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Research Article

IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL PHENOL DEGRADING BACTERIAL STRAINS ISOLATED FROM MUNICIPAL SEWAGE, BILASPUR, CHHATTISGARH

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Abstract

Phenol and its derivatives are consistently causing harmful effects to an aquatic ecosystem. The present study focused on the isolation and characterization of potential phenol degrading bacterial strains and subsequently optimization of media ingredients for efficient phenol degradation by potential bacterial strains. Bacterial strains were isolated from municipal sewage, Bilaspur (21'47 and 23'8 N 81'14 and 83'15 E). After optimization phenol degradation rate was increased by 1.84 fold for PDB 5 (from 40.37% to 74.67%) and 1.39 fold for PDB 11 (from 58.62% to 81.51%) at 500mg/l initial phenol concentration. PDB 5 and PDB 11 were identified as *Streptococcus sp.* PDB 5 and *Pseudomonas sp.* PDB 11 respectively as potential phenol degrading bacterial strains. These strains can further be used in microbially assisted phenol degradation to remove phenol derivatives present in industrial wastewater.

Keywords: Phenol; Aquatic ecosystem; Microbially assisted phenol degradation; Optimization; Municipal sewage; *Streptococcus sp.* PDB 5; *Pseudomonas sp.* PDB 11

Introduction

Effluents of Petrochemical, coke oven, dye industries, plastic and fiberglass manufacturing unit, pulp and paper production, herbicide manufacturing and oil refineries have been reported for higher amount of phenolic compounds (Paisio *et al.*, 2013, Mohite *et al.*, 2010, El-Ashtoukhy *et al.*, 2013, Veeresh *et al.*, 2005, Jadhav and Vanjara, 2004). These phenolic compounds enter into aquatic food chain when untreated or by improper treated industrial wastewater drained into natural water reservoirs e.g. ponds, lakes, rivers, and oceans. Further phenolic compounds cause harm to aquatic living organisms (e.g. fish) and related organisms which depend on them as a food source (e.g. Eagle and humans). WHO quoted that short-term exposure of excess phenol inside human body causes liver and kidney damage, harmful effects on respiratory system and growth retardation.

Physical methods (such as ultraviolet rays, ozonation), chemical methods (e.g. hydrogen peroxide and Fenton's reaction) and its combination (physio-chemical methods) have been applied to remove phenolic compounds from industrial wastewater (Lin *et al.*, 1994; Zilli *et al.*, 1993). Presently the chemical treatments have been used in most of the industrial wastewater treatments. But it has been found to produce harmful secondary pollutants and costly as well (Agarry *et al.*, 2008). The biological degradation of phenolic compounds is an alternative approach to overcome aforementioned issues. It is an eco-friendly and cost-effective method to remove excess phenolic compounds from the industrial wastewater. The pure or mixed cultures of microorganisms which are able to utilize phenolic compounds as an energy source can be used for biological degradation of phenols (Allsop *et al.*, 1993; Wang *et al.*, 1999; Yuan *et al.* 2000; Sa et al, 2001). Diverse microorganisms like bacteria, yeasts, algae and filamentous fungi have been reported for their metabolic capability to degrade phenol at high concentrations (Sivasubramanian and Namasivayam, 2014).

Microorganisms can metabolize phenolic compounds by either ortho- or meta-cleavage (Dagley, 1971; Agarry *et al.*, 2008). Phenol hydroxylase has known as the first enzyme in both metabolic pathways (ortho- or meta-pathway). The final products of both ortho- and meta-pathways are entering into the Tricarboxylic acid cycle (TCA) for complete mineralization (Shingler, 1996).

The present study is focused on the isolation and characterization of phenol-degrading bacteria from municipal sewage, Bilaspur and optimization of different parameters to enhance the rate of phenol degradation by potential bacterial isolates.

Materials and Methods

Sampling

Samples were collected from Municipal sewage water, Bilaspur (21'47 and 23'8 N latitudes and 81'14 and 83'15 E latitudes) for the isolation of Phenol degrading bacterial strains and stored in sterile plastic containers at 4ºC until used.

Isolation

Serial dilution method was adopted for isolation of bacterial strains. Serially diluted samples were inoculated in Nutrient Agar Media (NAM) plates and incubated at 37^0C for 24h. These NAM plates served as master plates for the isolation of phenol degrading bacterial strains. After incubation, the pure cultures of bacteria were prepared by streak plate method.

Acclimatization of Bacterial Strains

Pure cultures of bacteria were acclimatized for one month in mineral salt medium (MSM) containing phenol as the sole carbon source at a concentration of 500 mg/l at 37^0C and 140 rpm. MSM consisted of (g/l) $(NH_4)_2SO_4$-0.2, $CaCl_2$-0.007, $FeCl_3$-0.001, $MnSO_4H_2O$-0.1, $MgSO_47H_2O$-1.0, K_2HPO_4-0.50, KH_2PO_4-0.25 and glucose-2.0 (pH 7.0).

Phenol Degradation

The ability of the acclimatized bacterial strains to degrade phenol was determined. Experiments were carried out in 150 ml Erlenmeyer flask containing 50 ml of MSM with phenol concentration at 500 mg/1. A loop full bacterial culture (McFarland Standard-0.5; 1.5×10^8 cells) was aseptically inoculated in the flasks containing sterilized MSM and incubated at 37ºC in the shaker (150 rpm). Samples were withdrawn at every 24h time interval (till the completion of incubation period), centrifuged and analyzed for phenol concentration for the assessment of the rate of phenol degradation by respective bacterial strains. Initial phenol concentration was subtracted by residual phenol concentration (concentration of phenol remained after the microbial phenol degradation) for the assessment of phenol degrading efficiency of microbial strains. Percentage phenol degradation (g/l) was calculated as per formula is shown below:

$$Percentage\ phenol\ degradation\ (\%\ P_D) = \frac{Initial\ phenol\ concentration - Residual\ phenol\ concentration}{Initial\ phenol\ concentration} \times 100$$

Initial phenol concentration - initial amount (mg/l) of phenol
Residual phenol concentration- amount (mg/l) of phenol remained after biodegradation

Phenol Estimation Assay

Phenol concentration was determined quantitatively by a colorimetric method by using 4-aminoantipyrine (4- AAP) as the chromophore. Protocol was adopted from Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992) with some modification. Reaction mixture consisted of 0.9 ml double distilled water, 0.1 ml supernatant (sample), 50 µl of 2N NH_4OH (pH-10.0), 25µl of 2% 4- AAP and 25 µl 8% of $K_3Fe(CN)_6$ at 40^0C. Optical density was measured at 510 nm.

Estimation of Cell Biomass Concentration

Cell biomass concentration was estimated in terms of CFU/ml as per standard method.

Morphological and Biochemical Characterization

Morphological, Physiological and Biochemical characterization (as per described in Bergey's Manual) were done for the identification of potential phenol degrading bacterial isolates.

Results

Isolation

Total sixteen bacterial strains (PDB 1, PDB 2, PDB 3, PDB 4, PDB 5, PDB 6, PDB 7, PDB 8, PDB 9, PDB 10, PDB 11, PDB 12, PDB 13, PDB 14, PDB 15 and PDB 16) were isolated from municipal sewage (Table 1). PDB 3, PDB 4, PDB 5, PDB 6, PDB 7, PDB 8, PDB 9, PDB 10, PDB 11, PDB 14 and PDB 15 showed more than 10% of phenol degradation.

Table 1: Percentage phenol degradation rate by bacterial isolates

S.N.	Bacterial isolate	PD (± SD) %
1	PDB 1	*
2	PDB 2	*
3	PDB 3	14.75 (±1.29)
4	PDB 4	20.41(±0.40)
5	PDB 5	40.37 (±0.27)
6	PDB 6	32.33 (±0.44)
7	PDB 7	27.83 (±0.39)
8	PDB 8	19.96 (±0.55)
9	PDB 9	13.71 (±0.58)
10	PDB 10	35.88 (±0.26)
11	PDB 11	58.62 (±1.16)
12	PDB 12	*
13	PDB 13	*
14	PDB 14	22.30 (±1.10)
15	PDB 15	25.44 (± 0.45)
16	PDB 16	*

PD- Phenol degradation; * ≥ 10%;
Initial phenol concentration was 500mg/l

Screening of Phenol Degradation

Two bacterial strains PDB 5 and PDB 11 showed significant phenol degradation efficiency 40.37% and 58.63% respectively, after 96 h (Table 1).

Optimization of Bacterial Strains for Maximum Phenol Degradation

Physiochemical parameters and media composition for bacterial strain under un-optimized and optimized condition are shown in Table 2. PDB 5 and PDB 11 showed maximum phenol degradation at 35^0C and 37^0C respectively while pH 6.5 and 7.5 respectively (Table 2). Finally after optimization phenol degradation rate was found to increase from 40.37% to 74.67% for PDB 5 and from 58.62% to 81.51% for PDB 11 (Table 3).

Morphological and Biochemical Characterization

Morphological (Table 4) and Biochemical (Table 5) characterizations were done for the identification of bacterial isolates (PDB 5 and PDB 11). PDB 5 and PDB 11 were identified as *Streptococcus sp.* PDB 5 and *Pseudomonas sp.* PDB 11 respectively (Table 6). Percentage (%) Similarities were calculated as per Morphological and Biochemical characteristics similarity from existing database of *Streptococcus epidermis* and *Pseudomonas aeruginosa* to bacterial isolate PDB 5 and PDB 11 respectively (Table 6).

Table 2: Un-optimized and optimized condition for bacterial isolates

S.N.	Parameters	Un-optimized	Optimized	
			(PDB 5)	(PDB 11)
1	pH	7.0	6.5	7.5
2	Temperature (°C)	37	35	40
3	Time (hours)	96	96	96
4	Preinoculum (%)	1	5	5
	Media Composition			
1	Yeast extract (g/l)	NA	1.25	1.25
2	Glucose (g/l)	2.0	2.0	2.0
3	$(NH_4)_2SO_4$ (g/l)	0.2	0.2	0.2
4	$MnSO_4H_2O$ (g/l)	0.1	0.1	0.1
5	$MgSO_47H_2O$ (g/l)	1.0	1.0	1.0
6	K_2HPO_4 (g/l)	0.5	0.5	0.5
7	KH_2PO_4 (g/l)	0.25	0.25	0.25
8	$CaCl_2$ (mg/l)	7.0	7.0	7.0
9	$FeCl_3$ (mg/l)	1.0	1.0	1.0
10	$ZnSO4.7H_2O$ (mg/l)	NA	0.1	0.1
11	CuSO4. 5H2O (mg/l)	NA	0.2	0.2
12	$Na_2Mo(O)4$ (mg/l)	NA	0.1	0.1

NA – Not added in media

Table 3: Phenol degradation rate of bacterial isolates (Un-optimized and Optimized)

S.N.	Bacterial isolate	Initial Phenol (mg/l)	Un-optimized	Optimized	Fold increase in % phenol degradation rate
			Phenol degradation (±SD) %	Phenol degradation (±SD) %	
1	**PDB 5**	500	40.37 (±0.27)	74.67 (±0.39)	1.84
2	**PDB 11**	500	58.62 (±1.16)	81.51 (±0.89)	1.39

Initial phenol concentration-500mg/l; Time 96 h; pH (PDB 5; 6.5 and PDB 11; 7.5) and Temperature (PDB 5; 35 °C and PDB 11 ;40 °C)

Table 4: Morphological characteristics of bacterial isolates

SN	Morphological characteristics	PDB 5	PDB 11
1	Colony Color	Pale yellow	Cream
2	Surface Texture	Smooth	Ssmooth
3	Colony Elevation	Raised	Convex
4	Colony Diameter	1.2 mm	1.6 mm
5	Gram Stain	+	-
6	Shape	Coccid	Rod
7	Spore	+ (ND)	+
8	Capsule	+	+

ND- No data available in database when calculating percentage similarity.

Table 5: Biochemical characteristics of bacterial isolates

S.N.	Biochemical Characteristics	PDB 5	PDB 11
1	Catalase	+	+
2	Indole production	-	-
3	Acid Production	+	+
4	Gas Procution	+	-
5	Voges-Proskauer	+	+
6	Citrate utilization	+	+
7	Nitrate reduction	-	+*
8	H_2S production	+	-
	Hydrolysis		
9	Casein	**+ (ND)**	-
10	Urea	+	-
11	Glycerol	**-(ND)**	+
12	Gelatin	+	+
14	Lactose	+	-
15	Sucrose	+	-
16	Glucose	+	+
17	Fructose	+	+
18	Maltose	+	-
19	Cellobiose	**-(ND)**	-

*-Strain variation; ND- No data available in database when calculating percentage similarity.

Discussion

Two bacterial isolates *Steptococcus sp*. *Pseudomonas sp*. PDB 11 (40.37%) and PDB 11 (58.62%) were showed significant phenol degradation efficiency after 96 h under un-optimized condition (Table 1). Bacterial strains PDB 5 (74.67%) and *Pseudomonas sp.* PDB 11 (81.51%) showed significant phenol degradation efficiency after 96 h under optimized condition. Therefore after optimization phenol degradation rate increased by 1.84 fold for PDB 5 (from 40.37% to 74.67%), 1.39 fold for PDB 11 (from 58.62% to 81.51%) shown in Table 3. *Streptococcus sp.* PDB 5 and *Pseudomonas sp*. PDB 11 were identified as potential phenol degrading bacterial strains.

Table 6: Identification of bacterial isolates based on Morphological and Biochemical characteristics as per described in Bergey's Manual

S.N.	Bacterial Isolates	Identified (as per Bergey's Manual)	Percentage similarity
1	*Streptococcus sp.* PDB 5	*Streptococcus epidermis*	93%
2	*Pseudomonas sp.* PDB 11	*Psuedomonas aeruginosa*	91%

Several studies have been reported with the pure culture of *Pseudomonas sp.* (*Pseudomonas putida* and *P. aeruginosa*) (Allsop *et al.*, 1993; Ahmed, 1995; Annadurai *et al.*, 2002; Chao *et al.*, 2007) in which, phenols degraded via meta-pathway (Carme *et al.*, 1987). In 2009, Chakraborty *et al.*, were studied on biodegradation of phenol by native microorganisms isolated from coke processing wastewater and observed that maximum degradation of 33.46% occurred at 30°C after 6 h and at the end of 24 h (30°C) 76.69% of phenol degradation was recorded. Further, Chandana *et al.*, (2011) revealed that the concentration of 0.2 g/l ammonium chloride in media enhanced phenol degradation rate up to 73.28% with *Pseudomonas aeruginosa*. Pandimadevi *et al.*, (2014) optimized the concentration of media ingredients (Phenol, K_2HPO_4, KH_2PO_4, $MgSO_4$, $(NH_4)2SO_4$, $MnSO_4$, $FeSO_4$, NaCl and H_3BO_3) to enhance the rate of phenol degradation by *P. aeruginosa* (MTCC 7814). They optimized the concentration of media ingredients by Central composite Design (CCD) and Response Surface Methodology (RSM) and the observation revealed the optimal values of ingredients were KH_2PO_4 0.025 g/l, $(NH_4)2SO_4$ 0.45 g/l, $MnSO_4$ 0.05 g/l and phenol 1000 mg/l with maximum rate of phenol degradation (83.86%). In 2006, September, 9th International Conference on Environmental Science and Technology held on Rhodes Island, Greece where Wang *et al.*, revealed that in their experiment *Streptococcus* showed maximum phenol degradation rate at pH 6.5 and temperature 32^{o}C. Later (2010) Mohite *et al.*, revealed that *Streptococcus epidermis* isolated from soil was able to degrade 200mg/l of phenol and confirmed by both spectrophotometric and HPLC analysis. In 2015 Mohite again conducted the study on biotransformation of phenol and its derivatives and found that 4-nitrophenol was best transformed by *Streptococcus epidermis* at pH 7.0 and temperature 32^{o}C.

Conclusion

The present research was focused on the isolation and media optimization for phenol degrading bacterial strains. *Streptococcus sp.* PDB 5 and *Streptococcus sp.* PDB 11 were identified as potential phenol degrading bacterial strains. These bacterial strains would be effectively applied on microbially assisted phenol degradation of industrial wastewater which consisted of excess phenolic compounds. Further mutagenesis and kinetic study will be applied on *Streptococcus sp.* PDB 5 and *Streptococcus sp.* PDB 11 to enhance their phenol degrading efficiency at higher phenol concentration (≤500mg/l).

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