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Screening, isolation and Molecular characterization of Propanil degrading bacteria

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ABSTRACT

Microbial cultures able to degrade xenobiotic compounds are the key element for biological treatment of waste effluents and are obtained from enrichment processes. Propanil is an acylanilide herbicide introduced in the early 1960s to control dicotyledonous weeds and grasses. In this study a total number of 18 bacterial isolates were screened from the propanil contaminated rice fields. From the isolates 2 strains named, Isolate K & Isolate P were screened for propanil degradation on MS media under aspetic conditions. The isolate P was reported to degrade the lowest propanil (50mg/L) in 83.27% as well as high concentration (800mg/L) was degraded at 12.07% under aseptic conditions. The bacterial isolate P was biochemically characterized and the isolate was identified by amplification and sequencing of their 16s rDNA at species level. The isolate P was finally identified as *Pseudomonas sp.*

Keywords: Propanil degradation, *Pseudomonas* sp, Sequencing.

1. INTRODUCTION

The extensive usage of pesticides and herbicides in agriculture is a matter of global concern. These improper application and overuse of these chemicals damages the environment. The possibilities for environmental contaminants were may be due to spray drift, volatilization of sprayed herbicides, run-off and leaching, reported by many researchers [1-4]. These pesticides persist in the environment either unaltered or only partially degraded. Most of these compounds, as well as their metabolites, severely damage the human health and ecotoxicity threats and there should be a great concern to remove the excessive compounds from contaminated environment. Propanil is an anilide and postemergence herbicide, consists of molecular formula of C9 H9 Cl2 NO, used for weed control on potatoes, rice and other crop plants [5] and is ranked within the top 20 pesticides used in agriculture [6].
Propanil used for the control of broad-leaved and grass weeds in rice, and the herbicidal mechanism was to inhibit the Hill reaction in photosynthetic electron transfer (photosystem II [PSII]) [7]. Like other commercially used herbicides, propanil also affects non-target organisms and also cause several severe risks for birds, small mammals, and aquatic biota [8]; macro invertebrates [9], plankton communities [10], and fishes [11]. In the marine environment, PSII herbicides used in agriculture affect corals and their symbiotic dinoflagellate algae. The herbicide penetrates the coral tissues reducing the photochemical efficiency of the algal symbionts [12].

Propanil is also an important cause of death from acute pesticide poisoning, of which methemoglobinemia is an important manifestation [6]. Propanil absorbed into the body through ingestion, inhalation or dermal exposure, it cause central nervous system depression. The probable oral lethal dose (LD50) is 0.5 – 5.0 g/kg of body weight [13]. Propanil is cleaved enzymatically in plants, mammalian liver and soil into 3,4-dichloroaniline (3,4-DCA) and also during alkaline hydrolysis. The existence and potential ecotoxicity, water solubility (130–500 mg/L at 25^{0}C) [14],

persistence in soil and aquatic environments [15] and the possible accumulation of the toxic by-products, the complete removal of both Propanil and its by-product 3,4 DCA from contaminated sites is of today's environmental concern. The most common pesticide degradation reactions was the photodegradation, where sunlight was the source for breakdown of pesticides and several parameters which effects the rate of degradation [16].

In soil, biodegradation of propanil generates 3,4-DCA, which is also a product of the microbial transformation of other herbicides, such as diuron and linuron [17-20]. Compared with propanil, 3,4-DCA shows lower toxicity against fish and mammals [17, 21]. However, 3,4-DCA can be converted by microbial peroxidases to 3,3,4,4 tetrachloroazobenzene (TCAB) and other azo products. There is great concern about TCAB because it is a carcinogen and a potential genotoxin [17]. Hence the molecule 3,4-DCA is highly toxic and is classified as a secondary poisonous substance.

Degradation to more refractory monochloroanilines takes place more time under anaerobic conditions [22], and projections of mineralization rates observed in short-term experiments indicate that the residual life in soil of herbicide-derived chloroanilines may extend for up to 10 years [23]. The removal of non point source pollutants such as agrochemicals that affect soils and water bodies is difficult. To facilitate their removal, the use of wetlands, runoff holding ponds, or permeable reactive barriers could be a suitable technical choice.

Photocatalytic and electrochemical oxidation techniques are the physical methods could be used [24] for propanil degradation. Physicochemical processes are useful to degrade organic compounds, principally when they are concentrated, but for degradation of micropollutants, these methods could be uneconomic. Hence, Majority of researchers developed the strategy for micropollutant degradation by biological treatments [25, 26]. In case of agrochemical pollutants, a practical solution to remove them from water could be the use of specialized microbial consortia attached to porous material acting as a permeable reactive barrier. Besides the active compounds, the inclusion of additives considered inert in commercial formulation of pesticides has become a common practice in their production. However, the full extent of the toxicity of the adjuvants used in pesticide formulations to aquatic life has been rarely assessed [27]. Adjuvants include chemicals with diverse functions in the pesticide formulation. For example, wetting and dispersing agents such as polyoxethylated alkylphenols, polyoxethylated fatty alcohols, tridecyl alcohol polyglycol ether, alkylphenylsulfonates, salts of ligninsulfonic acid, dibutylnaphthalenesulfonic acid, swelling polysaccharides, defoamers including tributylphosphate or dialkylpolysiloxanes, antifreeze agents such as ethyleneglycol, propyleneglycol, or glycerol, oligoester surfactants, polyols such as sorbitol, maltitol, isomaltitol, or lactitol, and preservation agents such as benzoic acid, or formaldehyde [28, 29]. Beside pesticides, diverse adjuvants have been found in groundwater and top soils [30]. These chemicals may have various toxicological profiles, ranging from possible harmless products, to others that could represent a serious toxicological threat [31, 32]. Although a pure microbial strain would be able to metabolize a mixture of carbon sources such as propanil and adjuvants, a mixed microbial community would be more reliable to metabolize mixed substrates.

Effective break down of hazardous chemicals, toxic compounds into nontoxic compounds and, in some cases, back into their original elements is known as degradation of pesticides. The degradation of pesticides can take place in plants, animals and in the soil and water, or it may occur upon by physical exposure to ultra-violet (UV) radiation. In soil, there are some strictly chemical reactions which takes place by microbial degradation, especially the fungi and bacteria. There is nothing mysterious about microbial degradation of pesticides. Upon supplying the suitable energy source i.e food, essential elements for microbes, initiates the chemical reaction for pesticide degradation.

Many of the microorganisms possess the greatest enzymatic diversity found on earth and metabolize millions of organic compounds to capture chemical energy for their growth. Microorganisms are increasingly altered by genetic engineering methods to degrade the hazardous, xenobiotics compounds, an application commonly known as bioremediation [33, 34]. The rate of biodegradation was strongly influenced by various factor like, the chemical structure of the compound (molecular size, presence or absence of a certain substituent), the availability of the microorganisms, the type of environment (aerobic or anaerobic), and the degree of nutrients, light, pH and temperature [26]. Considering the potential of the bioremediation to reduce the environmental impact caused by the intensive use of pesticides, the purpose of this study was to isolate herbicide degrading microorganisms, by using various inoculum forms and enrichment techniques and evaluate the degradation power of these microorganisms in different conditions.

Hence, in this work, a continuous small-scale bioprocess to degrade propanil, its main catabolic by-product, 3,4- dichloroaniline, as well as the adjuvants of the herbicide, using a microbial consortium attached to the soil sample and the potential isolates were screened as well as molecular characterized.

2. MATERIALS AND METHODS

2.1 Chemicals, reagents and enzymes

All chemicals were obtained from Himedia Laboratories, Mumbai. Oxidase test discs were obtained from Fluka and other Biochemicals as well as dye Indicators were also procured from Himedia Laboratories, Mumbai, India. Taq polymerase used for PCR amplification was from New England Bio Labs.

Other PCR consumables like dNTP's, $MgCl_2$ etc. was purchased from Promega.

2.2 Herbicide

The main substrate used in this study was a commercial presentation of propanil (N-(3,4-dichlorophenyl) propanamide) provided by Bayer Crop Science, India and which was commercially sold by the named as Stam* 80 EDF.

2.3 Isolation of Propanil degrading bacteria from enriched soil sample by serial dilution technique

Propanil degrading bacteria was isolated from the soil samples of commercial crops like rice and cotton were extensively grown and Propanil pesticide was used intensively, by contemplating such soil would contain pesticide contamination and natural micro-flora experiencing pesticide stress. The collected samples were aseptically transferred into a sterile polythene bag to avoid external contamination and stored at 37^0C to maintain the biological activity of the soil microbes. The enrichment method was carried out by adding the pesticide propanil aseptically in Laminar air flow system in the ratio of 100:1(W/V). The soil moisture was maintained by adding 5 ml of sterile distilled water and the flask was incubated at 37^0C by temperature regulated shaking incubator [35] at 100 rpm/min for 4 weeks. Propanil degrading bacterial isolates were obtained by serial dilution of enriched soil sample on Nutrient Agar Medium (NAM), containing 5 ppm concentration of Propanil. 0.1 ml of 10^{-4} to 10^{-6} dilutions of enriched soil samples were inoculated into the nutrient agar plates and incubated at 37^0C for 24 hours. Propanil degrading bacterial colonies were developed on nutrient agar medium and the colonies were separately inoculated on enriched nutrient agar plates for obtaining pure cultures [36]. Two isolates were shown the degradation ability were labeled as isolate K & P, which further characterized for maximum efficiency by using spectrophotometer.

2.4 Screening of Propanil degrading bacterial isolates K and P in minimal salt medium

The two bacterial isolated colonies were pre cultured in 100mL of LB broth medium and incubated at 37°C for 8 hours. Bacterial cultures with 1 OD at 660 nm were used as inoculums for bacterial growth for propanil degradation assay. Degradation analyses were performed in 250 mL conical flask containing 100 mL of MSM supplemented. The compositions for minimal media used were contains KH2PO4 0.5g, K2HPO4 1.5 g, (NH4)2SO4 0.5 g, NaCl 0.5 g, MgSO4.7H2O 0.2 g, Glucose 4 g, pH 7.0 and the volume upto 1000mL with distilled water. The above chemicals sparing glucose and Propanil were dissolved in distilled water and adjusted to pH 7. Sterilized propanil (1 g/L) stock solution was used to prepare the working solution for Propanil degradation analysis. To the autoclaved MSM medium 5 µl to 80 µl of Propanil was poured in separate conical flasks and mixed to the medium to get different concentrations of Propanil from the range 50 mg/l to 800 mg/l [37, 38]. Media were inoculated with 5 ml/L biomass and incubated in the dark on an orbital shaker (100 rpm) at 30°C for 4 days of aerobic incubation. Two experimental sets for cell growth were prepared in duplicate. The biomass concentration was estimated by optical density measurement in 1 cm cuvettes at wavelength of 660 nm using UV-Visible spectrophotometer. The degradation ability of the screened bacterial isolates were observed and recorded.

2.5 Screening of propanil degrading bacterial isolates K&P

Various concentration of propanil was prepared from the stock solution which contains the concentration of 2g/L. From this by doing the serial dilutions concentration like 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L and 800 mg/L was prepared. These concentrations were transferred into LB broth media. The bacterial cultures were inoculated into the media and the tubes were incubated at 37oc for 48 hrs of aerobic conditions under strict aseptic conditions. After 48 hrs of aerobic degradation propanil degradation assay was carried out at 245 nm under aseptic condition by using Thermo Scientific Multiscan ES reader [25]. Because Propanil concentration was determined by its absorption at λ = 245 nm. The degradation assay was calculate based on the formula

$$\% \text{ of Propanil degradation} = \frac{\text{Concentration propanil in control} - \text{Concentration propanil in sample}}{\text{Concentration propanil in control}} \times 100$$

2.6 Biochemical characterization of Isolate P

Two staining techniques, simple staining and gram's staining were used for identification of isolated bacterium. Simple staining technique is used to recognize the three basic shapes of bacterial cells. Gram's staining technique is a differential staining method used to differentiate bacterial species into gram +ve and gram -ve bacteria. Based on the gram staining various biochemical tests like Indole production, Methyl red voges proskauer test, Citrate utilization test, Hydrogen sulphide production test, urease hydrolysis test, starch hydrolysis test, Oxidase production test, Casein hydrolysis test and catalase test were carried to characterize the bacterial isolate P under strict aseptic conditions [25].

2.7 Molecular characterization

2.7.1 Genomic DNA extraction and purification of isolated DNA

Bacterial isolate P was selected for molecular characterization, which possess the maximum propanil degradation when compared to the isolate K. 1.5 ml of overnight grown bacterial isolate P was spun at 8000 rpm for 5 minutes. Bacterial cells were lysed by adding 400 µl of lysis buffer (0.5% SDS, 1M NaCl) and incubated at 65^0C for 15 minutes. The solutions were cooled to room temperature and 130 µl of 3M potassium acetate (pH 5.2) was added to each solution and incubated for 5 minutes at room temperature. The samples were centrifuged at 10,000 rpm for 10 minutes

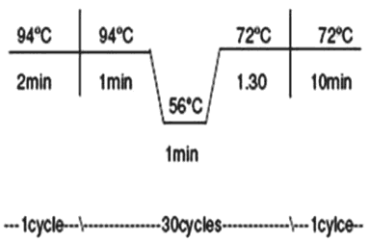
and clear supernatants were transferred into fresh 1.5 ml tubes. Nucleic acids were precipitated by adding equal volume of isopropanol and gently inverted, back and forth for 10 times and incubated on ice for 10 minutes. The samples were centrifuged at 10,000 rpm for 5 minutes and the pellets were washed with 75% ethanol. The pellets were dried at room temperature to evaporate ethanol completely and dissolved in TE buffer. The genomic DNA of bacterial isolate P was confirmed by 1% agarose gel electrophoresis along with λ Hind III digest DNA molecular weight marker (New England biolabs, USA). The isolated DNA was subjected to purification to remove proteins and other contaminants by addition of 3M sodium acetate (pH 4.6) and 100 µl of 95% ethanol were added separately and vortexed. The tubes were freezed at -20^{0}C for 30 minutes and centrifuged for 5 minutes and washed with 75% ethanol and were air dried completely and dissolved in 10 µl of sterile distilled water. Presence of pure DNA of bacterial isolate P was confirmed by UV spectrophotometric analysis. The purified bacterial genomic DNA was further used for Molecular characterization.

2.7.2 PCR amplification of 16S rRNA gene of bacterial isolate P

The partial sequence analysis of 16S rRNA gene of the bacterial isolate P was carried out by using Thermocycler (Corbet research Australia) by using 16S rRNA universal primers. The 1379 bp of 16S rRNA gene was amplified using two primers. The primers annealing at the 5' and 3' end of the 16S rRNA were (27f Forward Primer), 5'- AGAGTTTGATCCTGGCTCCAG -3' and (1492r Reverse primer) 5'- TACGGTTACCTTGTTACGACTT -3' were used.

The PCR was performed in 25 µl reaction volume containing Taq buffer (5µl), Taq DNA polymerase (1µl), dNTP mixture (1µl), 16S rRNA forward primer (1.5µl), 16S rRNA reverse primer (1.5µl), template DNA (2 µl), sterilized distilled water (13.0µl) Thermo-cycler was programmed as here under.

Program 1 (one cycle) (Initial de-naturation) 94°C for 2 minutes.
Program 2 (30 cycles) (amplification) Step one (denaturation) 94°C for 1 min Step two (annealing) 56°C for 1 min. Step three (extension) 72°C for 1min 30 seconds.
Program 3 (one cycle - final extension) 72°C for 10 minutes, then hold at 4°C
The programmed temperatures of the PCR reaction are represented in the diagram shown below:



The amplified PCR products were analyzed by 2% agarose gel electrophoresis and the bands were visualized under UV light, photographed and documented with an Alpha Imager (Alpha Innotech, California, USA). The PCR amplified product was further purified and used for sequence analysis. For sequence analysis, the amplified fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced by MWG Eurofins , Banglore, India (Taq DyeDeoxy Terminator Cycle Sequencing and ABI PRISM 310 capillary apparatus, Applied Biosystems) using 16S-specific primers and universal *lacZ* primers M13fwd or M13rev. The 16S rRNA gene sequences were aligned and edited with the BioEdit program (version 4.8.8) [39] and analysed using the programs SEQBOOT (100 iterations), NEIGHBOR and CONSENSE of the PHYLIP package [40]. BLAST searches and 16S rRNA sequence download were performed using the National Center for Biotechnology Information facilities (http://www.ncbi.nlm.nih.gov). Sequences have been deposited under GenBank Accession numbers JQ609306.

3. RESULTS AND DISCUSSION

3.1 Collection of soil sample

Soil samples were collected from different fields of paddy cultivated areas of Srikakulam, Andhra Pradesh where there was an intensive accumulation of the Propanil in the soil. The soil samples were collected from 15 inches depth below the surface for isolation of propanil degrading bacteria where there was moisture which supports ideal environment for the growth of the microbes. The diggers and other requirements were aseptically maintained for collecting the soil samples. The soil was collected in polythene bags that were sterilized with UV- rays and X- rays to avoid external cross contamination and transferred to the laboratory immediately. The collected soil sample bags were stored at 4^{o}C to maintain the biological activity of the soil microbes.

3.2 Enrichment of the Soil sample

The collected samples were thoroughly mixed and grinded well to maintain the equal particle size. Soil samples collected were enriched by adding 10ml of Propanil to 1000 grams of the soil sample in the ratio of 1:100 and incubated at 37°C, pH 7.0 and kept on a temperature regulated shaking incubator for 4 days at 100 rpm/min for bacterial growth enhancement.

3.3 Isolation of Propanil Degrading Bacteria

18 different microorganisms were isolated from the propanil contamination Soil samples by serial dilution method on nutrient agar media at temperature 37^{0}C and pH 7.0. Only two isolates were found to shown the ability to grow in the presence of herbicide, the strains named as K & P and stored in the glycerol stock for further studies (Figure 1).

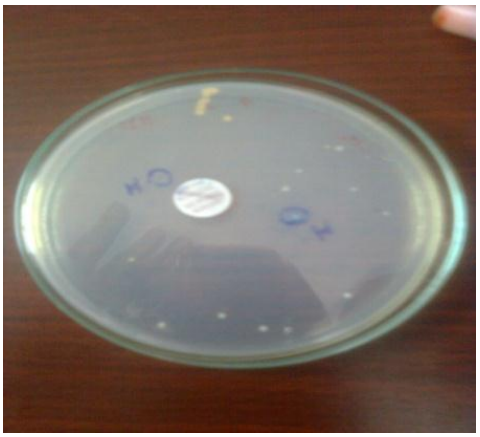


Figure 1: Enriched Bacterial isolates K & P

3.4 Growth by the Isolate K & P in Mineral Salt Medium (MSM):

The degradation of various concentration of propanil was performed under the aerobic condition by each strain for a period of 96 h in Mineral Salt Medium. Cell growth was measured by measuring OD 660 nm by following periodic interval under strict aseptic conditions. The bacterial growth increased during 6 h of incubation and followed by 36 and 42 hours for isolate K (Table 1). Whereas the bacterial growth was increased rapidly during 6 h of incubation followed by 48 and 60 hrs of aerobic incubation for the isolate P was recorded. The results for the isolates *Pseudomonas sp*. (Isolate P) showed best growth patterns for propanil degradation maximum at 60 h. (Figure 2).

Table 1: Bacterial growth curve at various time intervals on Minimal salt Media

S.No	Time in Hrs	OD at 660nm for Isolate K	OD at 660nm for Isolate P
1	0	0	0
2	6	0.084	0.121
3	12	0.123	0.194
4	18	0.155	0.269
5	24	0.198	0.418
6	30	0.259	0.689
7	36	0.382	0.884
8	42	0.578	1.266
9	48	0.427	1.516
10	60	0.246	1.624
11	72	0.169	1.584
12	96	0.117	1.349

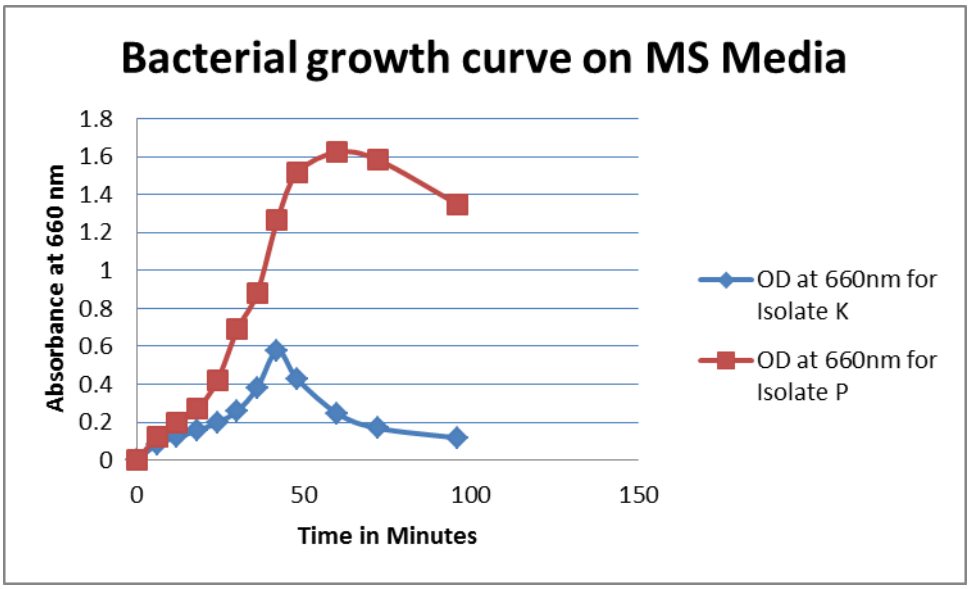


Figure 2: Bacterial growth kinetics of Isolate K & P at various time intervals on Minimal salt Media

3.5 Screening of Propanil degrading bacterial isolates in LB broth media:

By doing the bacterial growth assay for propanil degradation analysis at various concentrations from 50 to 800mg/L, bacterial isolate P showed the maximum degradation when compared to Isolate K. The bacterial isolate K showed the lowest pesticide degradation activity i.e 38% with lowest propanil concentration to 1.48% at highest propanil concentration (800mg/L). Whereas isolate P showed the maximum degradation, 83.27% at lowest propanil concentration and 12.07% at highest propanil concentration (800 mg/L). Based on the above reports, bacterial isolate P was selected for further investigations as it is found to be very effective in hydrolyzing Propanil in M9 minimal salt medium (Table 2).

Table 2: Propanil degradation of Bacterial isolates K& P on MSM media

S.No	Time in Hrs	OD at 250nm for Isolate K		% of degradation by isolate K	OD at 250 nm for Isolate K		% of Degradation by isolate P
		Control	Test		Control	Test	
1	50	0.269	0.165	38.66	0.269	0.045	83.27
2	100	0.453	0.339	25.17	0.453	0.121	73.28
3	200	0.768	0.599	22.00	0.768	0.311	59.50
4	300	0.952	0.774	18.69	0.952	0.433	54.51
5	400	1.129	0.938	16.92	1.129	0.589	47.03
6	500	1.298	1.148	11.56	1.298	0.823	42.04
7	600	1.486	1.351	9.08	1.486	1.023	31.16
8	700	1.609	1.526	5.15	1.609	1.340	16.72
9	800	1.823	1.796	1.48	1.823	1.603	12.07

3.6 Identification of Propanil Degrading Bacteria:

Morphological, Gram staining and biochemical test were performed with Bergey's Manual of Determinative Bacteriology for tentative identification of the isolates. The results of gram staining and biochemical test are shown in Table 2. It was found that, the isolates P was *Pseudomonas sp*. (Table 3).

Table 3: Biochemical examination of isolated bacterium P

S.No	Biochemical Reactions	Observation
1	Indole production Test	Negative
2	Methyl Red test	Negative
3	Voges-Proskauer Test	Negative
4	Citrate utilization Test	Negative
5	Carbohydrate catabolism test	Negative
6	Caesin Hydrolysis	Positive
7	Urease Hydrolysis	Negative
8	Hydrogen sulfide production test	Negative
9	Oxidase test	Positive
10	Starch hydrolysis test	Positive
11	Gelatin hydrolysis test	Positive
12	Catalase test	Positive

3.7 Molecular Characterization of 16s rRNA sequence:

The DNA isolated from the desired Propanil degrading bacterium was suspected to be *Pseudmonas* species and when checked for purity exhibited an absorbance ratio of 1.823 respectively (A_{260}/A_{280} ratio 1.8 to 2.0 to be pure), which can be concluded that the DNA isolated from the bacterial isolate P was pure and the same DNA samples when run on an agarose gel also confirmed to be pure as the bands of DNA are single and distinct and there is no evidence of traces of contaminants were found when observed under the UV transilluminator.

The genomic DNA was subjected for the isolation of the DNA coding for 16s rRNA by using Polymerase chain reaction (Figure 3).

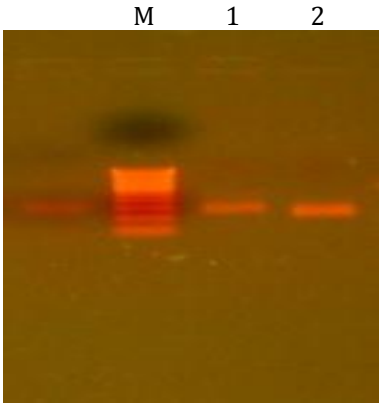


Figure 3: Agarose gel showing Amplified 16s DNA
Well M- Marker of 100bp; Well 1 and 2 -Pesticide degrading soil isolate 16s DNA

3.8 DNA Sequencing

The bands were cut and eluted and the DNA so obtained was subjected for sequencing. Upon sequencing of the amplified DNA, the obtained data corresponds to 1379 base pairs for *Pseudomonas putida*. The sequence so obtained was deposited at NCBI and its accession number is JQ609306.

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   1 tgctctctca aaacccccac aaggctggca cgtcgagcgg atgacgggag cttgctcctt
  61 gattcagcgg cggacgggtg agtaatgcct atgaatctgc ctggtagtgg gggacaacgt
 121 ttcgaaagga acgctaatac cgcatacgtc ctacgggaga aagaagcagg ggaccttcgg
 181 gccttgcgct atcagatgag cctaggtcgg attagctagt tggtggggta atggctcacc
 241 gcgacgatcc gtaactggtc tgagaggatg atcagtcaca ctggaactga gacacggtcc
 301 agactcctac gggaggcagc agtggggaat attggacaat gggcgaaagc ctgatccagc
 361 catgccgcgt gtgtgaagaa ggtcttcgga ttgtaaagca ctttaagttg ggaggaaggg
 421 cagtaagcta ataccttgct gttttgacgt taccgacaga ataagcaccg gctaactctg
 481 tgccagcagc cgcggtaata cagagggtgc aagcgttaat cggaattact gggcgtaaag
 541 cgcgcgtagg tggttcgtta agttggatgt gaaagccccg ggctcaacct gggaactgca
 601 tccaaaactg gcgagctaga gtacggtaga gggtggtgga atttcctgtg tagcggtgaa
 661 atgcgtagat ataggaagga acaccagtgg cgaaggcgac cacctggact gatactgaca
 721 ctgaggtgcg aaagcgtggg gagcaaacag gattagatac cctggtagtc cacgccgtaa
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 961 acatgcagag aactttccag agatggattg gtgccttcgg gaactctgac acaggtgctg
1021 catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgtaac gagcgcaacc
1081 cttgtcctta gttaccagca cgttatggtg ggcactctaa ggagactgcc ggtgacaaac
1141 cggaggaagg tggggatgac gtcaagtcat catggccctt acggcctggg ctacacacgt
1201 gctacaatgg tcggtacaga gggttgccaa gccgcgaggt ggagctaatc tcacaaaacc
1261 gatcgtagtc cggatcgcag tctgcaactc gactgcgtga agtcggaatc gctagtaatc
1321 gcgaatcaga atgtcgcggt gaatacgttc ccgggccttc acacgtacac accgcccgt
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3.9 Phylogenetic tree of the test bacterium constructed by BLAST of NCBI

The sequences obtained were made BLAST hit with the sequences of NCBI database. Basing on the alignment occurred, aligned sequences at the maximum degree were selected and the phylogenetic tree was constructed. The tree so obtained was as follows (Figure 4).

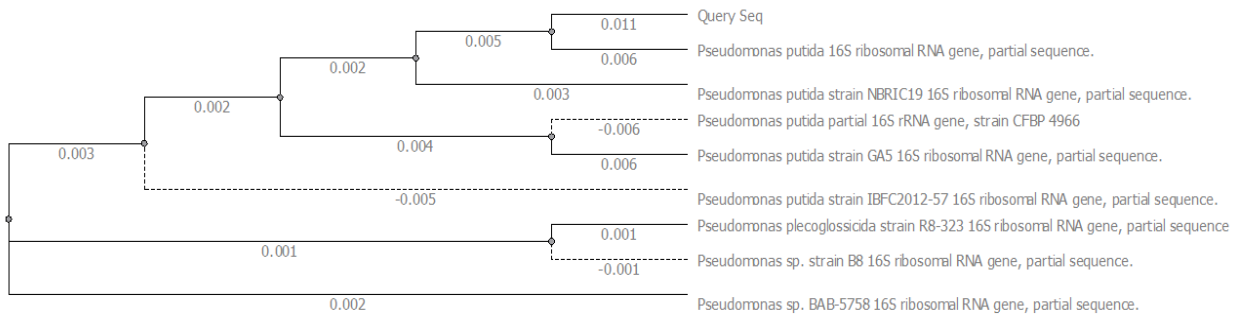

Figure 4: Phylogenetic tree of *Pseudomonas putida*

Microscopic, structural, growth characterization and molecular characterization basing on DNA coding for 16s rRNA sequences of the test organism P capable to degrade Propanil pesticides even at higher concentrations (800mg/l) can be concluded that the test bacterium P was *Pseudomonas putida.*

For enhancing the agricultural yield, herbicides play an important role. To ensure less than 1% of the pesticides are enough to kill the herbs, remaining pesticides enter into the ground, surface water and finally to food chain, which causes environmental pollution and affect human health also [41]. Because of this worse condition, some of the persistent pesticides were banned and some are chemically modified without damaging to the environment as well as humankind. However, excessive usage of herbicides was creating a lot of human health problems [23]. Researchers found that different kinds of bacteria present in soil are capable of degrading many persistent pesticides [42-45]. In the present research, a total of eighteen isolates were primarily screened and the 2 potential isolates were further screened in secondary screening which were isolated from Srikakulam region of Andhra Pradesh. The Secondary screened bacterial isolates K & P identification were done by various biochemical tests. These isolates were tested for the herbicides tolerance against various concentrations of Propanil ranged from 50mg/L to 800mg/L. The tolerant efficiency of isolates was tested by cell plat efficacy test. The growth curve of all two isolates were measured by spectrophotometry analysis at 248nm under aseptic conditions. The most tolerant strain P, which was able to degrade propanil 300mg/L at more than 50%. The isolate P was selected for further studies. The growth of bacteria was reported even in the presence of 800mg/L concentration of Propanil. The growth of isolate P was decreased by increasing the concentration of herbicide. The selected isolate P was Molecular characterized by using the 16s rRNA ribosomal characterized as *Pseudomonas putida*. Interestingly, the extracellular pesticide degrading bacterial enzymes in the *Pseudomonas putida* was very high.

4. CONCLUSION

Microorganisms play a potential role for environmental remediation and it has been increased in importance in the last several years due to its low cost and environmental impact. The most commonly used enrichment methods have successfully enriched microbial consortia, which can able to degrade Propanil from the rice fields of soil samples. The consortia contained different microbial strains that were enriched with 100:1 (W/V) of Propanil, as indicated by the bacteria recovered on solid media. From the microbial strains isolated from the batch degrading consortium two have shown capabilities to degrade propanil. The degradation rates of both the isolates K & P were carried out at various concentrations of Propanil. The effective degradation was reported by the ioslate P. This may be due to microbial interactions that are known to improve the degradation capacities. The continuous method seemed to favour microbial interactions. This work shows the potential of *Pseudomonas putida* for the bioremediation of propanil contaminated soil sample. This bacterial strain also promotes the plant growth by inhibiting the growth of other pests.

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