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RESPONSES OF LABORATORY STOCK CULTURE CONTAMINATED BACTERIA AGAINST PHENOL, pH, TEMPERATURE AND ANTIBIOTICS

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ABSTRACT

Responses of two bacteria isolated from the contamination of laboratory stock culture of phenol degrading bacteria against the stresses induced by phenol, temperature, pH and antibiotics were studied. The isolated bacteria were found to belong to the genus *Streptobacillus* and *Corynebacterium.* Growth of these two isolates on phenol resulted in changes in cell shape from rod to cocci as well as the reduction of cell size. Cell shape and size remained unchanged in nutrient broth. There was a decrease in optical density and increase in cell surface hydrophobicity when grown on phenol. Bacterial adherence to hydrocarbons assays showed cells grown on higher concentrations of phenol to be more hydrophobic than those grown on phenol with lower concentrations. Changes in growth of these two isolates were also evident for changing pH and temperature from their optimal as their response to these stresses. Antibiotic Susceptibility Test showed that both isolates were resistant to Amoxicillin and susceptible to tetracycline and phenoxymethylpenicilline.

Key words: *Streptobacillus*, *Corynebacterium*, Stress, Growth, Hydrophobicity

INTRODUCTION

Phenol is a very effective disinfectant, a 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms, that's why phenol is commonly used in hospitals and clinics as disinfectants. However, some bacteria can tolerate phenol and use it as carbon source, thus degrade phenol and phenolic compounds which give positive impact to minimize environmental pollution. In this study, two bacteria were isolated from the stock culture of phenol degrading bacteria. Those two identified bacterial isolates were *Streptobacillus* and *Corynebacterium.* It is often devastating to deal with these pathogens in the laboratory as well as in hospitals due to cross contamination and nosocomial infections (Emori and Gaynes 1993)**.**

In the natural environment bacteria and other microbes are constantly subjected to various stresses, such as nutrient supply, pH, temperature, salinity, osmotic pressure, oxygen supply and the presence of toxic or inhibitory substances in the environment. Bacteria use different tactics to overcome these stresses. Organic solvents can be toxic to microorganisms, depending on the inherent toxicity of the solvent and the intrinsic

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tolerance of the bacterial species (Ramos *et al*. 2002) and flocculated microorganisms display a high degree of resistance to biocidal compounds (Costerton *et al*. 1987, Giwercman *et al*. 1991, Anwer *et al*. 1992). The aim of the present study was to investigate the response of these two possible pathogens to solvent, temperature and pH stresses in respect of their cell size and shape, growth, and hydrophobicity. Antibiotic susceptibility test was also performed to investigate the susceptibility of these two possible pathogens to commonly used antibiotics.

MATERIALS AND METHODS

Organisms were isolated from the contamination of laboratory stock culture of phenol degrading bacteria. Bacteria were maintained on nutrient agar by plating on nutrient agar medium incorporating 2% Bacteriological Agar (w/v). The organism was kept at 4˚C for around one month and then sub-cultured. Phenol was obtained from Merck (Darmstadt, Germany).

On the basis of different morphological characteristics on solid agar plates bacteria were isolated and purified by repeated subculture on solid agar. Isolated bacteria were identified according to methods described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1984) and Manual for the identification of Medical Bacteria (Barrow and Feltham 1993).

The ingredients of the minimal medium (Goulding *et al*. 1988) were combined in distilled water and the pH was adjusted to 7.0 with 2 M NaOH. The trace salts solution was prepared separately in distilled water. The ingredients of the minimal medium were as follows: (K_2HPO_4, 4.36 g; NaH_2PO_4, 3.45 g; NH_4Cl, 1g; $MgSO_4 \cdot 6H_2O$, 0.912 g/l). The composition of per 100 ml trace salts solution was as follows: ($CaCl_2 \cdot 2H_2O$, 4.77 g; $FeSO_4 \cdot 7H_2O$, 0.37 g; $CoCl_2 \cdot 6H_2O$, 0.37 g; $MnCl_2 \cdot 4H_2O$, 0.10 g and $NaMoO_4 \cdot 2H_2O$, 0.02 g).

Bacteriological agar at a concentration of 2% (w/v), was added to the minimal medium. Following sterilization by autoclaving at 121˚C for 15 minutes, the medium was allowed to cool. Immediately prior to pouring, phenol was added to the agar to give the appropriate concentrations.

Bacteria were grown overnight in nutrient broth, centrifuged at 5000 rpm for 10 min and washed twice with 0.5 M potassium phosphate buffer. Five milliliter culture suspension was used to inoculate 95 ml sterile minimal medium and nutrient broth. After inoculation, flasks were incubated in an orbital shaker at 120 rpm at 32˚C. Samples were aseptically removed at regular intervals and analysed for phenol removal.

For growth measurement, inoculated flasks and control flasks were incubated in an orbital shaker at 120 rpm at 32˚C with different pH conditions and different phenol concentrations. For desired temperatures the shaker was adjusted at different

temperatures. Samples were aseptically removed to read OD (Optical density) for growth at 660 nm.

Pictures for documenting cell shape and size were taken by using a colour video camera attached to a microscope at 1000× magnification.

The hydrophobicity of both isolates during growth on phenol or other stress conditions was determined using the method of Rosenberg *et al*. (1980). Cells were removed by centrifugation (4000 rpm for 10 minutes) and washed twice with 0.5 ml potassium phosphate buffer to remove any interfering solutes. The cells were resuspended with potassium phosphate buffer to give a final OD at 400 nm. Four milliliters of the washed cells was added to acid-washed test tubes and overlaid with 1 ml of commercial octane (mixture of 2,2,4-trimethyl pentane 90% and n-heptane 10%). The resultant aqueous/organic mixtures were allowed to settle for 20 minutes. The aqueous phase was then carefully removed using Pasteur pipette and the OD at 400 nm was read. Hydrophobicity expressed as adherence to octane was calculated using the formula:

$$[(a - b)/a] \times 100$$

where, a is the initial cell concentration in the aqueous phase and **b** is the cell concentration in the aqueous phase after partitioning.

Susceptibility of the isolates was assessed by the multi-disk diffusion method (Ferguson 2007). The disk diffusion tests were completed using blank disks (Oxoid, UK) with desired antibiotic. Overnight cultures were streak plated using cotton swab on nutrient agar. The disks were placed on the agar surface using a sterile tweezers. The plates were then incubated at 37˚C for 24 hours. The clear zones (zones of inhibition) around the disks were noted and measured.

Phenol concentrations were determined by using the 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (APHA 1998).

RESULTS AND DISCUSSION

The bacterial strains which grew on phenol were identified by different morphological study accompanying staining procedure and some biochemical tests. Results of these experiments were shown in the Table 1. Morphological and phenotypic characteristics were examined for each isolate which were consistent with the description of typical *Streptobacillus* and *Corynebacterium* according to Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons 1984) and Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham 1993). Thus the isolates 1 and 2 were found to belong to the genus *Streptobacillus* and *Corynebacterium,* respectively.

Genus *Streptobacillus* is the inhabitants of throat and nasopharynx of wild and laboratory rats. This causes one form of rat bite fever in humans, type species: *Streptobacillus moniliformis* (Holt *et al*. 2000). Primarily the genus *Corynebacterium* is obligate parasites of mucous membranes or skin of mammals, but occasionally they are found in other sources; some species are pathogenic for mammals, *Corynebacterium diphtheriae* (Holt *et al*. 2000). *Corynebacterium diphtheriae* was identified as the etiological agent of diphtheria by Klebs in 1883.

Table 1. Cultural, morphological and biochemical characterization of bacterial isolates.

Characteristic	Isolate 1	Isolate 2
A. Colony characteristics:		
Colour	White	Cream
Shape	Round	Round
Edge	Irregular	Regular
Elevation	Flat	Raised
Texture	Non-mucoid	Mucoid
B. Cell characteristics:		
Gram +/-	-	+
Shape	Rod	Rod
Spores +/-	-	-
Motility	-	-
C. Biochemical properties:		
Oxidase	-	+
Catalase	-	+
Methyl red	+	-
Voges - Proskauer	-	+
Sugar fermentation:		
Fructose	+	-
Galactose	-	-
Glucose	+	-
Lactose	-	-
Maltose	+	-
Starch	+	-
Sucrose	-	-
Xylose	+	-

Changes in cell size and shape of the isolate *Streptobacillus* and *Corynebacterium* with time at different concentrations of phenol and different pH conditions are shown in Table 2. The rod shape of the organisms was observed initially. After 24 hours the shape of the cell was changed to round and there is a reduction in cell size. At 72 hours the cell became smaller than at 48 hours. Both organisms retained original rod shape and normal size of their cells at different temperatures with different time intervals.

Jan *et al*. (2001) worked on changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*. *P. freudenreichii* cells subjected to extreme acid environments were shown to undergo dramatic changes in morphology, while viability decreased. In contrast, cell integrity was preserved in adapted bacteria, while viability was not affected. Shrinkage of *E. coli* cells were also found in acid stress. Fakhruddin and Quilty (2005) studied on the response of the *Pseudomonas putida* CP1 cells to nutritional, chemical and environmental stresses. There was a change of bacterial shape from rod to round as well as the reduction of cell size when grown on phenol and chlorophenols.

In this study microscopic observation showed that the shape of the bacterial cell changed from rod to coccus when the organism was grown under phenolic and pH stress. Thus, it is suggested from the present study that the round morphology can be assumed by the rod-shaped bacteria *Streptobacillus* and *Corynebacterium* as a consequence of alterations in the shape-determining mechanism due to the nutritional and acid stress.

Different concentrations of phenol were used as the sole carbon source to investigate the growth of *Streptobacillus* and *Corynebacterium* on phenol. Effect of various incubation temperatures as well as different media pH on growth and hydrophobicity were also investigated and the results are presented in Fig. 1. The growth response of these bacterial strains was determined using optical density measurement. Growth of *Streptobacillus* after 72 hours and *Corynebacterium* after 48 hours is shown in Fig. 1. The effect of phenol on cell surface hydrophobicity during growth at different concentrations of phenol was investigated. When both organisms were grown on higher concentrations of phenol, the resultant biomass were more hydrophobic. Growth of both isolates decreased with increase or decrease of temperature from 32˚C. Hydrophobicity of both isolates also increased with increase or decrease of temperature from 32˚C.

Table 2. Changes in cell shape and reduction of cell size during growth of *Streptobacillus* and *Corynebacterium* on different phenol concentrations and pH conditions.

Applied stresses		*Streptobacillus*								*Corynebacterium*							
		Corynebacterium				Reduction in cell size (h)				Cell shape (h)				Reduction in cell size (h)			
		0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
Phenol	50 ppm	Rod	Coccus	Coccus	Coccus	-	-	+	+	Rod	Coccus	Coccus	Coccus	-	-	+	+
	100 ”	”	”	”	”	-	+	+	+	”	”	”	”	-	+	+	+
	200 ”	”	”	”	”	-	+	+	+	”	”	”	”	-	+	+	+
	300 ”	”	”	”	”	-	+	+	+	”	”	”	”	-	+	+	+
	400 ”	”	”	”	”	-	+	+	+	”	”	”	”	-	+	+	+
pH	5	”	”	”	”	-	+	+	+	”	”	”	”	-	+	+	+
	7	”	Rod	Rod	Rod	-	-	-	-	”	Rod	Rod	Rod	-	-	-	-
	9	”	Coccus	Coccus	Coccus	-	+	+	+	”	Coccus	Coccus	Coccus	-	+	+	+

The organisms grew in response to different pH reaching a maximum value at pH 7. Decrease of growth with increase or decrease of temperature was found. The effect of pH on cell surface hydrophobicity during growth at different pH was investigated. Both isolates increased their cell surface hydrophobicity as their response to higher or lower pH.

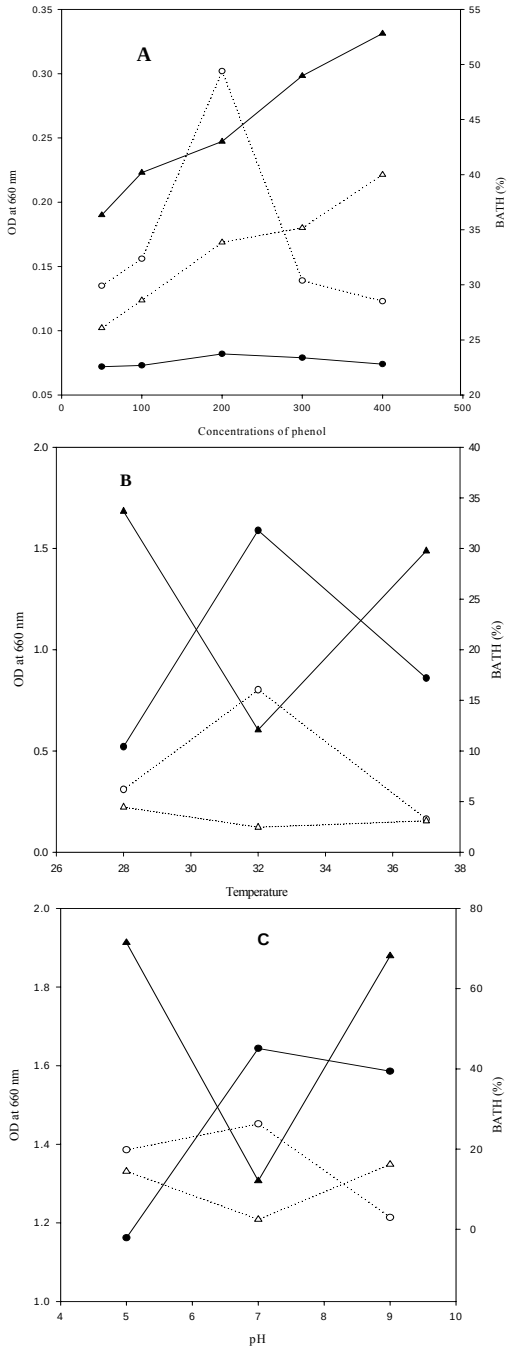

Fig 1. Changes in growth and hydrophobicity with phenol concentration (A), different incubation temperature (B) and different pH of growth media (C). Symbols: ●, growth of *Streptobacillus*; ○, growth of *Corynebacterium*; ▲, hydrophobicity of *Streptobacillus*; Δ, hydrophobicity of *Corynebacterium*.

Pseudomonas putida CP1 cells grown on higher concentrations of mono-chlorophenol were also more hydrophobic than those grown on lower concentrations (Farrell and Quilty 2002). When *P. putida* CP1 was grown on phenol at concentrations as high as 8.5 mM, cells were not hydrophobic, and no aggregation was observed, also suggesting a link between substrate toxicity and autoaggregation. Growth of *P. putida* CP1 on 4-chlorophenol, the least toxic of the mono-chlorophenols, resulted in formation of more numerous but smaller clumps than were formed during degradation of the more toxic 2- and 3- chlorophenols by *P. putida* CP1. Hydrophobicity assays showed the cells grown on 2- and 3-chlorophenols to be more hydrophobic than those grown on 4-chlorophenol. The results of that experiment suggested that *P. putida* CP1 flocculates as a result of chemical stress, as clumps formed in the presence of higher concentrations of monochlorophenols. Bacterial aggregation was also formed as a result of environmental stresses (Bossier and Verstraete 1996, Bossier *et al*. 1997).

In this experiment hydrophobicity of both isolates was measured by Bacterial Adherence to Hydrocarbon (BATH) test. From this experiment it was found that the hydrophobicity of both isolates was increased with the increase in concentrations of phenol and increase or decrease of temperature and pH from their optimal. Thus, this study concluded that the increase in hydrophobicity of these isolates were the consequences of the temperature and pH stresses and the toxic response of phenol.

Antibiotic susceptibility test for both isolates was performed using commonly used antibiotics and is presented in Table 3. From this experiment it was found that *Streptobacillus* was resistant to amoxicillin and trimethoprim-sulfamethoxazole; and susceptible to tetracycline and phenoxymethyl-penicilline; and showed intermediate resistant to Ciprofloxacin. On the other hand, *Corynebacterium* was resistant to amoxicillin, and sensitive to tetracycline, phenoxymethylpenicilline and ciprofloxacin, and showed intermediate resistant to trimethoprim-sulfamethoxazole.

The ability of these two possible pathogens to grow in the presence of antibiotics was tested. The disk diffusion method was performed to investigate the responses of *Streptobacillus* and *Corynebacterium* to six commonly used antibiotics. Typically, the raw data were interpreted based on the available Clinical and Laboratory Standards Institute data (Jorgensen and Hindler 2007) and zone diameter interpretive standards. The results will be reported out as: Susceptible, resistant, and intermediate. For the treatment of Streptobacillary in humans Tetracycline work well (Holden and Mackay 1964) and has good prognosis after appropriate antibiotic therapy, e.g., with Penicillin but resistant to trimethoprim-sulfamethoxazole (Machugh *et al*. 1985, Sens *et al*. 1989). Disk susceptibility tests for thirty three clinical isolates of *Corynebacterium jeikeium* were carried out by Traub *et al*. (1998). All isolates were resistant to trimethoprim-sulfamethoxazole varied in susceptibility to ciprofloxacin and tetracycline.

Table 3. Zone diameter interpretive standards used to determine interpretive categories (Mayer 2007).

Antimicrobial agent (per disc 15µgm)	Zone diameter (nearest diameter) for each interpretive category (mm)			Actual zone diameter (mm) *Streptobacillus*	Actual zone diameter (mm) *Corynebacterium*
	R	I	S		
Ciprofloxacin	≤15	16-20	≥21	19 (I)	22 (S)
Amoxicillin	≤14	-	≥15	7 (R)	13 (R)
Trimethoprim-sulfamethoxazole	≤10	11-15	≥16	7 (R)	15 (I)
Tetracycline	≤7	15-18	≥19	29 (S)	30 (S)
Azithromycine	-	-	-	12	14
Phenoxymethylpenicilline	≤14	-	≥15	15 (S)	28 (S)

R = Resistant, I = Intermediate, S = Susceptible.

CONCLUSIONS

The isolated bacteria were found to belong to the genus *Streptobacillus* and *Corynebacterium.* Growth of these two isolates on phenol resulted in changes in cell shape from rod to cocci as well as the reduction of cell size and also there was a decrease in optical density and increase in cell surface hydrophobicity when grown on phenol. Changes in growth, cell size and shape and hydrophobicity of these two isolates were also evident for changing pH and temperature from their optimal as their response to these stresses. Antibiotic Susceptibility Test showed that the both isolates were resistant to amoxicillin and sensitive to tetracycline and phenoxymethylpenicilline.

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