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Research Article

Molecular characterization and detoxification of phenol by haloalkaliphilic bacterium *Bacillus flexus*

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ABSTRACT
Phenols are the toxic pollutant and have hazardous effect on human health therefore attempt was made to isolate and characterized phenol detoxifying bacteria from halo-alkaliphilic environment. The most of the phenol bearing industrial effluent is alkaliphilic in nature; hence detoxification with the help of haloalkaliphilic organism is more suitable eco-friendly detoxifying technology. Lonar Lake, a halo-alkaliphilic environment, harbors many diversified microbes can degrade many toxic industrial effluents. In these study sediment, matt and water samples were collected from alkaline Lonar Lake and inoculated in 100 mL peptone water phenol medium. After enrichment isolation of bacteria was done on nutrient agar plate. Isolate was characterised by cultural, morphological, biochemically and 16S rRNA gene sequencing identified as *Bacillus flexus.* This *Bacillus flexus* was further screened for its ability to degrade phenol by 4-aminoantipyrine method. Data showed that the phenol-degrading *Bacillus flexus* appears to degrade 74% phenol in 96 h and found to have greater potential for removal of phenol from industrial effluent.

Keywords: Phenol, Lonar Lake and *Bacillus flexus*.

INTRODUCTION
Phenol and its substituent phenolic compounds contribute a remarkable adverse impact to the environment, which is often found in the effluents discharged from the industries such as paper and pulp, textiles, gas and coke, fertilizers, pesticides, steel and oil refineries etc., [8]. During the last two decades, phenolic compounds have become the subject of intense research in the preservation of our environment. The US Environmental Protection Agency [7] had classified the phenolic compounds as high priority pollutants due to their extensive impact on the deterioration of the water environment [2,10]. Phenol is produced both naturally and synthetically by chemical processes and such as leather, paint, pharmaceutical, coking plant petrochemical, oil refinery, plastic, explosives, steel, pesticides etc and disinfectants uses phenol and its derivative compounds as its products and raw materials. Phenol is toxic even at low concentrations and the toxicity of phenols for microbial cells has been investigated [11]. The environmentally unacceptable toxic effects and adverse effects of phenol on health are well documented [5]. Therefore, it is very important to employ appropriate strategies of wastewater treatment in order to counterbalance these growing environmental problems. Several treatment technologies have been employed in this regard. Most of phenols bearing industrial waste are alkaliphilic in nature; hence attempt was made to isolate a bacterium from halo-alkaliphilic environment of Lonar Lake. Alkaline Lonar Lake in India is a unique ecosystem and

wonder formed by meteorite impact on basaltic rock which is situated in a village Lonar in the Buldhana District of Maharashtra State, India. The lake water is alkaline having an average pH of 9.5- 10. Lonar Lake is a closed one without any outlet and unique due to its salinity, alkalinity and biodiversity. Due to the uniqueness, the lake has evoked much scientific value among researchers [12]. The Phenol degrading bacteria present in the Lonar Lake has not been studied in detailed hence attempt was made to apply culture dependent strategy to explore the diversity of phenol degrading bacteria from Lonar Lake and identification of this degrader based on morphological, biochemical, cultural characters and 16S rRNA analysis and evaluation of phenol degradation potential in different phenol concentrations.

MATERIALS AND METHODOLOGY

Collection of Sample: A total of twelve matt, sediment and water samples were collected from four different location of alkaline Lonar Lake during season September, 2014. The matt and sediment samples were collected in a zip lock polythene bags using a sterilized spatula and the water samples were collected in a sterile bottles. All samples were labelled and kept in sterile polythene bags at 4º C until analysis.

Isolation and Identification of Phenol degrading bacteria*:* To 90 mL of peptone water-phenol medium (in 250 mL conical flask) 10 mL of water sample or 1 g sediment sample was added separately. These flasks were shaken and incubated on rotary shaker for seven days at 120 rpm at 37º C. After seven days of incubation, 10 mL of cultured broth was sub-cultured in fresh 250 mL media and allowed to incubate in shaker for next seven days. Same procedure was repeated for five times [9]. Isolated bacteria were identified based on standard morphological, cultural, biochemical tests and 16S rRNA gene sequencing from Agharkar Institute of Pune.

Determination of phenol degradation potential: The isolates showing growth on nutrient agar slants were used for further studies on degradation of phenol. Hundred mL peptone phenol broths were inoculated for 24 h old culture of the isolates, grown on a respective agar medium and the flasks were incubated under shake culture condition on a rotary shaker for 48h at an ambient temperature. Phenol was estimated by 4-Aminoantipyrine method [15].

RESULT AND DISSCUSION

A total twelve samples comprising of sediment, matt and water were collected from Lonar Lake, from which one bacterium, *Bacillus flexus* was isolated in peptone water phenol medium having 0.5mg/mL concentration of phenol, the isolate was characterized biochemically by Hi-media Rapid Detection kit KB003 and KB009. The isolate is gram positive sluggishly motile rod shape and having arrangements (Table 1). The biochemically characterized isolate was identified by 16S rRNA sequencing from Agharkar Research Institute, Pune as *Bacillus flexus* (Table 2) and its phenol degrading capacity was determined by the 4-aminoantipyrin method. Tambekar *et al.*, [12] isolated the phenol degrading *Pseudomonas stutzeri*, from the samples collected from Lonar Lake, while Kanekar *et al.*, 1999 isolated alkaliphilic phenol degrading *Arthrobacter* sp., *Bacillus cereus*, *Citrobacter freundii*, *Micrococcus agilis* and *Pseudomonas putida* bacteria from the sediment of Lonar Lake. The phenol degrading *Staphylococcus aureus* strain was also isolated by Butani *et al.*, [4] from the effluent sample of Amla Khadi, located in Unkaleshwar, India.

The isolate *Bacillus flexus* (IFO 15715 (T) utilized 74% of phenol from the culture broth having 0.5mg/mL concentration of phenol (Fig 1). The rate of degradation of phenol was increasing for first 24 h to 96 h incubation as the bacterium was in log phase of division. The rate of degradation decreased slowly after the 24 h of incubation (Fig 2). Physiological parameters play a vital role in the growth and degradation behaviour of microorganism. The percent degradation was maximum at alkaline pH 10 (76%) while rate of degradation was 0.040 (Fig. 3 and 4). At high temperature the percent utilization of phenol by this organism was maximum at 40ºC (Fig.5 and 6).

Table 1. Morphological and biochemical characteristics of bacterium isolated from Lonar Lake					
TEST	**RESULT**	**TEST**	**RESULT**	**TEST**	**RESULT**
Shape	Rod	Catalase	+	VP	-
Colour of colony	Milky White	Oxidase	+	Arginine	+
Gram staining	+ve	citrate	-	Sucrose	+
Texture	Smooth	Nitrate reduction	+	Maltose	+
Arrangement	Single	Lactose	-	Fructose	+
Motility	Motile	Xylose	+	Dextrose	+
Growth at different temperature		Glucose	+	Melibiose	+
30°c	+	Arabinose	-	Mannose	+
40°c	+	Saccharose	-	Sodium Gluconate	+
50°c	+	Galactose	+	Glycerol	-
Growth at different pH		Raffinose	+	Salicin	-
pH 7	+	Trehalose	+	Dulcitol	-
pH 8	+	Mannitol	+	Inocitol	-
pH 9	+	Adonitol	-	Sorbitol	+
pH 10	+	Lysine Utilization	-	Erythritol	-
pH 11	+	Ornithine	-	Melezitose	-
Growth at different salt conc.		Esculin hydrolysis	-	α- Methyl-D-Glucoside	-
1%	+	Rhamnose	-	Xylitol	-
2%	+	Cellibiose	-	Sorbose	-
3%	+	ONPG	-	L-Arabinose	-
4%	+	Esculin	-	Inulin	-
5%	+	Malonate	-	MR	-
Note:- Positive(+) ; Negative(-)					

Table 2: The 16S rRNA gene sequencing Closest phylogenetic affiliation and pair similarity of isolated phenol degrading organism from Lonar lake		
Strain Designation	**Closest phylogenetic affiliation**	**Max ident**
DHT 13	*Bacillus flexus* IFO 15715(T) 16S ribosomal RNA gene partial sequence (AB021185)	99.70%

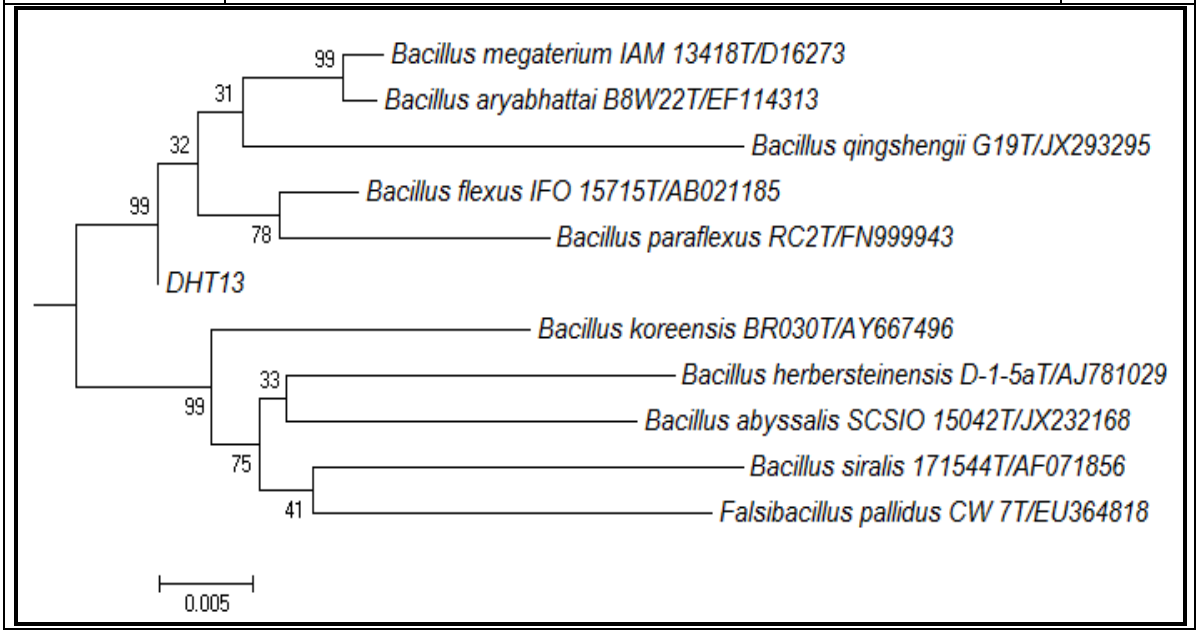


Fig. 1 Percentage utilization of Phenol

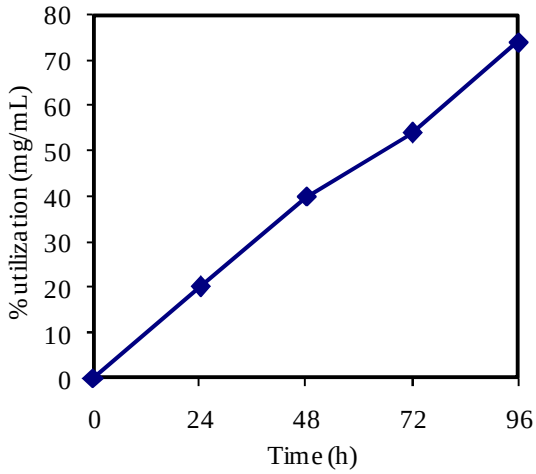


Fig 2: Rate of Degradation of Phenol

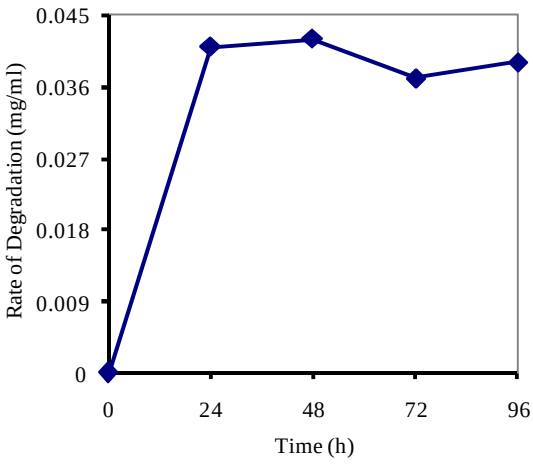


Fig 3: Effect of pH on percent utilization of Phenol

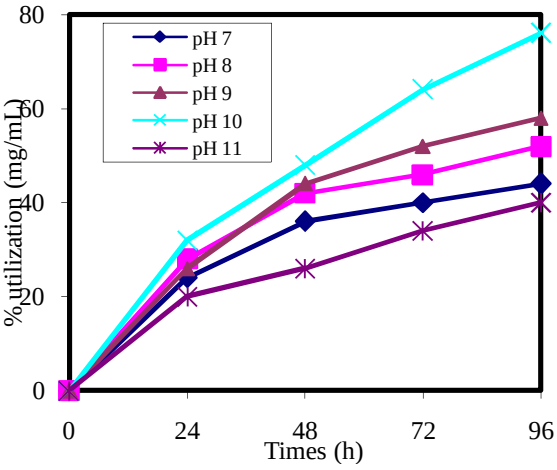


Fig. 4: Effect of pH on rate of degradation of Phenol

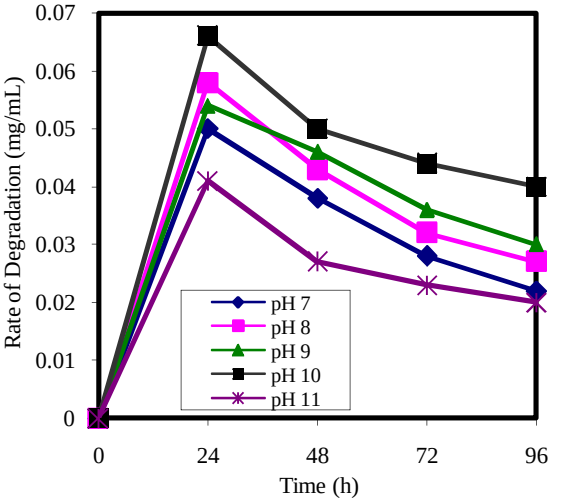


Fig 5. Effect of Temperature on percent utilization of phenol

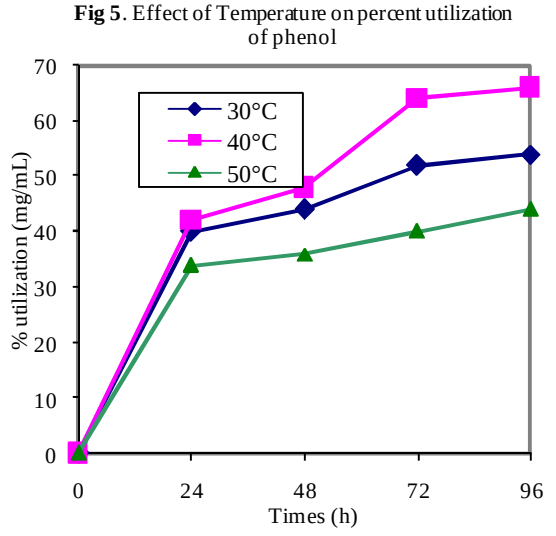
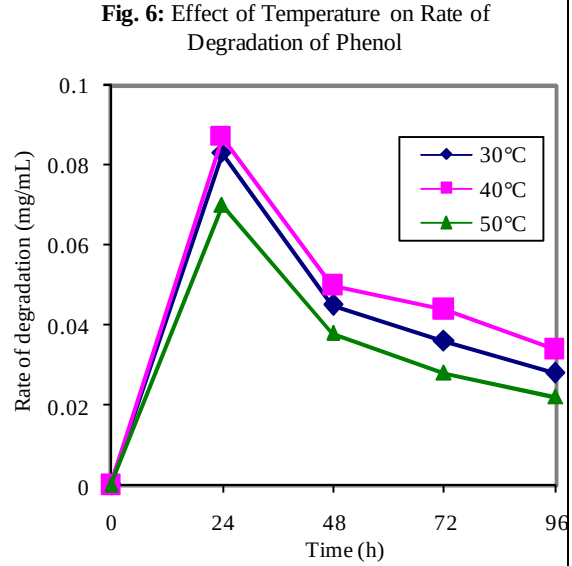


Fig. 6: Effect of Temperature on Rate of Degradation of Phenol



Tambekar *et al.,* [12] isolated the phenol degrading *Pseudomonas stutzeri*, from the sediment samples from Lonar Lake, and showed 87% phenol degrader. Abdullah *et al,* [1] reported phenol degradation by *Pseudomonas putida* and *Alva* and Peyton [3] by *Halomonas campisalis* that resulted in complete degradation of phenol (100 ppm) within 96 h. Tambekar *et al.,* in 2013 [11] reported *Staphylococcus arlettae* (SDT1), *Staphylococcus* species (SDT2) from the alkaline Lonar Lake which degrade phenol upto 64% and 75%. Mrozik *et al,* (2003), demonstrated that phenols and their compounds are the most recalcitrant and persistent organic chemicals in the environment. Tambekar et al in 2014 [13] also reported degradation of phenol upto 72% alkaliphilic by *Prolinoborus fasciculus*. The bioremediation potential of indigenous native microbes from cock waste water was studied by Chakraborty *et al*, in 2010 [6], in batch culture using synthetic phenol in water in concentration range of (100-500) mg/L as model limiting substrate.

CONCLUSION

Phenol is a major pollutants being discharged from the effluents of various sources and it mix in the water bodies and make them unusable. Several physical and chemical methods for removal or treatment of phenol are in use. The physicochemical removal or treatment technologies have been found to have inherent drawback owing to the tendency to form secondary toxic intermediates and also proven to be costly. Complete removal of the pollutants by the use of these processes is not possible. Hence biological method for degradation of phenol is adapted now days. Critical appraisal of the literature reveals that biological treatment is economical, practical and the most promising and versatile approach as it leads to complete mineralization of phenol producing non toxic end products.The aerobic phenol degrading isolate *Bacillus flexus* have greater potential to remove phenol as sole source of carbon and energy. They have ability to survive in harsh environmental condition and also can grow at normal condition. *Bacillus flexus* have high adaptability to wild range of environments. They can be directly used for such treatment where phenol is thrown by industries into the environment. They are cost effective less energy consuming and eco-friendly to the environment.

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