# Isolation, screening and polyphasic characterization of Methomyl degrading strain and assessment of its biodegradation potential by GC-MS

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**Abstract**— Dramatically increase in production and application of pesticides during last two decades for improvement in crop yield and good quality of agricultural crops has created potential risk for environment as pesticides comprise the largest group of xenobiotic compound. Consistent usage of these agrochemicals leads to soil and water pollution through incorporation of residual amounts of these components in soil and ground water. The present study was focused on the isolation of organisms from soybean field where Methomyl has been used as per the previous history. The isolate possessed an ability to degrade Methomyl (S-methyl-N[methylcarbamoyl)oxy]-thoacetimidate). Phylogenic characterization of the bacterial isolate was accomplished through 16S rRNA sequencing and FAME analysis which revealed it as *Rhodococcus kroppenstedtii* AHJ 3. Different analytical methods were used for quantitative and qualitative determination of degradation of Methomyl. Quantitative determination was based on spectrophotometric analysis and qualitative method GC-MS was applied for confirmation of biodegradation and metabolite formation in which there was formation of two different mass spectra. The possible degradation mechanism was revealed through GC-MS technique which exhibited Methomyl disappearance within four weeks.

**Index Terms**— Bioremediation, FAME analysis, GC-MS, Methomyl, phylogenetic analysis, *Rhodococcus kroppenstedii* AHJ3, 16S rRNA sequencing, specrophotometric.



## 1 INTRODUCTION

Agricultural production mainly relies on the use of chemicals to maintain high crop yields. The integral part for the success of agricultural industry has been gained due to use of these chemicals in modern farming practices [1]. However, less than five percent of these products were estimated to reach the target organisms [2]. To fight pest problem farmers resort pesticide application in order to achieve maximum production of crops. Various characteristic features of pesticides have been responsible for their activity in soil that how they can be acting there [3]. The major issue related to environmental pollution of used pesticides has been their capacity to leach down to subsoil and creating ground water contamination [2]. Some of these pesticides are immobile and they have been persist on the top soil where it could accumulate to toxic level in the soil and become harmful to man, plants, animals and microorganisms [4]. Excessive use of such chemicals leads to environmental deterioration [3]. The quality of soil, ground water, continental and coastal waters as well as air has been compromised by pesticide contamination [5]. Eighty billion pounds of hazardous organo-pollutants were produced annually in agricultural farms and only ten percent of these were disposed off safely from the environment [6]. Approximately $1 trillion cost has been used to decontaminate toxic waste sites in the agricultural farms using various traditional waste disposal methods like land filling and incineration [6]. Because of magnitude of this problem and lack of reasonable solution to this problem; a rapid, cost-effective, and ecologically reasonable method of cleanup has been greatly demanded [7]. Bioremediation is an efficient and economical approach which has been successful insitu [7], [8]. Microbes transform the applied xenobiotics by utilizing them as energy sources [9]. Otherwise, microbes have been developing new metabolic strategies and adapt to the changing environment through various processes like mutation, induction or by selective enrichment technique [9].

Methomyl is one of such compound which is persistent in the environment and has been known to be having carcinogenic and/or mutagenic effect. Thereby the present study was aimed towards isolation of bacterial cultures involved in Methomyl biodegradation. Methomyl, C5H10O2N2S (IUPAC name, S-methyl-N-[(methylcarbamoyl)oxy]thioacetimidate) is a broad spectrum insecticide introduced in 1966 [10], [11], [12] and is an insecticide of oxime carbamate group [12], [13] and

has been classified as a pesticide of Category-I toxicity [11], [13] and has been also considered as a restricted-use pesticide (RUP) as per the EPA [11]. It acts by inhibiting the acetylcholine esterase, an essential enzyme necessary for the functioning of nervous system, in both insects and mammals [14], [15]. WHO (World Health Organization), EPA (Environmental Protection Agency, USA) and EC (European Commission) have categorized this pesticide as a very toxic and hazardous material [14]. It is highly soluble in water (57.9 g dm-3 at 25 °C) and have low sorption affinity to soil it has potential to contaminate the ground and surface water in agricultural areas [14], [16]. Moreover Methomyl has been moderately to highly toxic to fish and highly toxic to invertebrates [16], [17], [18].

Microbial metabolism is the main mechanism responsible for the degradation of Methomyl in natural soils although it can also be degraded through chemical and physical processes [16]. Therefore, it is very much essential to develop such bioremediation technologies using bacteria capable of degrading Methomyl to reduce the soil and ground water contamination. The present investigation was undertaken for the isolation, screening and identification of bacterial strain which has an ability to degrade Methomyl up to 1000 ppm in Bushnell and Haas medium (BH medium). The characterization of metabolites formed during degradation was analyzed by GC-MS.

## 2 Materials and Methods

### 2.1 Isolation and screening of Methomyl degrading bacteria

The soil samples were collected from the rhizosphere of soybean field of Shahada Tehsil. With aim to isolate Methomyl degrading bacteria rhizospheric soil samples were serial diluted and suspended in BH (Bushnell and Haas medium) seeded with Methomyl (100 ppm). The flasks were incubated in an orbital shaker at $30^0$C at 150 rpm for seven days. In order to isolate a single species of Methomyl degrading bacterium, enrichment with BH medium was repeated several times. The screened isolates were maintained on nutrient agar slants at $4^0$C and labeled as MPP-A, MPP-B and MPP-C. To confirm the utilization and degradation ability of isolates, individual isolates were screened by re-suspending in BH medium containing Methomyl (100 ppm) for further fourteen days.

### 2.2 Identification of Methomyl degrading isolates

#### 2.2.1 Biochemical characterization

On screening of suspected Methomyl degrades, the isolates were further characterized and identified on the basis of physiological and biochemical characteristics. Physiological characteristics were studied using conventional methods like morphology and staining. In addition to this, biochemical characteristics were carried out as per Bergey's Manual of Systematic Bacteriology using pre-sterilized biochemical kits (Hi-Media, Mumbai, India). The ability of isolates to oxidize different carbon sources and enzyme production was investigated.

#### 2.2.2 Phylogenetic Identification

The phylogenetic identification was provided by National Center for Cell Science (NCCS), Pune carried out by 16S rRNA gene sequencing (Sambrook 1989). Universal primers were used for amplification and sequencing. The sequences were analyzed with gapped BLAST (http://www.ncbi.nlm.nih.gov) search algorithm. The 16S rRNA profile of this partially identified culture was compared with the available database of known strains for the classification of isolates at the species and higher levels. Phylogenetic analysis was performed by evolutionary distance and maximum likelihood with 1,000 bootstrap replicates using MEGA5 software. The evolutionary distances were computed using the neighbor-joining method and Kimura 2-parameter model. The 16S rRNA gene sequence of isolate MPP-A was submitted to a gene bank under accession no. JN873342.

#### 2.2.3 Whole cell fatty acid methyl ester (FAME) analysis

FAME analysis was carried out by using Chromatography [GC] with the Sherlock Microbial Identification System [MIDI, Inc. Newark, DE, USA]. The fatty acids from bacterial cultures grown in triplicate on trypticase soya agar for 24 hours at $28^0$C were separated from cell walls by saponification, as derivatives, and extracted with hexane and methyl tertiary butyl

ether. Extracts were analyzed on a GC 689 [Agilent Technologies, USA] fitted with Ultra 2 phenyl methyl silicone fused silica capillary column 25× 0.2 mm [Agilent Technologies, USA], using hydrogen as the carrier gas, nitrogen as the "make up" gas and air to support the flame. GC oven was programmed from 170 to 270$^0$C at 50$^0$C rise per minute with a 2-minutes hold at 300$^0$C. Fatty acids were identified and quantified by comparison to retention time and peak area obtained and compared with the fatty acid standards. Qualitative and quantitative differences in fatty acid profiles were used to compute the distance for each strain relative to other strains in the Sherlock bacterial fatty acid ITSA1 aerobe reference library [20].

### 2.3 Biodegradation of Methomyl

#### 2.3.1 Inoculum

The isolate MPP-A was initially grown in nutrient broth at 30$^0$C for 24 hrs on a rotary shaker with 150 rpm so as to reach at its exponential phase of growth. After centrifugation biomass was washed and transferred into in BH medium containing dipotassium phosphate 1.00, monopotassium phosphate 1.00, calcium chloride 0.02, ammonium nitrate 1.00, magnesium sulphate 0.20, ferric chloride 0.05 (gL$^{-1}$) up to the optical density 1.0 at 620 nm.

#### 2.3.2 Spectrophotometric analysis of Methomyl biodegradation

One percent inoculum was added to freshly prepared BH medium seeded from 200 to 1000 ppm L $^{-1}$ of Methomyl on a rotary shaker at 30$^0$C at 150 rpm throughout the experiment. The samples were harvested after every twenty four hours at regular intervals for growth and biodegradation studies. The samples were centrifuged at 12000 *g* for 15 minutes to remove the cells from the broth. Methomyl present in the supernatant was extracted by adding an equal volume of chloroform to it and the aqueous phase was dried with addition of enough anhydrous sodium sulphate and was analyzed at 245 nm using UV-visible spectrophotometer (Shimadzu, UVMINI- 1240) [21]. The growth was monitored regularly at 620 nm and the biodegradation was monitored on the basis of loss of Methomyl content in the medium with reference to the initial content provided.

#### 2.3.3 Gas chromatography-Mass spectroscopy

The process of Methomyl biodegradation is a continuous and time being process. The biodegradation was continuously monitored by using coupled gas chromatography/mass spectrophotometry [GC-MS], performed using Perkin Elmer gas chromatograph model Autosystem XL with turbomass GC+ equipped with an SGE forte GC capillary column BP20 [30 m X 0.250 µ internal diameter X 0.25 µ] [SGE analytical, Australia].

Samples for GC-MS analysis were prepared as per Xu Jing et al [21]. The broth samples were withdrawn regularly up to four weeks, centrifuged at 12000 *g* for 15 minutes. The supernatants were mixed with equal volume of acetone properly for 2 minutes, followed by addition of enough sodium chloride to saturate an aqueous phase. This mixture was warmed in hot water bath to take acetone out in which Methomyl was extracted. 1 µl of Methomyl extract was injected by split injection with a split ratio of 10:1 using 10 µl syringe [SGE analytical, Australia].

The column oven temperature was programmed from 120$^0$C for 2 minutes, increased at a rate of 20$^0$C per minute up to 230$^0$C, and was further held at this temperature for 10 minutes. The temperature of injector and detector was 225$^0$C and 230$^0$C respectively. Helium was used as carrier gas at a flow rate of 1 ml.min$^{-1}$. The mass spectra obtained were compared with the National Institute of Standards and Technology [NIST] mass spectra library.

## 3 Results and discussion

### 3.1 Isolation and screening of Methomyl degrading bacterial strain

Methomyl is an insecticide which was generally employed to control ticks and spiders of vegetable, fruits and field crops, cotton, soybean and commercial ornamental plants [11]. Its use for soybean crops was prevalent in this area. Therefore the soil samples were collected from rhizospheric soil of soybean field which reported previous history of use of Methomyl

application. Three bacterial strains were isolated by enrichment culture technique from soil samples. They were subjected for further screening and of these three isolates, MPP-A (figure 1) was found to be a Methomyl biodegrading isolate. It was used for further investigations and biodegradation studies.

### 3.2 Identification of Methomyl degrading isolate

#### 3.2.1 Morphological, Biochemical and cultural identification

On the basis of morphological, cultural and biochemical identification the strain MPP-A was identified as *Rhodococcus kroppenstedii* (Table 1).

#### 3.2.2 Phylogenetic identification

The partial sequence analysis of the amplified 16S rRNA gene exhibited highest degree of similarity (99%) to the genus *Rhodococcus* and was confirmed as a *Rhodococcus kroppenstedii*. The same isolates were submitted to genebank with name *Rhodococcus kroppenstedii* strain AHJ 3. The evolutionary history was inferred using the Neighbor-Joining method [22]. Optimal tree with the sum of branch length = 0.05125056 is shown. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [23]. Evolutionary distances were computed using the Kimura 2-parameter method using MEGA5 [24], which are in the units of the number of base substitutions per site. This analysis involved six nucleotide sequences. Here all positions containing gaps and missing data were eliminated. There were a total of 1301 positions in the final dataset. Phylogenetic tree based on all known related type of species of *Rhodococcus kroppenstedii* strain AHJ 3 are depicted in figure 2.

#### 3.2.3 Whole cell fatty acid methyl ester (FAME) analysis

Pesticide biodegrading strains differed in their whole-cell fatty acids composition and nature of fatty acid as well. MPP-A showed presence of 10:0, 11:0, 12:0, 11:03OH, 13:0 anteiso, 13:1, 13:0,14:0, 15:0 anteiso, 15:1 w8c, 15:1 w5c, 15:0, 16:0, 16:1 w9c, 17:0 anteiso, 17:1 w8c, 17:1 w6c, 17:0, 17:0 10 methyl, 18:1 w9c, 18:0, 18:0 10 methyl TBSA, 19:0 iso, 20:1 w7c, 20:0, Summ Feature 3, Summ Feature 6 and Summ Feature 9 which are the characteristic fatty acids of the genus *Rhodococcus-erythropolis/R.globerulus/N.globerula*. The fatty acid profile of isolate MPP-A has been illustrated in table 2.

### 3.3 Methomyl biodegradation

The biodegradation of Methomyl by *Rhodococcus kroppenstetii* strain AHJ 3 lasted for 4weeks. Growth pattern of *Rhodococcus kroppenstetii* strain AHJ 3 under different concentrations of Methomyl showed that, at minimum concentration (200 ppm) growth was slow as compared to highest concentration (1000 ppm). This indicated that as the concentration of pesticide increased the growth rate also increased. It can be reasoned that the ample substrate was available for the used inoculum level for remediation.

#### 3.3.1 Spectrophotometric analysis of degradation

The spectrophotometric analysis (245 nm) of Methomyl degradation revealed that degradation commenced within 24 hours and the concentration of pesticide subsided up to 48 hours but as the bacterial cell entered the exponential phase, the cell growth enhanced and the concentration of Methomyl also increased due to biomagnification of Methomyl in the bacterial cells. Thereafter at the end of logarithmic phase and in the initiation of stationary phase Methomyl concentration was reduced. The above discussion proved that the increase in the bacterial cell growth facilitated the enhancement of Methomyl biodegradation. The analysis pattern revealed the fact that degradation level was high in 200 ppm (96.22%) as compared to 1000 ppm (92.36%) concentration of Methomyl (Table 3).

#### 3.3.2 Degradation pattern and metabolite formation of Methomyl

The metabolite formation and the degradation analysis were carried out by GC-MS. The peak at m/z 162 disappeared, was inferred that Methomyl was degraded and other peaks were obtainable within thirty fourth days as depicted in figure 3 (a, b & c). Within fifteen days about ninety two percent loss in Methomyl provided was observed. The fragmentation pattern of Methomyl biodegradation and its degradation prod-

uct has been shown schematically in figure 4 (a & b) respectively.

The mass of Methomyl m/z 162 is shown in figure 3(a) as identified by mass spectra. In the figure 3(b) mass spectrum new peaks were obtained at m/z 105 which is a base peak having intensity100% (fragment I). Another fragment having major intensity was m/z 88 (fragment II) and m/z 58 (fragment III) is shown in figure 3(a). In case of another mass spectrum as in figure3(c) the peak at m/z 58 was base peak having hundred percent intensity (fragment I) and another peaks having major intensity of m/z 88 and m/z 47. The net reaction for Methomyl biodegradation was as assumed and shown in figure 5.

## 4 Conclusions

The main objective of the present investigation was to explore phylogenetic and FAME identification of potential Methomyl degrading isolate *Rhodococcus kroppenstedtii* AHJ 3. Methomyl has been reported to be persistent in soybean fields and thereby an isolate from such soils could prove beneficial to develop pesticide degradation process. In the present investigation the isolate was identified on the basis of cultural and biochemical characteristics as well as phylogenetic and FAME analysis. The biodegradation potential of the isolate was monitored by routine spectrophotometric analyses as well as the advanced technique involving the use of GC-MS. The analytical studies on the degradation products clearly revealed the fact that the fragmentation products were obtainable after the remediation of Methomyl and there was a considerable decrease in the quantity of Methomyl within a time period of four weeks. A further investigation on these lines can help to design a protocol for bioremediation of Methomyl which can assist in further studies co-related to bioremediation of other pesticides. This eco-friendly approach shall prove to be a contributory attempt towards environmental protection and preservation of natural fertility of soil.

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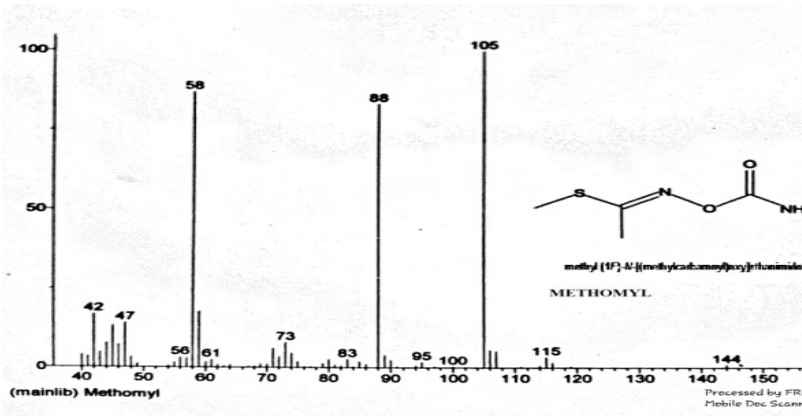


**Fig. 3a** Mass spectra of Methomyl

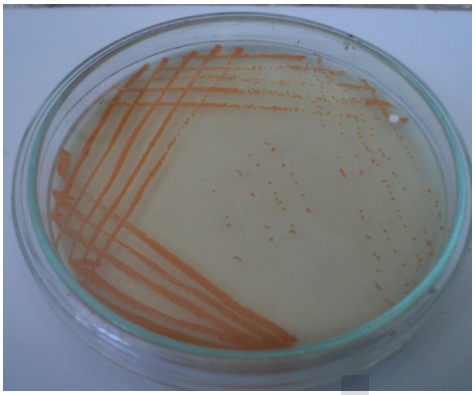

**Fig. 1** Photo plate of *Rhodococcus kroppenstedtii AHJ3*

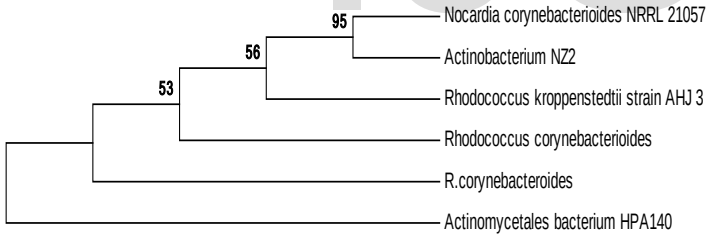


**Fig. 2** Phylogenetic analysis of MPP-1 and related species by the neighbor-joining method.

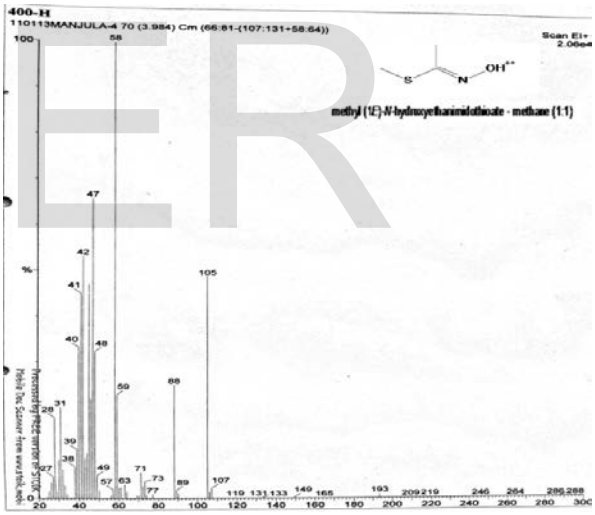


**Fig. 3b** Mass spectra of degradation product of Methomyl

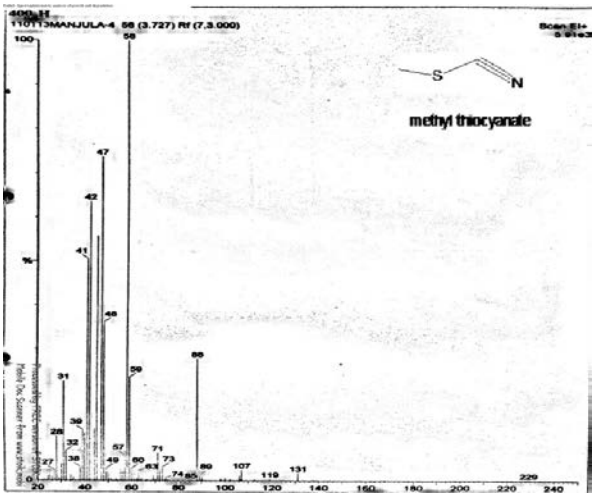


**Fig. 3c** Mass spectra of degradation product of Methomyl

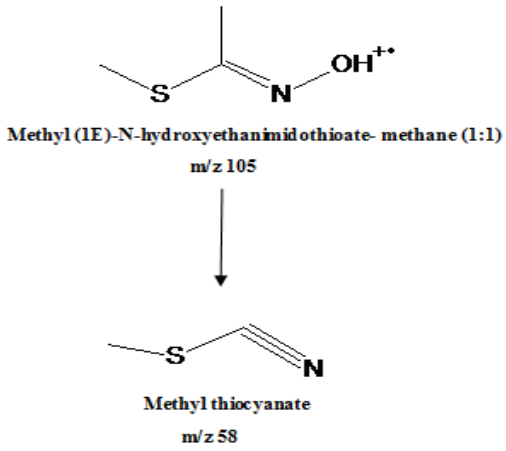


**Fig. 4b** Suggested fragmentation pattern of degradation product of Methomyl as per mass spectra obtained

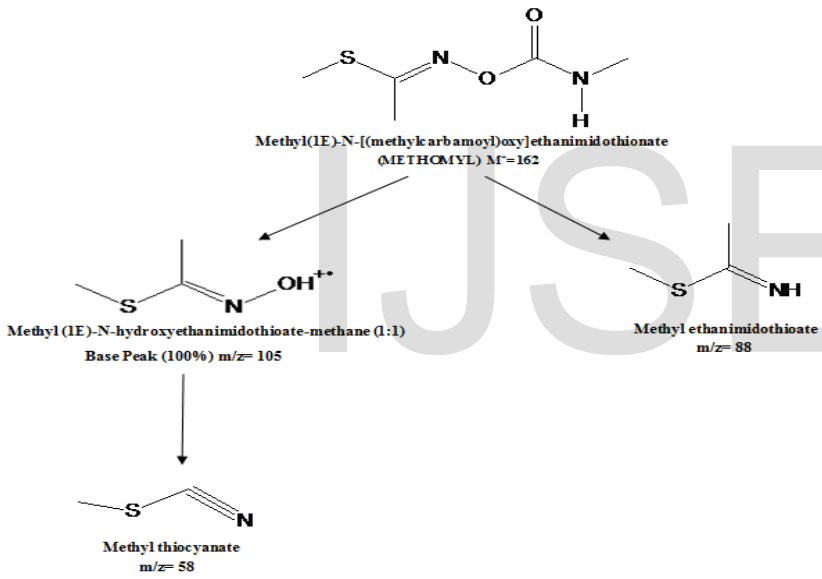


**Fig. 4a** Suggested fragmentation pattern of Methomyl biodegradation as per mass spectra obtained

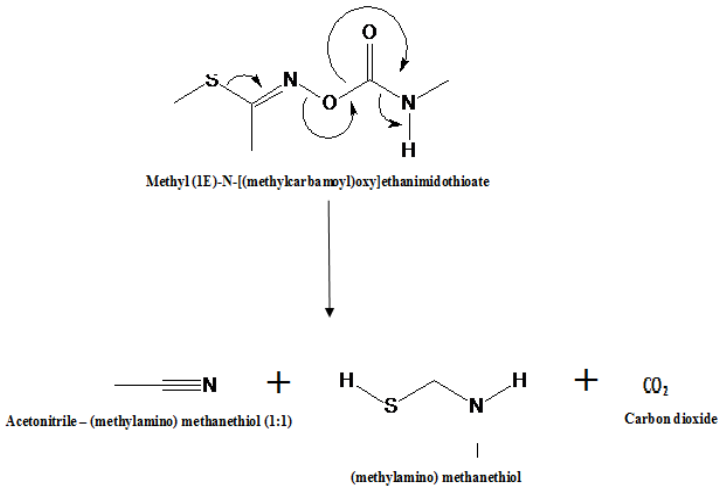


**Fig. 5** Net reaction for Methomyl biodegradation

**Table 1** Identification of bacteria on the basis of morphological and biochemical characteristics

| **Morphological characteristics** | |
|---|---|
| **Test** | **Culture** |
| **Colony Morphology** | |
| Size | Pin pointed |
| Shape | Circular |
| Colour | Orange |
| Margin | Entire |
| Elevation | Convex |
| Opacity | Opaque |
| Consistency | Butterous |
| Gram nature | Gram positive cocco bacil-li |
| Motility | Motile |
| **Biochemical Chatacteristics for IMViC** | |
| **Test** | **Result** |
| Indole | **-** |
| Methyl Red | **-** |
| Vogus Proskaur | **-** |
| Citrate utilization | **+** |
| **Biochemical Characteristics for some miscellaneous com-pounds** | |
| **Test** | **Result** |
| Mallonate | **-** |
| Esculin | **-** |
| ONPG | **+** |
| Lysine decarboxylase | **-** |
| Ornithine decarboxylase | **+** |
| Phenylalanine deamination | **+** |
| Nitrate reduction | **-** |
| **Biochemical Characteristics for some enzymes** | |
| **Enzyme** | **Test** |
| Oxidase | - |
| Amylase | - |
| Cellulose | + |

| | |
|---|---|
| Casein | - |
| Catalase | + |
| Urease | - |
| $H_2S$ | - |
| **TSI** | |
| Slant | Yellow |
| Butt | Pink |
| **Biochemical Characteristics for Different sugar sources** | |
| **Carbohydrates** | **Result** |
| Glucose | - |
| Adonitol | - |
| Lactose | + |
| Arabinose | - |
| Sorbitol | - |
| Xylose | + |
| Rhamnose | - |
| Cellobiose | - |
| Melibiose | + |
| Saccharose | - |
| Raffinose | + |
| Trehalose | - |
| Maltose | + |
| Fructose | + |
| Dextrose | - |
| Galactose | + |
| Sucrose | + |
| L-arabinose | - |
| Mannose | - |
| Inulin | - |
| Na-gluconate | - |
| Glycerol | - |
| Salicin | - |
| Glucosamine | - |
| Dulicitol | - |
| Inocitol | + |
| Mannitol | - |
| Ribose | - |
| Melezitose | - |
| $\alpha$-methyl-D-mannose | - |
| $\alpha$-methyl-D-glucoside | - |
| Xylitol | - |
| Sorbose | - |



**Table 2** Identification of bacteria on the basis of their Whole Cell Fatty Acid Methyl Ester profile

| | **AHJ 3** | | **AHJ 3** |
|---|---|---|---|
| 10:0 | 0.22 | Summ Feature 3 | 20.92 |
| 11:0 | 0.26 | Summ Feature 6 | 0.41 |
| 12:0 | 2.22 | Summ Feature 9 | 0.70 |
| 11:03OH | 0.17 | | |
| 13:0 anteiso | 0.40 | | |
| 13:1 | 0.12 | | |
| 13:0 | 0.56 | | |
| 14:0 | 6.98 | | |

15:0 anteiso 0.10
15:1 w8c 0.40
15:1 w5c 0.30
16:0 27.39

| No. of Days | 200 ppm | | 400 ppm | | 600 ppm | | 800 ppm | | 1000 ppm | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ABS 600nm | ABS 245nm | ABS 600nm | ABS 245nm | ABS 600nm | ABS 245nm | ABS 600nm | ABS 245nm | ABS 600nm | ABS 245nm |
| 1 | 0.120 | 3.122 | 0.179 | 3.186 | 0.233 | 3.222 | 0.258 | 3.319 | 0.624 | 3.407 |
| 2 | 0.674 | 2.941 | 1.310 | 3.087 | 1.655 | 3.087 | 1.772 | 3.164 | 2.020 | 3.251 |
| 3 | 0.760 | 0.201 | 1.330 | 0.211 | 1.630 | 0.606 | 1.816 | 0.758 | 2.125 | 1.021 |
| 4 | 1.446 | 0.400 | 1.370 | 0.150 | 1.640 | 0.518 | 1.880 | 0.749 | 2.230 | 1.046 |
| 6 | 1.662 | 0.405 | 2.313 | 0.299 | 2.646 | 0.649 | 3.091 | 1.004 | 3.336 | 2.645 |
| 11 | 1.550 | 0.262 | 1.893 | 0.367 | 2.447 | 0.379 | 2.792 | 0.656 | 3.136 | 1.695 |
| 15 | 0.800 | 0.182 | 1.619 | 0.247 | 1.846 | 0.355 | 2.339 | 0.495 | 2.731 | 0.522 |
| 24 | 0.358 | 0.149 | 1.362 | 0.175 | 1.862 | 0.241 | 2.305 | 0.480 | 1.430 | 0.374 |
| 30 | 0.017 | 0.118 | 0.077 | 0.156 | 0.259 | 0.166 | 0.442 | 0.419 | 0.538 | 0.260 |

16:1 w9c 0.95
17:0 anteiso 0.12
17:1 w8c 2.59
17:1 w6c 0.18
17:0 1.02
17:0 10 methyl 0.63
18:1 w9c 21.99
18:0 0.27
18:0 10 methyl TBSA 10.02
19:0 iso 0.53
20:1 w7c 0.26
20:0 0.30



**Table 3** Spectrophotometric analysis of growth and methomyl biodegradation

**Figure captions**

**Fig. 1** Growth pattern of *Rhodococcus kroppenstedii* on plate

**Fig. 2** Phylogenetic analysis of MPP-1 and related species by the neighbor-joining method

**Fig. 3a** Mass spectra of methomyl

**Fig. 3b** Mass spectra of degradation product of Methomyl

**Fig. 3c** Mass spectra of degradation product of methomyl

**Fig. 4a** Suggested fragmentation pattern of methomyl biodegradation as per mass spectra obtained

**Fig. 4b** Suggested fragmentation pattern of degradation product of Methomyl as per mass spectra obtained

**Fig. 5** Possible net reaction for methomyl biodegradation

**Table titles**

**Table 1** Identification of bacteria on the basis of morphological and biochemical characteristics

**Table 2** Identification of bacteria on the basis of their Whole Cell Fatty Acid Methyl Ester profile

**Table 3** Spectrophotometric analysis of growth and methomyl biodegradation

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