Batch Biodegradation of Phenol of Paper and Pulp Effluent by *Aspergillus Niger*

Neha Sharma and Vikas. C. Gupta

***Abstract*—Biodegradation of industrial phenol by a fungal isolate *Aspergillus niger* was studied in batch flask system with synthetic & industrial effluent. *Aspergillus niger*, was efficiently immobilized on sodium alginate beads. The immobilized cells were used in the batch culture flasks for paper & pulp industry as well as synthetic effluent phenol removal. All the flasks were operated at temperature 25℃ at 125 rpm for five days in continuous mode. The immobilized cells showed over all better performance as compared to free cells. The highest cell growth and the amount of phenol degraded were observed on 5 day. average overall pH, Temp., Conductivity, B.O.D, C.O.D, T.S, T.D.S, T.S.S, Chlorides and phenol were upto 7.5, 34.75 ℃, 39.6 µmohs/cm, 139.5 mg/l, 430.5 mg/l, 1490 mg/l, 900 mg/l, 590 mg/l, 281.25 mg/l and 268 mg/l respectively. While culture with immobilized cell reached 110 mg/L whereas in free cell it is 119 mg/L in industry effluent whereas in synthetic effluent culture with immobilized cell reach 28 mg/L whereas in free cell it is 150 mg/L with the same conditions. Reduction in phenol level proved the biodegradation. Results from this study showed that *Aspergillus niger* has potential to be used in biodegradation of wastewater containing phenol.**

***Index Terms*—*Aspergillus niger*, batch culture, Biodegradation, immobilized cells, phenol.**

I. Introduction

Phenol and its derivatives is the basic structural unit in a wide variety of synthetic organic compounds [1]. Phenol and its higher homology are aromatic molecules containing hydroxyl group attached to the benzene ring structure. The origin of phenol in the environment is both industrial and natural. Phenol pollution is associated with pulp and paper mills, coal mines, refineries, wood preservation, plants & various chemicals industries as well as their wastewaters. Due to their high inhibitory and antibacterial activity, phenols may create problems in the operation of biological treatment plants. They also add odour to drinking and food processing water and have mutagenic and carcinogenic effects [2]-[5]. Phenol is also a priority pollutant and is included in the list of EPA (1979).

Biological processes using microbial systems provide an alternative to the existing physical/ chemical technologies (expensive and commercially unattractive) because they are more cost-effective, environment friendly and do not produce large quantities of sludge [6]. No. of microorganisms can utilize phenol under aerobic conditions as source of carbon & energy [7]-[9]. The biological treatment of industrial wastewaters usually depends upon the oxidative activities of microorganisms. Filamentous fungi can be an important source of phenol-degrading species as they grow frequently in wood where phenolic structures are present. Nevertheless filamentous fungi are not frequently used due to difficulties in their cultivation in liquid media and their slow growth rate in comparison with most of the other microbial species.

During this oxidation process, contaminants and pollutants are broken down into end products such as carbon dioxide, water, nitrates, sulphates and biomass (microorganisms). The most efficient *Aspergillus Niger* is capable of using phenol as the sole source of carbon and energy for cell growth and metabolism degrade phenol via metapathway. That is the benzene ring of phenol is dehydroxylated to form catechol derivative and the ring is then opened through meta-oxidation.

Biodegradation is used to describe the complete mineralization of the starting compound to simpler ones like CO_2, H_2O, NO_3 and other inorganic compounds. Microbial degradation of phenol with different initial concentration ranging from 50-2000mg/L have been actively studied using shake flask, fluidized- bed reactor, continuous stirred tank bioreactor, multi stage bubble column reactor, air lift fermenter and two phase partitioning bioreactor methods [10]-[16] and these studies have shown that phenol can be aerobically degraded by wide variety of fungi and bacteria cultures such as *Candida tropicalis* [17] and *Pseudomonas putida* [18].

The efficiency of the phenol degradation could be further enhanced by the process of cell immobilization. Various methods have been described for the immobilization [19]. Alginates represent however several advantages such as high porosity and chemical stability with a mild, fast, simple and low cost immobilization method [20]. Under many conditions, immobilized cells have advantages over either free cells or immobilized cells. The biodegradation of phenol by immobilized cells had been investigated for several microorganisms but the use of immobilized *Aspergillus niger* cells for phenol biodegradation is scanty. This study has the results obtained from the biodegradation of phenol by free and immobilized cells of *Aspergillus sp*. Batch experiments were carried out in order to obtain the maximum phenol biodegradation rates by analyzing the influence of the immobilization in sodium- alginate gel beads on biodegradation performance.

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II. Material & Methods

A. Chemicals and Reagents

All the chemicals used were of analytical grade and the chemicals were supplied by Qualigens Fine chemicals (Mumbai, India).

B. Characterization of Industrial Effluent

The effluent was collected from Anand tissues ltd. (Meerut) which is a paper industry. The effluent collected from industry, was analyzed for different physico- chemical properties (Table I) viz. Temperature, pH, Conductivity, B.O.D, C.O.D, T.S, T.D.S, T.S.S, Chlorides, Phenol. The concentration of each of the component was determined as per the procedure outlined in APHA (2005). [3].

TABLE I: Physic-Chemical Parameters of Effluent.

S.No	Physico – chemical parameters	Test	Standard
1	Temp	34.75 ℃	_
2	pH	7.5	-
3	Conductivity	39.6 µmohs/cm	5 µmohs/cm
4	B.O.D	139.5 mg/L	30 mg/L
5	C.O.D	430.5 mg/L	250 mg/L
6	T.S	1490 mg/L	1200mg/L
7	T.D.S	900 mg/L	1000mg/L
8	TSS	590 mg/L	200 mg/L
9	Chlorides	281.25 mg/L	1000 mg/L
10	Phenol	268 mg/L	1 mg/L

C. Synthetic Effluent

Sterile synthetic effluent composition (mg/l) was proposed by Passos et al. (2009), [21]: KH_2PO_4; 200 $MgSO_4.7H_2O$; 100 NaCl; 25 $Cacl_2.2H_2O$; $3MnSO_4.H_2O$; 500 $NH_4NO_3.H_2O$; 500 Glucose; Phenol (250 or 500).

D. Isolation and Identification of Predominant Microorganisms

The sample was serially diluted using sterile pipettes from 10^{-1} to 10^{-8} dilution. Five different types of bacteria were predominant in the raw effluent and single type of fungi was found to be present. For enumeration of bacteria nutrient agar medium containing peptone (5 g/L), yeast extract (1.5 g/L), sodium chloride (5 g/L), agar (15 g/L) was used and for enumeration of fungi Potato dextrose agar containing potato (200 gm), dextrose (20 gm), agar (15 gm), distilled water (1000 ml) at *pH* 5.6 was used. To obtain pure culture, the cultures were repeatedly streaked on nutrient agar medium and incubated at 37 ℃ for 24 hrs. The isolated bacteria were identified by colony morphology, gram staining, microscopic observation and confirmation test. The identified bacteria were *Pseudomonas alkaligenes, Bacillus pumilus, Bacillus subtilis, Klebsielle sp, Proteus sp*. The isolated fungal culture was identified as *Aspergillus niger* using Lactophenol cotton blue staining method.

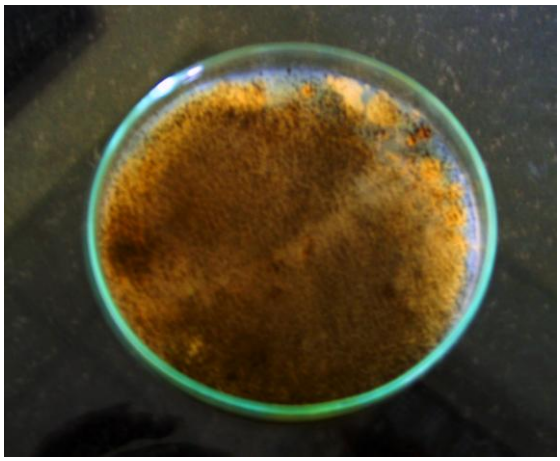

Fig. 1. *Aspergillus niger* a fungal strain growing on PDA agar medium.

E. Isolation, Growth and Processing of Cells

Aspergillus niger previously isolated from paper industry effluent, and was capable of using phenol as carbon source [22]. Strain was maintained on potato dextrose agar (PDA) at 4 ℃. For mass culturing, liquid broth was used as a culture medium which was having the following composition (g l^{-1}): Dextrose (20 g l^{-1}), Peptone (10 g l^{-1}), *NaCl* (0.2 g l^{-1}), $CaCl_2.2H_2O$ (0.1 g l^{-1}), *KCl* (0.1 g l^{-1}), K_2HPO_4 (0.5 g l^{-1}), $NaHCO_3$ (0.5 g l^{-1}), $MgSO_4$ (0.25g l^{-1}) and $FeSO_4.7H_2O$ (0.005 g l^{-1}). The liquid broth pH was adjusted to 4.5. The liquid broth was inoculated with a loop of culture grown on PDA medium and incubated on an orbital shaker (Orbitek, sci, Genics Biotech Ltd) at 125 rpm and 25 ℃ for 5 days in 500 ml conical flasks. The biomass produced was collected by filtration and washed twice with extra pure double distilled water.

Fig. 2. *Aspergillus niger* biomass produced

F. Fungal Spore Immobilization

Spores were immobilized in calcium alginate according to Ellaiah et al. [23]. About 20 ml of sterile sodium alginate solution (3% W/V) and 5 ml of spore suspension (5 $\times 10^6$ spores/ml) were mixed fully and the slurry was dripped into 0.2 M $CaCl_2.2H_2O$ solution at room temperature. The beads were maintained in $CaCl_2$ solution for 1 hour at 4 ℃ and then

the beds were washed three times with sterile distilled water.

G. Inoculum

The free and immobilized spores were inoculated in 250 ml flasks containing 100 ml of synthetic medium and industry effluent and were incubated at 25 ℃ for 5 days to spores germination.

H. Phenol Biodegradation Experiment

Experiments in triplicate were carried out. Flasks containing synthetic and industry effluent were inoculated with free and immobilized cultures. Temperature and agitation were set at 25 ℃ and 200 rpm on a rotary shaker. Control assay (without inoculum) were performed under the same experimental conditions in order to verify abiotic losses. Samples were withdrawn at regular intervals for phenol determination.

Fig. 3. Phenol biodegradation free by and immobilized cells of *Aspergillus niger*

I. Phenol Determination

For Phenol determination, the Folin-Ciocalteau phenol reagent was used, involving the successive addition of 1ml sodium carbonate (200mg/l) and 0.5 ml Folin- Ciocalteau phenol reagent to 10 ml sample. After 60 minutes at 20 ℃, the absorbance was measured at 725 nm against a distilled water and regent blank [24].

J. Growth Determination

The growth determination was done by measuring the absorbance at 600 nm using UV-Vis spectrophotometer (Shimadzu).

III. Result and Discussion

A batch cultivation experiment was carried out using phenol as limiting substrate for *Aspergillus niger*. Initial phenol concentration in synthetic effluent was taken as 250 mg/L.

The extent of phenol concentration was investigated for several batch residence times by intermittent sampling, showing the biodegradation potential of *Aspergillus niger* in degrading synthetic phenol waste. The initial phenol concentration observed in industrial effluent is 268 mg/L.

The measured absorption at 600 nm gives us valuable information about the biomass accumulation (Table II). Dynamics of cell number has the major importance for explanation of *Aspergillus niger* interactions with the available substrate. The variation of the concentration of microbial cells is lowest at the starting hour of the experiment. This result confirms the existence of very similar initial conditions in the batch cultivation process, which is crucial for the objectives of our investigations. The fluctuations of the number of fungal cells were higher at the 5 day. Growth of *Aspergillus niger* in industrial wastewater has been shown in Fig. 3. The free cell growth curve has a typical exponential and stationary phase whereas the growth curve of the immobilized cells of *Aspergillus niger* showed that the cell under immobilized conditions could make a better growth.

Immobilized cells in synthetic & industrial wastewater.

The *Aspergillus niger* cells exhibits diauxic type of growth (Fig. 4) in industrial wastewater. During incubation time of 5 days it is observed that there is slight increase in the biomass but the maximum growth is observed on the fifth day as shown in Fig. 4. When the growth was studied on synthetic wastewater free cell show slight lag phase whereas immobilized cells has a typical exponential phase as shown in Fig. 4.

Since degradation proceed with biomass growth as shown in the respective growth curves. It could be seen from Fig. 4. that the phenol concentration (250 mg/L) was maximum degraded on fifth day but in the industrial wastewater maximum degradation was also observed on fifth day (Fig.4). The control was observed constant throughout the study.

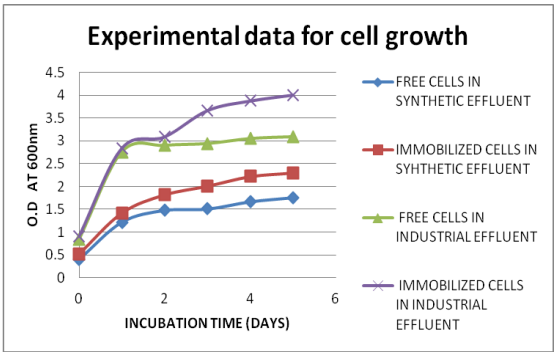

Fig. 4. Growth kinetics of free and immobilized cells in industrial and synthetic effluent.

TABLE II: Growth Kinetics Of Free Cells In Industrial Wastewater

Incubation time (days)	SYNTHETIC EFFLUENT		INDUSTRY EFFLUENT	
	O.D at 600 nm(Free cells)	O.D at 600 nm(Immobilized cells)	O.D at 600 nm(Free cells)	O.D at 600 nm(Immobilized cells)
0	0.4	0.52	0.84	0.9
1	1.22	1.42	2.76	2.84
2	1.48	1.82	2.9	3.09
3	1.51	2.01	2.94	3.67
4	1.67	2.22	3.05	3.88
5	1.76	2.3	3.09	4.01

A batch cultivation experiment was carried out using phenol as single limiting substrate for *Aspergillus niger*. Initial phenol concentration of 250 mg/L was used. The extent of phenol degradation using this initial phenol concentration was investigated for several batch residence times by intermittent sampling Fig. 3 shows the biodegradation potential of *Aspergillus niger* in degrading synthetic phenol waste. Since degradation proceed with biomass growth as shown in the respective growth curves. It could be seen from

Fig. 5 that the phenol concentration (250 mg/L) was maximum degraded on fifth day but in the industrial wastewater maximum degradation was also observed on fifth day Fig. 6

After five days of incubation with *Aspergillus niger* tremendous reduction in phenol concentration is observed. The culture with immobilized cell reached 110 mg/L whereas in free cell it is 119 mg/L. As shown in Table II phenol concentration in synthetic effluent initially provided was 250 mg/L, after five days of incubation the culture with immobilized cell reach 28 mg/L whereas in free cell it is 150 mg/L. Immobilize cell of *Aspergillus niger* results in better performance than the free cell in batch process by reducing the adaptation and consequently the time for complete phenol biodegradation.

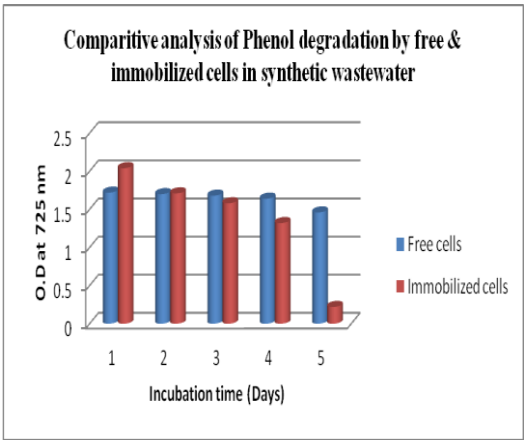

Fig. 5. Comparative analysis of phenol biodegradation by free and immobilized cells in synthetic wastewater

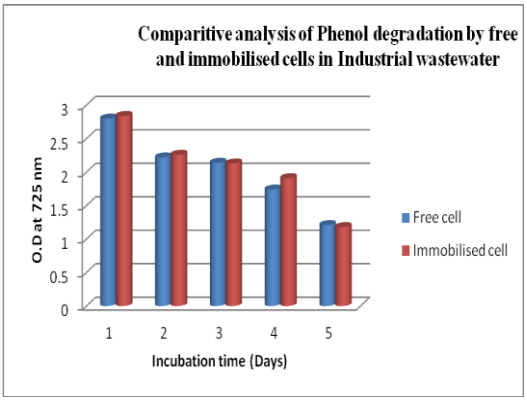

Fig. 6. Comparative analysis of phenol biodegradation by free and immobilized cells in industrial wastewater

TABLE III: COMPARATIVE ANALYSIS OF PHENOL BIODEGRADATION

Sample	Synthetic wastewater		Industrial wastewater	
Phenol degradation time (Days)	Free cell (O.D at 725 nm)	Immobilized cell (O.D at 725 nm)	Free cell (O.D at 725 nm)	Immobilized cell (O.D at 725 nm)
1	1.72	2.04	2.8	2.84
2	1.7	1.71	2.22	2.26
3	1.68	1.58	2.14	2.13
4	1.64	1.32	1.74	1.91
5	1.46	0.22	1.21	1.18

In encapsulated cell culture, the carrier material act as a protective cover against toxicity of phenol by forming networks of the beads, a diffusion barrier for phenol is build up which is not present in free cell culture [17]. The present finding will be useful to treat the waste containing phenol to convert the toxicant into nutrient, biomass and CO_2 via biodegradation through their intermediates. This technology will be useful to the Paper industry which generates the waste containing compounds such as phenol. The present technology will also be efficient and beneficial to treat the waste generated by paper industry.

Our work shows that *Aspergillus niger*. immobilized cells in calcium alginate is promising for application in bio-degradation schemes in order to degrade phenol and possibly other related aromatic compounds at high concentrations in industry generated wastewater which leads to a reduction in time for complete phenol removal in relation to free cells.

IV. CONCLUSION

In the present work the phenol degrading fungi *Aspergillus niger* was isolated from industry effluent. Investigated culture of *Aspergillus niger* are able to eliminate almost entirely the introduced quantity of phenol as sole source of carbon and energy for a period of 5 days. The present finding will be useful to treat the waste containing phenol to convert the toxicant into nutrient, biomass and CO_2 via biodegradation through their intermediates. This technology will be useful to the Paper industry which generates the waste containing compounds such as phenol. The present technology will also be efficient and beneficial to treat the waste generated by paper industry

Our work shows that *Aspergillus niger*. immobilized cells in calcium alginate is promising for application in bio-degradation schemes in order to degrade phenol and possibly other related aromatic compounds at high concentrations in industry generated wastewater which leads to a reduction in time for complete phenol removal in relation to free cells.

Better biodegradation rate of phenol was observed in immobilized cells due to absence of internal and external mass transfer resistance.

An immobilized cell is one of the approaches for incorporating fungal biomass into an engineering process. The advantage of the process based on immobilized biomass include enhancing microbial cell stability, allowing continuous process operation and avoiding the biomass – liquid separation requirement.

The present work open up new dimensions in research in the areas of biological treatment of phenolic effluents and also into the mechanism of phenol biodegradation. The fungus used in the present study is an efficient one and can further be exploited for industrial scale applications.

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