RESULTS
3. RESULTS

In the present work a phenol degrading bacteria \( d_2 \) was isolated by soil enrichment technique and was identified as *Alcaligenes* sp. The conditions for maximum degradation of phenol were optimized and the growth of the selected strain in the optimum conditions was monitored. The growth and the capacity for degradation of the immobilized *Alcaligenes* sp \( d_2 \) were also investigated.

In order to understand the mechanism of degradation the ortho/meta cleavage pathway confirming tests were conducted. The degradation products were extracted and analyzed by GCMS and FTIR.

The degradation analysis suggested that along with the complete degradation of phenol, the *Alcaligenes* sp \( d_2 \) was capable of producing alkyl resins like poly \( \beta \) hydroxy butyric acid. As there were no products detectable in the ortho or meta pathway by the GCMS or FTIR analysis, the possible involvement of polyphenol oxidase was evaluated in the biodegradation of phenol by the *Alcaligenes* sp. \( d_2 \). The presence of this enzyme was confirmed which was purified and characterized. The degradation of phenol was also attempted with partially purified poly phenol oxidase.

The phenol degrading efficiency of the selected strain was applied in the treatment of a phenolic paper factory effluent. The efficiency of the immobilized cells in the treatment of the phenolic effluent was also evaluated. A three-stage bioreactor was designed and was used with charcoal, immobilized cells and with chitosan for the effective treatment of the effluent.
3.1. ISOLATION, IDENTIFICATION AND SELECTION OF AN EFFICIENT PHENOL DEGRADING BACTERIA

3.1.1. Isolation of phenol tolerating strains by soil enrichment technique

The soil extract enriched with phenol at 1mM concentration gave a colony count of $46 \times 10^5$ on serial dilution followed by a colony count of $71 \times 10^1$ at 2mM concentration. Serial dilution of the extract on further enrichment gave a colony count of $60 \times 10^1$ at 5 mM phenol concentration and $40 \times 10^1$ at 10 mM concentration (table 5).

Table 5: Isolation of Phenol tolerating strains by soil enrichment technique

<table>
<thead>
<tr>
<th>Concentration of Phenol (mM) in the soil extract</th>
<th>Serial Dilution Factor</th>
<th>Colony Forming Unit (CFU) in nutrient agar with phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^5$</td>
<td>46±8</td>
</tr>
<tr>
<td>2</td>
<td>$10^1$</td>
<td>71±6</td>
</tr>
<tr>
<td>5</td>
<td>$10^1$</td>
<td>60±5</td>
</tr>
<tr>
<td>10</td>
<td>$10^1$</td>
<td>40±4</td>
</tr>
</tbody>
</table>

3.1.2. Isolation of phenol degrading strains by enrichment technique with the Mineral Salt Phenol Medium (MSPM)

All the 40 ± 4 colonies isolated by soil enrichment technique were individually subjected to enrichment with MSPM. In the MSPM with 1mM phenol, only 32 isolates showed positive growth. These isolates were designated as $a_1$-$a_{32}$. When these 32 isolates were individually tested in MSPM with 2mM phenol concentration only 20 isolates gave growth and were designated as $b_1$-$b_{20}$. On inoculating these 20 strains individually in MSPM with 5mM phenol concentration, growth was exhibited by only 11 isolates and were designated as $c_1$-$c_{11}$. All these isolates were individually
inoculated into MSPM enriched with 10mM phenol and only 7 isolates showed growth. These isolates were designated as d₁-d₇ (table 6).

Table 6: Isolation of phenol degrading strains by enrichment technique using mineral salt phenol medium

<table>
<thead>
<tr>
<th>Stages</th>
<th>No. of isolates inoculated</th>
<th>Concentration of phenol in Mineral salt Phenol (mM) Medium</th>
<th>Number isolates grown in mineral salt phenol agar medium</th>
<th>Designation given</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st stage</td>
<td>40</td>
<td>1</td>
<td>32</td>
<td>a₁-a₃₂</td>
</tr>
<tr>
<td>2nd stage</td>
<td>32</td>
<td>2</td>
<td>20</td>
<td>b₁-b₂₀</td>
</tr>
<tr>
<td>3rd stage</td>
<td>20</td>
<td>5</td>
<td>11</td>
<td>c₁-c₁₁</td>
</tr>
<tr>
<td>4th stage</td>
<td>11</td>
<td>10</td>
<td>7</td>
<td>d₁-d₇</td>
</tr>
</tbody>
</table>

3.1.3. Identification of the screened isolates and the selection of the potential strain for the biodegradation of phenol.

All the seven isolates were tested for their consistency of growth in MSPM for five days and all the stable isolates were identified by performing various morphological and biochemical tests according to Bergey's manual of Systematic Bacteriology (Buchanan and Gibbons, 1974). The isolates d₁ and d₄ showed inconsistent growth in MSPM and were discarded (table 7). The isolate d₂ gave a maximum phenol degradation of 99.7% after 32 hours of incubation and was identified as *Alcaligenes* sp. The morphological and biochemical characteristics of the d₂ strain were as follows. The margin was round and entire. The colonies were small and slightly whitish and the surface didn't show any elevation. There was no pigment production. The d₂ strain was a small rod shaped gram -ve strain. They mostly showed single arrangement. The bacteria were non-spore forming and were found to be motile. The isolate was -ve in Oxidation/Fermentation test, Indole test, MR
test, VP test, Gelatin hydrolysis, Starch hydrolysis, H₂S production and was +ve in Citrate test and Catalase test. The identity was confirmed by sending the isolate to Microbial Type Cultural Collection Center (MTCC), Indian Institute of Microbial Technology (IMTECH), Chandigarh, India.

The other strains d₃, d₅ d₆, and d₇ resulted in a phenol reduction of 16.4 %, 95.6%, 91% and 92.5 % respectively after 96 hours of incubation. The isolates were identified as, Acinetobacter sp (d₃) and Pseudomonas sp (d₅, d₆, d₇) (table 7).

Table 7: Identification and selection of potential strain for the biodegradation of phenol

<table>
<thead>
<tr>
<th>Designation of the strain</th>
<th>Identity of the strain</th>
<th>Percentage of phenol degradation in MSPM</th>
<th>Incubation period</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>d₁</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Inconsistent growth</td>
</tr>
<tr>
<td>d₂</td>
<td>Alcaligenes sp</td>
<td>99.7</td>
<td>32</td>
<td>Selected for further studies</td>
</tr>
<tr>
<td>d₃</td>
<td>Acinetobactor sp</td>
<td>16.4</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>d₄</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Inconsistent growth</td>
</tr>
<tr>
<td>d₅</td>
<td>Pseudomonas sp</td>
<td>95.6</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>d₆</td>
<td>Pseudomonas sp</td>
<td>91</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>d₇</td>
<td>Pseudomonas sp</td>
<td>92.5</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

3.2. OPTIMIZATION OF THE CONDITIONS FOR THE BIODEGRADATION OF PHENOL

3.2.1. Effect of phenol concentration on specific growth rate of Alcaligenes sp. d₂

The growth of the Alcaligenes sp d₂ was studied at different concentrations of phenol such as 20mM, 40mM, 60mM, 80mM, 100mM, 120mM. The specific growth rate of the strain at each of this concentration
was found out and was plotted against the respective phenol concentration. The specific growth rate of *Alcaligenes* sp d₂ decreased along with the increase in the phenol concentration from 20mM to 120mM and 120mM phenol concentration was found to be inhibitory to the growth of the organism (Fig. 2).

**Fig. 2** Effect of phenol concentration on specific growth rate of *Alcaligenes* sp d₂

![Graph showing specific growth rate against phenol concentration](image)

3.2.2. **Effect of substrate (phenol) concentration on phenol biodegradation by *Alcaligenes* sp d₂.**

The effect of substrate concentration on the phenol degradation (Fig. 3) showed that up to 60mM concentration there was 100% degradation and any further increase in the phenol concentration from 60mM, resulted a decrease in the percentage of phenol degradation. However more than 90% degradation of phenol was attained even at 100mM concentration.
3.2.3. Optimization of the incubation period for the biodegradation of phenol by *Alcaligenes sp d₂*

The Fig. 4 indicated a steady increase in the degradation of phenol along with the increase in the incubation time and showed 100% degradation after 32 hours, which was considered as the optimum condition for the biodegradation of phenol. Optimum incubation period was the minimum contact time for 100% degradation.
3.2.4 Optimization of the pH for the biodegradation of phenol by *Alcaligenes* sp $d_2$

The optimum pH for the biodegradation of phenol by *Alcaligenes* sp $d_2$ was found to be 6 (Fig. 5). However the organism showed more than 97% phenol degradation in the pH range from 5 to 8.5.
3.2.5. Growth curve of free cells of *Alcaligenes* sp d₂ in Mineral Salt Phenol Medium (MSPM)

The only carbon source in the mineral salt phenol medium was phenol and hence the growth rate is limited by the availability of phenol (Fig.6). The logarithmic phase of the strain in MSPM extended from 24 hours to 32 hours. The maximum yield in cell number was only in the order of $10^7$ in MSPM within 32 hour. The biomass remained constant upto 44 hours.

Fig.6: Growth curve of free cells of *Alcaligenes* sp d₂ in mineral salt phenol medium (MSPM)

3.2.6. Growth curve of immobilized cells of *Alcaligenes* sp d₂ in MSPM

Growth curve of the immobilized cells (Fig. 7) showed a logarithmic phase extending upto 12 hours and the maximum biomass was in the order of $10^{10}$. The biomass remained constant upto 24 hours.
3.2.7. Biodegradation of phenol in MSPM by immobilized cells of *Alcaligenes sp d2*

Immobilized cells of *Alcaligenes sp d2* (activation time, 6 hours) showed a better performance in the biodegradation of phenol by achieving 100% degradation even at 20 hours of incubation (Fig. 8). The immobilized system was able to show the same performance up to 44 hours of incubation.
3.3. ANALYSIS OF THE PRODUCTS OF PHENOL BIODEGRADATION

3.3.1. Meta /Ortho cleavage test

The ortho/meta cleavage test conducted with catechol solution gives yellow color for meta cleavage and deep blue color for ortho cleavage. The meta/ortho cleavage test for the biodegradation of phenol by *Alcaligenes* sp d₂ did not give any colour.

3.3.2. Analysis of the extract of uninoculated MSPM (control) by Gas chromatography

The gas chromatogram of the extract of uninoculated MSPM (control) showed only few peaks at 2.951 min, 5.881 min, 7.272 min, 7.5 min, 7.9 min, 9.3 min, 14.997 min, 16.5 min, and 18.8 min. There were no significant peaks after 20 min of retention time (Fig. 9).
3.3.3. Mass spectroscopic analysis of the peak at 14.997 min. in the gas chromatogram of the extract of uninoculated MSPM.

The peak at 14.997 min of the gas chromatogram of the extract of the uninoculated MSPM on mass spectroscopic analysis gave a fraction of maximum molecular weight as 94. This corresponds to phenol and hence the peak at 14.997 min in the gas chromatogram corresponds to that of phenol (Fig. 10)
3.3.4. **Analysis of the extract of biodegraded phenol in MSPM by Gas chromatography**

Gas chromatogram of the extract of biodegraded phenol in MSPM showed many peaks at 2.951 min, 5.881 min, 7.272 min, 7.5 min, 7.9 min, 9.3 min, 16.5 min, 18.6 min, 23.394 min, 26.567 min, 36.204 min, 36.783 min, 37.962 min, 38.988 min and at 39.084 min (Fig. 11). However, the peak at 14.997 min corresponding to that of phenol in the gas chromatogram of the extract of uninoculated MSPM, was totally absent in the gas chromatogram of the extract of biodegraded phenol MSPM by *Alcaligenes* sp d2. The biodegraded sample showed many additional peaks after 20 min of retention time and all these peaks were absent in the control.

**Fig 11:** Analysis of the extract of biodegraded phenol MSPM by Gas chromatography
3.3.5. Mass spectroscopic analysis of the additional peak at 23.394 min. in the gas chromatogram of the extract of biodegraded MSPM

The additional peak at 23.394 on mass spectroscopic analysis showed that the fraction with highest molecular weight is that of 87 (Fig. 12). The molecular weights of the other fractions were 74 and 40.

Fig 12: Mass spectroscopic analysis of the additional peak at 23.394 min. in the gas chromatogram of the extract of biodegraded MSPM

3.3.6. Mass spectroscopic analysis of the additional peak at 26.567 min. in the gas chromatogram of the extract of biodegraded MSPM

The additional peak at 26.567 min on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 86 (Fig. 13). The molecular weights of the other fractions were 75 and 43.

Fig 13: Mass spectroscopic analysis of the additional peak at 26.567 min. in the gas chromatogram of the extract of biodegraded MSPM
3.3.7. Mass spectroscopic analysis of the additional peak at 36.204 min. in the gas chromatogram of the extract of biodegraded MSPM

The additional peak at 36.204 on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 282 (Fig. 14). The molecular weights of the other fractions were 149, 71 and 56.

Fig 14: Mass spectroscopic analysis of the additional peak at 36.204 min. in the gas chromatogram of the extract of biodegraded MSPM

3.3.8. Mass spectroscopic analysis of the additional peak at 36.783 min. in the gas chromatogram of the extract of biodegraded MSPM

The additional peak at 36.783 on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 278 (Fig. 15). The molecular weights of the other fractions were 167, 150, 70 and 41.

Fig 15: Mass spectroscopic analysis of the additional peak at 36.783 min. in the gas chromatogram of the extract of biodegraded MSPM
3.3.9. **Mass spectroscopic analysis of the additional peak at 37.962min. in the gas chromatogram of the extract of biodegraded MSPM**

The additional peak at 37.960 on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 166 (Fig. 16). The molecular weights of the other fractions were 149 and 113.

**Fig 16:** Mass spectroscopic analysis of the additional peak at 37.962min. in the gas chromatogram of the extract of biodegraded MSPM

3.3.10. **Mass spectroscopic analysis of the additional peak at 38.988min. in the gas chromatogram of the extract of biodegraded MSPM**

The additional peak at 38.998 on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 278 (Fig. 17). The molecular weights of the other fractions were 167, 149, 70 and 57.

**Fig 17:** Mass spectroscopic analysis of the additional peak at 38.988min. in the gas chromatogram of the extract of biodegraded MSPM
3.3.11. Mass spectroscopic analysis of the additional peak at 39.084 min. in the gas chromatogram of the extract of biodegraded MSPM

The additional peak at 39.084 on mass spectroscopic analysis showed that the fraction with highest molecular weight was that of 279 (Fig. 18). The molecular weights of the other fractions were 165, 149 and 71 and 57.

Fig 18: Mass spectroscopic analysis of the additional peak at 39.084 min. in the gas chromatogram of the extract of biodegraded MSPM

3.3.12. Analysis of the extract of uninoculated MSPM by FT-IR

FT-IR analysis of the extract of uninoculated MSPM showed many bands at wave numbers of 3286 cm$^{-1}$, 3045 cm$^{-1}$, 1593 cm$^{-1}$, 1499 cm$^{-1}$, 1472 cm$^{-1}$, 1361 cm$^{-1}$, 1225 cm$^{-1}$, 810 cm$^{-1}$, 749 cm$^{-1}$ (Fig. 19). The 3286 cm$^{-1}$ band corresponds to intermolecular hydrogen bonded O-H. The band at 3045 cm$^{-1}$ represented aromatic C-H stretching. The bands at 1593 cm$^{-1}$, 1499 cm$^{-1}$ and 1472 cm$^{-1}$ represented C=C stretching. The band at 1361 cm$^{-1}$ represented O-H bend and the band at 1225 cm$^{-1}$ represented C=O stretching. The bands at 810 cm$^{-1}$ and 749 cm$^{-1}$ represented out of plane C-H bend.
3.3.13. Library search report of the FT-IR analysis of the extract of uninoculated MSPM

Library search report of the FT-IR analysis of the extract of uninoculated MSPM showed the presence of phenol in the control (Fig. 20). The library search gave 80% match with the phenol. The only organic compound in the control was also phenol.
Fig 20: Library search report of the FT-IR analysis of the extract of uninoculated MSPM

Search results for: "PHENOL"  
Date: Tue Apr 05 12:37:41 2005 (GMT-07:00)  
Search algorithm: Correlation  
Regions searched: 3456 26-465.13

<table>
<thead>
<tr>
<th>Index</th>
<th>Match</th>
<th>Compound Name</th>
<th>Library Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
<td>PHENOL 99+</td>
<td>Aldrich Condensed Phase Sample Library</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>2,3-DIETHOXYPHENOL 98%</td>
<td>Aldrich Vapor Phase Sample Library</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>PHENYL-D-GLUCOSIDE</td>
<td>Sigma Biological Sample Library</td>
</tr>
</tbody>
</table>

3.3.14. FT-IR analysis of the extract of biodegraded MSPM

The FTIR analysis showed the disappearance of band at 3045 cm\(^{-1}\). This band corresponds to aromatic compound (Fig.21). There was also a shift in the absorbance from 3286 cm\(^{-1}\) to 3331 cm\(^{-1}\). The spectrum showed the disappearance of bands at 1593 cm\(^{-1}\), 1499 cm\(^{-1}\) and 1472 cm\(^{-1}\) in the aromatic region. Also there was a broad absorption at 3333 cm\(^{-1}\) which conclusively indicated the presence of a polymeric structure.
Fig 21: FT-IR analysis of the extract of biodegraded MSPM

3.3.15. Library search report of the FT-IR analysis of the extract of biodegraded MSPM

Library search report of the FT-IR analysis of the extract of biodegraded MSPM suggested the highest similarity to alkyl resin (Fig 22). This represented aliphatic polymeric compounds like poly β hydroxy butyric acids in the extract of biodegraded MSPM.
3.4. POLYPHENOL OXIDASE FROM *Alcaligenes* sp d₂

3.4.1. Purification profile of the polyphenol oxidase extracted from *Alcaligenes* sp d₂

The supernatant after the removal of biomass showed a polyphenol oxidase activity of 1.68U/ml (table 8). The crude extract was subjected to ammonium sulphate precipitation at 30-40% fractionation followed by dialysis. The dialyzed fraction showed an enzyme activity of 20 U/ml. The dialyzed fraction was purified by ion exchange chromatography in DEAE cellulose column at a pH of 5 and using a gradient concentration of phosphate citrate buffer from 60-360 mM. The purity of the column fraction was confirmed with native PAGE (plate 12). The partially purified fraction showed an activity of 62 U/ml. The overall yield was 37% and the specific activity was 620.
Table 8: Purification profile of polyphenol oxidase isolated from phenol degrading *Alcaligenes* sp d2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total volume</th>
<th>Enzyme u/ml</th>
<th>Protein mg/ml</th>
<th>Total Enzyme</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>fold</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>500ml</td>
<td>1.68</td>
<td>0.056</td>
<td>840</td>
<td>28</td>
<td>25.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate fraction after dialysis</td>
<td>20ml</td>
<td>20</td>
<td>0.063</td>
<td>400</td>
<td>1.26</td>
<td>317</td>
<td>12.5</td>
<td>38</td>
</tr>
<tr>
<td>Ion exchange fraction partially purified enzyme</td>
<td>5ml</td>
<td>62</td>
<td>0.1</td>
<td>310</td>
<td>0.5</td>
<td>620</td>
<td>24.8</td>
<td>37</td>
</tr>
</tbody>
</table>

Plate 12

Native Page of the Partially Purified Enzyme
3.4.2. Effect of substrate concentration (ABTS) on the activity of polyphenol oxidase from *Alcaligenes* sp d₂

The effect of substrate concentration on the activity of the polyphenol oxidase was demonstrated in Fig 23. When the concentration of the substrate was increased from 0.25mM, the enzyme activity also got increased and it reached a maximum value of 75 units at 0.75mM ABTS concentration.

**Fig 23**: Effect of substrate (ABTS) on the activity of polyphenol oxidase from *Alcaligenes* sp d₂

![Graph showing effect of substrate concentration on enzyme activity](image)

3.4.3. Line weaver Burk plot of polyphenol oxidase from *Alcaligenes* sp d₂ with ABTS as the substrate.

On analyzing the effect of substrate concentration on enzymatic action by, Line weaver Burk plot, the Km was found to be 0.34 mM for ABTS as the substrate (Fig.24).
3.4.4. Effect of pH on the activity of polyphenol oxidase from *Alcaligenes* sp d2

The enzyme activity was very less at acidic pH of 3. However it got increased when the pH was slightly enhanced from 3. At a pH of 4.5-6.5 the enzyme showed highest activity of 64 units (Fig.25). Further increase in pH resulted in the decrease of enzyme activity and finally the enzyme gave only 20 units at a pH of 9.5.
3.4.5 Biodegradation of phenol using partially purified polyphenol oxidase from *Alcaligenes sp. d₂*.

The partially purified enzyme with an enzyme activity of 62 U/ml at a concentration of 1% (ml/ml) could degrade 11.4% phenol. On increasing the enzyme concentration the rate of degradation also got increased (Fig.26). At an enzyme concentration of 4% (ml/ml) there was 65% reduction in the degradation of phenol, which remained as 67.5% even at 5% (ml/ml) enzyme addition.
3.5. APPLICATION OF THE SELECTED PHENOL DEGRADING ORGANISM, *Alcaligenes* sp d₂, IN WASTEWATER TREATMENT

3.5.1. Characteristics of the phenolic paper factory effluent

The characteristics of the phenolic paper factory effluent are presented in the table 9. The effluent was collected from the industry at different times and the characteristics of the effluent were analyzed. The phenolic paper factory effluent used in the present study gave a COD of $1100 \pm 8.15\, \text{mg/l}$, BOD of $425 \pm 5.2\, \text{mg/l}$, total suspended solids of $1620 \pm 15.5\, \text{mg/ml}$, total heterotrophic bacterial population of $48 \pm 12 \times 10^2$, pH $9 \pm 0.5$ and a phenol concentration of $100 \pm 5\, \text{mM}$. The effluent was used in the study after bringing the pH to 6.
Table 9: Characteristics of the phenolic paper factory wastewater

<table>
<thead>
<tr>
<th>Characteristics of the wastewater</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical oxygen demand</td>
<td>$1100 \pm 8.15 \text{mg/l}$</td>
</tr>
<tr>
<td>Biological oxygen demand</td>
<td>$425 \pm 5.20 \text{mg/l}$</td>
</tr>
<tr>
<td>Total suspended solids</td>
<td>$1620 \pm 15.5 \text{mg/l}$</td>
</tr>
<tr>
<td>Total heterotrophic bacterial population</td>
<td>$48 \pm 12 \times 10^2$</td>
</tr>
<tr>
<td>pH</td>
<td>$9 \pm 0.5$</td>
</tr>
<tr>
<td>phenol concentration</td>
<td>$100 \pm 5 \text{mM}$</td>
</tr>
</tbody>
</table>

3.5.2. Growth curve of the free cells of *Alcaligenes* sp $d_2$ in the phenolic effluent.

The growth curve of the free cells of *Alcaligenes* sp $d_2$ in effluent showed a diauxic type of growth (Fig.27). The first logarithmic phase was from 8 hours to 16 hours and the second logarithmic phase was from 32 hours to 36 hours. The maximum yield of biomass in effluent was in the order of $10^9$ in 36 hours.
Fig. 27. Growth curve of free cells of *Alcaligenes sp* d₂ in phenolic effluent

![Growth curve of free cells of *Alcaligenes sp* d₂ in phenolic effluent](image)

3.5.3. Treatment of phenolic effluent with free cells of *Alcaligenes sp* d₂

Treatment of the phenolic effluent with free cells *Alcaligenes sp* d₂ showed that the strain could reduce both phenol and Chemical oxygen demand of the effluent (Fig. 28). When the phenolic effluent was treated with free cells *Alcaligenes sp* d₂, a maximum of 99% phenol removal was effected in 32 hours. The corresponding COD reduction was 55%. The strain could further reduce chemical oxygen demand by a maximum of 69% within 40 hours of treatment.
3.5.4. Growth curve of immobilized cells of *Alcaligenes* sp d₂ in phenolic effluent.

The growth curve of the immobilized cells of *Alcaligenes* sp d₂ showed that the cells under immobilized condition could make a better growth in the effluent. Here the cells exhibited diauxic type of growth (Fig. 29). The first logarithmic phase extended from 4 hours to 24 hours and the second extended from 28 hours to 44 hours. The maximum biomass of $10^{14}$ was attained at 44 hours of incubation.
3.5.5 Treatment of phenolic effluent with immobilized cells of *Alcaligenes* sp \( d_2 \)

The immobilized cells could also reduce the phenol and COD of the phenolic effluent in a better way. 99% phenol removal was effected in 20 hours of treatment where the corresponding COD reduction was 70% (Fig. 30). A maximum COD reduction of 85% was attained in 38 hours of treatment by immobilized cells and there was enhancement of COD reduction even after 99% phenol removal.

Fig 30: Treatment of phenolic effluent with immobilized cells of *Alcaligenes* sp in batch process

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Fig 29: Growth curve of immobilized *Alcaligenes* sp \( d_2 \) in phenolic effluent

![Growth curve](image)
3.5.6 Continuous treatment of phenolic effluent with immobilized cells of *Alcaligenes* sp d₂ in a packed bed reactor.

The continuous treatment of the effluent was done with immobilized cells of *Alcaligenes* sp d₂ in a packed bed reactor. In the continuous treatment of phenolic effluent at different flow rates, 99% removal in phenol and 92% reduction in COD were attained at 2.5 ml/hr flow rate (Residence time 8 hrs). On increasing the flow rate there was subsequent decrease in the performance of the reactor (Fig.31). At the flow rate of 10 ml/hour the phenol reduction was only 6% and the corresponding COD reduction was only 28%. Continuous treatment of the effluent in packed bed reactor gave maximum reduction in both phenol content (99%) and COD (92%) in 8 hours.

Fig.31. Phenol degradation and COD reduction in effluent by immobilized cells of *Alcaligenes* sp d₂ by continuous treatment in a packed bed reactor.

3.5.8. Performance of the packed bed reactor with immobilized cells of *Alcaligenes* sp d₂ in the treatment of phenolic effluent

Half-life period of the packed bed reactor system in treating the phenolic effluent was also evaluated. On assessing the performance of the packed bed reactor continuously for 10 days, 76 hours was observed as the
half-life period of the reactor. Half-life period was same in both COD removal and phenol removal performances (Fig. 32). Even though the system was able to maintain 49% of COD reduction even after 3 days, the phenol removal efficiency got reduced to 32%.

Fig 32: Performance of the packed bed reactor with immobilized cells of *Alcaligenes* sp d₂ in the treatment of phenolic effluent by continuous process.

3.5.9. Scanning of the coloured phenolic paper factory effluent for the absorption maxima by spectrophotometric analysis.

The phenolic paper factory effluent was coloured. In order to express the reduction in the color of the effluent upon treatment, an attempt was made to find out the absorption maxima of the effluent after removing the suspended particles of the effluent. On scanning the effluent for absorption from 200 nm to 1100 nm spectrophotometrically, a spectrum was attained with absorption maxima nearly at 400nm (Fig. 33).
Fig 33: Scanning of the coloured paper factory effluent for the absorption maxima by spectrophotometric analysis

3.5.10. Continuous treatment of phenolic effluent individually with powdered activated charcoal (PAC) packed in the reactor

On continuous treatment of the colored phenolic paper factory effluent with PAC packed reactor there was reduction in the color, the phenolic content and the COD of the effluent (Fig.34). The treatment was conducted at five flow rates and the results were consistent. Highest performance was achieved at 25 ml/hour flow rate. At this flow rate the reduction in color was 40±1%, reduction in phenolic content was 12±1.7% and the reduction in the COD was 20±1% (plate8).
Fig 34: Continuous treatment of the phenol paper factory effluent with powdered activated charcoal (PAC) packed in the reactor

3.5.11. Continuous treatment of phenolic effluent individually with immobilized cells of Alcaligenes sp d2 packed in the reactor (plate 9)

The immobilized cells packed in the reactor could make a highest performance at 25ml/hour of the flow rate and could bring down the color by 5±1.1%, phenolic content by 98±1.5% and COD by 92±1% (Fig.35).

Fig 35: Continuous treatment of phenolic paper factory effluent using immobilized cells of Alcaligenes sp d2 packed in the reactor
3.5.12. Continuous treatment of phenolic effluent with chitosan coated in the reactor (plate 9)

The chitosan coated reactor could make a highest performance at 25ml/hour flow rate of the effluent and could bring down the color by 36±1.9%, phenolic content by 22±1.8% and COD by 31±1% (Fig.36).

Fig 36: Continuous treatment of the phenol paper factory effluent using chitosan coated reactor

% reduction in colour, phenol and COD

% of color removal
% of phenol removal
% of COD removal

Flow rate(ml/h)

3.5.13. Continuous treatment of phenolic effluent using the three stage reactor with PAC, immobilized cells of *Alcaligenes* sp d2 and chitosan packed in each stage of the reactor

Continuous treatment of phenolic effluent using the three stage reactor could finally result in a good performance as far as the color, the phenolic content and the COD reduction of the phenolic paper factory effluent were concerned. PAC was packed as the first stage of the reactor (first reactor), immobilized cells were packed as the second stage of the reactor (third reactor) and the chitosan coating was made as the third stage (second reactor) of the reactor. The three stage bioreactor on operation at 25ml/hour flow rate of the effluent could finally result in the color removal by 91±1.6%, phenolic content removal by 99.7±0.01% and COD removal by 98±0.1%.(Fig.37).
Fig 37: Continuous treatment of the phenolic paper factory effluent using the three stage activated charcoal; immobilized cell and chitosan packed reactor in series.

- % of color removal
- % of phenol removal
- % of COD removal

1 - First stage, 2 - Second stage, 3 - Third stage