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Benzene Biodegradation By A Novel Species of Bacillus Megaterium

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ABSTRACT

In the present study, an attempt was made to evaluate the biodegradation profile of benzene by Bacillus megaterium. The bacterial culture was incubated at 6 intervals of time (8, 16, 24, 32, 40 and 48hr) in Mineral Salt Benzene Medium (MSBM). The intermediates were identified by TLC and HPTLC. Functional groups of the intermediates were confirmed by FT/IR analysis. Peak at 3300 cm-1 region of 8hr sample represented O-H group in aromatic ring of benzene. The spectrum represented new peaks in the range 1271 cm-1 -1039 cm-1 of 16hr indicating C-O bond, in 1726 cm-1 representing the C=O and in 900 cm-1 – 1000 cm-1 indicating shift in the C=C of aromatic ring. 24hr FT/IR gave evidence to suggest the presence of C-O bond (1100-1800 cm-1) and O-H (2880-3400 cm1). O-H stretching and C=C shift in the aromatic ring was further confirmed by the FT/IR at 32 hr incubation. FT/IR also helps in the analysis of the nature of the intermediates formed. The product of the degradation process was analyzed with GC/MS.

KEYWORDS : Benzene Degradation, Bacillus megaterium, FT/IR, GC/MS

INTRODUCTION

Infrared spectroscopy is certainly one of the most important analytical techniques available to the scientific community to confirm the structural changes taking place in an organic compound. The most important advantage of the technique is that it gives strong indication of any change effected in any one of the functional groups present in the sample. When exposed to infrared radiation, sample molecules selectively absorb radiation of specific wavelengths which causes the change of dipole moment of the sample molecules. Consequently, the vibrational energy levels of sample molecules transfer from ground state to excited state [1]. The number of absorption peak is related to the number of vibrational freedom of the molecule. The intensity of the absorption peaks is related to the change of dipole moment and the possibility of the transition of energy levels. Therefore, by analyzing the infrared spectrum, one can readily obtain abundant structure information of a molecule. The common used region for infrared absorption spectroscopy is 4000 ~ 400 cm^{-1} because the absorption radiation of most organic compounds and inorganic ions is within this region [2].

Biodegradation is defined as the biologically catalyzed reaction in the complexity of chemical compound. It is based on two processes; growth and co-metabolism. In the case of growth, organic pollutants are used as sole source of carbon and energy. This process results in a complete degradation of organic pollutants. Large amount of contaminants enter the soil most commonly from leaking underground storage tanks, landfills, waste disposal ponds etc [3]. The objective of the present study is to explore the utility of FT/IR technique in tracing the biodegradation of Benzene. The work also aims at the analysis of the products of benzene biodegradation. The present study is to develop a bioprocess for the effective decontamination of benzene.

MATERIALS AND METHODS

Organism and Biodegradation process

The organism used in the study, *Bacillus megaterium* was collected from the culture collections of School of Biosciences, MG University, Kottayam and the biodegradation was carried out in mineral salt benzene medium [4] and was subjected to solvent extraction. 100ml supernatant was mixed with 100ml of di ethyl ether in a separating funnel. It was allowed to stand for 30 minutes for the separation of the two layers. Repeat the procedure (3 cycles) with equal amount of di ethyl ether. After pooling the organic fraction it was evaporated at 40°C to 2 ml as control and sample. After measuring the absorption maximum the residues were resuspended for TLC and FT-IR.

Thin layer chromatography

The ether extract thus obtained of test and control (reaction mixtures) was spotted on the bottom of silica gel in thin layer chromatography (TLC). The sample was chromatographed on the TLC silica gel plate using the solvent systems benzene / hexane / acetic acid in the ratio of 8.5:1.5:0.5v/v. The silica gel plate was placed in the solvents in a developing chamber, so that only the very bottom of the plate was in the liquid. It was mobile phase and was slowly rises up by capillary action. When the solvent has reached about the 3/4th of the plate, plate was removed from the chamber, dried at room temperature and the separated components were visualized by using UV analyzer.

Fourier Transform Infra Red Spectroscopy (FT/IR)

The ether extract of test and control prepared in diethyl ether were subjected to FT/IR analysis. IR spectrometer passes infrared red radiation through the control and sample of unknown compound and uses a detector to plot percent transmission of the radiation through the molecule versus the wave number of the radiation. Some of the radiation was absorbed by the sample and some of it was passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular finger prints of the sample.

High Performance Thin Layer Chromatography (HPTLC)

The ether fraction of the control and sample were taken for HPTLC. For carrying out the procedure the control and sample were first filtered and then spotted on the TLC plate. Solvent mixture (Benzene: Hexane: Acetic acid) was prepared in the ratio of 8.5: 1.5: 0.5 and the plate was kept for band formation in the HPTLC chamber. The bands formed were visualized in UV illuminator at 254 nm and 366 nm. The plate was kept in TLC scanner and peaks were viewed using WinCATS software.

Gas Chromatography and Mass Spectrometry (GC/MS)

The ether extract of both control and sample were subjected to GC/MS. GC/MS was done at Department of Applied Chemistry, Cochin University of Science and Technology, Cochin.

Results and Discussion

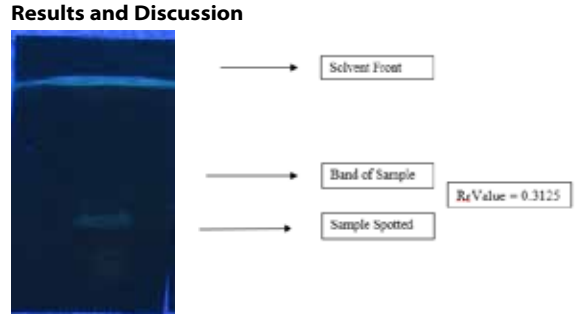

Fig.1. TLC of the ether extracts of biodegraded benzene in 16 hr incubation.

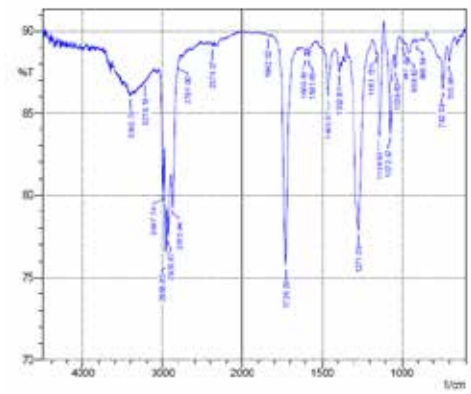

Fig.2. FT/IR Absorption peaks of ether extracts of biodegraded benzene after 16 hr incubation and 32 hour incubation.

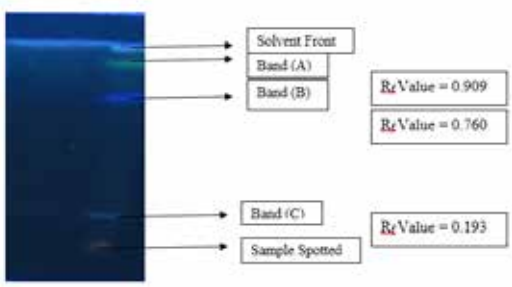

Fig.3. TLC analysis of ether extracts of biodegraded sample in 32 hr incubation

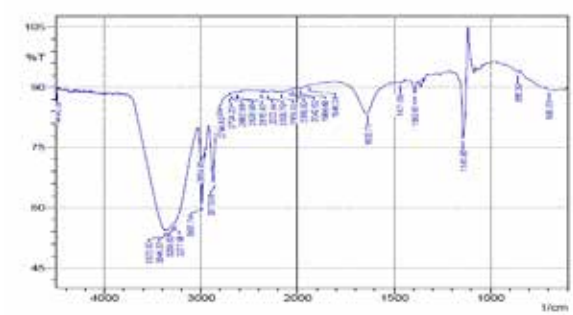

Fig.4. FT/IR Absorption peaks of ether extracts of biodegraded sample in 32 hr incubation

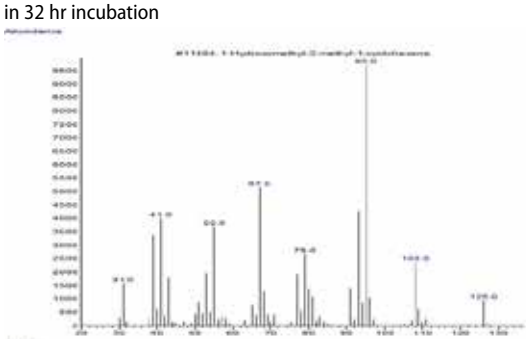

Fig. 5 The MS of the additional peak observed in 48 hour biodegraded sample.

Biodegradation is the very effective mechanism to remove the benzene from the contaminated area. Benzene is considered as one of the main human carcinogen. Benzene cause many health hazards and environmental pollution. [5,6] So it is very necessary to degrade or remove the benzene. In the present study *Bacillus megaterium* is used to degrade the benzene by aerobic degradation mechanism. *Bacillus megaterium* used in this study, have the capability to grow on benzene and it can utilize benzene as their sole source of carbon. The pathway of degradation and the degradation products could be analyzed by TLC, FT/IR, HPTLC and GC/MS.

The nature and number of the formed intermediates/metabolites were analyzed by TLC (Thin Layer Chromatography) in the 6 interval of time (8hour, 16hour, 24hour, 32hour, 40hour and 48hour). The solvent system used for doing TLC is Benzene: Hexane: Acetic acid in the ratio of 8.5: 1.5: 0.5.

Fourier transform spectroscopy is emerging as the most reliable quick analytical tool to explain the functional group changes taking place in an organic compound during biodegradation [7]. In the present investigation on benzene biodegradation, FT/IR analysis could establish the progressive changes effected in the structure of the compound during the presence of biodegradation. In the FT/IR spectrum of 8hr biodegraded sample the new peak at 3300 cm^{-1} region represented O-H group introduction into the aromatic ring of benzene. This was followed by the presence of many more peaks in the FT/IR of 16hr sample. The spectrum represented many new peaks in the range 1271 cm^{-1} – 1039 cm^{-1} indicating new C-O bond, in the range 1726 cm^{-1} strongly representing the –C=O and in the range 900 cm^{-1} – 1000 cm^{-1} indicating shift in the – C=C- of aromatic ring due to the introduction of 'OH' group 24hr FT/IR result gave strong evidence to suggest the presence C-O bond (1100-1800 cm^{-1}) and O-H (2880-3400 cm^{-1}). –O-H stretching and C=C shift in the aromatic ring was further confirmed by the FT/IR at 32 hr of incubation. The same trend was continued in the FT/IR of 40 hr and in 48 hr sample FT/IR spectrum, there was the significant presence of carboxylic acid along with the –C-O and 0-H representations confirming the degradation of benzene. Therefore FT/IR is a valuable tool to trace and to confirm the processes of biodegradation. This approach not only helps in the confirmation of biodegradation but also helps in the analysis of the nature of the intermediates formed.

Benzene degradation has been initiated in 16 hr incubation by *Bacillus megaterium*. Hence the degradation rate is faster than 8 hr incubation.In the FT/IR spectrum of 16 hour treated sample there were peaks representing –OH stretching along with that of –O-H bending. The most significant observation was that of ketone group represented by peak at 1760 cm^{-1} as a very strong band. This has even resulted in the shifting of double bonds in the benzene ring.[8,9] A progressive structural change in the oxidative degradation of benzene is well reflected in these FT/IR spectrums. Three bands were formed in the ether extracts of sample in 32 hr incubation. Formation of intermediates indicated the presence of bands on the TLC plate. Majority of benzene is degraded in 32 hr incubation period compared to other incubation times. Hence the degradation rate is very fast in 32 hr incubation by *Bacillus megaterium*. 32 hour FT/IR spectrum supported the existence of –OH stretching in carboxylate ion by giving a representation at 2615 cm^{-1}.The electron density change in the benzene ring was also reflected as the band at 1633 cm^{-1}. On comparing the GC of the control with that of 16 hour treated sample it is evident that the peak at 1.792 minute was absent in the GC of the 16 h sample whereas a new peak was present at 4.49 minute.

The MS resolution of the peak at 4.4 minute supported the existence of an intermediate compound with molecular weight 125.This may be indicative of the hydroxylated derive of the intermediates of meta pathway. In the spectrum of 48 hour sample there was considerable increase in the representation at 4.4 minutes as similar to the observation made in the case of 16 hour sample .Both the MS spectrum represented the fraction with highest molecular weight as 126 this might be representing the intermediates of benzene degradation probably representing muconic semialdehyde, the intermediate in the meta pathway degradation of benzene.

Conclusion

Analytical techniques used in the study of biodegradation of organic compounds are of high cost and often requires immense skill in performing the analysis. For preliminary confirmation of the process

of biodegradation FT/IR is a convenient tool. If it could be supported by TLC analysis, the interpretation would become consistent and dependable. More than that in the tracing of the fate of biodegradation one has to closely observe the conversion of the substrate to product through large number of intermediates. Naturally, any biodegradation profiling would necessitate large number samples for instrumental analysis which signifies the requirement of easy and cheap analytical methods like FT/IR .However the results need to be confirmed with sophisticated analytical techniques like GC/MS or MS/MS.

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