PATNA WOMEN'S COLLEGE 1940

Industrial Microbiology

Explore–Journal of Research for UG and PG Students
ISSN 2278 – 0297 **(Print)**
ISSN 2278 – 6414 **(Online)**

http://www.patnawomenscollege.in/journal

Isolation and Characterization of phenol degrading bacteria and fungi used for bioremediation

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Received : November 2014
Accepted : March 2015
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Abstract : *Phenol is a major pollutant of environment, especially waste water discarded from many industries. The present study was carried out to isolate phenol degrading bacterial and fungal strains from various sources. Samples used for the isolation of bacterial strains were cow dung and cow dung polluted soil while oil polluted soil was used for fungal strains. The isolated bacterial strains were screened for its phenol degradation ability in phenol broth. Total of six different bacterial strains were isolated out of which two most potential phenol degrading bacterial strains (SIV, SV) were selected. A comparative study was performed between bacterial and fungal strain on the basis of their ability to tolerate phenol and it was concluded that bacterial strains have more phenol tolerance than fungal strains. The isolated organisms were then subjected to biochemical tests for further identification and results showed that bacterial strains may be Staphylococcus intermedius and Bacillus carboniphilus whereas fungal strains may be Aspergillus flavus and Aspergillus niger. Staphylococcus intermedius and Bacillus carboniphilus showed 76.70% and 76.60% of phenol degradation at pH 7.5 and 6.5 respectively, within one week. The maximum degradation of phenol was observed in optimized condition. With the increase in NaCl concentration, rate of phenol degradation decreases.*

Key words: *Bacillus carboniphilus, Staphylococcus intermedius, Aspergillus flavus, Aspergillus niger, Phenol degradation, Phenol tolerance, NaCl concentration.*

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Introduction :

Phenol, also known as carbolic acid, is an aromatic organic compound with the molecular formula, C_6H_5OH. It is volatile in nature and is mildly acidic. Melting point is 40-43°C and boiling point is 182°C. It is moderately soluble in water. It is a highly toxic compound. It is an important industrial commodity, used as a precursor to many materials and useful compounds. Its major uses involve its conversion to plastics or related materials.

The environment has been contaminated by variety of toxic compounds through industrial effluents. Phenol is one of the major aromatic pollutants among all the toxic compounds derived from pulp mills, coal mines, gasoline, petrochemicals, wood preservation plants, pesticides, insecticides, herbicides, domestic waste, agricultural run-off and chemical spills (Bulbul *et. al.,* 1997; Aksu *et. al.,*1998; Gupta *et. al*.,1998;Loh *et. al*.,2000).

Among all the aromatic compounds, phenol and their derivatives are common constituent of waste water originating from many industries including pharmaceutical, petroleum, oil refineries, textiles and coal refining (Nuhoglu and Yalcin, 2004). Therefore, because of their toxicity, biodegradation of aromatic compounds are receiving great attention from industrialists and researches. Bacterial strains are capable of utilizing phenol as sole carbon source for growth and therefore, various phenol-degrading microorganisms, *Pseudomonas* have been intensively studied in the past for treating waste water by various physiochemical methods (Annadurai *et. al.*, 2002).

Degradation of phenol occurs through both aerobic and anaerobic pathways. Aerobic biodegradation has been studied in the early 19th centuries. The organisms which utilize phenol by aerobic pathway are Acinetobacter calcoceticus, Pseudomonas *and* Candida tropicalis*. Phenol can also be degraded in the absence of oxygen and it is less advanced than the aerobic process. The organisms capable of degrading phenol under anaerobic conditions were* Desulphobacterium phenolicum.

In addition to bacteria, fungi are known for their diversity and remarkable ability to degrade phenolic compounds. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth. They are able to grow under environmentally stressed conditions such as low nutrient availability, low water activity and at low pH values where bacterial growth might be limited (Davis, J.S. and Westlake, D.W.S., 1978).

Parameters like pollutant concentrations, viable biomass, concentrations, existence of inhibitor, temperature, pH, microbial completion and microbial adaptation are the most important parameters that affect phenol biodegradation rate which depends on the period in which the culture was adapted to phenol.

Materials and Methods :

Sample Collection : Cow dung and cow dung polluted soil were collected from Punaai Chak, Patna while oil contaminated soil was collected from Krishna Nagar, Patna. The samples were collected and all the microbiological analysis was performed within 24 hours of collection of samples.

Isolation of phenol degrading bacteria and fungi : Serial dilution of the samples was done upto10^{-6} dilution and the sterile Phenol agar media plates were inoculated with $10^{-4}$and 10^{-6} dilution by using enrichment culture technique under aseptic conditions, followed by incubation at 37°C and 26°C respectively for 24 - 48 hours until growth was observed. The isolates were maintained by repeated sub culturing and preserved on slants of the same medium.

Screening of the isolated strains : The isolates were screened for their phenol tolerance ability by observing their growth patterns on phenol agar media after incubation. Out of six bacterial and two fungal colonies isolated, two isolates from each group were selected based on their phenol tolerance abilities.

Identification : The potential phenol degrading strains (SIV, SV and FI, FII) isolated from various samples on Phenol agar plates were identified on the basis of their cultural and morphological observation and biochemical characterization. Fungal strains were identified on the basis of Lacto phenol-Cotton blue staining. The tests involved for the identification of bacterial isolates were gram staining, amylase and gelatinase production, citrate utilization, indole test etc. (Nagamani *et. al.*, 2009).

Biochemical Characterization : For biochemical characterization isolated bacterial strains were tested for catalase, hydrolysis of urea, casein, starch, gelatin, hydrogen sulphide production, nitrate reduction and sugar fermentation tests.

Phenol Tolerance : To determine the phenol tolerance ability of the isolated strains, Phenol agar media plates were prepared with different phenol concentration *i.e.*, (1%, 2%, 3%, 4%, 5% and 6%) and inoculated with six bacterial and two fungal strains, followed by incubation at 37p C and 28p C, respectively. Then, the growth was observed.

Phenol Degradation : The assessment of phenol degradation potential of the isolated strains was done by preparing the Phenol broth (1% phenol) and inoculating it with the pure bacterial strains. A control was also prepared without inoculum. Then, the tubes were incubated at 37ºC and at 150 rpm in shaker incubator for one week, followed by its centrifugation at 5000 rpm for 10 minutes. The supernatant was taken from the centrifuged broth and its OD was taken at 272nm (Quintana *et. al.*, 1997). Then, the percentage of phenol degradation or phenol elimination was calculated.

Comparative Study between phenol degrading bacteria and fungi: A comparative study was performed to determine the phenol tolerance potential of bacteria and fungi isolated from various sources.

Optimization: Several growth parameters were taken into the consideration for the optimization of the isolated bacterial strains (SIV, SV) such as- temperature, pH and NaCl Concentration etc**.**

Temperature Optimization: For the optimization of temperature, Phenol broth was prepared and inoculated with pure strains, followed by incubation at various temperatures (04p C, 15p C, 30p C, 35p C, 37p C, 40p C, 50p C). Then, growth was observed.

pH Optimization: pH optimization was performed by preparing phenol broth of different pH (05, 5.5, 06, 6.5, 07, 7.5, 08 and 8.5) and inoculating it with the pure strains. Then, test tubes were incubated at 37°C and growth was analyzed spectrophtometrically at 600nm.

NaCl Optimization: The isolates were optimized with respect to NaCl concentration by preparing the Phenol broth with different NaCl concentrations (0.5%, 01%, 1.5%, 02%, 2.5% and 3%). Then, the phenol broth was inoculated with pure strains and incubated at 35p C and 37p C respectively. Finally, growth rate was observed spectrophtometrically at 600nm.

Results :

Bacterial and fungal colonies were isolated from the dilution (10^{-6}) and (10^{-4}) respectively on mineral salt medium (MSM), supplemented with 1% phenol and no growth was observed on mineral salt medium.

Table 1: Isolation of phenol - degrading strains on Phenol agar medium

Sample used	No. of bacterial isolates Obtained	No. of fungal isolates Obtained
Cow dung	02	–
Cow dung polluted soil	04	–
Oil polluted soil	–	02

Fig: 1.a. S IV

Fig: 1.b. SV

Fig: 1.c. FI

Fig: 1.d. FII

Fig.1: Phenol agar plates showing isolated bacterial strains (SIV, SV) and fungal strains (FI, FII).

Table 2a: Morphological Characteristics of isolated bacterial strains

S.N	Characteristics	S IV	S V
1.	Colour	Red	White
2.	Margin	Entire	Entire
3.	Texture	Smooth	Rough
4.	Elevation	Convex	Convex
5.	Optical characteristics	Opaque	Opaque

Table 2b:Morphological Characteristics of isolated fungal strains

S.N	Characteristics	FI	FII
1.	Colour		
	i. Front view	Light	Black
	ii. Back view	Buff	Cream
2.	Margin	Irregular	Irregular
3.	Texture	Cottony	Cottony

Table 3: Biochemical tests

S.N	Biochemical Tests	S IV	S V
1.	Sugar fermentation test		
	(i) Glucose	+	–
	(ii) Lactose	+	+
	(iii) Sucrose	+	–
2.	Amylase test	+	+
3.	Gelatinase test	+	+
4.	Casein hydrolysis test	+	–
5.	Urease test	+	–
6.	H_2S production test	+	–
7.	Catalase test	–	–
8.	Nitrate reduction test	–	–
9.	IMViC Tests		
	(i) Indole production test	+	+
	(ii) MR Test	–	–
	(iii) V-P Test	–	–
	(iv) Citrate utilization test	+	–

Table 4: Microscopic characteristics of isolated bacterial strains

S. N.	Strains	Gram reaction	Shape	Identification
1.	SIV	Gram negative	coccus	*Staphylococcus*
2.	SV	Gram positive	rod	*Bacillus*

Table 5: Microscopic characteristics of isolated fungal strains

S.N	Strains	Foot cell	Identification
01	FI	Present	*Aspergillus flavus*
02	FII	Present	*Aspergillus niger*

Table 6: Phenol degradation by the bacterial strains

S.N	Strains	OD of Control	OD of Sample
01	SIV	3.018	0.703
02	SV	3.018	0.706

6.1a) Amount of phenol removed from phenol broth was studied and calculated with the help of following equation:

$$\text{Percent of Phenol degradation} = \frac{\text{Control absorbance - Sampleabsorbance}}{\text{Control of absorbance}} \times 100$$

6.1b) <u>Calculation of phenol degradation</u>:

$$\text{Percent of Phenol degradation by SIV} = \frac{3.018 - 0.703}{3.018} \times 100 = 76.70\%$$

$$\text{Percent of Phenol removed by SV} = \frac{3.018 - 0.706}{3.018} \times 100 = 76.60\%$$

Table 7: Phenol tolerance of bacterial and fungal strains

S. N.	Strains	Phenol Concentration					
		1%	2%	3%	4%	5%	6%
1.	S I	+	–	–	–	–	–
2.	S II	+	+	–	–	–	–
3.	S III	+	+	–	–	–	–
4.	S IV	+	+	+	+	+	–
5.	S V	+	+	+	+	–	–
6.	S VI	+	–	–	–	–	–
7.	F I	+	+	–	–	–	–
8.	F II	+	+	–	–	–	–

Table 8: Optimization of growth parameters

S. No.	Optimization parameters	S IV	S V
1.	Temperature	37°C	35°C
2.	pH	7.5	6.5
3.	NaCl Concentration	1.5%	1.5%

Table 9: Comparative study between bacteria and fungi

S.No.	Strains	Tolerance limit
01	SIV	5%
02	SV	4%
03	FI	2%
04	FII	2%

Discussion:

Phenol tolerance and degradation : The results showed that isolates SIV and SV have maximum level of phenol tolerance *i.e.,* 5% and 4%, respectively, among all the six bacterial isolates while FI and FII can also tolerate certain limit of phenol *i.e.,* 2% present in media.

The result showed that Strain SIV and SV both have phenol degradation ability. SIV have comparatively more potential than SV as it removes almost 76.70% phenol within one week while SV removes 76.60% phenol present in medium.

Optimization : The isolates show maximum phenol degradation on optimum growth parameters and the results are as follows – Strains SIV and SV are mesophillic in nature having optimum growth temperature as 37°C and 35°C respectively while they can tolerate temperature range of 30°C -50°C also. Strain SIV is slightly acidic in nature as it grows best at pH 6.5 while SV is neutral showing maximum growth at pH 7.5.Both strains can grow at wide range of pH but fail to grow at alkaline pH. Strain SIV and SV can tolerate certain limit of salt concentration and shows maximum growth as well as phenol degradation at 1.5% salt concentration. These are slightly halophilic in nature.

Comparative study : On the basis of comparative study performed result can be concluded that bacteria has greater potential of phenol degradation than fungi that's why bacteria is most commonly used for bioremediation than fungi.

Identification : From the cultural, morphological and biochemical analysis the strains were identified using Bergey's manual of determinative bacteriology and ABIS Online Software System. SIV and SV may be identified as *Staphylococcus intermedius* and *Bacillus carboniphilus* respectively, while FI and FII may be identified as *Aspergillus flavus* and *Aspergillus niger*.

Conclusion:

The result of this study indicated that indigenous microorganisms with good phenol degradation ability which may enhance the bioremediation and minimize cost of biodegradation than physical methods could be obtained from soil. Since the toxicity of phenol is causing adverse effects on the environment, therefore, degradation of phenol with the help of phenol-degrading microorganisms can be an effective tool for the bioremediation of phenol, persisting in the environment as a hazardous pollutant.

Acknowledgement:

This project was carried out at the Department of Industrial Microbiology, Patna Women's College, Patna. We would like to express our gratitude towards our Principal, Dr. Sister Doris D'Souza A.C., Patna Women's College, the Head of Department, Prof. Sheila Bedi and University Grants Commission, India for their constant support.

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