Biological degradation of toluene and benzene by indigenous bacteria isolated from petroleum contaminated sites

4.1 Introduction

Petrochemical pollutants generated from various industries have gained more attention worldwide. Benzene, toluene, ethyl benzene and xylene are together known as BTEX compounds and discharged in significant amount in the water bodies through the effluents from petrochemical and other industries such as refineries, paint, textile, paper, rubber, etc. (El-Naas et al., 2014a; Huang and Li, 2014; Mathur et al., 2013a; Xin et al., 2013; Zhang et al., 2013). The exposure to BTEX compounds and other hydrocarbons is extremely harmful for living organisms, including human beings (Jean et al., 2008; Zhang et al., 2013). According to Chen et al. (2008), various types of DNA damages were observed in human lymphocytes on being exposed to hydrocarbons like BTEX. Benzene is considered to be carcinogenic to human beings, causing various disorders like leukaemia, lymphoma, chromosomal breakage and interference with their segregation, etc. (Bird et al., 2005). Toluene is homologous to benzene; however, it shows different properties than benzene. Inhalation is the prime route of toluene exposure in addition to dermal and gastro-intestinal mode. Being highly lipid-soluble in nature, poly aromatic hydrocarbons (PAHs) are readily absorbed in the gastrointestinal tract of mammals and distributed in the body tissues, especially in adipose tissues (Cerniglia, 1984). They show bioaccumulation and biomagnifications in food chains and causes serious health problems. Also, these compounds adversely affect the aquatic organisms by reducing their chlorophyll

content, thus photosynthetic efficiency (Peng et al., 2015). Saturated aromatic hydrocarbons are released in huge amounts into the environment by both natural seepage of subsurface reservoirs and anthropogenic activities, which adversely affects soil and water ecology due to their toxic nature (Kim et al., 2014; Stasik et al., 2015; Vogt et al., 2016). These organic compounds have been categorized in list of hazardous wastes because most of these are mutagenic or carcinogenic in nature (Goldman et al., 2001; Mastrangela et al., 1997). Various studies have reported the genotoxic effects of these hydrocarbons in human beings as well as in plants (Gopinath et al., 2016). The benzene and toluene are the most widely used industrial solvents for organic synthesis and instrumental cleaning. It is the major aromatic component in many petroleum products, and they are often found in groundwater as a result of leakage in underground storage tanks and pipelines, improper waste disposal practices, inadvertent spills, and leaching from landfills and some disastrous oil spill accidents. These compounds, occurring in all the segments of environment, have adversely affected human health through various mode of interaction i.e. soil, water and air. Therefore, its remediation is an important issue and application of eco-friendly indigenous practices for the clean up the environment is the need of hour.

Different physical, chemical and biological treatment technologies have been proposed to treat these petrochemical contaminants at source as well as remediate soil and water systems contaminated by them. In the category of physical methods, adsorption (El-Naas et al., 2014a) and coagulation (El-Naas et al., 2014b) are the most commonly used processes. Dissolved organics can be easily adsorbed on activated carbon, organoclay, co-polymers, zeolites and other resins. The most common

chemical process used for treatment of effluents is oxidation (Abdelwahab et al., 2009) but there are various problems associated with it, such as requirement of excessive quantity of chemicals (Guo and Al-Dahhan, 2005), low reaction rate (Shu and Huang, 1995) and production of sludge which requires further treatment. Advanced oxidative processes (AOPs) such as photocatalytic degradation, microwave assisted catalytic wet air oxidation (Sun et al., 2008), Fenton's process, etc. have gained popularity. But the associated problems include high maintenance cost, requirement of skilled manpower and generation of more toxic intermediates, leading to further increase in environmental contamination. Besides these conventional physical and chemical methods, various biological methods such as activated sludge, trickling filters, sequencing batch reactors, chemostat reactors, biological aerated filters, bioremediation, bioaugmentation, etc. are also used to treat petrochemical wastes. Under natural conditions, very few studies have reported bioremediation as a sustainable and successful approach for spilled oil degradation (Greenwood et al., 2009; Hii et al., 2009; Tian et al., 2016).

Most of the studies performed on bioremediation of petrochemical wastes have employed bacteria for the degradation purpose. However, some studies have also made use of different types of algae, fungi, yeast, genetically modified organisms (GMOs), biosurfactants, and consortium of microbes. Biological methods employ the diverse metabolic and enzymatic capabilities of microbes for detoxification and mineralization of pollutants. These microbes utilize the carbon contained in the petrochemical wastes for their metabolism, and in the process they degrade the pollutants to less harmful products. Thus, the harmful pollutants undergo

biotransformation to generate less toxic compounds. Biological methods have low operating costs and since they involve direct degradation of pollutants, there is no threat of generation of more toxic intermediates. Majority of microbial community possess the ability to degrade petrochemical pollutants among which bacteria are considered to be the most active and primary agents in petrochemical waste degradation (Floodgate, 1984; Atlas, 1985). Additionally, different bacteria were used solely and in the form of microbial consortium for the degradation of petrochemical wastes. For example, microbial consortium of *Arthrobacter*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas* and *Rhodococcus* has been more effective for alkyl aromatic degradation in marine sediments as compared to single strains (Jones et al., 2007). This is due to the broad enzymatic capability of the consortium. The biodegradation process of aromatic compounds also depends on number of physical, chemical, and biological factors. It includes pollutant concentration, temperature, pH, availability of inorganic nutrients, soil and water composition, and microbial adaptation (Mohan et al., 2006; Singh and Celin, 2010).

The aim of present study was to investigate, isolate, and indentify the potential bacterial strains which effectively biodegrades toluene and benzene in aqueous medium at specific concentration. The potential toluene and benzene degrading microbes were isolated from the refinery contaminated soils and identified by 16s rDNA technology. Further, the toluene and benzene degrading capabilities of identified bacterial strains were assessed and intermediates were identified by GC-MS.

4.2 Materials and methods

4.2.1 Sample collection and isolation, screening and identification of bacteria

Soil and effluent samples were collected in sterile poly bags from the oil refinery situated in Mathura district, Uttar Pradesh, India. Two different soil solutions (1%) were incubated with 100 mg l^{-1} concentration of toluene and benzene, respectively in nutrient broth maintained at 37±2 °C for 4 week under both static and shaking conditions. The resultant broth samples with visible bacterial growth were streaked on the fresh solid nutrient agar (NA) plates, having 100 mg l^{-1} concentration of toluene and again incubated at 37±2 °C for a week. Different bacterial colonies were selected and picked up from this agar plate and were again streaked on fresh NA plate for isolation of pure culture of bacteria. The toluene and benzene degrading bacteria were identified on the basis of morphological details of colony, its colour, Gram's staining and biochemical test of isolated strains according to the Bergey's manual of determinative bacteriology. For molecular identification, DNA was isolated from the pure culture of bacterium and quality was evaluated on 1.2% agarose gel. A single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified from the above isolated DNA segment by PCR using 8F and 1492R. A single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified and processed for the sequencing. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1488 bp 16S rDNA gene was generated from forward and reverse

sequence data using Aligner software. The 16S rDNA gene sequence was used to carry out BLAST (Basic Local Alignment Search Tool) of NCBI GenBank database. Based on maximum identity score, first 15 sequences were selected and aligned using multiple alignment software program. Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA (Version 6).

4.2.2 Preparation of mineral salt medium (MSM)

All the chemicals used for the preparation of MSM were of analytical grade procured from **S**igma Aldrich, Merck, Himedia, Thermo Fisher Scientific. The MSM was prepared by mixing of the following chemicals: For 1000 ml of medium, KH_2PO_4 (0.5 gm), K_2HPO_4 (0.5 gm), $(NH_4)_2SO_4$ (2.0 gm), NaCl (0.01 gm), $MgCl.6H_2O$ (0.2 gm), $CaCl_2$ (0.02 gm), $MnSO_4$ (0.0003 gm), $ZnSO_4$ (0.0004 gm), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0003 gm), $CoCl_2.6H_2O$ (0.0004 gm), and EDTA (0.001 gm) was used and then autoclaved for 30 minutes at 15psi pressure and 121°C temperature.

4.2.3 Toluene and benzene batch degradation studies by pure and mixed bacterial culture

Batch studies were carried out to understand the biodegradation of toluene and benzene at different pH ranging from 4.5 to 8.5 by using fixed concentration. Teflon liner screw cap flasks were used for the batch biodegradation studies. The mixed culture were grown overnight and inoculated in the flasks. The flasks were kept on

orbital shaker at 37 °C with 150 rpm. Batch experiments were performed in triplicate along with the control. For the sampling purpose, flasks were removed from the shaker and 2 ml of sample was collected from the sampling port with air tight syringe to estimate the cell density and toluene degradation.

4.2.4 Cell growth measurement

The cell growth was determined by measuring absorbance of bacterial culture at 600 nm using UV–Vis spectrophotometer (Systronics, India) (Padhi and Gokhale, 2016; Sahoo et al., 2014).

4.2.5 Scanning Electron Microscope (SEM) Analysis

The pure culture of *Acinetobacter junii, Klebsiella pneumoniae* and *Serratia marcescens* was observed under scanning electron microscope (SEM) for surface characterization and structure. Sample preparation techniques as adopted by Paje et al. (1998) were used for the SEM analysis. The specimen's fixations were performed in 2% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) for one hour followed by washing with the buffer to eliminate any unbound chemicals. Thereafter, the specimens were dehydrated in a series of alcohol and acetone for 10 minutes. Further drying was performed in a critical point drying chamber. Dried specimens were carefully mounted onto stubs and coated with gold prior to observation under the scanning electron microscope (ZEISS, model EVO-18 research; Germany).

4.2.6 Analytical investigation

HPLC investigations of benzene and toluene biodegradation were performed as per Rajamanickam et al. (2017). The detailed processes are briefly described here. Sample in the liquid phase was analyzed by HPLC (Thermo Scientific Ultimate 3000) chromatograph with UV detector at 254 nm. The column used was C18 bond packed 3 µm (25 × 4.6 mm) with mobile phase consisting of methanol and water (70:30). The flow rate was set at 1 ml min^{-1}. The samples were also analyzed by GC−MS. Inoculated samples were centrifuged for 10 minutes at 10,000 rpm to separate cell mass and the supernatant. Supernatant was extracted in an organic solvent (n-Hexane) for analysis through mass spectrometry to identify toluene, benzene and its degraded products. The samples were analyzed by mass spectrometer (Shimadzu Gas Chromatograph attached with single quadruple Mass Spectrometer (GCMS-QP2010 Plus)) using ionization mode, ESI (electron spray ionization) and masrlynx 4.0V software.

4.2.7 Detection of intermediate compounds produced after toluene biodegradation

GC-MS was performed in order to determine the metabolic intermediates formed during the toluene and benzene degradation. Samples were extracted in n-hexane in order to know the degradation products. GC–MS analysis of the extractions was performed in Shimadzu GC-MS-QP-2010 plus system. The column RTx-5 Sil MS having internal diameter of 30 m × 0.25 mm and 0.25 µm film thickness fitted in instrument was used for the intermediate compound analysis produced after bacterial

degradation. The operational programmes of the column were as follows: oven temperature increase from 80 to 210 °C at 4 °C min^{-1} rise with hold time of two minutes and from 210 to 300 °C at 15 °C min^{-1} rise with stand time of 5 minutes. Final temperature was maintained for 20 min. The injector temperature was kept at 270 °C. A total 0.2 µl volume of sample was injected into column. Pressure, total flow and column flow was 85.4 kPa, 76.8 ml min^{-1}, 1.21 ml min^{-1}, respectively. The identification of compounds was performed by comparing their mass spectra with data available from NIST05 (National Institute of Standards and Technology, US) and WILEY 8 libraries.

4.3 Results and Discussion

4.3.1 Culture identification

The culture, which was labeled as CH005 was very much close to *Acinetobacter junii* isolate OTU-a6 (GenBank Accession Number: KJ147060.1) based on nucleotide homology and phylogenetic analysis. Culture labelled as CH007 was identified as *Serratia marcescens* strain 35 dr (GenBank Accession Number: KJ729606.1) while CH010 was identified as *Klebsiella pneumoniae* strain GX120222 (GenBank Accession Number: KP091888.1). List of some other organisms along with their accession numbers that have been used for degradation of toluene and benzene are included in following table 4.1.

Table 4.1. List of some other organism identified or isolated in the study

S. No.	Micro-organisms	Accession Number	Isolation condition /place of isolation	Degradation condition	Compounds /Pollutants
1	*Klebsiella pneumoniae* CH001	**KT630867**	Temperature 28 °C Mathura refinery sludge and effluent	Batch studies, shaking at 150 rpm, temperature 37 °C	Benzene /Toluene
2	*Klebsiella pneumoniae* CH002	**KT630868**	Temperature 40 °C Mathura refinery sludge and effluent	Batch studies, shaking at 150 rpm, temperature 37 °C	Benzene/Toluene
3	*Klebsiella* spp.	**KT630869**	DLW, Varanasi	Batch studies, shaking at 150 rpm, temperature 37 °C	Toluene
4	*Acinetobacter junii*	**KT630871**	Temperature 28 °C Mathura refinery sludge and effluent	Batch studies, shaking at 150 rpm, temperature 37 °C	Benzene /Toluene
5	*Serratia marcescens*		Temperature 28 °C Mathura refinery sludge and effluent	Batch studies, shaking at 150 rpm, temperature 37 °C	Benzene /Toluene

6.	*Klebsiella pneumoniae*	**KT630872**	DLW, Varanasi	Batch studies, shaking at 150 rpm, temperature 37 °C	Benzene /Toluene
7	*Trichoderma harzianum*	**KM058117**	Temperature 25 °C Mathura refinery sludge and effluent	Batch studies static condition Temperature 28 °C	Toluene

4.3.2 Phylogenetic relationship of the isolated bacteria

The evolutionary history was interpreted using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to show the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. All positions containing gaps and missing data were eliminated. There were a total of **1447** positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The phylogenetic tree of isolate CH005, CH007 and CH010 is shown in figures 4.1, 4.2 and 4.3.

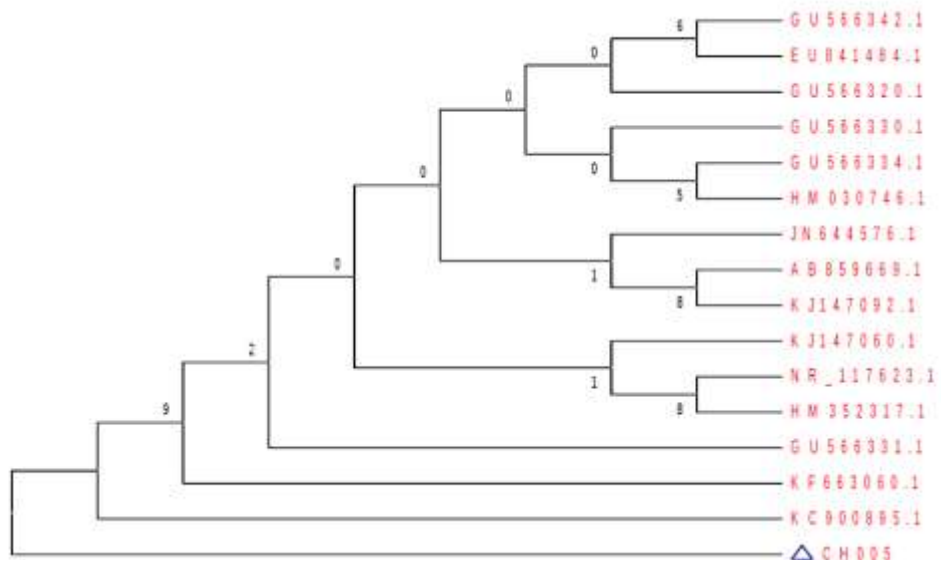


Fig. 4.1: Phylogenetic relationship of *Acinetobacter junii* isolate CH005 with other closely related taxa.

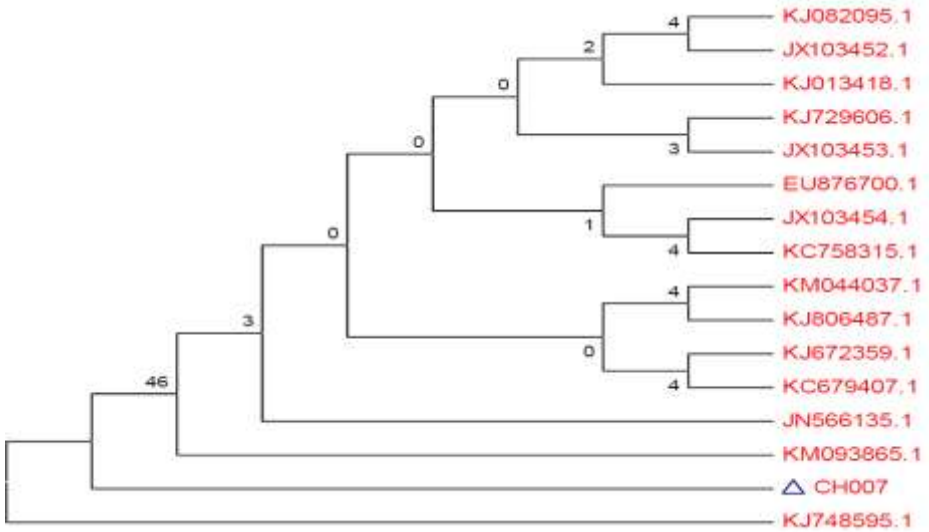


Fig. 4.2: Phylogenetic relationship of *Serratia marcescens* isolate CH007 with other closely related taxa.

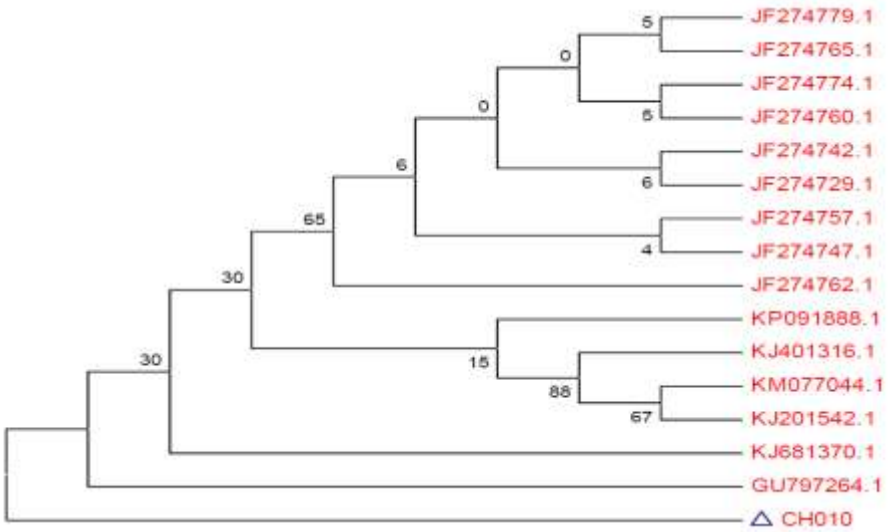


Fig. 4.3: Phylogenetic relationship of *Klebsiella pneumoniae* isolate CH010 with other closely related taxa

4.3.3 Effect of pH on bacterial biodegradation

The pH is one of the most important factor controlling the growth and enzymatic activities in microorganisms. Range of pH (4.5 to 9.5) was selected to observe the toluene degradation efficiency of bacteria. There was an enhancement in the percent degradation by mixed culture with an increase in pH from 4.0 to 7.5. Beyond pH 7.5, a reduction in degradation activity was observed which might be due to lowered enzymatic activity. At pH 4.0, 6.5, 7.5, 8.5 and 9.5 toluene degradation by mixed culture was 12%, 48%, 52%, 46% and 30%, respectively. In the present study, the bacterium demonstrated the best biodegradation performance at pH 7.5. Thus, optimum pH 7.5 was selected for further degradation studies. However, most of the microorganisms are reported to preferably grow at neutral or near neutral pH

(Margesin and Schinner, 2001). The results of the effect of pH on biodegradation are shown in following figure 4.4.

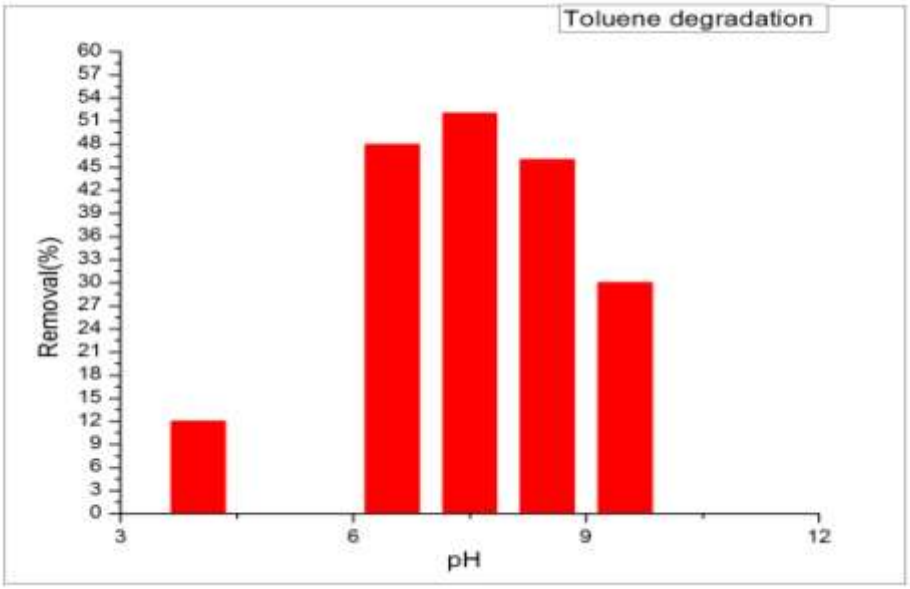


Fig. 4.4: Effect of pH on toluene biodegradation by bacteria.

4.3.4 Biodegradation studies of toluene by pure and mixed bacterial culture and benzene degradation by mixed culture

Majority of the studies on toluene biodegradation have been performed with anaerobic microorganisms. In the present study, we evaluated the toluene degradation ability under aerobic conditions. With an increase in time gap, concomitant increase in degradation by both single and mixed culture was observed. Pure culture of *Acinetobacter junii* was able to degrade 69, 73 and 80% of 150, 100, and 50 ppm toluene, respectively within 72 hours (Fig. 4.5), while pure culture of *Serratia marcescens* was able to degrade 74, 77, and 82% of 150, 100, and 50 ppm toluene

within 72 hours (Fig. 4.6). However, *Klebsiella pneumoniae* cells degraded 65, 79, and 89% of 150, 100, and 50 ppm toluene, respectively within 72 hours (Fig. 4.7). It indicates that *Serratia marcescens* cells were most effective amongst all the tested pure cultures, as they were able to degrade 74% of 150 ppm toluene. It was observed that 80% of toluene was degraded within 72 hours by the mixed bacterial culture (Fig. 4.8). The effectiveness of microbial consortium of bacteria has been found for alkyl aromatic degradation in marine sediments as compared to single strains (Jones et al., 2007). This is attributed to the broad enzymatic capability of the consortium, which enhances the rate of degradation.

Toluene degradation with time in the present study shows that the degradation occurs due to the enzymatic activities resulting from bacterial growth. Non-linear increase in percent degradation of toluene was determined up to 72 hours. The percent removal was very low when cells reached near to 72 hours, showing saturation of enzymes involved in degradation. The increase in toluene concentration resulted into diminished percent biodegradation which may be explained by toxic impact of toluene (Dorer et al., 2016). For benzene, we selected 150 ppm concentration and evaluated the degradation performance of mixed bacterial culture under optimum conditions (Fig. 4.9). Benzene degradation by mixed culture in mineral salt media (MSM) was determined to be 63% within 72 hours of incubation. Cells reached to saturation stage after 60 hours of incubation period. No further degradation was observed up to 72 hours probably due to cellular enzyme saturation (Zhang et al., 2013).

Degradation trend for different concentration of toluene by individual and mixed culture and benzene degradation by mixed culture only is shown in following figures 4.5-4.9.

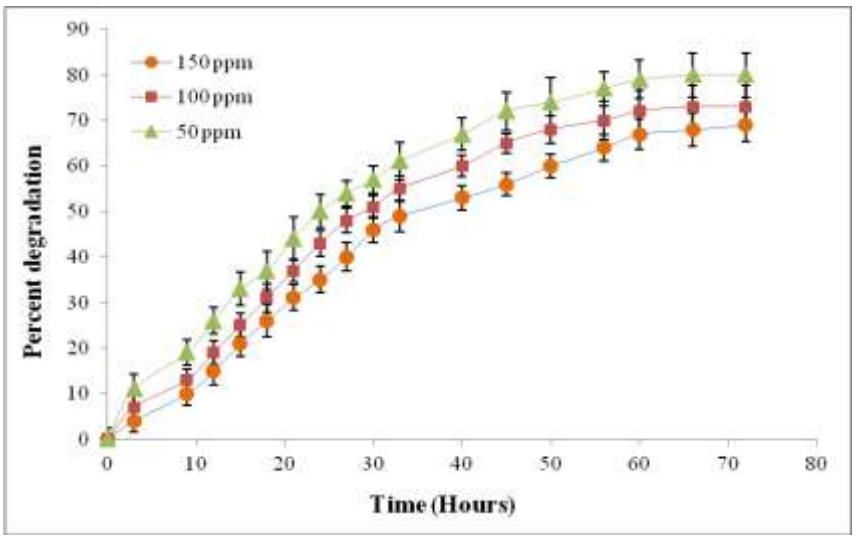


Fig. 4.5: Percent degradation of different concentrations of toluene by *Acinetobacter junii* cells.

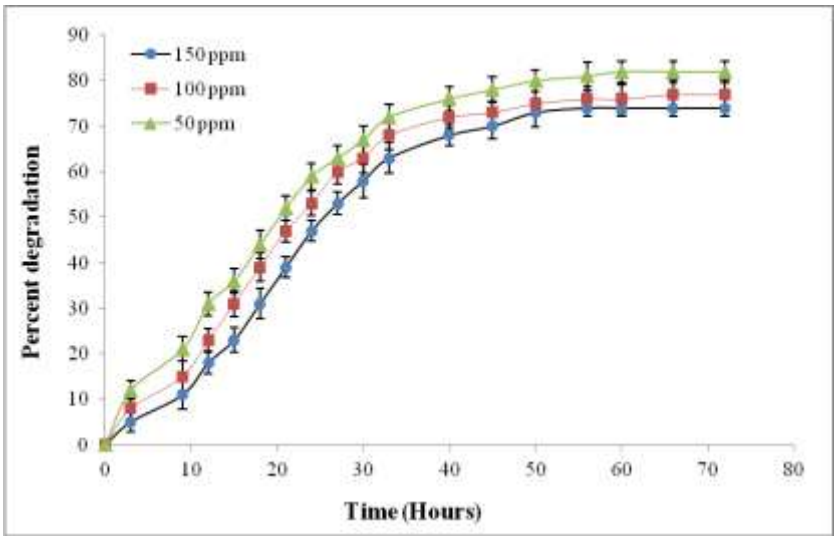


Fig. 4.6: Percent degradation of different concentrations of toluene by pure culture of *Serratia marcescens*.

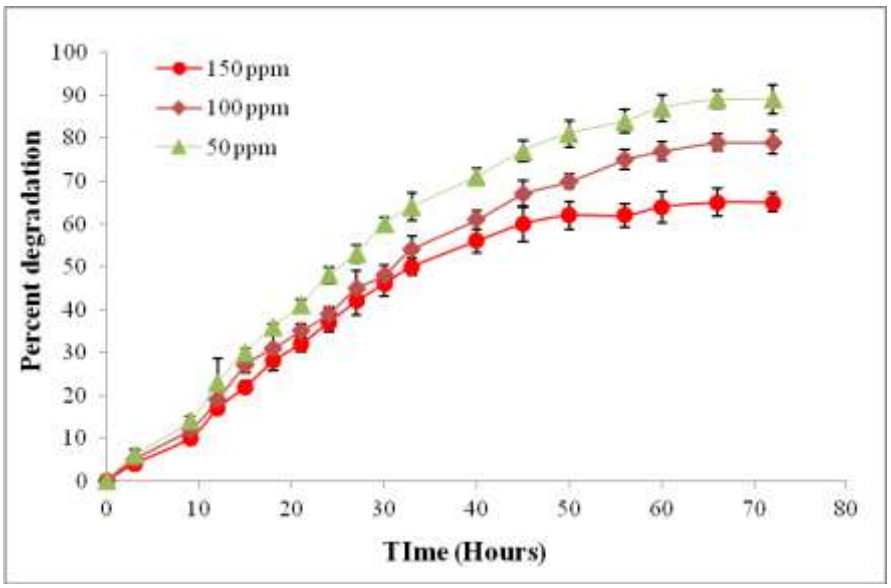


Fig. 4.7: Percent degradation of different concentrations of toluene by pure culture of *Klebsiella pneumoniae*.

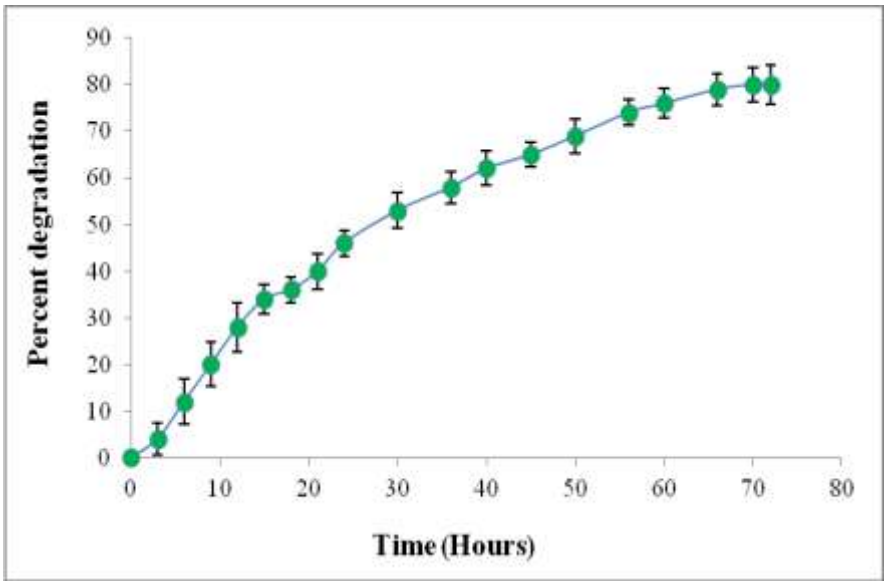


Fig. 4.8: Degradation of 150 ppm toluene by mixed bacterial culture (*Acinetobacter junii*, *Klebsiella pneumoniae* and *Serratia marcescens*).

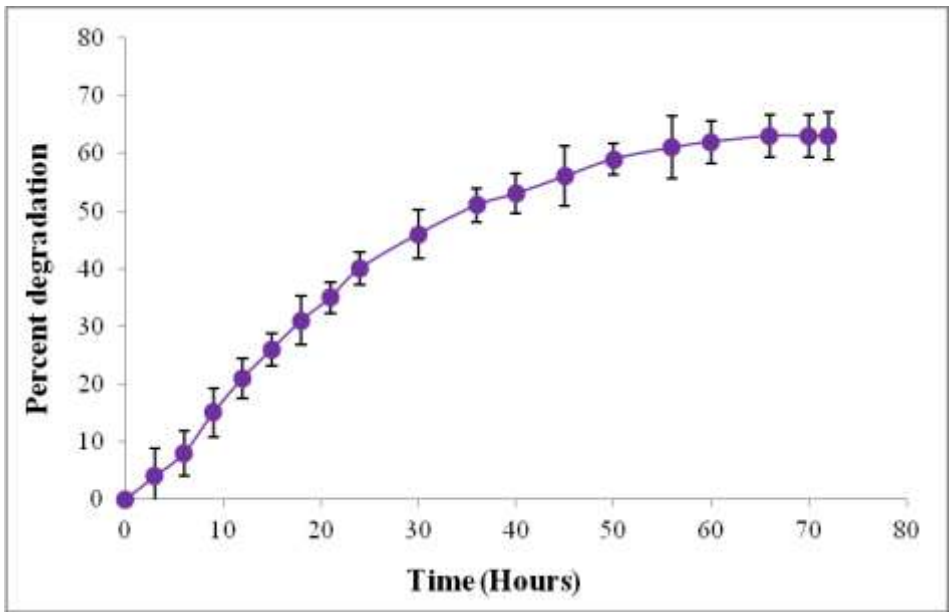


Fig. 4.9: Percent degradation of 150 ppm benzene by mixed bacterial culture of *Acinetobacter junii*, *Klebsiella pneumoniae* and *Serratia marcescens*.

Growth of bacteria in the presence of aromatic compounds indicates its applicability for bioremediation of contaminated sites. Studies on the use of mixed bacterial culture for biodegradation of toluene has already been reported by Rajamanickam et al. (2017). Studies on biodegradation of toluene under both aerobic as well as anaeobic conditions have been reported worldwide. Studies on the biodegradation under aerobic conditions are lesser as compared to the anerobic degradation. We studied the toluene degradation by pure as well as mixed bacterial culture. Under optimum laboratory conditions, mixed culture of *Acinetobacter junii*, *Serratia marcescens* and *Klebsiella pneumoniae* proved most efficient. The present study will be beneficial in the industrial level treatment using biological treatment technologies.

4.4 General degradation pathway for petroleum hydrocarbon

BTEX compounds are mostly degraded by aerobic pathway. Figure 4.10 shows the main principle for aerobic degradation of hydrocarbons by microorganisms. Aerobic conditions provide O_2 as a powerful oxidant to oxidize and cleave the ring of aromatic petrochemical compounds. Oxygen not only serves as the final electron acceptor but also as a co-substrate for some key catabolic processes (Díaz et al., 2013; Fuchs et al., 2011). Aerobic degradation of aromatic petrochemical waste mostly starts with the attack of dioxygenase enzymes on aromatic rings yielding cis-dihydrodiols. This is followed by dehydrogenation of cis-dihydrodiols to 1,2-dihydroxy compounds by a dehydrogenase, followed by metabolism of 1,2-dihydroxy compounds (Goyal et al., 1997).

Benzene gets transformed into catechol. Toluene can be degraded by many biodegradation pathways, some involving 3-methylcatechol as an intermediate. The derivative of catechol was also found in our study, indicating the degradation by the same pathway as used in the benzene degradation. Similarly, many biodegradation pathways exist for degradation of ethyl-benzene, in which some involves 3-ethylcatechol as an intermediate. However, degradation of xylene is a complex process because all xylenes get metabolized to mono-methylated catechols (*e.g.*, m-xylene gets metabolized to 3-methylcatechol). The aromatic ring of all these substituted catechols is later cleaved under the action of dioxygenase enzymes.

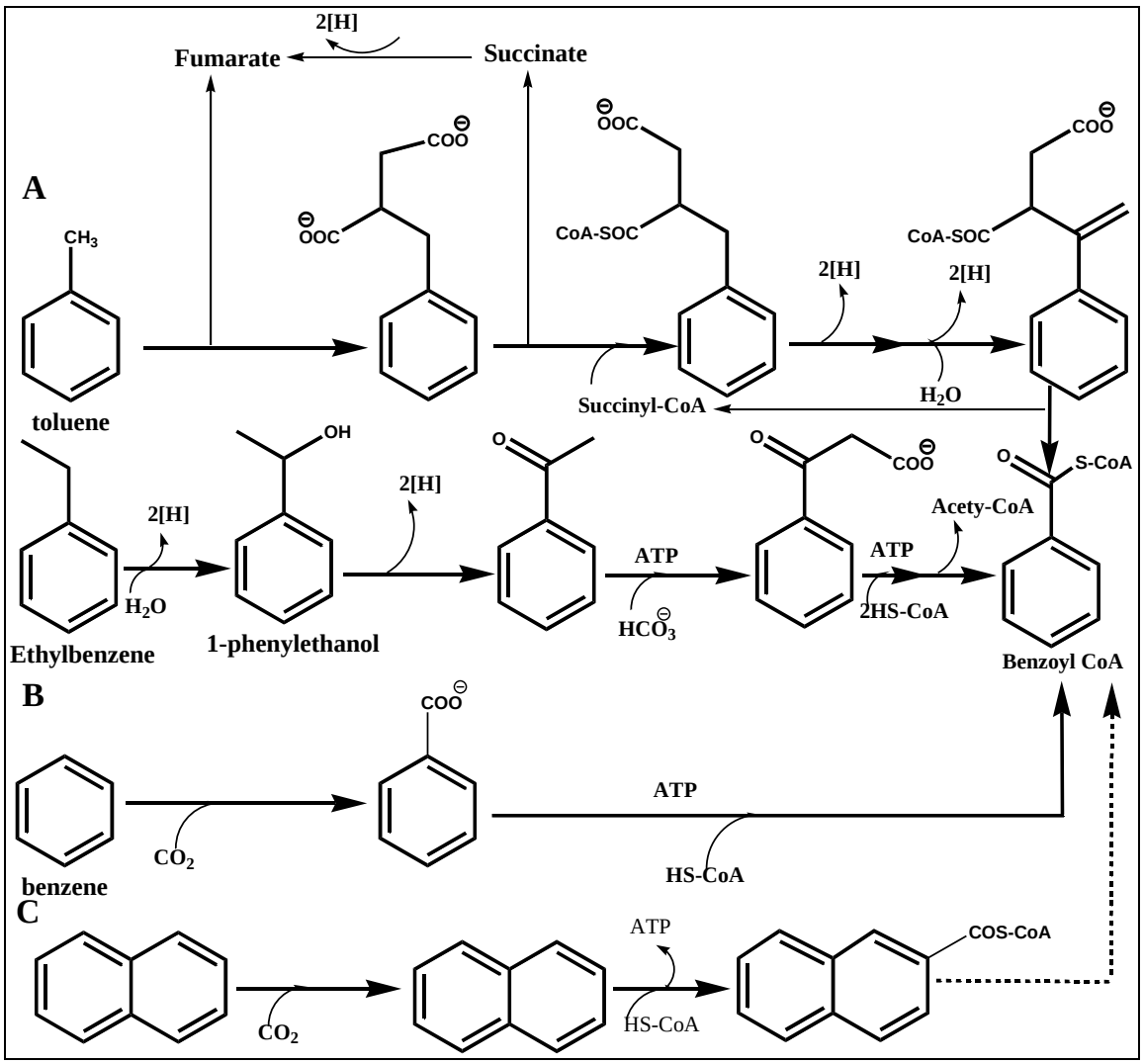


Fig. 4.10: General aerobic biodegradation pathway of benzene, toluene and ethylbenzene.

4.4.1 Determination of intermediates formed after toluene degradation by *Acinetobacter junii*

Formation of intermediate compounds such as 1-Isopropenyl-4-methyl-1,3-cyclohexadiene, 1,3-Cyclohexadiene, 2-Methyl-5-(1-Methylethyl), 4-Methoxycarbonyl-4-butanolide (Dobslaw and Engesser, 2015), Vinyl (2E,4E)-2,4-

hexadienoate (Cho et al., 2000) was observed after toluene degradation by *Acinetobacter junii* isolate CH005. GC-MS chromatogram of biodegradation products are shown in figure 4.11.

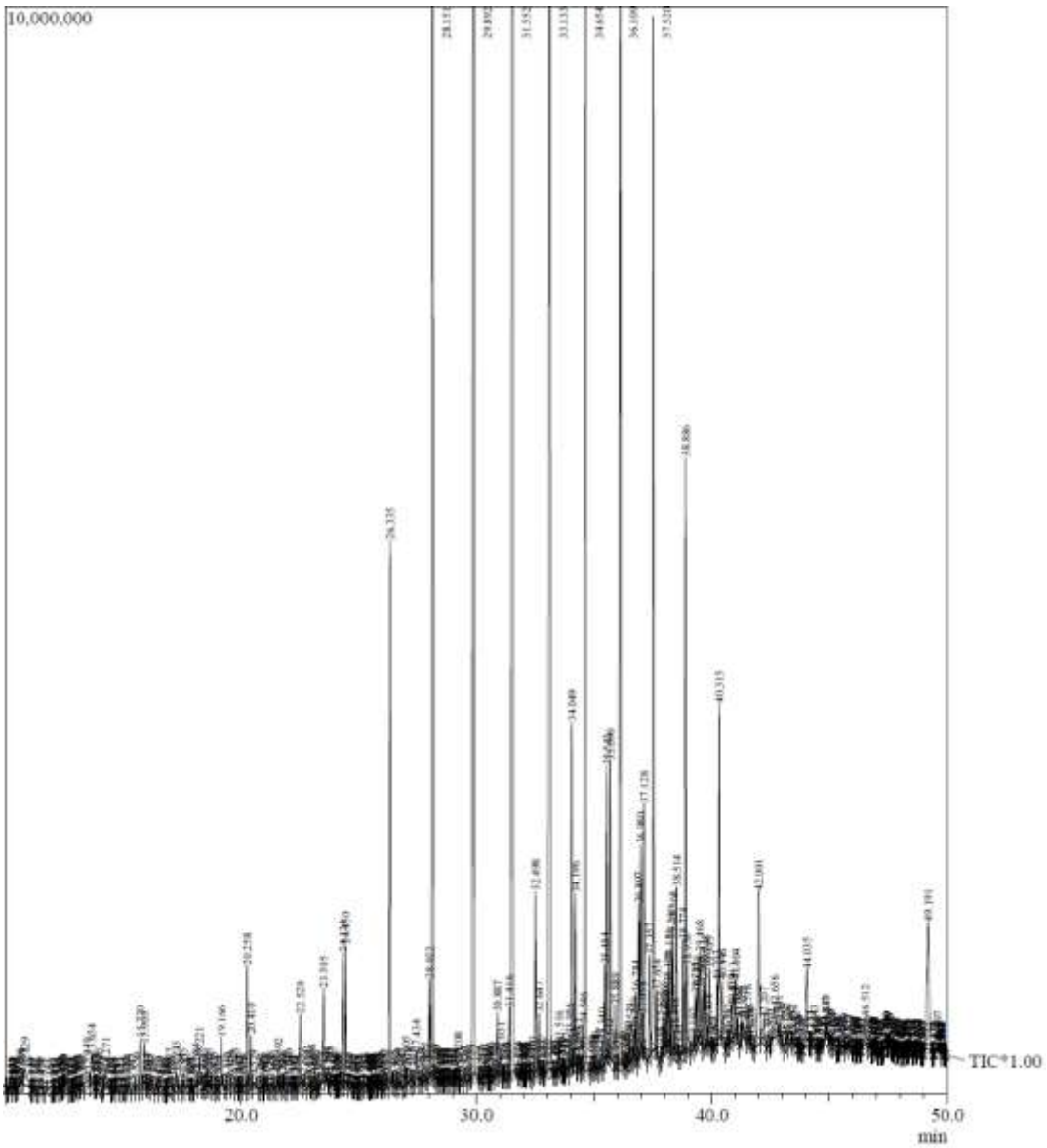
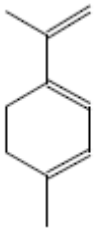
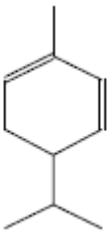


Fig. 4.11: GC-MS chromatogram of toluene degradation product after treatment with *Acinetobacter junii*.

Structures of intermediate compounds formed are presented below:



1



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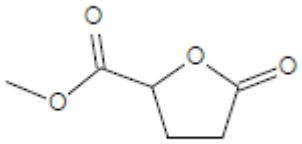

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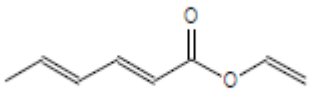
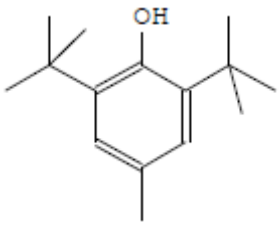


4

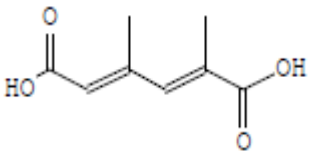
1. (1-Isopropenyl-4-methyl-1,3-cyclohexadiene)
2. 1,3-Cyclohexadiene, 2-Methyl-5-(1-Methylethyl)
3. 4-Methoxycarbonyl-4-butanolide
4. Vinyl (2E,4E)-2,4-hexadienoate

4.4.2 Determination of intermediates formed after toluene degradation by *Serratia marcescens*

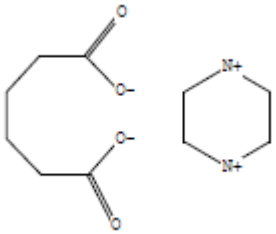
Toluene degradation by *Serratia marcescens* resulted into formation of toluene derivative i.e. butylated hydroxytoluene also known as p-Cresol, 2,6-di-tert-butyl (structure given below).



Muconic acid derivative i.e. dimethylmuconic acid (structure given below) was also observed to be formed into media after enzymatic degradation as also reported by Dobslaw and Engesser (2015).



Piperazinium adipate was also noticed after bacterial action, which has also been reported previously by Parales et al. (2008). The structure of compound is shown below:



GC-MS chromatogram of biodegradation products formed after *Serratia marcescens* treatment is shown in figure 4.12.

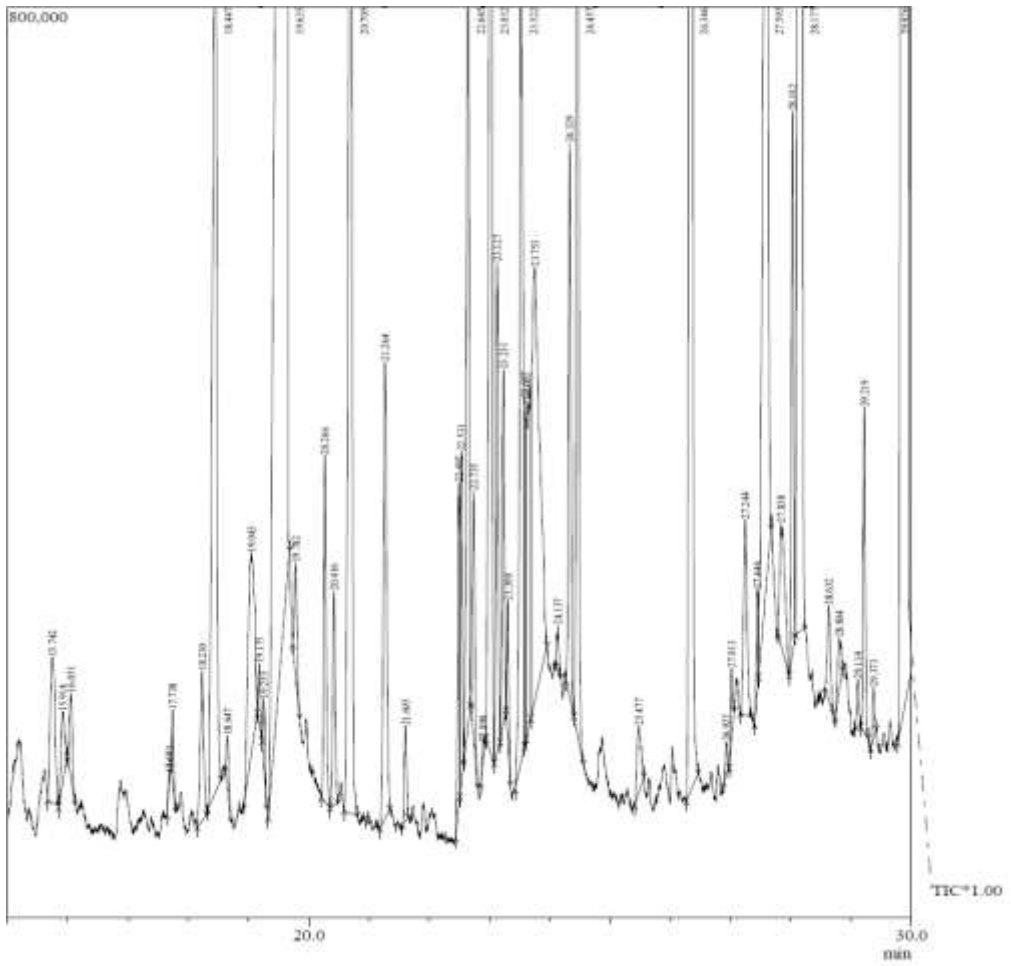
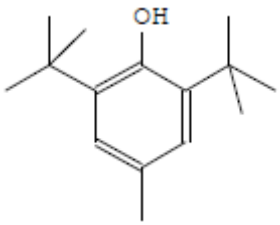


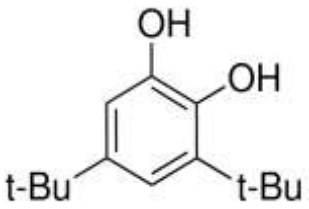
Fig. 4.12.: GC-MS chromatogram of toluene degradation product after treatment with *Serratia marcescens*.

4.4.3 Determination of intermediates formed after toluene degradation by *Klebsiella pneumoniae*

Toluene degradation by *Klebsiella pneumoniae* resulted into formation of toluene derivative i.e. butylated hydroxytoluene also known as p-Cresol, 2,6-di-tert-butyl



3,5-di-tert-Butylcatechol was also noticed after toluene degradation by *Klebsiella pneumoniae*. The compound is catechol derivative and commonly formed after biodegradation as also reported by Parales et al. (2008). The structure of compound is given below:



GC-MS chromatogram of toluene degradation product after treatment with *Klebsiella pneumoniae* is shown below in figure 4.13.

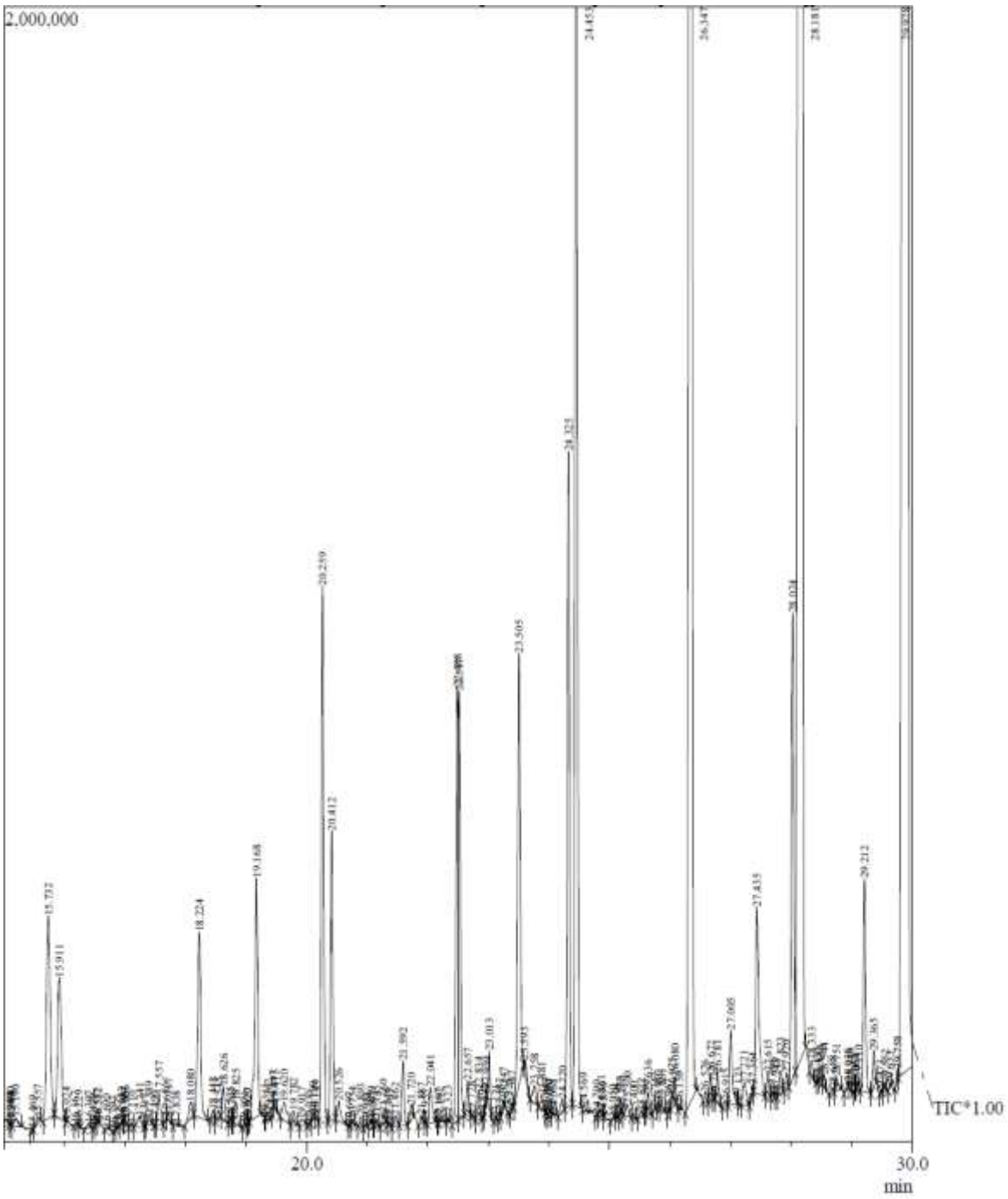
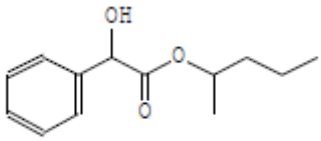


Fig. 4.13: GC-MS chromatogram of toluene degradation product after treatment with *Klebsiella pneumonia*.

4.4.4 Determination of intermediates formed after toluene degradation by mixed bacterial culture activity

1-Methylbutyl hydroxy(phenyl)acetate formation by mixed culture after toluene degradation is evident in the medium as also reported by Kuhner et al. (2005). The structure is presented below:



1,3-Cyclohexadiene, 2-Methyl-5-(1-Methylethyl) and Vinyl (2E,4E)-2,4-hexadienoate formation was also observed after toluene degradation by mixed culture as also evidenced in toluene degradation by *Acinetobacter junii* (as reported by Dobslaw and Engesser, 2015). GC-MS chromatogram of degraded product produced after bacterial degradation is presented below in figure 4.14.

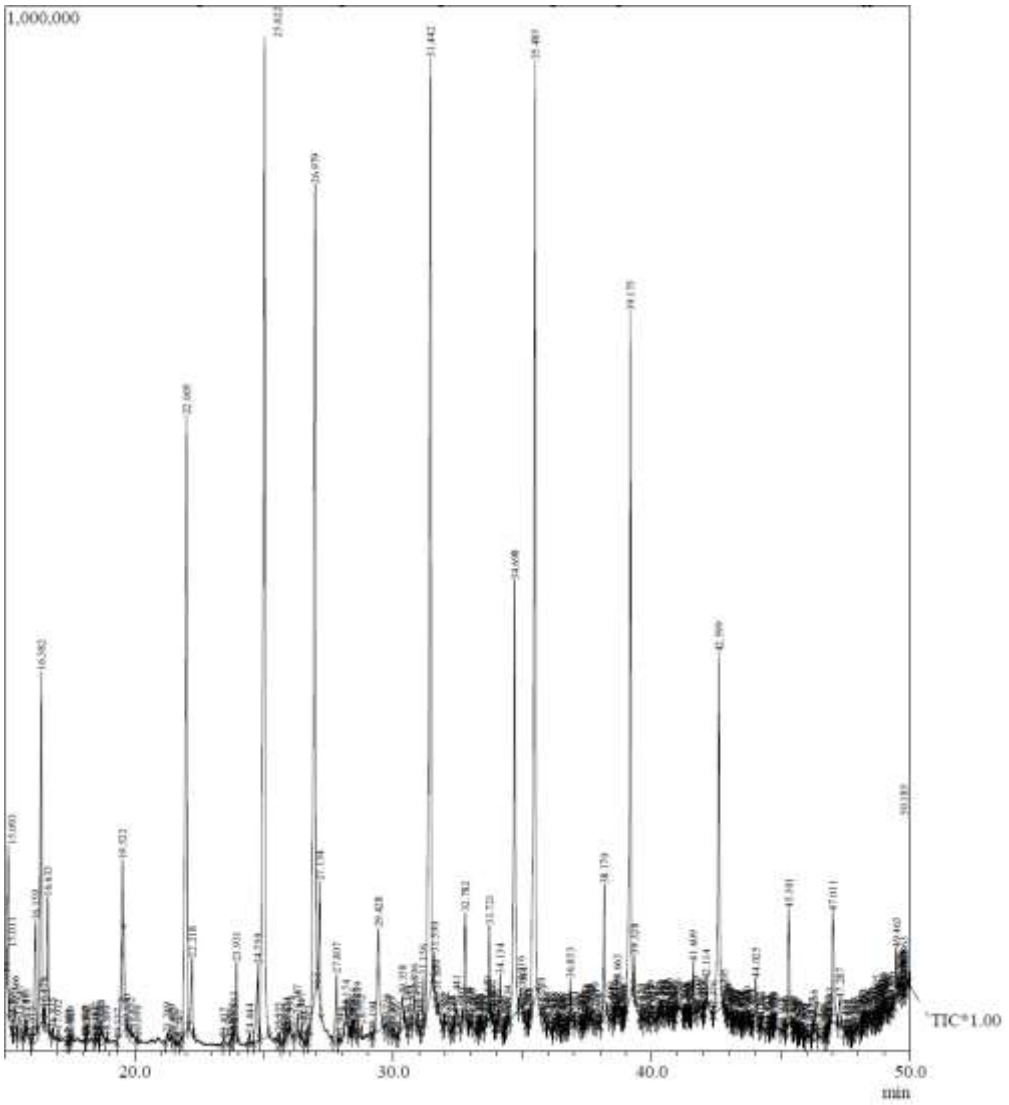
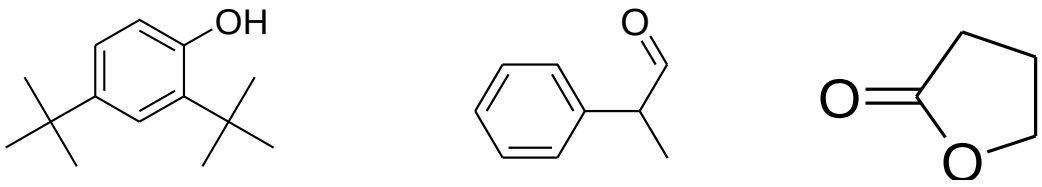


Fig. 4.14: GC-MS chromatogram of toluene biodegradation products formed after mixed culture treatment.

4.4.5 Determination of intermediates formed after benzene degradation by mixed bacterial culture activity

Benzene degradation by mixed bacterial culture resulted into formation of different compounds. The formed compounds are of general occurrence produced after aerobic bacterial degradation. The compounds produced were phenol, 2,4, bis

(1,1 dimethyl ethyl) (Chakraborty, 2005); Benzene acetaldehyde, alpha methyl (Trigueros, 2010) and Gamma butyrolactone (Iyer, 2013). The structure of each compound is represented below:



Phenol, 2,4, bis (1,1 dimethyl ethyl) Benzene acetaldehye, alpha methyl Gamma butyrolactone

GC-MS chromatogram of benzene degradation product formed after mixed bacterial culture treatment is presented below in figure 4.15.

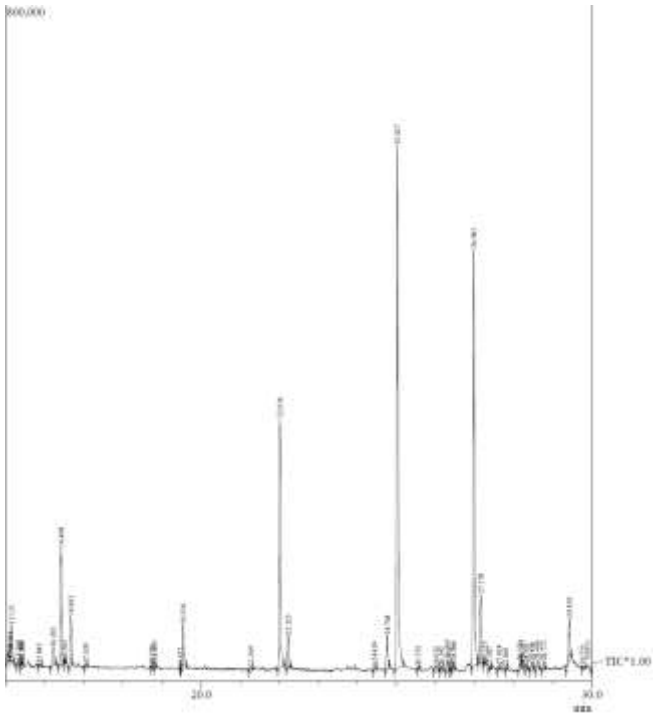


Fig. 4.15: GC-MS chromatogram of benzene biodegradation products formed after mixed culture treatment.

4.5 Scanning Electron Microscopy

Surface morphology of untreated and toluene treated *Acinetobacter junii*, *Klebsiella pneumoniae*, *Serratia marcescens* and mixed culture was revealed by SEM as shown in Figs 4.16-4.19. Under general condition, all bacterial cells are cylindrical which was clearly visible under scanning electron microscope image. However, some long cylindrical cells were transformed into ovoid and spherical structure after treatment, probably to escape themselves from toxicity as evidenced under SEM microphotographs. Such morphological changes confirmation has also been reported by Affandi et al. (2014). Bacterial systems have developed several types of changes at the biochemical and physiological level which makes them suitable to survive under stressed conditions such as presence of organic compounds including benzene, toluene, ethylbenzene and xylene (Michael et al., 2016). These protective mechanisms include changes in characteristics of cell membrane, alteration in cell shape, size, formation of vesicular structure on membrane, and protein associated with stress tolerance, efflux pumps and energy pool maintenance.

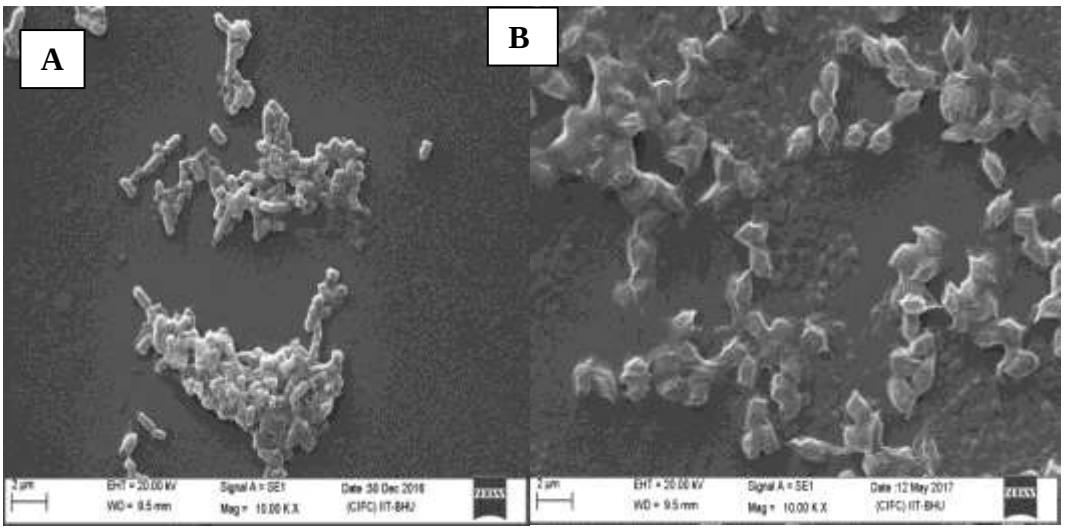


Fig. 4.16: SEM image of *Acinetobacter junii* isolate CH005 (A) before treatment of toluene (B) after treatment of toluene.

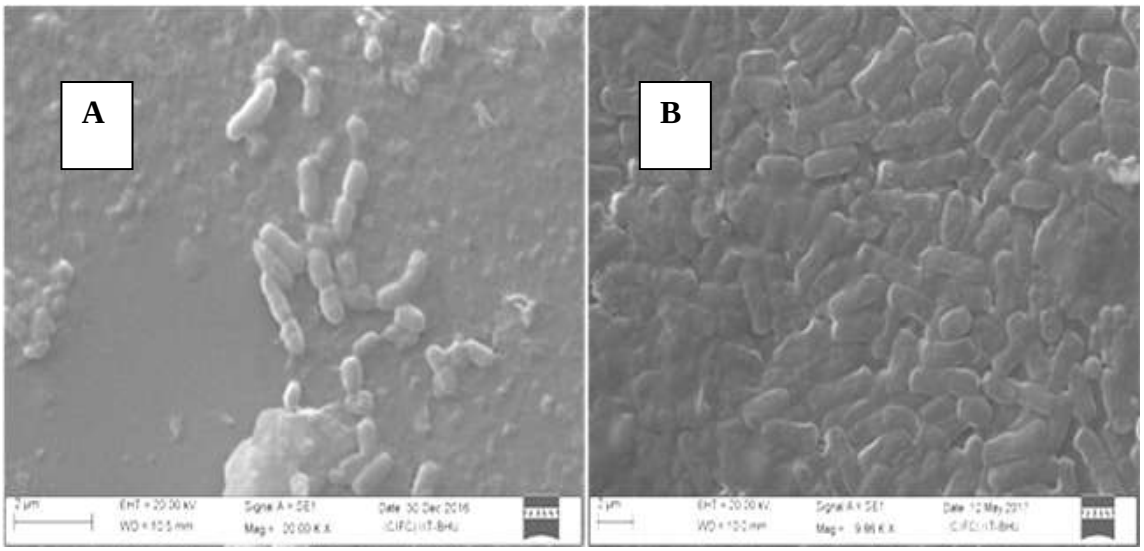


Fig. 4.17: SEM image of *Serratia marcescens* isolate CH007 CH005 (A) before treatment of toluene (B) after treatment of toluene.

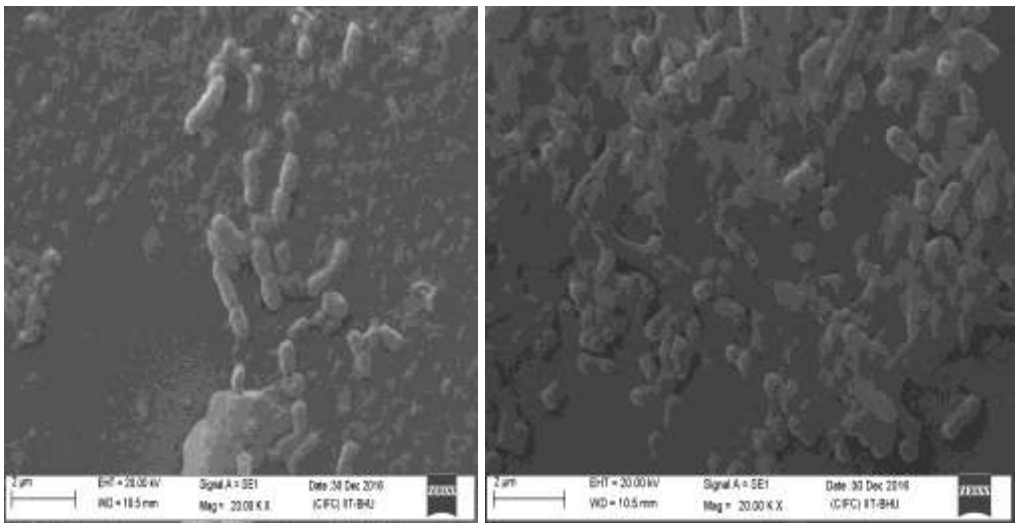


Fig. 4.18: SEM image of *Klebsiella pneumoniae* isolate CH010 CH005 (A) before treatment of toluene (B) after treatment of toluene.

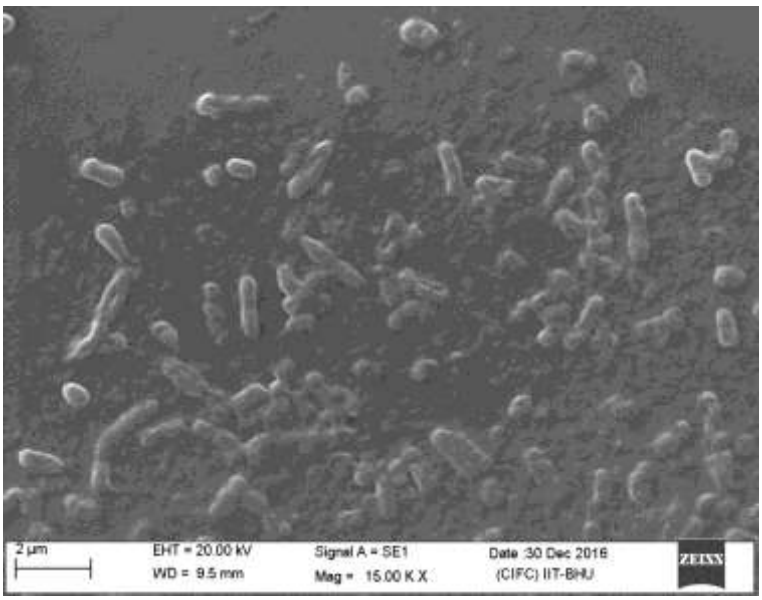


Fig. 4.19: SEM image of mixed bacterial culture of *Acinetobacter junii*, *Klebsiella pneumoniae* and *Serratia marcescens* before treatment.

4.6 Conclusion

Petrochemical waste management is one of the most serious concerns faced by environmentalists today. Bioremediation practice has proved to be a huge success in the laboratory and also in many cases, in the natural environment. Most of the studies done on bioremediation of petrochemical waste employed bacteria for the degradation purpose owing to its very fast growth and easy adaptation in changing environmental conditions. Therefore, environmental microbiologists and biotechnologists have the challenging objective of solving these problems using microorganisms in bioremediation technologies.

In the present experiment, the biodegradation of toluene and benzene by bacteria *Acinetobacter junii* isolate CH005, *Serratia marcescens* isolate CH007 and *Klebsiella pneumoniae* isolate CH010 from petroleum-contaminated water and soil showed that percentage removal of toluene and benzene was best at pH 7.5 and at a temperature of 37 °C. Bacterial species showed varying degree of ability in toluene degradation. Furthermore, mixed bacterial culture was found most suited for the degradation purpose with highest efficiency of degradation. Moreover, it is necessary

to support the activities of these indigenous microorganisms by bioaugmentation and biostimulation in the polluted biotopes to enhance their degradation abilities.

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