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Pathway of 1,2,4-Trichlorobenzene Degradation by *Bacillus mucilaginosus* Isolated from Petroleum Polluted Soils

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Abstract: An indigenous bacterial strain MAM-24 was isolated from petroleum polluted soil by selective enrichment with 1,2,4-trichlorobenzene (1,2,4-TCB) as the sole source of carbon and energy. During growth on 1,2,4-TCB, growth by measuring O.D., protein measurement and bacterial count were determined. A stoichiometric amount of chloride ions is released. The degradation percentage of 1,2,4-TCB was quantified by high performance liquid chromatography (HPLC). *Bacillus mucilaginosus* MAM-24 which have been identified by 16 S rRNA in a previous study was the best 1,2,4-TCB degrader. It could degrade 99.3%, 98.0%, 98.6%, 99.3% and 14.4% of 5, 10, 15, 25, 50 µM 1,2,4-TCB respectively. Degradation products of 1,2,4-TCB was identified by gas chromatography mass spectrometry (GC-MS) analysis which revealed that isolate MAM-24 dechlorinated 1,2,4-TCB as a first step. The corner stone of the intermediates was acetophenone.

Keywords:1,2,4-trichlorobenzene, Bacterial isolates, Degradation, Gamma radiation induced mutant, Pathway.

1 Introduction

Soil contamination by petroleum products is widespread problem, with many hotspots of pollution arising from individual spills [1]. Cleanup of these contaminated sites is an important goal and bioremediation is a low input and cheap approach to remove hydrocarbons [2].

Pesticides are typically applied as a mixture or sequentially to soils and plants during crop production [3, 4].

1,2,4-Trichlorobenzene (1,2,4-TCB) is one of the most widely used chlorobenzenes. Due to its use as dye carrier, pesticides intermediate, heat-transfer medium, dielectric fluid in transformers, degreaser, lubricant and solvent in chemical manufacturing it has become a ubiquitous environmental pollutant [5]. The toxicity of halogenated benzoic acids was found to be directly related to the compound's hydrophobicity [6].

Many groups worked on the enrichment and isolation of bacterial strains from the polluted environment with the hope that natural selection had done some of the pathway assembly [7].

Bacteria that are able to use chloroaromatic compounds as sole carbon and energy source have been isolated from polluted environments and are described as members of the genera *Alcaligenes*, *Pseudomonas*, *Burkholderia*, *Xanthobacter* and the species *Acidovoraxavenae* and *Rhodococcus phenolicus* [8-19].

So, the aim of this study was to investigate the ability of five bacterial strains isolated from petroleum polluted soils to degrade the chloroaromatic compound 1,2,4-Trichlorobenzene (1,2,4-TCB) since this compoundconstitute a main part of petroleum oil components.

2 Materials and Methods

2.1 Bacterial strains

Five bacterial isolates (MAM-24, -27, -29, -33 and -3) were isolated from deposits of petroleum oil on soils which are either chronic or recent from Cairo Oil Refining Company, Al-Qalyubia, Egypt. Bacterial isolate MAM-24 isolated from agricultural region around the company and having a history of pesticide exposure.

Enterobacter cloaceae MAM-4 was isolated and identified from waste-water contaminated by heavy metals and provided by Dr. Abo-State.

2.2 Culture medium

The composition of basal salt medium (BSM) was (g/L): $(NH_4)_2SO_4$ 1.1, K_2HPO_4 2.2, KH_2PO_4 0.9, $MgSO_4.7H_2O$ 0.1, $MnSO_4.6H_2O$ 0.025, $FeSO_4.7H_2O$ 0.005, L-ascorbic acid 0.005, deionized water 1000 ml. For use, the following supplements were added to 1 liter of the cooled basal medium: 1 ml of trace elements and 0.1 ml of vitamin

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solution. Trace element (mg/L): H_3BO_3 0.3, $CoSO_4$ 0.4, $ZnSO_4.7H_2O$ 0.1, $MnCl_2.4H_2O$ 0.03, $NaMoO_4.2H_2O$ 0.03, $NiSO_4.6H_2O$0.02, $CuSO_4.5H_2O$ 0.01, HCl 50 ml, deionized water 950 ml. Vitamin solution (mg/L): Biotine 2.0, Folic acid 2.0,Pyridoxal hydrochloride 10.0, Riboflavine 5.0, Thiamine 5.0, Nicotonic acid 5.0, Ca-Panthothenate 5.0, Cyanocobalamine 5.0, P-aminobenzoic acid 5.0, Deionized water 1000 ml [20].

The medium was sterilized by autoclaving at 121°C for 20 min. for use 1 ml of trace element plus 0.1 ml vitamin solution was added to every 1 liter of sterilized cooled basal medium. The Luria broth medium (L.B.) composed of the following (g/L): Tryptone 10.0, Yeast extract 5.0, NaCl 5.0, distilled water 1000 ml. The pH was adjusted to 7.1 $\pm$ 0.2 before sterilization [21].

2.3*Preparation of bacterial inoculum for biodegradation studies*

The five most potent isolates and *E. cloaceae* MAM-4 were grown on L.B broth medium for 48 hours in shaking incubator (150 rpm) at 37°C. The well grown cultures were centrifuged at 8000 rpm for 10 minutes. The pellets were washed twice by sterilized BSM. Washed pellets were suspended in BSM and used for inoculation.

2.4 Biodegradation of chloroaromatic compounds

Fifteen ml of each of the six selected isolated bacterial strains was used to inoculate 150ml BSM, which was free from any chloride ions. The BSM was amended by five concentrations (5, 10, 15, 25 and 50µM) of 1,2,4-trichlorobenzene (1,2,4-TCB) product of Aldrich, Germany. Three replicates were used for each treatment. Inoculated BSM were incubated in the dark on shaker (150 rpm) at 30°C for 0, 1, 2, 3, 4, 5, 6, 7, 15 and 21 days [22]. Growth was determined by measuring optical density (O.D.) at 600 nm using spectrophotometer (LW-V-200-RS UV/VIS, Germany). Extracellular protein was determined according to Lowry *et al.* [23] at 720 nm spectrophotometrically. Chlroide ion was determined according to Bergmann and Sanik [24] by spectrophotometer at 480 nm.

2.5 Analytical methods

(i) High Performance Liquid Chromatography (HPLC):

The quantitative determination of various chloroaromatic compounds was performed using High-Performance Chromatography (HPLC) in Egyptian Petroleum Research Institute- Cairo-Egypt.

The various chloroaromatic compounds were quantified by (HPLC pump No. 2360, gradient programmer No. 2360 and detector No. UA-5 with a 280nm fitter [Isco, Inc.]; integrator No. SP4600 [Spectra-Physics]; and HPLC auto sampler No. 738 [Alcott Chromatography] with the 150 mm reversed phase column hypersil ODS-C18, 5µm, [Altech; No. 9876]). The mobile phase consisted of methanol, water and 0.5% acetic. The methanol/water ratio varied from 70:30 to 5:95 [25].

(ii) Gas Chromatographic/Mass Spectrometry (GC/MS):

The qualitative and quantitative determination of various chloroaromatic compounds was performed using Gas Chromatographic/ Mass Spectrometry (GC/MS) in Central Water Quality Laboratory- Holding Company for Water and Waste-Water, Cairo, Egypt.

The GC is a 3800 Varian USA, EI-ITS 1200L Varian USA (Electron Impact Ion Source, Quadrupole MS, and EMD Detector). The capillary column was a VF-5-MS capillary (30m × 0.25mm i.d., 0.25µm film thickness). Helium 5.0 was used as carrier gas for the system (75Psi, 1ml min^{-1}). The chromatographic temperature programme for GC-MS was: start (t = 0) at 60^0C followed by a 10^0C min^{-1} increase to 160^0C and 4-250^0C maintaining this final temperature for 10 min. Temperature of the injector was set to 250^0C, transfer line: 270^0C. The injection volume was 1µl in the splitless mode.

A measured volume of sample, approximately 1liter, is serially extracted with methylene dichloride at a pH greater than 11 and again at a pH less than 2 using a separatory funnel or a continuous extractor. The methylene dichloride extract is dried, concentrated to a volume of 1ml, and analyzed by GC/MS. Qualitative identification of the parameters in the extract is performed using the retention time and the relative abundance of three characteristic masses (m/z). Quantitative analysis is performed using internal standard techniques with a single characteristic m/z [26].

3 Results and Discussion

Growth of isolate MAM-24 on 5µM of 1,2,4-TCB as Fig. (1) showed an increase till the third day and began to decreased. As the concentration became 10 or 15µM the growth increased till the fourth day. However, at 25 and 50µM the growth was increased till the fifth day then began to decrease.

Extracellular protein was increased as the incubation period increased at the first three concentrations (5, 10 and 15µM) of 1,2,4-TCB till the fourth day then began to decrease. At 25µM, the secreted protein continued to increase till the fifth day. As the concentration increased more (50µM), the secreted protein continued increasing till the sixth day as Fig. (2). From the previous results, it was clear that as the concentrations of 1,2,4-TCB increased, there were an increase in time for extracellular protein secreated by the cells. This may be explained on the bases that higher concentrations of 1,2,4-TCB need more enzyme to be secreted to degrade the compound and this increasing in enzymatic activity needs more time to face the increasing demand (stress).

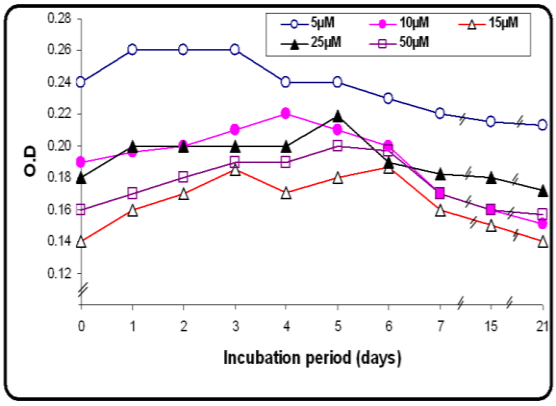

Figure (1): Growth of isolate MAM-24 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

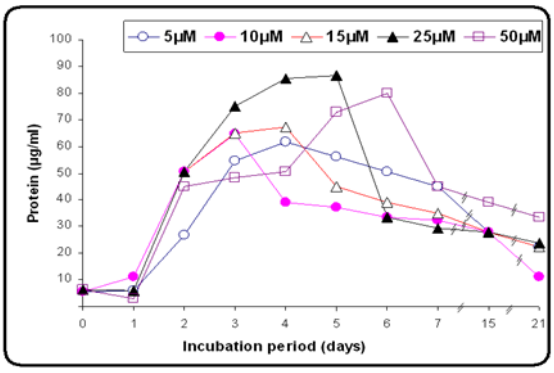

Figure (2): Concentration of extracellular protein of isolate MAM-24 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Concentration of Cl^- was increased till the first and second day for the first four concentrations of 1,2,4-TCB as in Fig. (3).

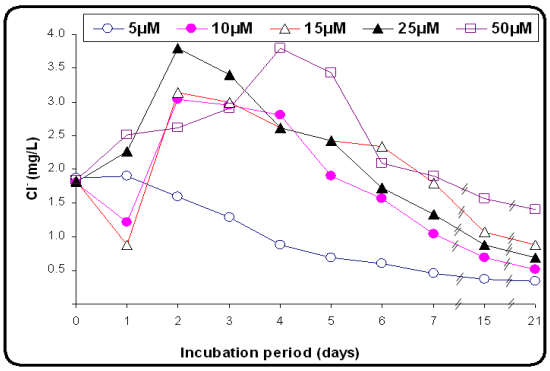

Figure (3): Concentration of Cl^- of isolate MAM-24 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

At the higher concentration (50µM), Cl^- concentrations continued increasing till the fourth day and began to decrease as the incubation period increased.

Growth of isolate MAM-27 showed an increasing till the third day of incubation on the five concentrations of 1,2,4-TCB as in Fig. (4). Then growth began to decrease. Extracellular protein of this isolate increased as the incubation period increased till the fifth day on the five concentrations as in Fig. (5).

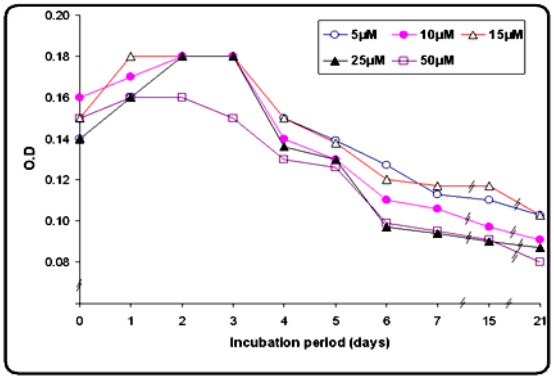

Figure (4): Growth of isolate MAM-27 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

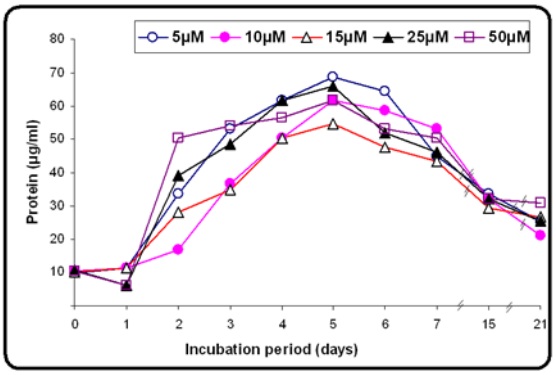

Figure (5): Concentration of extracellular protein of isolate MAM-27 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Concentrations of Cl^- increased as the incubation period increased till the third day and then began to decrease in case of the first four concentrations (5, 10, 15 and 25µM). At higher concentration (50µM), Cl^- concentration continued to increase till the sixth day as in Fig. (6). The results revealed that, as the concentration of 1,2,4-TCB increased, Cl^- concentration increased.

Trend of growth of isolate MAM-29 showed an increase for the first day, and then began to decrease as indicated in Fig. (7) for all concentrations. However, its extracellular protein for the five concentration of 1,2,4-TCB showed an increase till the fifth day of incubation and then began to decrease as more incubation had been recorded as in Fig. (8).

In case of Cl^- concentration, isolate MAM-29 continued to increase Cl^- till the third day at the lower concentrations (5, 10 and 15µM) as shown in Fig. (9). As the concentrations of 1,2,4-TCB increased (25 and 50µM), the increase in Cl^- concentration increased till the fifth and sixth days respectively.

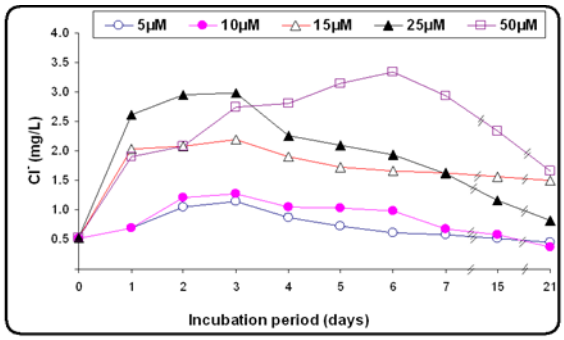

Figure (6): Concentration of Cl^- of isolate MAM-27 on different concentrations of 1,2,4-trichloro-benzene (1,2,4-TCB).

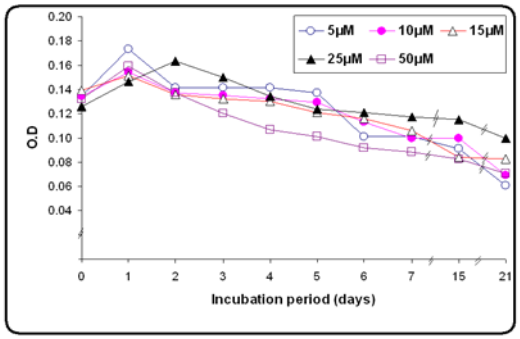

Figure (7): Growth of isolate MAM-29 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

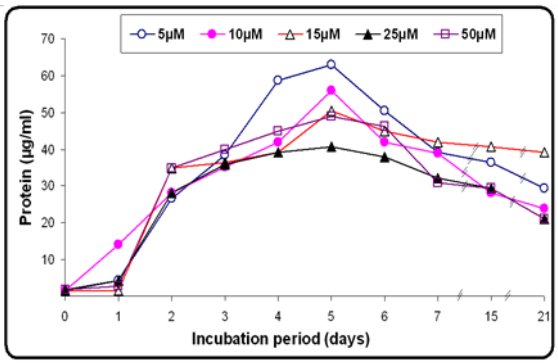

Figure (8): Concentration of extracellular protein of isolate MAM-29 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Trend of growth of isolate MAM-33 showed an increase as the incubation period increased till the third day in all the five concentrations as in Fig. (10). Then the growth began to decrease. Extracellular protein secreted by MAM-33 increased as the incubation period increased till the fifth day, and then began to decrease as shown in Fig. (11) for the five concentration of 1,2,4-TCB used. Concentration of Cl^- of isolate MAM-33 increased till the fourth day at 5 and 10µM of 1,2,4-TCB. As the concentrations of 1,2,4-TCB increased (15,25 and 50µM), Cl^- concentration continued to increase till the sixth, seventh and fifteen days respectively as in Fig. (12).

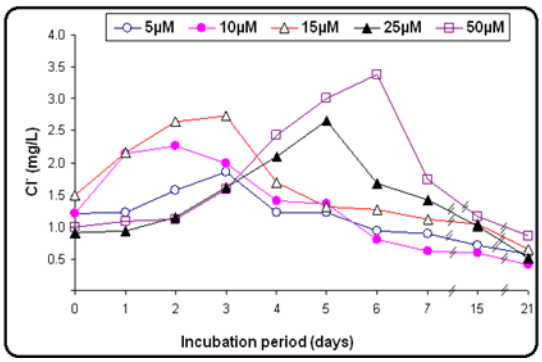

Figure (9): Concentration of Cl^- of isolate MAM-29 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

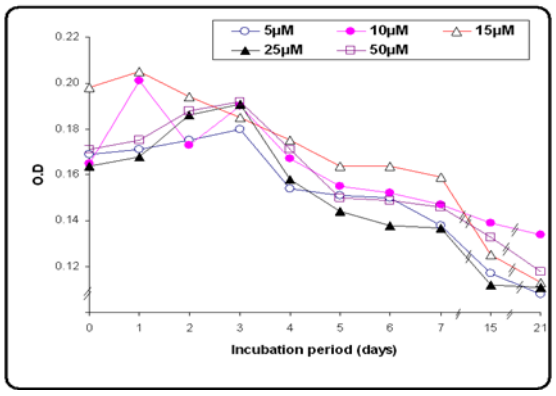

Figure (10): Growth of isolate MAM-33 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

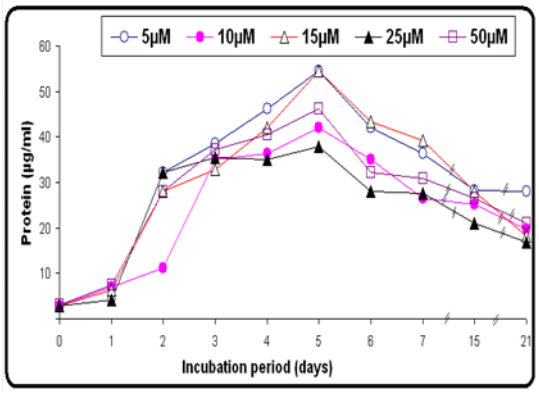

Figure (11): Concentration of extracellular protein of isolate MAM-33 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Trend of growth of the Gram-negative isolate MAM-3 showed an increase till the second day for the first three concentrations (5,10 and 15µM) of 1,2,4-TCB. The increase in growth was recorded for the first day and then began to decrease as the incubation increased at the higher concentrations (25 and 50µM) as indicated in Fig. (13). Extracellular protein secreted by isolate MAM-3 showed an increase in protein as the incubation period increased till the sixth day as in Fig. (14) for the five concentrations of 1,2,4-

TCB.

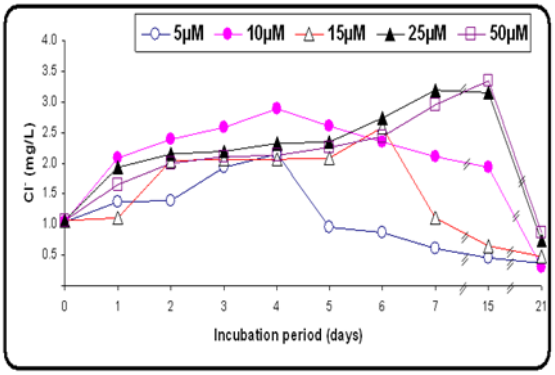

Figure (12): Concentration of Cl^- of isolate MAM-33 on different concentrations of 1,2,4-trichloro-benzene (1,2,4-TCB).

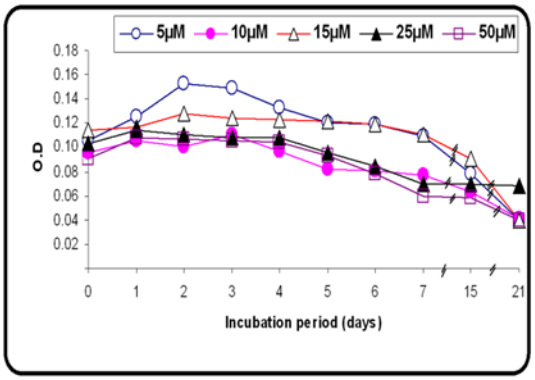

Figure (13): Growth of isolate MAM-3 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

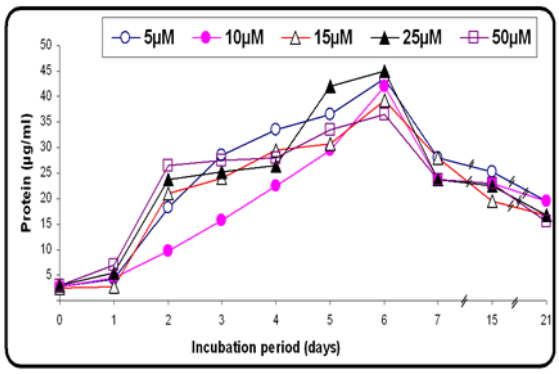

Figure (14): Concentration of extracellular protein of isolate MAM-3 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Concentration of Cl^- increased till the second day at 5µM of 1,2,4-TCB, till the fourth day for 10 and 15µM, till sixth day for 25µM and till seventh day for 50µM. This means, as the concentration of 1,2,4-TCB increased, the dissociation of Cl^- increased as in Fig. (15). And means also, as concentrations of 1,2,4-TCB increased more time was needed for Cl^- releases i.e. more time was needed for degradation of 1,2,4-TCB.

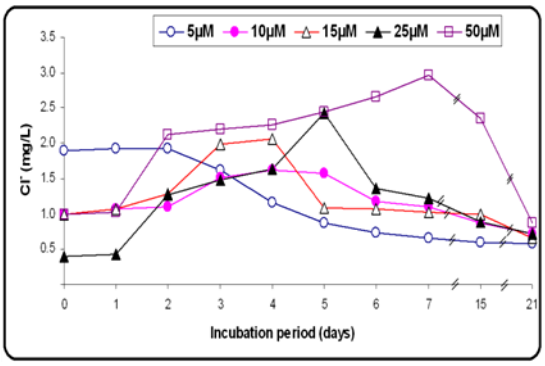

Figure (15): Concentration of Cl^- of isolate MAM-3 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

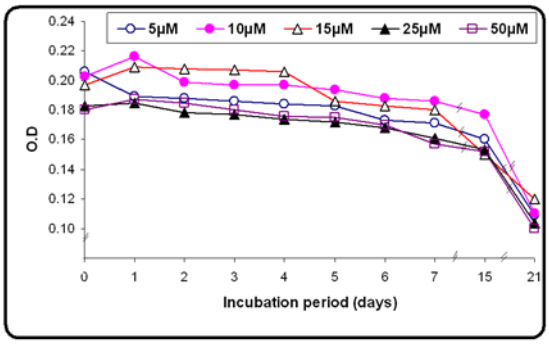

Figure (16): Growth of *E. cloaceae* MAM-4 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

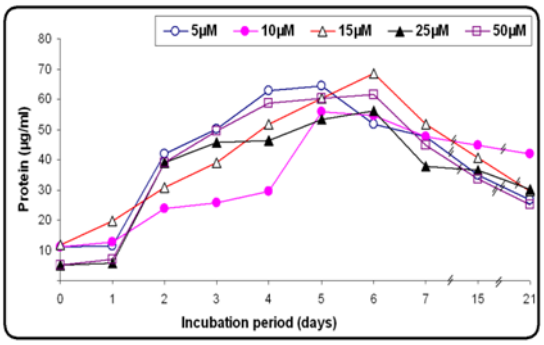

Figure (17): Concentration of extracellular protein of *E. cloaceae* MAM-4 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Growth trend of Gram-negative *E. cloaceae* MAM-4 on 5µM of 1,2,4-TCB as in Fig. (16) showed decrease in growth as incubation period increased. At the higher concentrations (10, 15, 25 and 50µM), growth increased for the first day of incubation and then began to decrease. Extracellular protein of *E. cloaceace* MAM-4 increased as the incubation period increased till the fifth day for (5 and 10µM) of 1,2,4-TCB and till sixth day for the higher concentrations (15, 25 and 50µM) as shown in Fig. (17). This means that *E. cloaceae*need more time to degrade 1,2,4-TCB as the concentration increased by secreting more enzymes (extracellular protein).

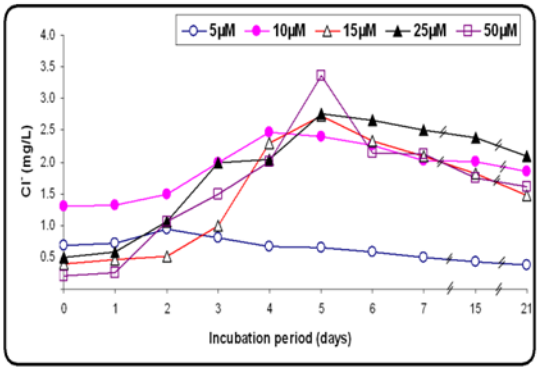

Figure (18): Concentration of Cl^- of *E. cloaceae* MAM-4 on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Concentrations of Cl^- increased as the incubation period increased till the second day for 5µM OF 1,2,4-TCB, till the fourth day for 10µM and till the fifth day for 15, 25 and 50µM as in Fig. (18). The results revealed that, as the concentrations increased, bacterial cells of *E. cloaceae* MAM-4 needs more time to secret more enzymes to stipe off the chlorine and degrade the chlorinated compound. So the results of extracellular protein and Cl^- concentration confirmed each other. The results of previous investigators also confirmed the above results as the following.

Several pure strains were isolated which used chlorobenzenes as sole source of carbon and energy [8-12, 15, 27].

The ability to degrade 1,2,4-TCB has been previously described for *Pseudomonas* sp. P51[7], *Ralstonia sp.* PS12 and *Ralstonia* sp. PS14 [12, 28], *Rodococcus sp.* MS11 [16] and *Bordetella* strains [19], but there are no previous reports on biodegradation of 1,2,4-TCB by *Bacillus*.

Inoculation with *Pseudomonas aeruginosa* strain RHO1, a mono and 1,4-dichlorobenzene-degrading organism, to a titre of $1x10^5$ cells/g soil led to rapid and complete degradation of 0.8mM growth substrate within 30h. In addition, the strain was able to degrade 1,2-dichloro and 1,2,4-trichlorobenzene with stoichometric release of chloride in the presence of acetate, ethanol, monochloro- or 1,4-dichlorobenzene as growth substrates[13].

Aerobic mineralization of mono-, di-, tri- and even tetrachlorobenzenes is reported [5, 28-35].

The reported data show that biodegradation of 1,2,4-trichlorobenzene in natural samples occur in very low rates due to insufficient degradation capacity and slow adaptation of the indigenous microorganisms [5]. The reinoculated strain kept its biodegradation capability: ^{14}C-labeled 1,2,4-TCB applied to this inoculated soil was mineralized to about 40% within one month of incubation. This indicates a possible application of the isolated *Bordetella* sp. for bioremediation of 1,2,4-TCB contaminated sites [19].

Utilization of 1,4-dichlorobenzene, 1,2,3-TCBs and 1,3,5-TCBs by the two strains SA-2 and SA-6, was visually indicated by increased turbidity and slight darking of the growth medium, with increase in population densities. Growth rates for both organisms were quite similar regardless of whether the cells grown on 1,2,3-TCB or 1,3,5-TCB. This may suggest that Cl-substitution patterns have little effect on the ability of this organism to utilize these two trichlorobenzene isomers. Consistent with earlier publications, the carbon for growth may have been derived from cleavage of the catechol intermediate with spontaneous release of organic chloride. With stoichiometric release of chloride, the amount of chloride released during metabolism of chlorobenzenes by strains SA-2 and SA-6 was not determined but the extent of growth suggested either or total mineralization of the chlorobenzenes [36].

The measurement of a specific product of biodegradation of chloroaromatics i.e., chloride is a good indicator for microbial degradation of the chemicals. About 70% of organic bound chlorine was eliminated after 25 days from soil, where $2\text{-}3x10^5$ cells/g soil with each of the strains were added to the slurries [31].

On, 1,4-DCB, *Xanthobacterflavus* 14P1, grew withdoubling time of 8h, which is similar to those reported for other DCB-degrading *Pseudomonas* or *Alcaligenes* species and the stoichiometric amount of two chloride ions per molecule of 1,4-DCB was released. At concentrations higher than 0.1mM 1,4-DCB, bacterial growth was inhibited [14].

The number of dechlorinating bacteria was strictly limited by the amount of TCB supplied in the medium [37].

Count of different bacterial isolates on different concentrations of 1,2,4-TCB had been shown in Table (1). The initial count was ranging from 3.9×10^5 to 9.3×10^7 CFU/ml. Isolate MAM-24 showed good growth on all the five concentrations of 1,2,4-TCB after 7 days and 28 days incubation. Its initial count was 7×10^6 CFU/ml. However, the range of growth was 8.0×10^6 to 2.1×10^8. This means an increase in count ranging from 0.1 to 1.5 log cycles.

Table (1): Count of different indigenous isolates on different concentrations of 1,2,4-trichlorobenzene (1,2,4-TCB).

Isolate code	Initial		After 7 Days										After 21 Days									
	5µM		5µM		10 µM		15µM		25µM		50µM		5µM		10 µM		15µM		25µM		50µM	
	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N	Count (CFU/ml)	Log.N
MAM-24	7×10^6	6.8	26×10^6	7.4	12×10^7	8.0	21×10^7	8.3	12×10^7	8.0	46×10^6	7.7	77×10^6	7.9	57×10^6	7.8	57×10^6	7.8	55×10^6	7.7	8×10^6	6.9
MAM-27	39×10^4	5.6	13×10^7	8.1	18×10^7	8.3	14×10^7	8.1	13×10^7	8.1	9×10^7	8.0	13×10^7	8.1	12×10^7	8.1	2×10^7	7.3	5×10^7	7.7	3×10^7	7.5
MAM-33	37×10^6	7.6	53×10^7	8.7	84×10^7	8.9	29×10^7	8.5	14×10^7	8.1	12×10^7	8.1	13×10^7	8.1	7×10^7	7.8	5×10^7	7.7	9×10^6	7.0	10×10^6	7.0
MAM-3	21×10^6	7.3	12×10^7	8.1	60×10^6	7.8	4×10^7	7.6	96×10^6	8.0	68×10^6	7.8	7×10^7	7.8	6×10^7	7.8	5×10^7	7.7	4×10^7	7.6	2×10^6	7.3
MAM-29	93×10^6	8.0	6×10^7	7.8	11×10^7	8.0	10×10^6	7.0	2×10^6	6.3	72×10^6	7.9	4×10^7	7.6	8×10^7	7.9	13×10^6	7.1	6×10^6	6.8	28×10^6	7.4
E. cloaceae MAM-4	83×10^6	8.0	96×10^6	8.0	12×10^7	8.1	91×10^7	8.0	74×10^6	7.9	69×10^7	8.8	2×10^7	7.3	3×10^7	7.5	3×10^7	7.5	7×10^7	7.8	2×10^7	7.3

Isolate MAM-27 its initial count was 3.9×10^5 CFU/ml and showed an increase in count ranging from 2×10^7 to 1.8×10^8 CFU/ml. This increase in count was ranging from 1.7 to 2.7 log cycles. Isolate MAM-29, its initial count was 9.3×10^7 but their growth was lower or equal that of the initial count in all the five concentrations after 7 and 28 days incubation as shown in Table (1).

Isolate MAM-33 showed good growth on different concentrations of 1,2,4-TCB after 7 days incubation. Its initial count was 3.7×10^7 CFU/ml. Increasing in count after 7 days was ranging from 1.2×10^8 to 8.4×10^8 CFU/ml (i.e. 0.5 to 1.3 log cycles). However, after 28 days incubation at the lower concentration (5µM), there was an increasing in count, but at higher concentration the count was equal or lower than the initial. The Gram-negative bacteria isolate MAM-3 and *E. cloaceae* MAM-4 showed no increasing or lowering in count compared with their initial count after 7 and 28 days.

The chosen bacterial isolate MAM-24 which identified previously as *Bacillus mucilaginosus*HQ013329 was exposed to different doses of gamma radiation. The change in morphological characters of the irradiated MAM-24 was picked up as probable mutant and inoculated in BSM supplemented with different concentration of 3-CBA and the growth was recorded at the initial time (zero) and after 7 days incubation as indicated in Table (2). The chosen mutant No. "9" which exposed to 4.0 kGy of gamma radiation was used for further investigation. This mutant was used with the parent strain MAM-24 to study their abilities to degrade 3-CBA and determined their intermediates.

The ability of different isolates to degrade 1,2,4-TCB had been indicated in Fig. (19). The results showed that isolate MAM-24 was the best 1,2,4-TCB degrader. MAM-24 could degrade 99.3%, 98.0%, 98.6%, 99.3% and 14.4% of 5,10,15,25 and 50µM 1,2,4-TCB respectively. The other isolates could degrade between 1.3% to 28.4% of 1,2,4-TCB along the five different concentration.

Degradation percent of 1,4-DCB, 1,2,3-TCB and 1,3,5-TCB was 80%, 84% and 88% by strain SA-2 while it was 89%, 91% and 91% by strain SA-6 [36].

The isolated strains E3 and F2 of *Bordetella* showed nearly as high mineralization capacities as the enriched mixed culture with 58% and 46% mineralization of 1,2,4-TCB respectively within 30 days incubation. Highest mineralization rate were observed 6 days after incubation [19].

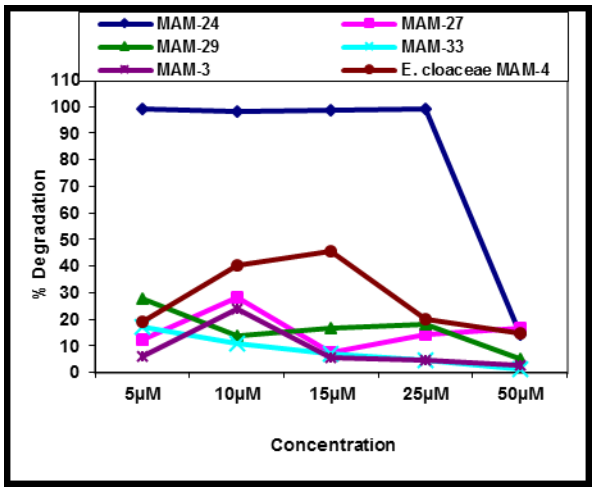

Figure (19): Degradation percentage of 1,2,4-trichlorobenzene (1,2,4-TCB) after 21 days by HPLC.

Biodegradation of 1,2,4-trichlorobenzene (1,2,4-TCB) by *Bacillus mucilaginosus* MAM-24 and its mutant MAM-24 (9) as shown in Table (3) and Fig.(20) revealed that, both the parent strain MAM-24 or the mutant MAM-24 (9) dechlorinated the aromatic compound as a first step and the resulted intermediates undergo either direct cleavage to the benzene ring or oxidation to give acetophenone and the intermediate acetophenone undergo cleavage of the ring to give aliphatics, followed by polymerization process to give a mixture of intermediates with variable polymer length of aliphatic chains.

Several investigators have proposed that bacterial PCBs degradation is initiated by attack of dioxygenase at carbon positions 2,3 (or 5,6). Available evidence suggests that a necessary requirement is the availability of 2,3 (or 5,6) site

Table (2): Growth of the parent strain *(B. mucilaginosus*MAM-24*)* and its mutants exposed to different doses of gamma radiation on different chloroaromatic compounds.

Mutant No.	Dose (KGy)	1,2,4- Trichlorobenzene	
		Initial	After 7 DAYS
Parent strain	0	1.698	1.282
1	1	1.741	1.267
2	1	1.710	1.274
3	1	1.801	1.302
4	1	1.742	1.316
5	2	1.781	1.300
6	2	1.745	1.297
7	2	1.683	1.232
8	2	1.777	1.275
9	4	1.751	1.297
10	4	1.722	1.304
11	4	1.638	1.234
12	6	1.751	1.243
13	6	1.939	1.270
14	6	1.836	1.291
15	6	1.684	1.209
16	8	1.681	1.280
17	8	1.562	1.183
18	8	1.967	1.223
19	10	1.561	1.152
20	10	1.752	1.243
21	10	1.956	1.317
22	10	1.987	1.308
23	15	1.775	1.288
24	15	1.635	1.301
25	15	1.558	1.234

free of chlorines [38-41].

High chlorinated benzene can only be degraded by preliminary reductive dechlorination [5, 42-49].

Repeated subculturing maintaining high dechlorination of 1,2,3- and 1,2,4-TCB supplied in the medium. This indicated that reductive dechlorination of TCB was the primary conservating process [37].

Cl^- released with hexachlorobenzene as electron acceptor; with pentachlorobenzene, the growth yield was 2.9 g/mol Cl^-. Hexachlorobenzene was reductively dechlorinated to pentachlorobenzene, which was converted to a mixture of 1,2,3,5- and 1,2,4,5-tetrachlorobenzene.

Formation of tetrachlorobenzene was not detected. The final end products of dechlorination of hexachloro- and pentchlorobenzene were 1,3,5-trichlorobenzene, 1,3- and 1,4-dichlorobenzene. As reported previous, *Dehalococcoides* sp. strain CBDB1 converted 1,2,3,5-tetrachlorobenzene exclusively to 1,3,5-trichlorobenzene and 1,2,4,5-trichlorobenzene exlcuisvely to 1,2,4-trichlorobenzene.

The organism therefore catalyzes two different pathways to dechlorinate highly chlorinated benzenes. In the route leading to 1,3,5-trichlorobenzene, only double flanked chlorine substituents were removed, while in the route leading to 1,3-, 1,4-dichlorobenzene via 1,2,4-trichlorobenzene single flanked chlorine substituents were also removed [50].

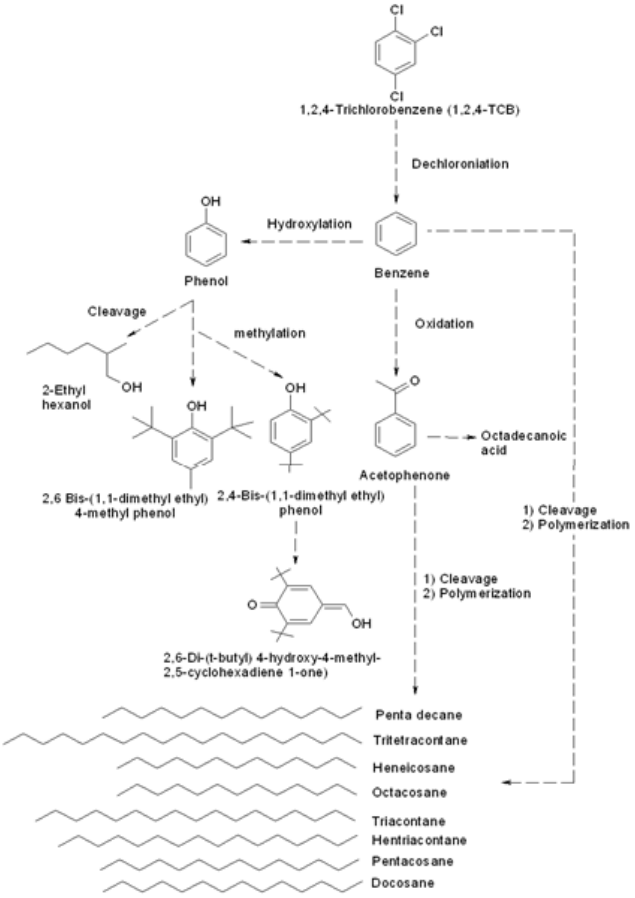

Figure (20): Proposed pathway of 1,2,4-trichlorobenzene (1,2,4-TCB) degradation by *Bacillus mucilaginosus*MAM-24.

These intermediates were pentadecane, pentadecanoic acid, tritetracontane, hentriacontane, pentacosane, docosane and dicyclohexylphathalate. These intermediates could be undergoing further degradation to CO_2 and H_2O to give complete mineralization.

Pseudomonas putida GJ31 has a meta cleavage enzyme which is resistant to inactivation by the acylchloride providing this strain with the exptional ability to degrade both toluene and chlorobenzene via meta cleavage pathway [51].

Previously-described bacteria which can use chlorobenzenes as their sole carbon and energy degrade CB2 via the modified ortho-cleavage pathway, and it is generally accepted that the meta-cleavage pathway is not suitable for the degradation of haloaromatics [36, 51].

Table (3): Intermediates determined by GC-MS analysis resulted from biodegradation of 1,2,4-trichlorobenzene (1,2,4-TCB) by *Bacillus mucilaginosus*MAM-24 and its mutant MAM-24 (9).

R.T	MAM-24 after 24 hours	MAM-24 after 48 hours	MAM-24 (9) after 24 hours
12.107	-	-	Phenol
15.126	4-methyl phenol	-	-
18.29	1,2,4-Trichlorobenzene	1,2,4-Trichlorobenzene	1,2,4-Trichlorobenzene
25.824	2,6-Di-(t-butyl) 4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one	-	-
26.951	2,4-Bis-(1,1 dimethyl ethyl phenol)	-	2,6-Bis-(1,1-dimethyl ethyl) 4-methyl phenol
29.158	Nonadecane	-	-
31.418	Eicosane	-	-
32.778	Octadecanoic acid	-	-
33.548	Pentadecane	-	Penta decane
34.825	Pentadecanoic acid	Pentadecanoic acid	-
35.658	Tritetracontane	-	Tritetracontane
36.793	Dibutylphathalate	Dibutylphathalate	Dibutylphathalate
37.531	Henicosane	Heneicosane	Heneicosane
39.384	Octacosane	Octacosane	Octacosane
41.161	Triacontane	Triacontane	Triacontane
42.866	Hentriacontane	Hentriacontane	Hentriacontane
43.757	Benzylbutylphathalate	-	-
44.501	Pentacosane	Pentacosane	Pentacosane
48.115	Docosane	Docosane	Docosane
46.524	Dicyclohexylphathalate	-	Dicyclohexyl phthalate
46.772	Di (2-ethyl hexyl) phathalate	Di (2-ethyl hexyl) phathalate	Di (2-ethyl hexyl) phathalate
48.015	-	Hexadecane	-
50.383	-	Tetratetracontane	-

Mutant MAM-24 (9) showed 2-ethyl hexanol and acetophenone intermediates which had not been recorded in case of parent strain. Also, the parent strain showed two intermediates after 48 hours which had not found in the early stage (24 hours). These intermediates were hexadecane and tetracontane.

Conclusion

The isolated bacterial strain *Bacillus mucilaginosus* MAM-24 from soil polluted with petroleum oil, was able to degrade more than 98% of 5,10 , 15 and 25 µM of 1,2,4 trichlorobenzene. This strain dechlorinated 1, 2, 4 TCB as first step in degradation pathway. The most important intermediate (metabolite) was acetophenone. The results of GC/MS revealed that strain MAM-24 was able to mineralize 1, 2, 4 TCB.

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