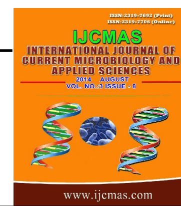

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Original Research Article

High strength phenol degradation by CSMB4 at microaerophilic condition

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A B S T R A C T

Industrial effluents containing phenolic compounds are toxic to biological systems and pose problems with regard to meeting the environmental regulations. In this study, a bacterial strain was isolated from an anaerobic sludge by enriching with phenol as sole carbon at microaerophilic conditions, using online controlled biosensors. Biochemical analysis showed that the bacterial strain CSMB4 belonged to the genus Alcaligenes and 16S rRNA gene analysis identified it as Alcaligenes faecalis subsp. faecalis strain AE1.16, with genbank accession number GQ284565.1. The strain CSMB4 degrades 1000 mg/l concentration of phenol as sole carbon and energy within 24 h of incubation time. Incubation time increases with increase in phenol concentration and it can tolerate phenol even at an elevated concentration of 2000 mg/l, achieving 60% degradation. CSMB4 showed metabolic diversity by degrading a wide spectrum of phenolic compounds like Nonyl phenol, Phenolic formaldehyde resin, Wattle extract, Naphthalene sulfonic acid, Catechol, Benzoate, Hydroxy benzoate by utilising them as sole carbon. Biodegradation of phenol was confirmed by the intracellular specific activity of phenol hydroxylase with 547 U/mg. The presence of 1568 U/mg of intracellular specific activity of catechol 1, 2 oxygenase, confirms the biodegradation of phenol by CSMB4 is through ortho cleavage pathway. FTIR spectrum also confirmed the ortho cleavage of phenol, by a complete shift of bands in the aromatic to aliphatic region, with the appearance of bands corresponding to acids, aldehydes and ketones due to the presence of intermediary product catechol and cis,cis.muconate by this bacterial strain CSMB4 *Alcaligenes faecalis subsp. faecalis* strain AE1.16.

Keywords

Alcaligenes faecalis subsp. faecalis strain. Phenol. Microaerophilic Ortho cleavage. FTIR.

Introduction

Phenolic derivatives are used as raw materials in textiles, pulp and paper, petrochemical, pharmaceutical, oil-refineries and leather industries. These heterocyclic aromatic chemicals are among the most frequently found pollutants in industrial effluents. Since these compounds are toxic either through ingestion, inhalation or contact even at low concentrations, biodegradation of the effluent is an important issue to meet the environmental regulations (Song , 2005; Santos, 2009; Basha, 2010; Mohite, 2010; Chandra 2011).

In the leather industry, substituted phenols are used for making synthetic tannins. Consequently, considerable amount of 5 to 250 mg/l of phenolic wastes are found to be present in the wastewater from this industry. Conventional methods like physico-chemical treatment are not economically

viable and also produce toxic by-products (Agarry et al., 2008). Biological treatment is one of the methods of choice since it is environment friendly and economically viable. It is cited that many genera of microorganisms like *Acinetobacter* (Cordova-Rosa et al., 2009), *Alcaligenes* (Nair et al., 2007) *Comamonas, Pseudomonas*, (Neumann, 2004; Dong, 2008) *Brevibacillus*, (Yang and Lee, 2006) *Serratia*, (Pradhan and Ingle, 2007) *Rhodococcus*, (Rehfuss and Urban, 2005) *Bacillus*, (Đokić et al., 2011) and *Candida tropicalis* (Yan et al., 2006) are able to degrade phenol. However, it is very difficult for them to survive in a conventional biological treatment plant, , unless they are susceptible to the stress conditions caused by the presence of fluctuation in the concentration and composition of phenolic derivatives present in industrial wastewater due to their recalcitrant nature (Moosvi and Madamwar, 2007).

Microaerobic conditions have possible advantages such as less energy requirement for blower operation, less bio-solid production, quick recovery from organic shock loads when compared with the present conventional treatment systems (Zitomer and Shrout, 2000). Recently, increasing interest is being given to microaerobic conditions in wastewater treatment, for bioremediation of toxic compounds (Fuchs et al., 2011). It is reported by Krooneman (1996) that aerobic *Alcaligenes sp. strain L6*, metabolize 0.5 mM chlorobenzoate, with 10 mM succinate as co-substrate under both oxygen limitation ($O_2 < 0.1$ mM) and low oxygen concentrations (3 mM O_2). *Pseudomonas putida F1* degrades toluene at 0.1 mg/l of Dissolved Oxygen (DO) concentration indicating that toluene dioxygenase is expressed even at this low DO level [Costura and Alvarez, 2000]. Degradation of dibenzothiophene, naphthalene and anthracene at less than 0.2 ppm DO concentration by *Xanthobacter polyaromaticivorans* has also been reported (Hirano et al., 2004) Degradation of 2, 4, 6-tricholorophenol (2,4,6-TCP) by co-immobilization of anaerobes and aerobes in upflow sludge blanket reactors was reported by Gardin, (2001). The shielding of facultative bacteria help methanogens located in the anaerobic granules, to tolerate high oxygen ranging from 7 to 41% in the headspace. (Kato et al 1993). It was hypothesized that with respect to their physiological characteristics, the aerobic cultures were located at the more oxidized outer parts, while the anaerobes in the oxygen-free inner parts as a survival mechanism (Ergüder 2005). But degradation of high strength phenol at microaerobic condition is yet to be reported. Hence, in the present study, anaerobic sludge, from a pilot scale UASB reactor that was fed with leather industrial wastewater was used as inoculum. It was then enriched with phenol as sole carbon at microaerophilic condition. The isolated bacterial strain was evaluated for the degradation of high strength phenol and its potential on metabolic versatility on phenolic derivatives by utilising them as sole carbon is discussed in detail.

Materials and Methods

Chemicals

Nonyl phenol, Alkyl phenol, Naphthalene sulfonic acid, Resorcinol, Catechol, Benzoate, Hydroxy benzoate, was procured from Sigma-Aldrich Bangalore, India. Phenolic formaldehyde resin, Wattle extract was kindly provided by a tannery in Chennai and used without further purification. The phenol enrichment medium used for isolation of microorganisms was a Mineral Salt Medium (MSM) with phenol (Atlas,

2005). All medium components and phenol were procured from E.Merck Mumbai (India). All the solutions were prepared in Milli-Q water.

Enrichment

For the enrichment of phenol, a specially designed laboratory scale bioreactor (3.5 l) with automatic control biosensors for pH, temperature and Dissolved Oxygen (DO) were used. Constant DO level was maintained with a DO 1000 Controller. The controller consists of an air pump with air vapour condenser and a solenoid control valve which introduces air into the reactor to achieve the designated DO level. The oxygen concentration in the liquid phase was continuously measured using an oxygen probe (InPro6000; Mettler Toledo, Germany). For initial enrichment, the inoculum was grown in the presence of 50 mg/l of phenol concentration and increased to a phenol concentration of 1000 mg/l at an interval of 100 mg/l at a DO concentration of 0.9 ± 0.1 mg/l. Once the culture became turbid with bacterial growth and the phenol concentration reduced to about 90%, the next dose of 100 mg/l of phenol was transferred and equal volume of the culture was replaced.

Isolation

Bacterial strains from the phenol enriched microaerophilic bacterial consortium that grew well in mineral agar by utilising 1000 mg/l concentration of phenol were purified by repetitive streaking. Six were selected based on their potential to degrade high strength phenol and also its derivatives used in the leather industry. The selected six bacterial isolates designated as CSMB1 to CSMB6 were deposited in MTCC, IMTECH, India and a patent has been awarded for the invention entitled 'A microaerophilic bacterial consortium and use thereof for the simultaneous biodegradation of mixture of recalcitrants present in water' (Umamaheswari et al., 2012). Among the six selected CSMB strains, CSMB4 *Alcaligenes faecalis subsp. faecalis strain AE1.16* has not been cited so far in the degradation of high strength phenol. Hence it was selected as a model bacterial strain for further studies.

Analytical methods

For growth determination, turbidity was measured at 600 nm. The extent of % phenol degradation was determined using 4-aminoantipyrine at 510 nm (APHA, 2005). The absence of aromaticity was confirmed by a spectrum at 280 nm (Lofrano et al., 2008), using a UV-Vis spectrophotometer (Shimadzu UV2450).

Degradation assay

Phenol degradation and growth measurements were conducted under different growth conditions, using MSM with 1000 mg/l phenol as the sole carbon source in the same bioreactor used for enrichment of bacterial strains. The CSMB 4 was inoculated into the culture medium to give an initial cell concentration of 0.034 optical density (OD) at 600 nm. Phenol degradation studies were carried out with DO level ranging from 0.2 to 1.2 mg/l by increasing the aeration flow rate. The studies were conducted at different initial temperatures ranging from 25 to 55 °C and pH ranging from 3 to 10 to find out the optimum conditions for the degradation of phenol. The effect of different initial concentration of phenol on degradation was evaluated from 1200 to 2,000 mg/l phenol under optimum conditions. To evaluate the metabolic diversity of the CSMB4, phenolic derivatives used in leather industrial process

namely, nonyl phenol, naphthalene sulfonic acid, wattle extract, phenolic formaldehyde resin, catechol, benzoate and hydroxy benzoate were amended independently in MSM as sole carbon. For each experiment, samples were taken at regular intervals for the measurement of growth and % phenol degradation. The reported values are the average of three replicate measurements.

Enzyme activity

Cells of strain CSMB4 were grown in MSM medium supplemented with phenol and harvested at mid-log phase by centrifugation (8,000 g, 4 °C for 20 min). The cells were washed twice with phosphate buffer (pH 7.0) and sonicated on ice using a Digital Sonifier Model 250 (Branson, USA). The lysate was centrifuged at 20,000×g for 20 min. Phenol hydroxylase activity (EC 1.14.13.7) was measured by monitoring the disappearance of the NADPH at 340 nm in a spectrophotometer (Shimadzu UV2450). Standard assay mixture (3ml) contained 42 mM Na^+K^+ phosphate buffer, (pH 7.1), 0.15 mM NADPH, 0.5 mM phenol and cell extract (100 µl). One enzyme unit is defined as the amount of enzyme which in the presence of phenol causes the oxidation of 1 µmol NADPH per min. (Jones et al., 1995).
Catechol 1, 2 oxygenase (EC 1.13.11.2) and catechol 2, 3 oxygenase (EC 1.14.13.1) activity were measured respectively by monitoring the formation of *cis,cis.*muconic acid or 2-HMSA at 260 nm or at 375 nm respectively (Neujahr and Varga 1970). The reaction mixture (3 ml) contained Tris HCl buffer (50 mM, pH 8.0), catechol (1 mM) and cell extract (100 µl). Readings were taken at 30 second intervals for 5 min. Controls without substrate or cell extracts were prepared for each assay. One enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 µmol of the product per minute. Protein concentration was determined according to the method of (Bradford, 1976), using bovine serum albumin as the standard. Specific activities were expressed as units (U) per milligram protein.

Fourier Transform Infrared Spectrometry (FTIR) analysis

The product obtained after 24 h of incubation of CSMB4 in MSM supplemented with 1000 mg/l phenol was analysed through FTIR Spectrometry (ABB MB 3000). For this, the culture broth was centrifuged at 12,000 g for 20 min and the supernatant was lyophilized. KBr pellets of the lyophilised samples were subjected to FTIR analysis using transmission mode. The measurements were carried out in the mid-infrared range from 4000 to 500 cm^{-1}. Pure phenol was used as control.

Results and Discussion

Isolation and Identification of CSMB4

The morphology of the bacterial strain CSMB4 obtained after repeated streaking on MSM phenol agar plates, appeared as a thin matt with irregular colonies. Microscopic examination revealed it as rod shaped, gram-negative, non-sporing, non-capsulated and motile in nature. Through biochemical results, CSMB4 was observed to be of *Alcaligene species* by showing positive reaction for catalase, oxidase, citrate utilization and nitrate reduction and negative to Voges-Proskauer and urease activities. CSMB4 also showed a broad range for carbohydrate utilization. Inspection of 16S rRNA gene tree clearly demonstrated that the strain CSMB4 is a member of the genus *Alcaligenes*, forming a cluster with *Alcaligenes sps*. The highest degree of similarity of 99% was observed with *Alcaligenesfaecalis subsp. faecalis strain*

AE1.16 and hence, the strain CSMB4 was identified as *Alcaligenesfaecalis subsp. faecalis strain AE1.16* with the Genbank accession number GQ284565.1.

Effect of Dissolved Oxygen

To find out the effect of DO, pH, and temperature on phenol degradation by CSMB4, experiments were conducted using MSM with 1000 mg/l phenol as sole carbon in the online controlled bioreactor. Growth and phenol degradation by CSMB4 under a wide range of DO concentration was conducted by stepwise increase from 0.2 to 1.2 mg/l (Fig. 1). Under the DO concentration range of 0.6 to 1.2 mg/l, phenol degradation was found to be between 85 to 99 %. When aeration was below 0.4mg/l, degradation of phenol decreased from 72 to 66 %. At the DO concentration of 1.0 mg/l, growth by turbidity was corresponding to an OD of 1.355 at 600 nm with 99 % phenol degradation. By increasing DO concentrations to 1.2%, decrease in growth and 97% phenol degradation was observed. So, the DO concentration of 1.0 mg/l was used as optimum for further studies. The critical oxygen concentration to initiate the biodegradation of monoaromatic hydrocarbons was reported to be in the range of 1.0-1.5 mg O_2/l (Laleh Yerushalmi et al., 2002). Similarly, to treat municipal wastewater, DO of 0.4 mg/l was used for the COD load of 6.2 kg/m^3 and a COD reduction of 62% was achieved. To improve the nitrification efficiency, DO concentration was further increased to above 1.0 mg /l (Zheng et al., 2011, 2012).

Effect of pH and Temperature

CSMB4 was able to utilize phenol in mineral medium, under a wide range of pH and temperature (Fig 2 and 3). The bacterial strain CSMB 4 at microaerophilic condition showed degradation efficiency of 95 %, 99 % and 93 % at pH 6, 7 and 8. The efficiency was reduced only by 30% at pH 5 and pH 9. However, the % phenol degradation was 31 % lower at pH 5 and at pH above 9 it was inhibited. Maximum phenol degradation of 97 % and 99 % were observed at a temperature of 30 and 40 °C, confirming the mesophilic nature of CSMB4. With the temperature at 25 and 50 °C, it had shown 65 and 53% phenol degradation. However, above 55 °C, phenol degradation was inhibited. Though, neutral pH is optimum for mesophilic bacterial strains, (Clintia et al., 2012) CSMB 4 showed high removal efficiencies with a wide temperature range of 25°C to 45°C and pH of 6 to 8.

Effect of initial phenol concentration

Detection of phenol at 510 nm in the culture supernatant confirmed that 99 % of degradation was obtained in MSM with 1000 mg/l of phenol within 24 h of incubation with concomitant growth by CSMB4 *Alcaligenes faecalis* subsp. *faecalis strain AE1.16* (Fig. 4a and b). Reduction in aromaticity at 280 nm confirmed the cleavage of phenol. When the concentration of phenol was increased to 1200 mg/l, the incubation time increased to 48h for 95% phenol degradation. It was capable of degrading 90 % of 1400 mg/l phenol within 72 h and was also able to tolerate phenol even at an elevated concentration of 2000 mg/l with 60% degradation (Fig. 5). However, for the same 2000 mg/l phenol concentration, in activated sludge reactor, the genus Pseudomonas (Felföldi et al., 2010) needed 21 days of incubation to degrade 72%. In aerobic treatment plants, α-*Proteobacteria* and β-*Proteobacteria* isolates (members of the genera *Comamonas, Alcaligenes* and

Castellaniella) require seven days even to reduce 250 mg/l of phenol (Watanabe, 2002; Zhang, 2004).

Metabolic versatility

Turbidity measurements were conducted after growing CSMB4 *Alcaligenes faecalis subsp. faecalis strain AE1.16* in MSM medium supplemented with nonyl phenol, catechol, benzoate, hydroxy benzoate and naphthalene sulfonic acid independently, at optimized conditions (Table 1). The optical density values of 24 hour culture was observed to be between 0.9 to 1.1, confirming that these aromatic compounds can also be used as sole carbon. However, in the case of wattle extract, the optical density values were 0.5 to 0.7 after 24 h of incubation, which indicated that the organism may need an additional carbon source to enhance the growth. It is cited that by pre-adaption, the bacteria have the ability to co-metabolize various similar compounds, by becoming more efficient than pure culture due to their synergetic effects (Lambo and Patel, 2006; Wang, 2007). Since the isolated CSMB4 *Alcaligenes faecalis subsp. faecalis strain AE1.16*, was already exposed to phenolic compounds present in leather industrial wastewater, it demonstrated the potential to degrade the phenolic compounds used in leather processes, utilizing them as sole carbon and energy.

Enzyme activity

Phenol hydroxylase activity (PH) and catechol 1, 2 oxygenase activities (C12O) was detected in the presence of 1000 mg/L phenol at optimum conditions by *CSMB4 Alcaligenes faecalis* subsp. *faecalis strain AE1.16* (Table 2). The intracellular activity of PH was found to be 30.1 U/ml and its specific activity observed to be 547 U/mg. On the other hand, the intracellular specific activity of C12O was observed to be 3 times higher than phenol hydroxylase with 1568 U/mg and its activity observed to be 86.25 U/ml. However, no significant catechol 2, 3 oxygenase (C23O) activity or specific activity was detected. It had been shown that PH, the mono oxygenase enzymatic system, played an important role in the biodegradation of aromatic ring under microaerophilic conditions, since the low concentration of oxygen suppressed the activity of dioxygenase enzymatic systems (Olsen, 1994; Krooneman, 1996). It was found that even with a minimum of DO concentration the dioxygenases which were involved in ring fission processes incorporate molecular oxygen into the aromatic ring (Viliesid and Lilly, 1992). PH is responsible for converting phenol to catechol, which is the initial and rate-limiting step in phenol degradation pathways (Stiborová, 2003, Djokic, 2011). Our results are substantiated by the report of Krastanov (2013) on the preferential induction of pH in the presence of phenol. Presence C12O and absence of C23O activity confirms the phenol degradation through ortho cleavage (Ambujom, 2001).

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The products obtained by the action of *CSMB4 Alcaligenes faecalis subsp. faecalis strain AE1.16* was investigated using FTIR and compared with the spectrum of pure phenol (Fig. 6a, b) The FTIR spectra of pure phenol showed the characteristic bands in five regions of the mid infrared spectrum The C–H stretching bands of aromatic compound at 3,045 cm^{-1}, and O–H band at 3,323 cm^{-1} correspond to the intermolecular hydrogen bonding. The bands at the regions of 1593, 1499, and 1472 cm^{-1} represent C=C stretching, which is a series of weak combination and overtone bands of the benzene ring. C–O stretching in phenols

produces a strong band in the 1300–1000 cm^{-1} regions. The C-H in-plane bending bands appear in the regions of 1023, 1069, 1151 and 1166 and 1217 and out of plane bending of strong vibrations at the region of 616, 686, 747, 808 and 886 cm^{-1} (Silverstein, 1988; Stuart,2004). No single characteristic peak representing phenol was observed through FTIR measurement in the lyophilized powder obtained after 24 h growth of CSMB4. A complete shift of bands in the aromatic to aliphatic region was observed. Intense bands in the region of 2956 cm^{-1}, 2923 cm^{-1} representing O-H stretching of carboxylic acids and 2,853 cm^{-1} representing symmetric C-H stretching frequencies of aldehydes was obtained. A band at 1667 cm^{-1} representing C=O stretch of keto groups (Silverstein et al., 1988) was obtained, which confirmed that phenol was degraded into catechol followed by the formation of cis, cis.muconate through ortho cleavage pathway.

In conclusion, a phenol degrading bacterial strain CSMB4 that can grow at microaerophilic conditions has been isolated. Through 16s rRNA analysis, CSMB4 was identified as *Alcaligenes faecalis subsp. faecalis strain AE1*.16. To our knowledge this is the first time, degradation of phenol by this organism is being reported. Enzyme and FTIR studies proved that CSMB4 is able to express the aerobic enzymes even under microaerophilic condition and initiate the ortho-cleavage of the aromatic ring. The microaerophilic properties of CSMB4 *Alcaligenes faecalis subsp. faecalis strain AE1.16* make it a possible candidate for a viable commercial application with minimum energy requirement for its growth in industrial wastewater treatment plants, where phenol and its derivatives are a common occurrence.

Table.1 Metabolic diversity

Phenolic compounds	Growth (Optic Density) at 600 nm
Nonyl phenol	++
Phenolic formaldehyde resin	++
poly phenol (wattle)	+
Naphthalene sulfonic acid	++
Hydroxy benzoate	++
Catechol	++
Benzoate	++

Note: OD at 600nm: ++ 0.9 to 1.1, + 0.5 to 0.7

Table.2 Enzyme activities in cell extracts during the degradation of 1000 mg/l phenol by *CSMB4 Alcaligenes faecalis subsp. faecalis strain AE1.16.*

Enzyme assayed	Intracellular activity (U/ml)	Intracellular Specific activity (U/mg)
Phenol hydroxylase	30.1	547
Catechol 1,2 oxygenase	86.25	1567
Catechol 2,3 oxygenase	1.37	24.09

Fig.1 Effect of dissolved oxygen on growth and % phenol degradation at different time intervals

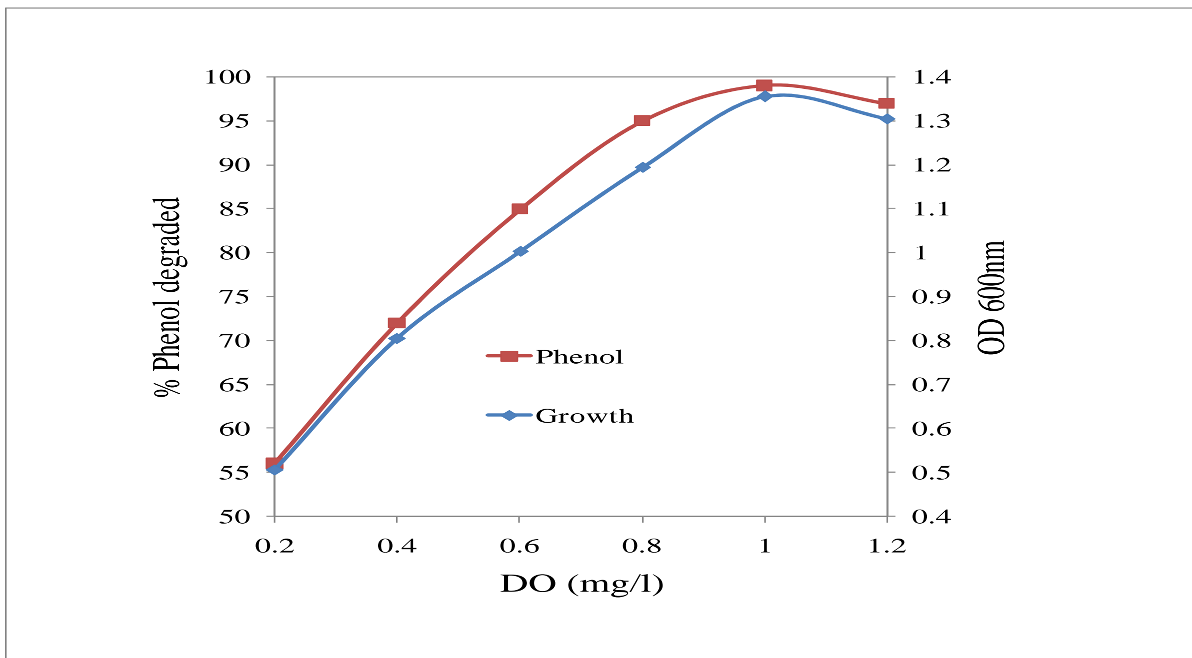


Fig.2 Effect of % phenol degradation at different time intervals using different initial pH

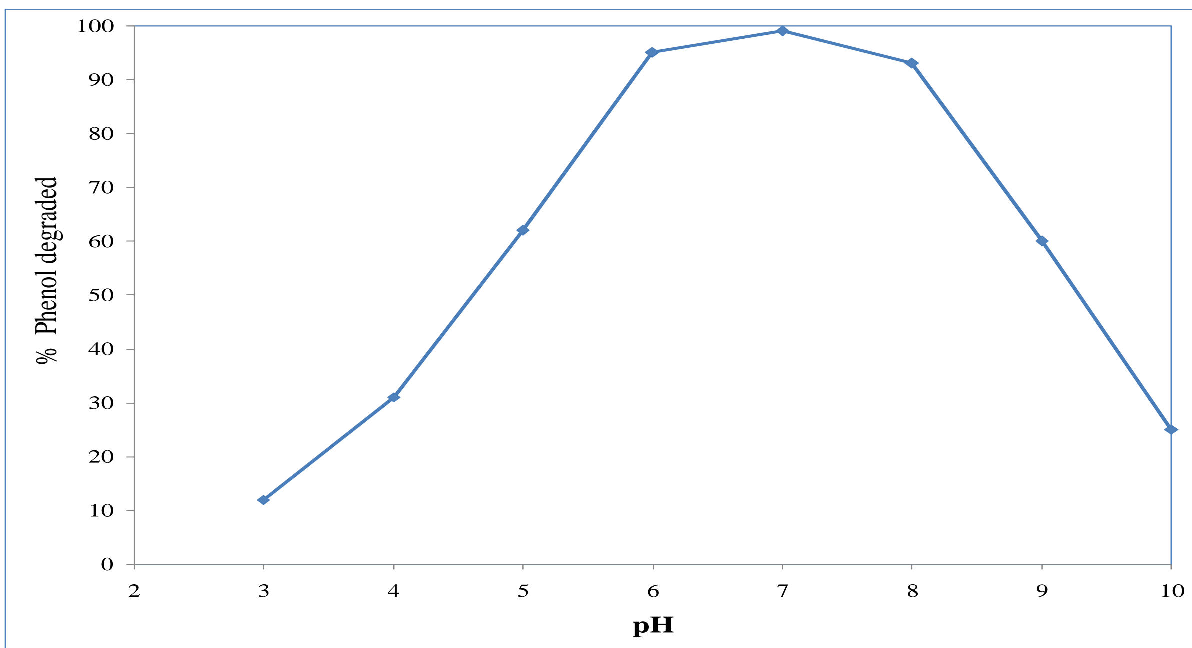


Fig.3 Effect of % phenol degradation at different time intervals using different initial Temperature

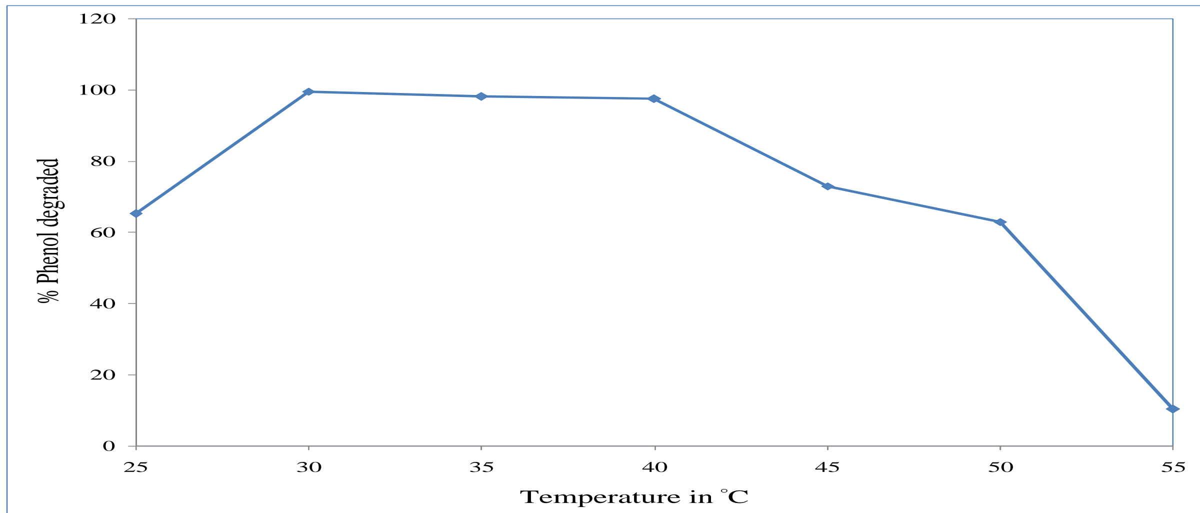


Fig.4(a) Growth and phenol degradation profile

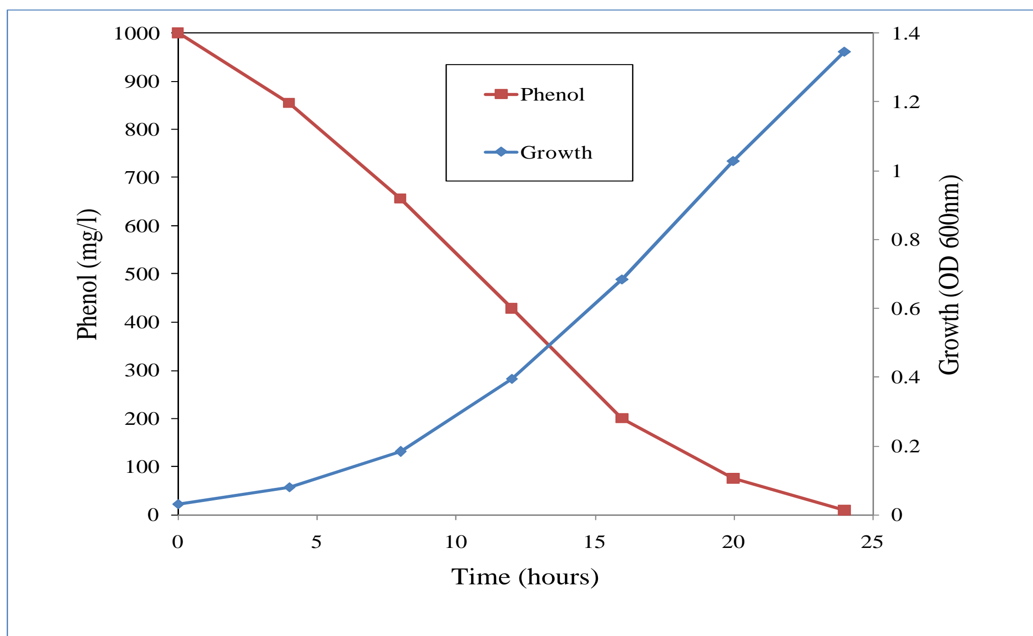


Fig.4 (b) Reduction in Aromaticity Spectrum (λ 280) with initial phenol concentration of 1000 mg/l at different time intervals at optimised condition

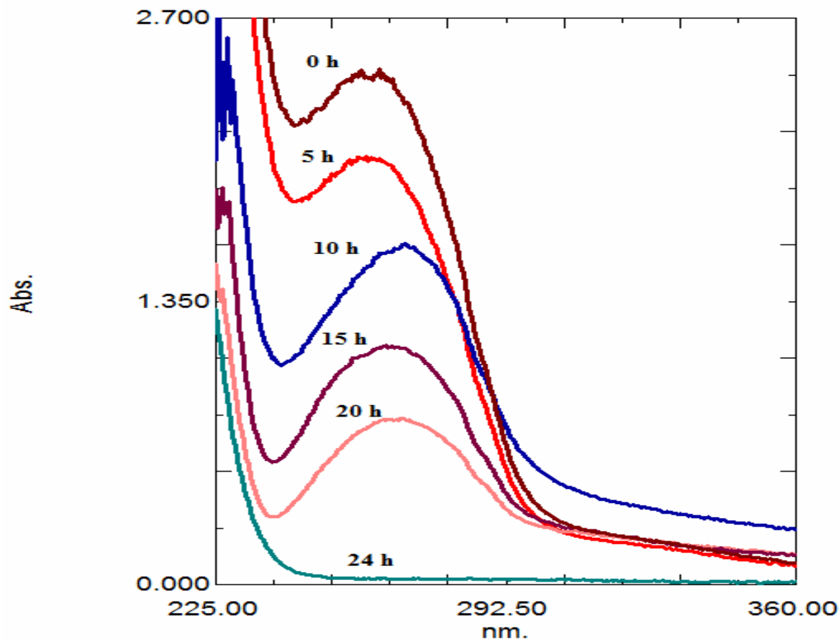


Fig.5 Effect of different initial phenol concentration on phenol degradation

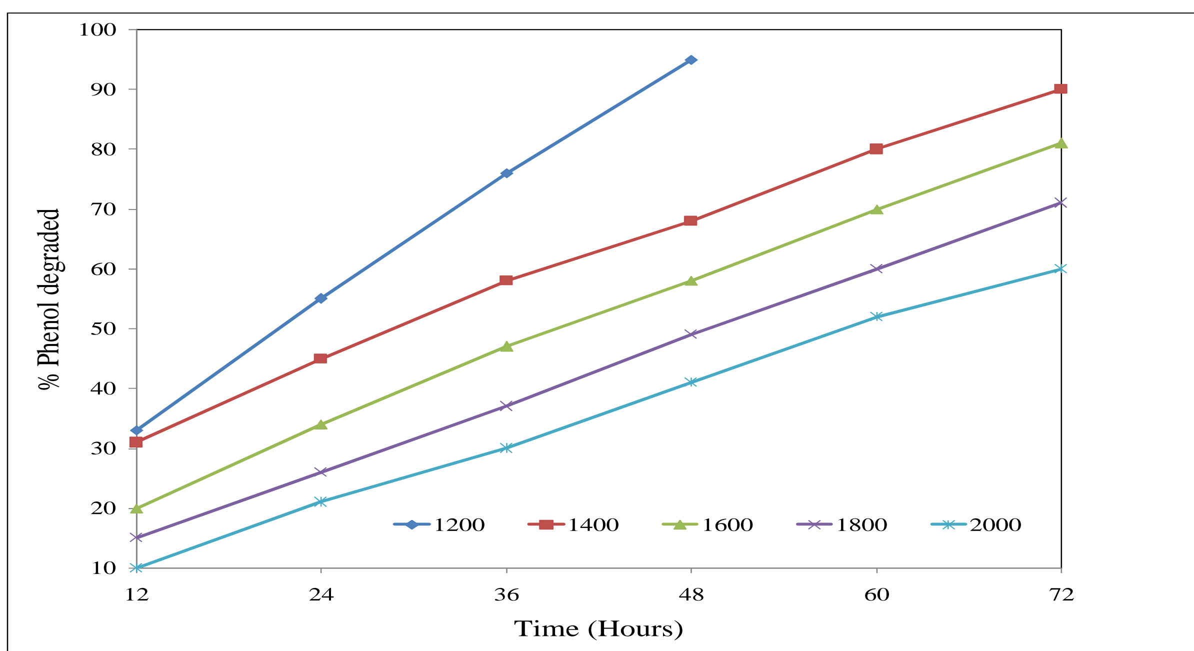


Fig.6 (a) FT-IR spectrum at 0 h

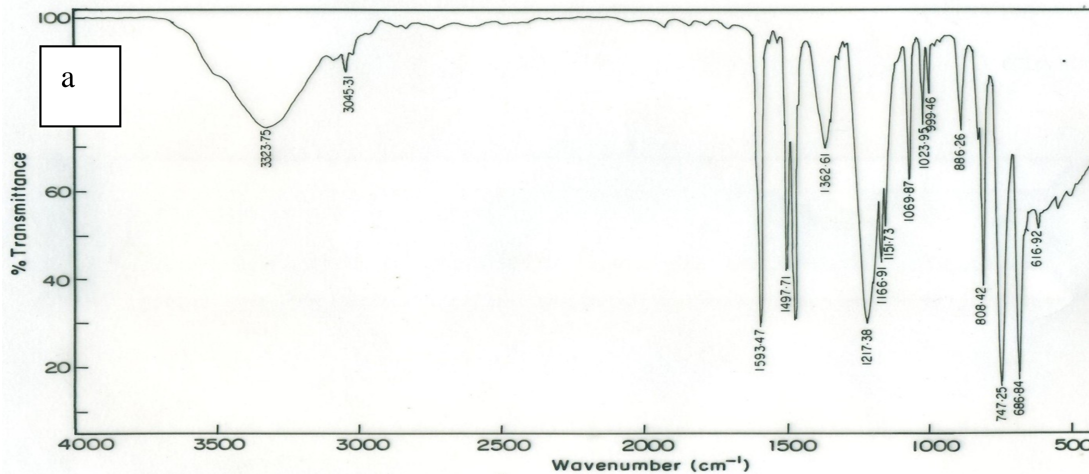
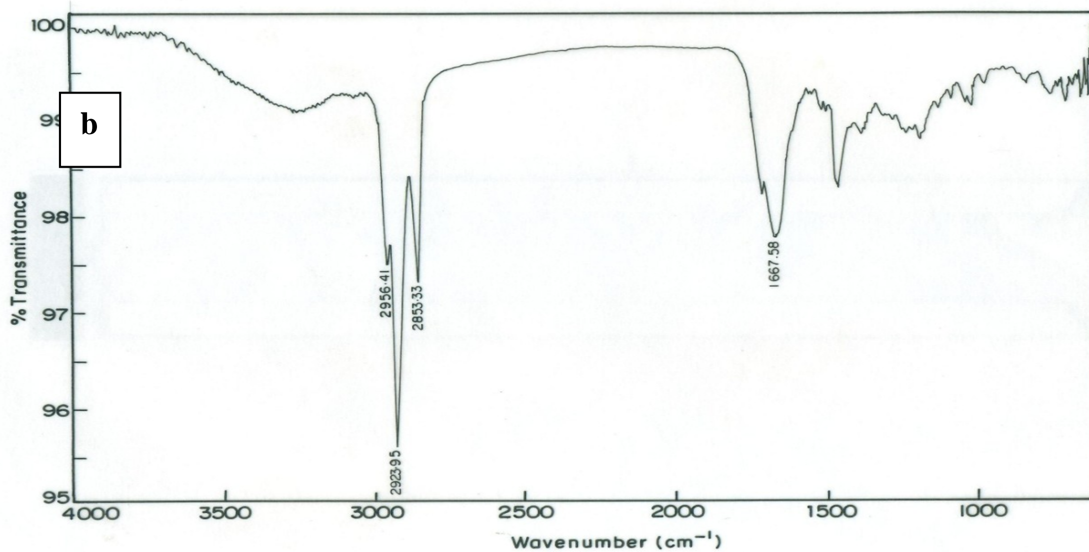


Fig.6 (b) FT-IR spectrum at 24 h



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