HP 2	0.00	0.00	0.423	11.37	0.461	31.65	0.683	45.62
HP 3	0.00	0.00	0.365	7.86	0.407	27.28	0.465	43.99
HP 4	0.00	0.00	0.310	5.90	0.423	28.27	0.526	36.66
HP 5	0.00	0.00	0.296	7.64	0.333	30.54	0.540	45.41
HP 6	0.00	0.00	0.315	14.98	0.345	25.59	0.662	44.40
HP 7	0.00	0.00	0.540	18.99	0.660	45.41	0.889	62.10
HP 8	0.00	0.00	0.213	8.43	0.345	31.67	0.421	48.24
HP 9	0.00	0.00	0.215	6.55	0.362	29.78	0.565	46.85
HP 10	0.00	0.00	0.235	13.25	0.341	33.55	0.465	44.62
HP 11	0.00	0.00	0.325	9.47	0.425	35.31	0.585	43.07
HP 12	0.00	0.00	0.368	14.70	0.465	28.27	0.512	50.82
HP 13	0.00	0.00	0.364	14.30	0.412	35.43	0.523	48.30
HP 14	0.00	0.00	0.253	8.88	0.356	32.92	0.456	43.74
HP 15	0.00	0.00	0.402	13.51	0.465	25.67	0.598	42.05
HP 16	0.00	0.00	0.412	11.75	0.520	28.96	0.612	47.58
HP 17	0.00	0.00	0.457	10.44	0.525	28.25	0.595	45.58
HP 18	0.00	0.00	0.401	14.16	0.487	24.43	0.594	50.25
HP 19	0.00	0.00	0.417	19.22	0.512	40.50	0.854	58.90
HP20	0.00	0.00	0.323	6.81	0.445	30.48	0.598	46.83
HP21	0.00	0.00	0.320	10.95	0.415	28.17	0.615	45.04
HP22	0.00	0.00	0.368	11.03	0.496	29.38	0.523	48.18
HP23	0.00	0.00	0.214	5.05	0.389	24.19	0.444	50.55
HP24	0.00	0.00	0.299	14.24	0.356	26.80	0.475	47.84
HP25	0.00	0.00	0.314	14.81	0.452	28.69	0.587	47.75
HP26	0.00	0.00	0.326	13.95	0.440	29.55	0.596	43.15

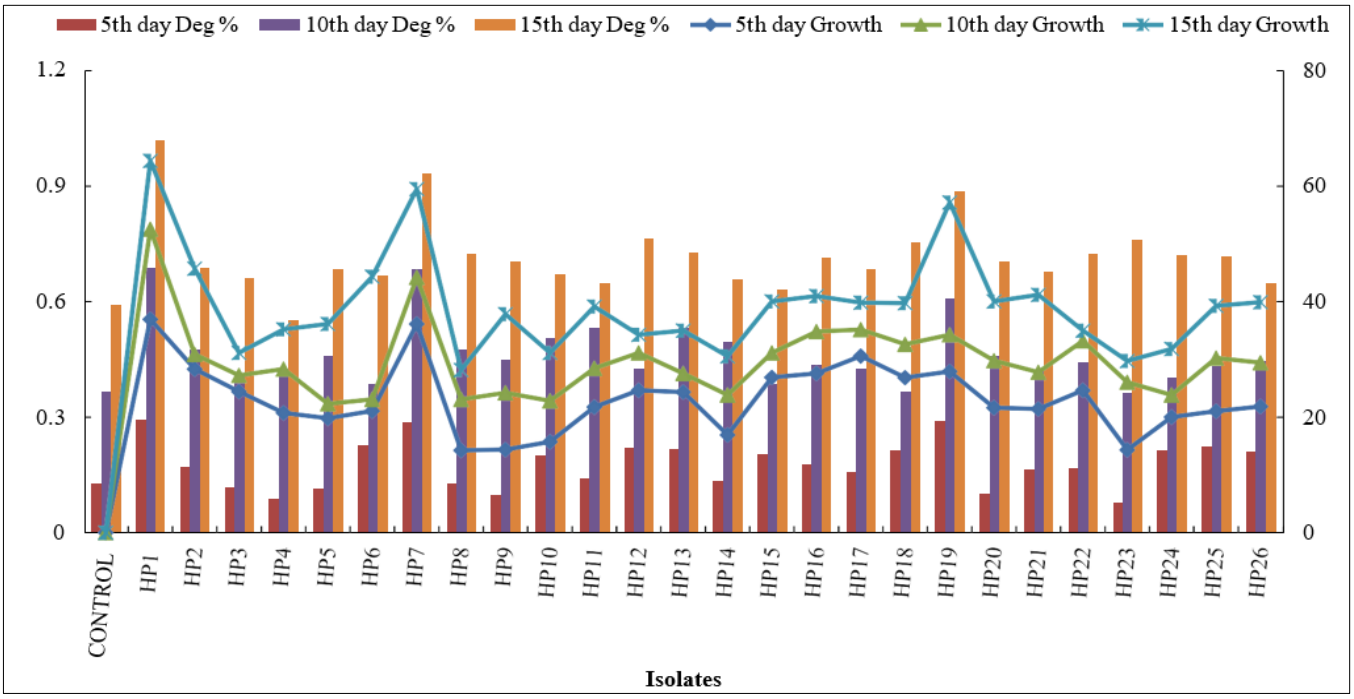


Fig1: Relative pyroxasulfone degradation by isolated bacterial species HP 1-HP 26 in BHM broth

3.2 Screening for Biosurfactant Production

3.2.1 Blood Agar

Positive strains seeded on blood agar showed a translucent, colorless ring surrounding the colonies after a 24-hour incubation period (Table 2).

3.2.2 Blue Agar (CTAB agar)

All isolates were able to produce a complete blue colony or blue ring around the edge of colony after 24-hour of incubation which indicating the formation of extracellular glycolipids or other anionic surfactants.

The results obtained by biosurfactant production on HP 1 and HP 7 showed weak hemolytic activity produced on blood agar and also blue colony was observed on CTAB agar (Table 3).

Table 2: Biosurfactant production on Blood agar by different isolates

Isolates	Blood agar haemolysis	Isolates	Blood agar haemolysis
HP 1	γ	HP 14	γ
HP 2	β	HP 15	γ
HP 3	γ	HP 16	γ
HP 4	γ	HP 17	γ
HP 5	γ	HP 18	γ
HP 6	γ	HP 19	γ
HP 7	γ	HP 20	γ
HP 8	γ	HP 21	γ
HP 9	γ	HP 22	β
HP 10	γ	HP 23	γ
HP 11	α	HP 24	β
HP 12	γ	HP 25	β
HP 13	γ	HP 26	γ

Where,
(Gamma) γ= weak hemolytic activity
(Beta) β= produce completely hemolysis with zone around colony.
(Alpha) α= reduction of red color hemoglobin to appear green color colony

Table 3: Biosurfactant production on blue agar (CTAB) by different isolates

Isolates	CTAB observation
HP 1	Blue colony
HP 2	Helo blue ring with zone
HP 3	Blue colony
HP 4	Blue colony
HP 5	Blue colony
HP 6	Blue colony
HP 7	Blue colony
HP 8	Blue colony
HP 9	Blue colony
HP 10	Blue colony
HP 11	Blue colony
HP 12	Blue colony
HP 13	Negative
HP 14	Blue ring around the edge of colony
HP 15	Blue ring around the edge of colony
HP 16	Blue colony
HP 17	Blue colony
HP 18	Blue colony
HP 19	Negative
HP 20	Blue halo ring
HP 21	Negative
HP 22	Blue halo ring
HP 23	Negative
HP 24	Blue colony
HP 25	Blue halo ring
HP 26	Blue colony

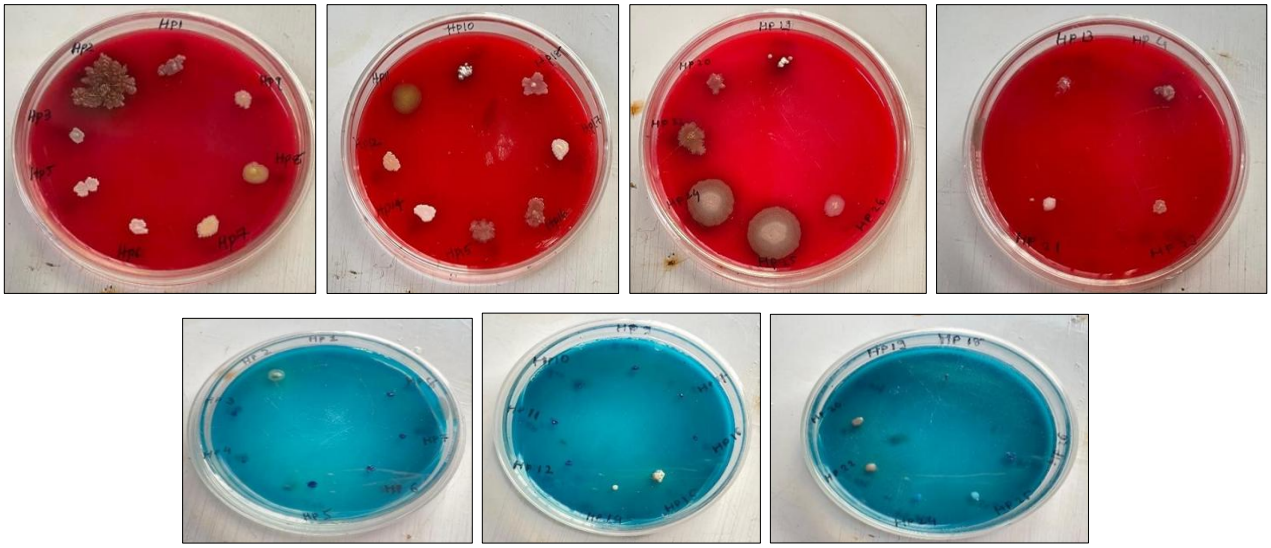
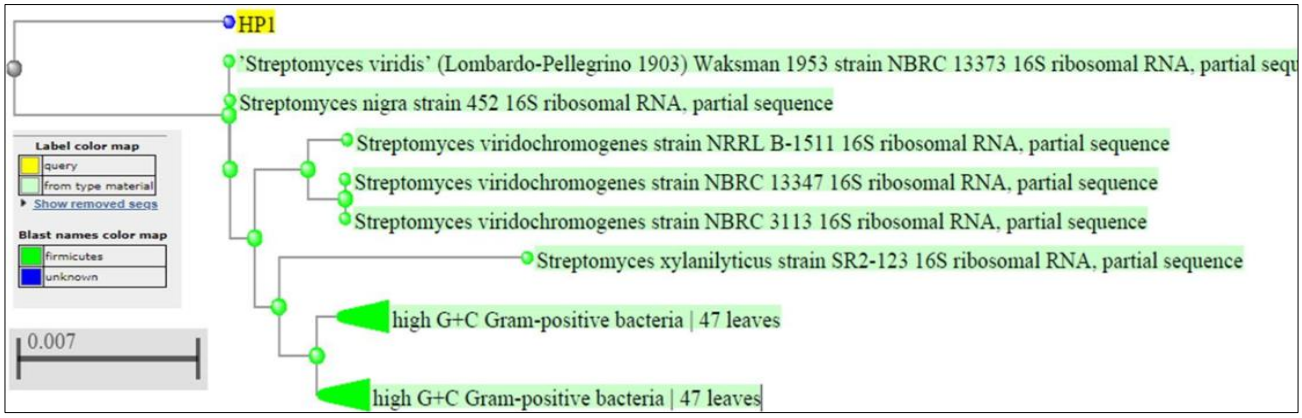


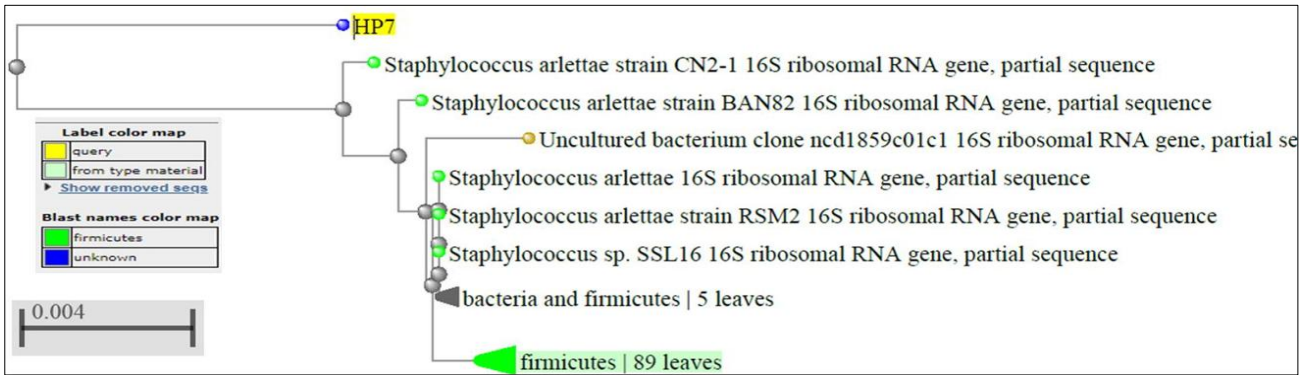
Photo 3.1: Biosurfactant production on blood agar and CTAB agar of different Isolates

Identification of Isolates Showing Highest Pyroxasulfone Degradation

Among the 26 culture, HP 1 and HP 7 culture recorded highest pyroxasulfone degradation as compared to other. These two cultures *viz*. HP 1 and HP 7 were identified by 16S rRNA gene sequencing. Based on sequence homology, the cultures HP 1and HP 7 were identified as *Streptomyces* sp. HP 1 and *Staphylococcus* sp. HP 7. The sequence of above isolates were submitted to GenBank for accession number and these were assigned the accession number PQ069794 and PQ069798 for HP 1 and HP 7 cultures, respectively. Their phylogenetic tree is given in Photo 3.2.



(A) *Streptomyces* sp. HP 1: Phylogenetic tree



(B)*Staphylococcus* sp. HP 7: Phylogenetic tree HP 7 cultures

Photo 3.2: Phylogenetic tree of HP 1 and HP 7 cultures

4.1 Conclusion

4.1.1 Screening of Pyroxasulfone Degrading Cultures

In present study, 26 bacterial cultures were screened for their capability to degrade the pyroxasulfone in BHM broth which were previously isolated from farm soil with history of pyroxasulfone application at Food Quality Testing Laboratory of Navsari Agriculture University, Navsari, Gujarat. DCM based liquid extraction method adopted for pyroxasulfone residue analysis from BHM broth and quantified on GC-MS/MS. The BHM broth were spiked with pyroxasulfone at the rate of 10 mg/l and incubated for 15 days with sampling on 0, 5, 10 and 15th days. The overall pyroxasulfone reduction for each of these species increased gradually throughout the duration of the incubation period although it was maximum for two bacterial species, *i.e.*, *Streptomyces* sp. HP 1(67.74%) followed by *Staphylococcus* sp. HP 7 (62.10%) on 15th day. All isolates showed positive results on blood agar. Similarly, all of the isolates were positive results for biosurfactant synthesis on CTAB blue agar except of HP 13, HP 19, HP21 and HP23.

4.1.2 Identification of Isolates Showing Highest Pyroxasulfone Degradation

The potential isolates, HP 1 and HP 7 which showed highest degradation identified by molecular characterization as *Streptomyces* sp. HP 1 and *Staphylococcus* sp. HP 7. Genetic sequences were submitted to GenBank and accession numbers of HP 1 and HP 7 cultures were assigned the accession number PQ069794 and PQ069798 for HP 1 and HP 7 cultures, respectively.

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