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Formulation of a novel bacterial consortium for the effective biodegradation of phenol

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Abstract. Phenol is frequently present as the hazardous pollutant in petrochemical and pesticide industry wastewater. Because of its high toxicity and carcinogenic potential, a proper treatment is needed to reduce the hazards of phenol carrying effluent before being discharged into the environment. Phenol biodegradation with microbial consortium offers a very promising approach now a day's. This study focused on the formulation of phenol degrading bacterial consortium with three bacterial isolates. The bacterial strains *Bacillus cereus* strain VCRC B540, *Bacillus cereus* strain BRL02-43 and *Oxalobacteraceae* strain CC11D were isolated from detergent contaminated soil by soil enrichment technique and was identified by 16s rDNA sequence analysis. Individual cultures were degrade 100 µl phenol in 72 hrs. The formulated bacterial consortium was very effective in degrading 250 µl of phenol at a pH 7 with in 48 hrs. The study further focused on the analysis of the products of biodegradation with Fourier Transform Infrared Spectroscopy (FT/IR) and Gas Chromatography-Mass Spectroscopy (GC-MS). The analysis showed the complete degradation of phenol and the production of Benzene di-carboxylic acid mono (2-ethylhexyl) ester and Ethane 1,2- Diethoxy- as metabolic intermediates. Biodegradation with the aid of microorganisms is a potential approach in terms of cost-effectiveness and elimination of secondary pollutions. The present study established the efficiency of bacterial consortium to degrade phenol. Optimization of biodegradation conditions and construction of a bioreactor can be further exploited for large scale industrial applications.

Keywords: bacterial consortium; fourier transform infrared spectroscopy; gas chromatography-mass spectroscopy; phenol biodegradation

1. Introduction

The chief pollutants of the ecosystem are the chemicals used in day to day life activities and these pollutants are produced as by-products through different industries. Recalcitrant Xenobiotics compounds are non-degradable and resistant to being broken down through chemical processes. Therefore these xenobiotics are treated using microorganisms into smaller, more manageable set of compounds. Since the start of the industrial revolution with poor management practices, a large variety of aromatic compounds has been introduced into the ecosystem through anthropogenic activities. Aromatic pollutants are considered as the most toxic environmental pollutants because

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they are comparatively persistent in the environment due to the high thermodynamic stability of its benzene moiety and its unusual properties. Most important aromatic pollutants like phenol and related compounds are produced now a days and the disposal of effluents containing these hazardous chemical without proper treatment cause negative impact on ecosystem (Chakraborty and Coates 2004).

Many reviews reported the success of pure and mixed cultures of *Pseudomonas* genus in phenol biodegradation. From a site contaminated with medical wastes and wastewater, Mohanty and Jena (2017) isolated a pure culture *Pseudomonas sp.* Strain NBM11 which can degrade up to 1000 mg/L of phenol completely within a pH range of 6.8 to 7.2 and an incubation temperature between 30℃ and 32℃. Mishra and Kumar (2017) used a high efficiency bacterium *Pseudomonas putida* for the biodegradation of phenol. Many researchers reported the isolation of many bacteria from different regions (Deng *et al.* 2018, Sepehr *et al*. 2019). Samimi and Moghadam (2020) reported the isolation of *Halomonas elongata* strain O-CH1for the effective degradation of phenol. Investigation by Bui *et al*. (2012) formulated an aerobic bacterial consortium from soil contaminated with dioxin and the consortium degrades phenol very effectively. Maulana *et al*. (2017) reported the formulation of bacterial consortium from cattle rumen for phenol degradation. A bacterial consortium with two *Ochrobacterium sp.* and *Pseudomonas sp.* formulated from industrial effluent was effective to use phenol and able to degrade phenol at haloalkaline condition (Chandrasekaran *et al*. 2017).

Many times a single microorganism is not so efficient in degrading many organic compounds. The mixture of different strains exhibits better degradative capacities than single strain. In nature, synchronized biodegradation of aromatic pollutants typically involves a series of microbial species and therefore emphasis has been given on the development of microbial consortia with broad enzymatic capabilities. The main objective of the study was to formulate a bacterial consortium for the better degradation of phenol and analyse the metabolic products during biodegradation process. Based on the available information, this is the first report discussing the phenol biodegradation potential of *Bacillus cereus* strain VCRC B54*0, Bacillus cereus* strain BRL02-43 and *Oxalobacteraceae* strain *CC11D* as a consortium.

2. Material and methods

2.1 Enrichment and screening of phenol degrading bacterial isolates

Phenol degrading bacterial strains were isolated through soil enrichment procedure (Nair and Shashidhar, 2004) from a homogenized mixed soil sample collected from laundry area in Kakanad, Kerala, India. The collected soil sample was progressively enriched with phenol. The enrichment was initiated at a concentration of 25 µl of phenol in 100 ml soil extract and enriched up to a maximum concentrations of 100 µl of phenol. The culture was kept on a rotary shaker at 150 rpm at 37ºC up to 5 days. The soil sample was pour plated with nutrient agar medium. All the individual colonies isolated by soil enrichment technique were individually inoculated into 100 ml Mineral Salt (MS) medium with 100µl of phenol as the only carbon source. The composition of the basal medium used was 1 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Mg $SO_4.7H_2O$ and 0.001 g $CaCl_2$ in 1 litre of the distilled water at pH 7. The isolates which showed growth in MS-P medium were selected as the phenol degrading strains.

2.2 Identification of phenol degrading strains

Pure isolates were sent to Scigenome labs, Pvt Ltd, Cochin, Kerala, to amplify and sequence 16S rDNA gene for identification using the universal forward primer sequence (5´-AGA GTT TGA TCM TGG CTC-3´) and the reverse sequence (5´-AAG GAG GTG WTC CAR CC-3´). The final concentration of the reagents were 1 mM $MgCl_2$, 200 µM dNTP, 100 pmol primers and 50 ng DNA (Chun *et al.* 1995). Polymerase Chain Reaction (PCR) was carried out in Mycycler™ (Bio-Rad, USA) with the following PCR Cycle: one cycle at 94ºC for 2 min, followed by 35 cycles at 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min, followed by final 2 min incubation at 72ºC and the PCR products were sequenced. The sequence similarity was analysed by sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST (Basic Local Alignment Search Tool) analysis and the strains were identified on the basis of the best match in the database.

2.3 Inoculum preparation and formulation of consortium

One loopful of each of the selected cultures was individually inoculated to 50 ml nutrient broth containing 25 µl phenol and the flasks were incubated over night at 37ºC at 150 rpm. After the incubation, cells were harvested by centrifugation and suspended separately in physiological saline (0.85% NaCl) to obtain the inoculum of 1.OD concentration (Vijayan *et al*. 2014). Microbial consortia formulation for the degradation of phenol was done by evaluating the degradation capacity of individaul isolates through analytical technique FT/IR**.** Individual bacteria were grown in nutrient broth and the flasks were incubated over night at 37ºC. From the culture the cells were harvested by centrifugation and resuspended in sterile saline to yield an absorbance reading of 0.5 at 540 nm (Ghazali *et al.* 2004). The consortium was constituted by mixing equal proportions of all the three isolated bacterial strains.

2.4 Evaluation of phenol degradation

The inoculum prepared from individual cultures and the formulated consortium were used for biodegradation of MS medium with 250 µl of phenol. The biodegradation was continued up to 72 hrs. After removing the cells by centrifugation at 10,000 rpm for 10 minutes, the supernatant was subjected to solvent extraction with diethyl ether and the biodegradation was monitored by FT/IR analysis.

2.5 Phenol degradation assay

The efficiency of bacterial consortium to degrade phenol was carried out in different 250 ml conical flasks contain 100 ml Mineral Salt media. Add 250 µl of phenol to each flask. All the flasks were inoculated with 3 ml of formulated consortium except the control sample. The uninnoculated mineral salt (MS) medium with 250 µl phenol at pH 7 was taken as the control sample. The control MS-P medium was scanned between 200-300 nm in a UV spectrophotometer (Hitachi) immediately after the medium preparation (0 hr) to determine the particular wavelength at which the sample showed maximum absorption. The absorption maximum ie, the λ max shown by the control medium was used for the biodegradation assay. All inoculated media were incubated at room temperature on a rotary shaker at 150 rpm for different time intervals like 8, 16, 24, 32, 48 and 72 hrs and scan all the media between 200 to 300 nm.The λ max shown by the

control MS-P medium was used for the estimation of biodegradation rates of phenol at various intervals.

2.6 Analysis of the products of phenol degradation

The supernatant after biodegradation was extracted with the solvent diethyl ether. The extract was concentrated by evaporation and used as the biodegraded sample. The sample was subjected to FT/IR and GC/MS. FT/IR was done at mid IR region (400–4000 cm−1) using Shimadzu-8400 FT/IR spectrophotometer. GC-MS wa done using Agilent 6890 GC/5973 MS with J and W HP5-MS column (300 x 2.5 mm) at Department of Chemical Oceanography, Cochin University. This was done by using 1 µl of each samples and Helium with flow rate1 ml/min was used as a carrier gas. The temperature gradient program used was;initial 40ºC, 2 min holds and at a rate of 10ºC per min to 280ºC, hold for 4 minute for control phenol and initial 60ºC, 1 min holds and at a rate of 10 ºC per minute to 130ºC, hold for 4 min, then at a rate of 3ºC per min to 280ºC and final hold for 15 min for the bacterial treated samples. The MS conditions used were: Scan range 12-600 m/z; Source temperature: 200ºC.

3. Results and discussion

3.1 Identification of phenol degrading strains

The experimental technique enrichment culture has been used widely by microbiologists to isolate microorganisms with specific desirable characters in pure culture. The isolation of microorganisms by enrichment method is mostly based on the specific metabolic capability. Selection of specific growth conditions allow the favoured type to grow faster than other types of microorganisms. This basic techniques and later refinements have been used extensively to obtain pure cultures of many microorganisms which participated in the biodegradation of many toxic chemicals. This technique has given key information's on the metabolic characteristics of thousands of microorganisms.

In this study, three different baterial isolates were selected for the effective biodegradation of phenol. In an attempt to screen phenol degrading strains through soil enrichment technique, three isolates viz., *Bacillus cereus* strain *VCRC B540, Bacillus cereus* strain *BRL02-43* and *Oxalobacteraceae* strain *CC11D* were selected as the potent degraders. The identity of the three strains isolated by soil enrichment technique was confirmed by molecular identification based on 16S rDNA analysis. The size of the amplicons were found to be 1500 bp in 1% agarose. The 1500 bp amplicons were sequenced and the strains were identified by examined the top-score sequences from the BLAST search results.The gene sequences of the isolated strains were compared with the existing NCBI database.

3.2 Inoculum preparation and formulation of consortium

Currently there is little guidance with respect to the preparation of the inoculum that is participated in the biodegradation of xenobiotic pollutants. The pelletized inoculum sources were washed with physiological saline to limit the presence of carbon sources used during inoculation preparation. According to many studies inoculum samples with particular cell densities

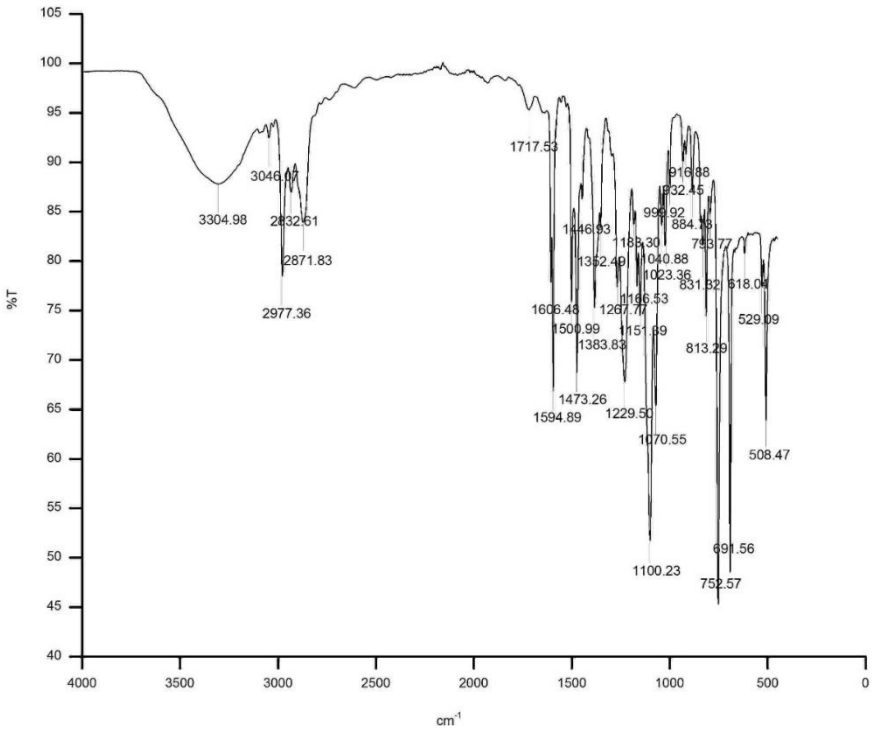
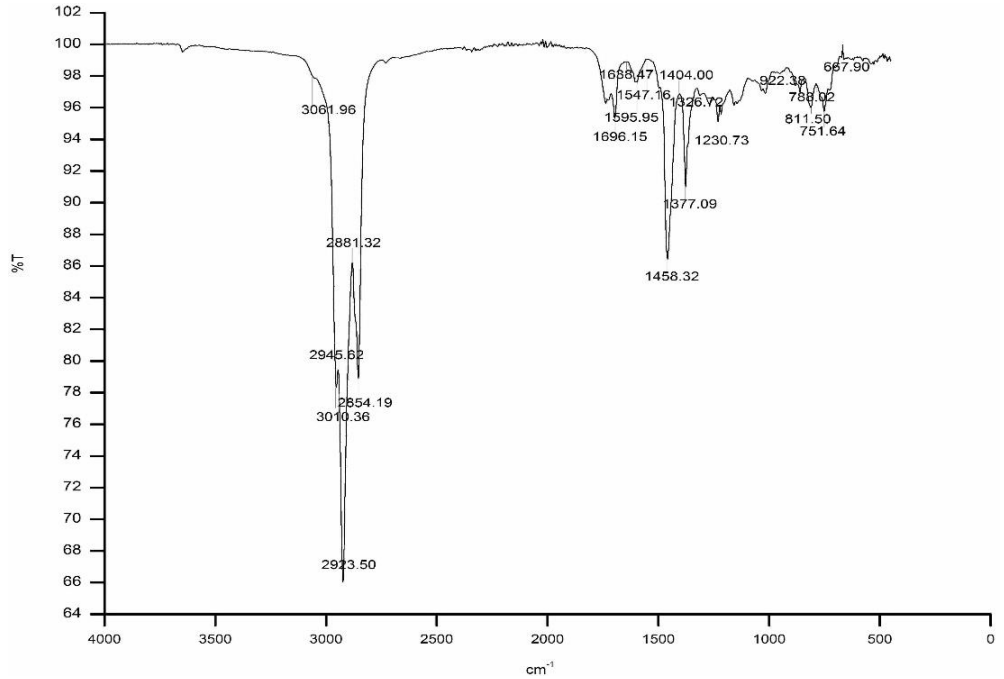


Fig. 1 FT/IR spectrum of phenol control-uninoculated

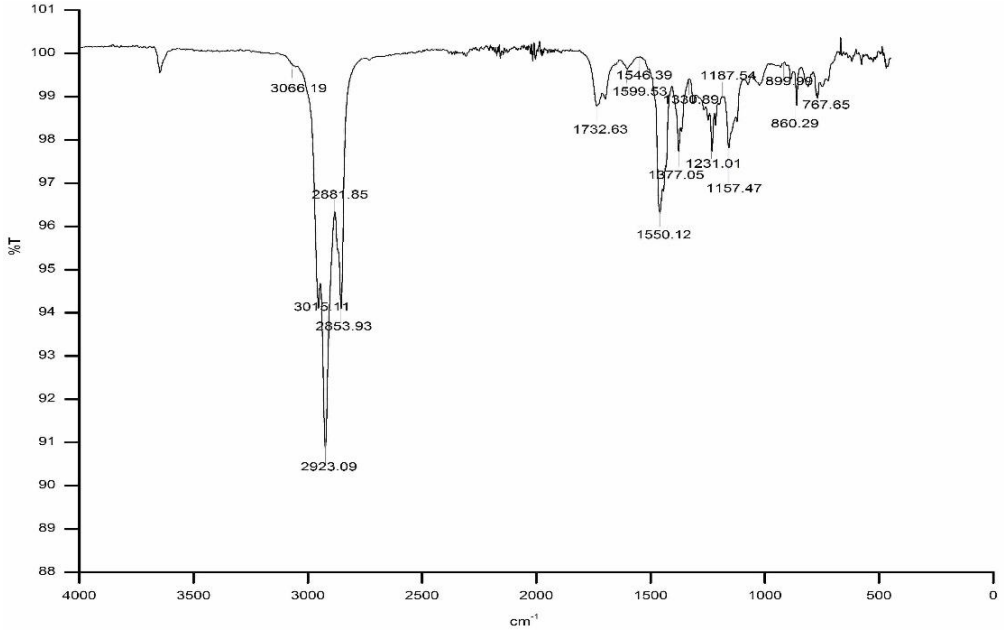
were essential for the success of bioremediation. A small inoculum density required more time for cell multiplication (Jin *et al*. 1998).The low cell density led to insufficient number of microbial cells and reduced the production of sufficient amount of enzymes for biodegradation. But very high inoculums densities act as the reason for the lack of oxygen and depletion of nutrients (Abusham *et al*. 2009). In this study a standardized inoculum with 0.5 absorbance reading was used successfully to determine the effectiveness of each strain during biodegradation. The microbial consortium for biodegradation studies was formulated by mixing equal proportions i.e., 3 ml of each culture.

3.3 FT/IR analysis of the biodegradation of Phenol by individual inoculum

During the biodegradation of mixture of organic compound by a microbial consortium, the determination of individual compound degradation is essential. When compared with the un-inoculated control sample (Fig. 1) FT/IR analysis of the mineral salt phenol medium [MSPM] individually inoculated with *Bacillus cereus* strain *VCRC B540, Bacillus cereus* strain *BRL02-43* and *Oxalobacteraceae* strain *CC11D* showed the shift or disappearance of many of the specific bands represents phenol (Figs. 2(a)-2(c) with in 72 hrs. This disappearance indicated the structural changes in the functional groups H bonded O-H stretch, C-H stretch, C=C stretch, C-O stretch, C-H bend. The shift or disappearance of corresponding vibrations indicated the effective participation of individual bacterial strains *Bacillus cereus* strain *VCRC B540, Bacillus cereus* strain *BRL02-43* and *Oxalobacteraceae* strain *CC11D* on the degradation of phenol.

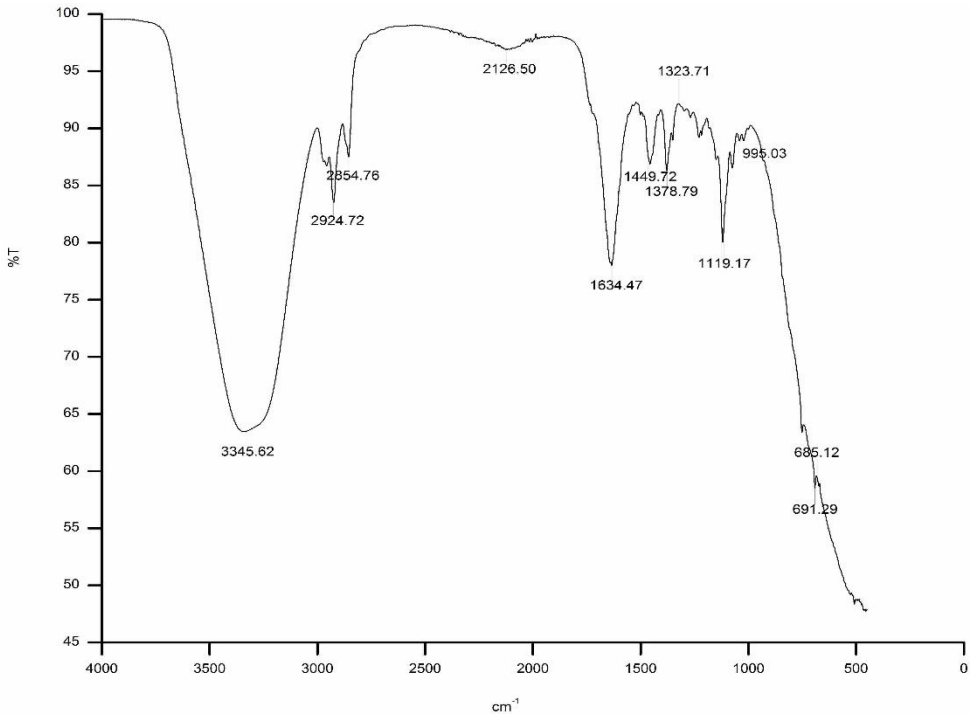


(a)



(b)

Fig. 2 (a)-(c) FT/IR spectra of biodegraded phenol by individual strains. (a) FT/IR spectrum of Bacillus cereus strain VCRC B540, (b) FT/IR spectrum of Bacillus cereus strain BRL02-43 and (c) FT/IR spectrum of Oxalobacteraceae strain CC11D



(c)

Fig. 2 Continued

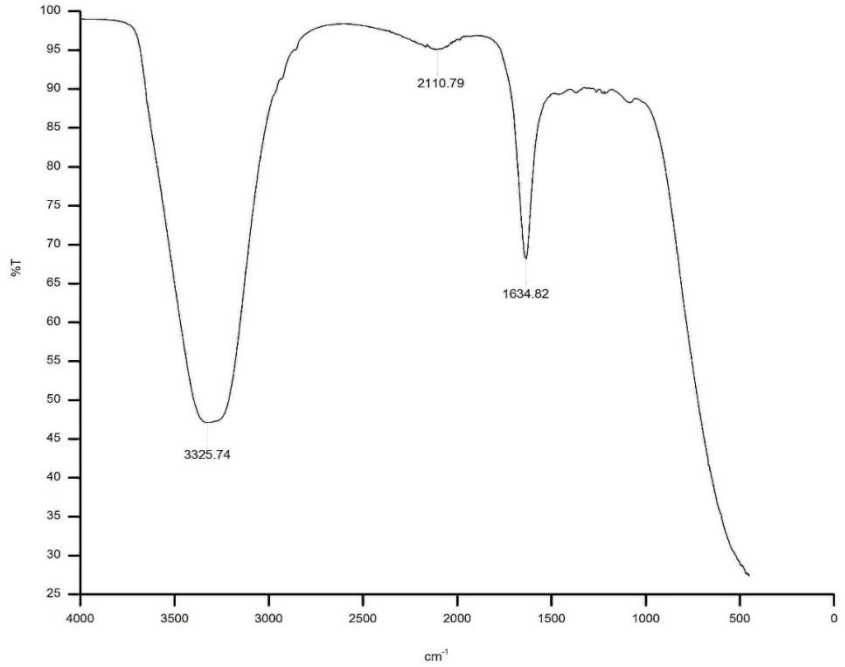


Fig. 3 FT/IR spectrum of biodegraded phenol by the formulated bacterial consortium

Table 1 Percentage degradation of phenol at different incubation time with the formulated consortium

Wave length: 251 nm	**0 hr**	8 hrs	16 hrs	24 hrs	32 hrs	**48 hrs**	72 hrs
Absorption at 251 nm	**3.98**	2.055	1.117	0.717	0.697	**0.511**	1.232
% degradation of Phenol	-	3.98-2.055 / 3.98 =48.366	3.98-1.117 / 3.98 = 71.93	3.98-0.717 / 3.98 = 81.98	3.98-0.697 / 3.98 = 82.89	3.98-0.511 / 3.98 **= 87.16**	3.98-1.232 / 3.98 = 69.05

3.4 FT/IR analysis of the biodegradation of Phenol by formulated consortium

The advantages of developing mixed cultures during bioremediation have been widely demonstrated by many reiewers (Rambeloarisoa *et al*. 1984, Prpich and Daugulis 2005, Sarkar *et al*. 2011).The higher diversity of microbial strains in a mixed culture had often been postulated as the main reason for the success of biodegradation of toxic compounds. It could be attributed to the effects of synergistic interactions among members of the association. During such interactions one species removes the toxic metabolites that are produced by other species in the consortium. So each individual strains got benefited during such interactions which may led to the complete degradation of toxic chemicals (Mukred *et al*. 2008). In this study, FT/IR spectral analysis (Fig. 3) strongly supported the fact that these three isolates *Bacillus cereus* strain *VCRC B540, Bacillus cereus* strain *BRL02-43* and *Oxalobacteraceae* strain *CC11D* could be used for the formulation of an effective microbial consortium for the effective biodegradation of phenol with in 48 hrs.

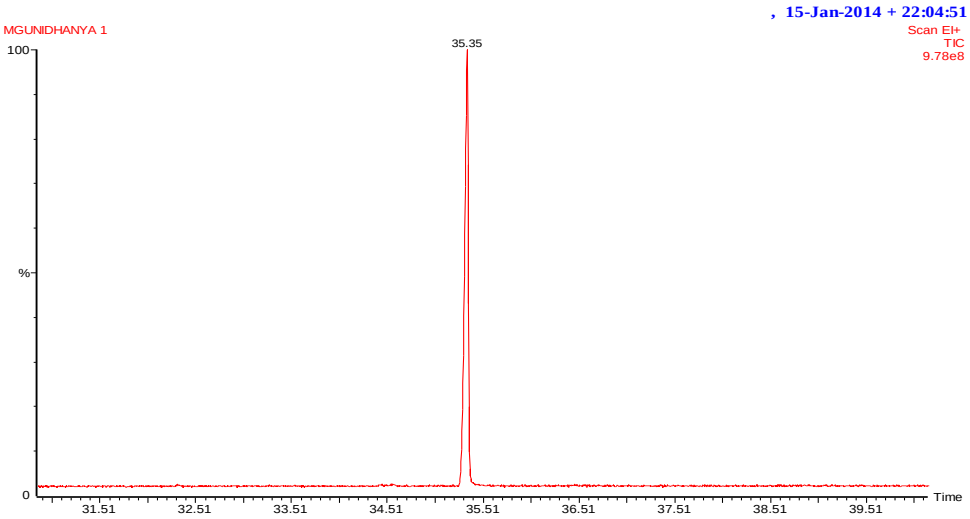
3.5 Phenol degradation assay

The presence of phenol and the rate of phenol biodegradation were again confirmed by using spectroscopic analysis. This method is faster, less expensive and more available than any other methods for the analysis of volatile organic compounds. The absorbance of sample pollutant before and after treatment was measured using UV/Vis spectrometer. MS medium with 250 µl of phenol was taken as the control. Samples treated with bacterial consortium at different time intervals were used as the test samples. All the samples were scanned from 300 nm- 200 nm. The UV absorption spectra of control sample shown a very good absorbance in the wave length 251 nm with a reading 3.98 was used for the detection of phenol degradation rate during different time intervals. Table 1 indicated the increase in the % degradation of phenol along with the increase in the incubation time. The degradation rate of phenol was gradually increased from 24 to 48 hrs and the maximum was at 48 hrs and then showed a decrease after this optimum incubation time.

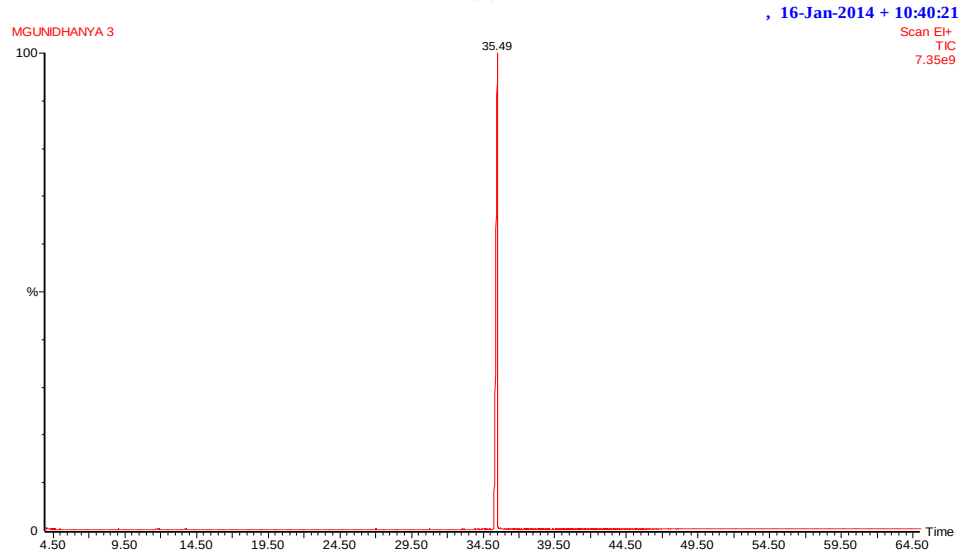
3.6 Analysis of the products of phenol degradation

Product analysis during biodegradation is important to get an idea about the mechanism of degradation and the type of products formed through the biodegradation process. In this study the product formation after phenol biodegradation was analysed by FT/IR, TLC and GC-MS analysis.

FT/IR analysis of consortium inoculated medium showed the absence of many of the specific bands of phenol on biodegradation. The structural changes indicated in the representation of C-H stretch, C=C stretch, C-O stretch and C-H bends in the FT/IR analysis spectra indicated the



(a)



(b)

Fig. 4 (a) and (b) Analysis of the extract of biodegraded MS - P medium ((a): spot 1 and (b): spot 2) by Gas chromatography

biodegradation of phenol. FT/IR analysis of the biodegraded phenol medium indicated the introduction of a ketonic group which was represented in the wave number range 1625-1750 cm^{-1}. The library search report (Correlation Table for Characteristic Raman and Infrared frequencies), indicated that vibrations, 1625 cm^{-1} – 1649 cm^{-1} are representing symmetric C=O stretch of ketones. The FT/IR spectrum of samples also showed the presence of vibration in the range 1000 cm^{-1}- 1300 cm^{-1} indicating the presence of C-O stretch of esters and carboxylic acids formed by the biodegradation of phenol in the medium. The characteristic changes in the vibration range indicated the production of ketonic, carboxilic acid/esters in the biodegraded sample.

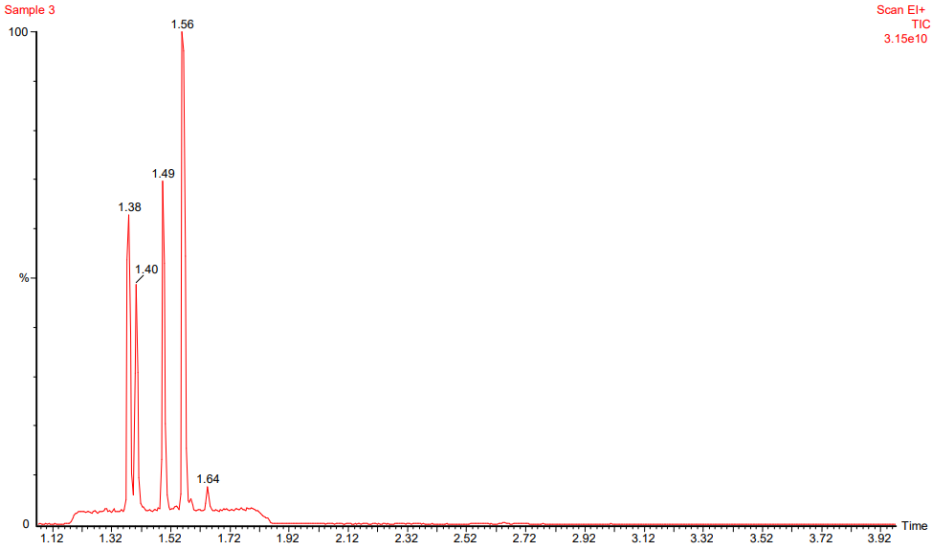
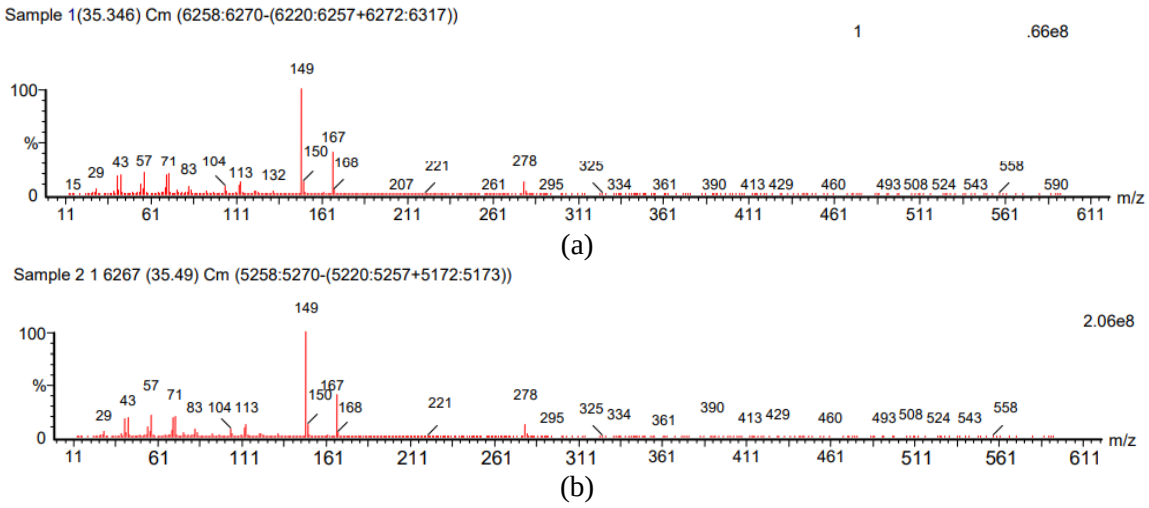


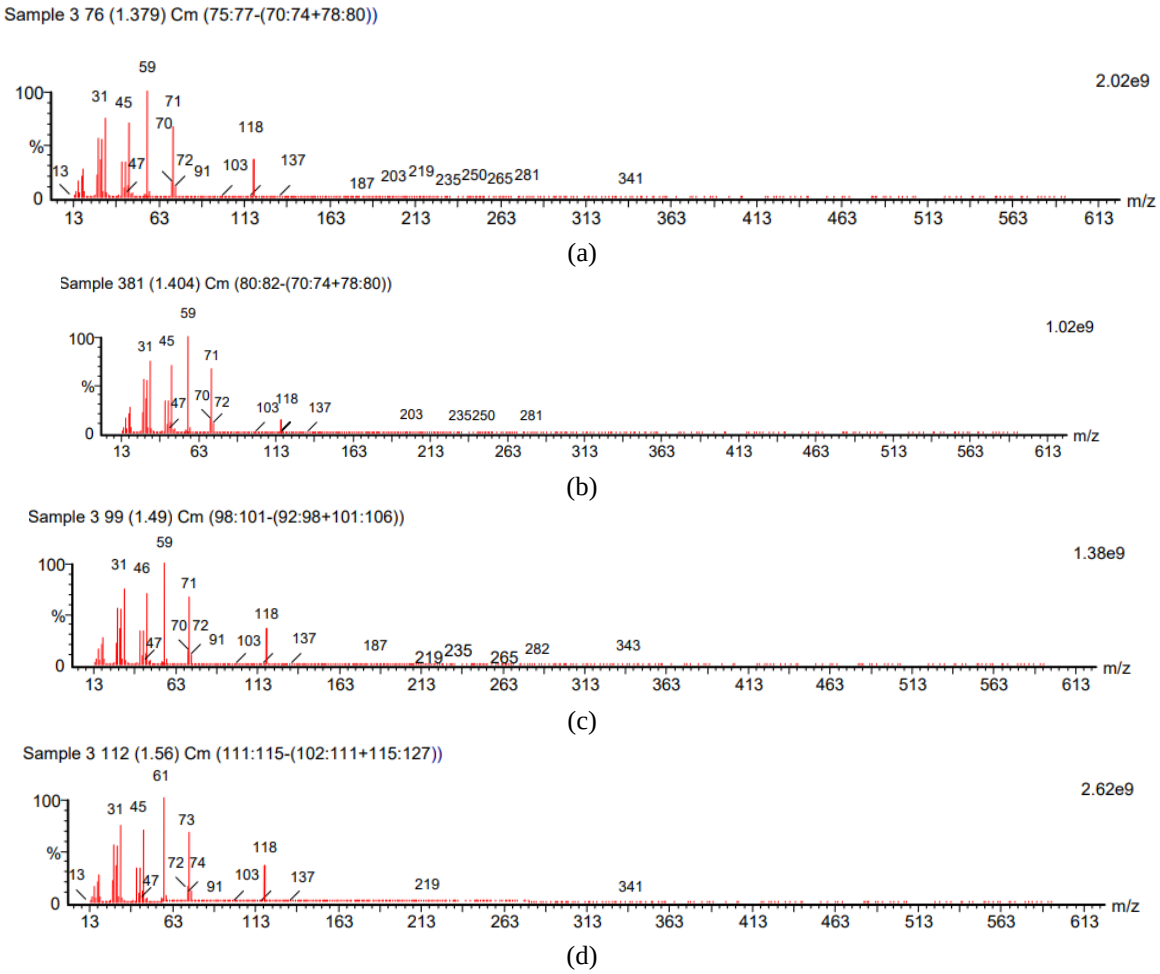
Fig. 5 Analysis of the extract of biodegraded MS-P medium (spot 3) by Gas chromatography



Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	986	972	1,2-BENZENEDICARBOXYLIC ACID, MONO(2-ETHYLHEXYL) ESTER	278	C16H22O4	4376-20-9
2	982	962	1,2-BENZENEDICARBOXYLIC ACID, DIISOOCTYL ESTER	390	C24H38O4	27554-26-3
3	976	964	DI-N-OCTYL PHTHALATE	390	C24H38O4	117-84-0
4	968	948	BIS(2-ETHYLHEXYL) PHTHALATE	390	C24H38O4	117-81-7
5	963	956	1,2-BENZENEDICARBOXYLIC ACID, DIISOOCTYL ESTER	390	C24H38O4	27554-26-3
6	958	949	DI-N-OCTYL PHTHALATE	390	C24H38O4	117-84-0
7	951	910	BIS(2-ETHYLHEXYL) PHTHALATE	390	C24H38O4	117-81-7

Fig. 6 (a) and (b) Mass spectroscopic analysis of the peaks at 35.35 ((a): spot 1) and 35.49 min. ((b): spot 2) in the gas chromatogram of the extract of biodegraded MS phenol medium

Thin Layer Chromatography is the simplest, sensitive and inexpensive method used for the determination of different products formed during biodegradation process. The mobile phase used



Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	938	932	ETHANE, 1,2-DIETHOXY-	118	C6H14O2	629-14-1
2	885	882	ETHANE, 1,2-DIETHOXY-	118	C6H14O2	629-14-1
3	749	748	2-PROPANOL, 1-(1-METHYLETHOXY)-	118	C6H14O2	3944-36-3
4	710	653	CYCLOSERINE	102	C3H6O2N2	68-41-7
5	707	705	ACETIC ACID, (2-ETHOXY)ETHOXY-, METHYL ESTER	162	C7H14O4	16326-34-4
6	660	655	1-PROPENE, 3-[(1-METHYLETHYL)THIO]-	116	C6H12S	50996-72-0
7	648	493	13,13-DIMETHYL-3,6,9-TRIOXA-13-SILATETRADECAN-1-OL	264	C12H28O4Si	900213-34-9
8	619	615	ETHANE, 1,2-DIETHOXY-	118	C6H14O2	629-14-1
9	618	617	1,3-OXATHIANE, 2,2-DIMETHYL-	132	C6H12OS	5809-68-7
10	612	609	CYCLOSERINE	102	C3H6O2N2	68-41-7
11	610	604	SILANE, TRIMETHYLPROPYL-	116	C6H16Si	3510-70-1
12	601	597	DL-ERYTHRO-O-METHYLTHREONINE	133	C5H11O3N	900214-70-7
13	593	549	XYLOPYRANOSIDE, METHYL 4-AZIDO-4-DEOXY-3-O-METHYL-, .ALPHA.-D-	203	C7H13O4N3	18390-81-3

Figs. 7(a)-(d) Mass spectroscopic analysis of the peaks at 1.38, 1.40, 1.49 and 1.56 (spot 3) mints in the gas chromatogram of the extract of biodegraded MS phenol medium

in TLC separation was selected based on the migration of the products through the TLC plate. Benzene: Hexane: Acetic acid in the ratio 9:1.5:0.5 was chosen and used for TLC analysis for the screening of products. The three bands representing the formed degraded metabolites were scraped from the TLC plates and subjected to GC-MS analysis.

The GC analysis of three spots obtained during TLC separation of the products formed during the biodegradation of phenol showed peaks at retention times 35.35 min, 35.49 min (Figs. 4(a)

and 4(b) and 1.38, 1.40, 1.49, 1.56 (Fig. 5).

The MS analysis of each of the peak obtained during GC analysis gave clear knowledge about the products formed due to the biodegradation of phenol with the bacterial consortium. The MS analysis spectrum of peaks at retention times 35.35 min and 35.49 min (Figs. 6(a) and 6(b) indicated the production of Benzenedicarboxylic acid, mono (2-ethylhexyl) ester as the metabolite. This phthalate esters are compounds containing 16 carbon atoms and with a high molecular weight 278.The study by Kumar *et al.* (2013) indicated the production of Benzenedicarboxylic acid, mono (2-ethylhexyl) ester during the biodegradation of mobil oil hydrocarbons by a biosurfactant producing bacterial consortium.

Mass spectroscopic analysis of the peaks in the gas chromatogram of the extract of biodegraded MS-P medium-spot 3 (Fig. 5) showed that the fraction with highest molecular weight is that of 118 (Figs. 7(a)-7(d) and hence the peak corresponds to that of Ethane 1,2-Diethoxy-.

There was no indication of phenol in the gas chromatogram after biodegradation. This confirmed that the phenol supplemented in the mineral salt (MS) medium was completely degraded by the formulated consortium.

The new compounds might have formed as products by the bacterial consortium during its restricted growth in MS-P medium where the four bacterial strains were forced to use phenol as the only carbon and energy sources. 1, 2- Benzenedicarboxylic acid, mono (2- ethylhexyl) esters are used as effective drugs against life threatening diseases (Krishnan *et al*. 2014). The same compound used as antimicrobial, antifouling, plasticizer and PVC softening materials which are less toxic to human health. In short, the bacterial consortium used in this study, along with the degradation of phenol was capable of synthesizing biodegraded products which can also used in industrial and pharmaceutical applications.

4. Conclusions

In the present study bacterial consortium of *Bacillus cereus* strain *VCRC B540*, *Bacillus cereus* strain *BRL02-43* and *Oxalobacteraceae* strain *CC11D* enriched from detergent condaminated area was able to utilize phenol as carbon and energy source, and degradae phenol effectively than individual bacterial isolates. Analysis of the product of biodegradation with TLC, FT/IR and GC-MS showed the complete degradation of phenol and the production of Benzenedicarboxylic acid, mono (2-ethylhexyl) ester and Ethane 1,2-Diethoxy- as intermediate products.Through this study, the formulated bacterial consortium can serve as versetile participant for the treatment of phenol present in many of the industrial effluents.

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