ISOLATION AND GROWTH CHARACTERISTICS OF *Bacillus* sp. SD12 IN MEDIUM CONTAINING MIXTURES OF 4-CHLOROPHENOL AND ZINC

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Abstract: A bacterial strain tolerance to 4-Chlorophenol and Zinc was isolated from sediment of Okrika River that receives effluent from petroleum refinery plant. Out of fifteen (15) bacterial strains isolated, only one of the isolates identified as *Bacillus* sp. SD12 with highest adhesion to soil (47.4 %) was found to be tolerant to high concentrations of both 4-CP (400 ug/ml) and Zn (200 ug/ml), and hence was selected for further studies. In BH agar medium with mixture of 4-CP and Zn, growth stimulation was observed at 50 ug/ml. The isolate exhibited tolerance to mixtures of 4-CP and other heavy metal ions (Cu, Co and Ni) with growth values of 3.6, 4.7 and ^ 0 cfu/ml for Cu, Co and Ni respectively. Therefore from the results obtained, the organism was found to be good candidate for detoxification of industrial phenolic wastewaters.

Keywords: Sediment Bacteria, Tolerance, 4-Chlorophenol, Zinc, Heavy metals,

Introduction

The production and usage of man-made chemicals in industry has led to the entry of many chemicals into the environment. One such group of chemicals is phenol of which chlorinated phenols are its derivatives. Chlorinated phenols are a well known class of soil pollutants as a result of their use in large scale synthesis of pharmaceuticals, azo dyes, explosives and pesticides (Farrell and Quilty, 2002; Megharaj *et al.*, 1992). The main sources of chlorinated phenols in the environment include production of chlorine from the bleaching of pulp, combustion of organic matter, treatment of wood against fungi and insects, and preservation of raw hides in leather tanning industries (Shukla *et al.*, 2001).

Therefore in the last few decades, contamination of the environment by chlorinated phenolic compounds has been the subject of increased concern due to their acute toxicity and resistance to degradation (Haggblom, 1990). The recalcitrance of chlorophenols results from the carbon-halogen bond which is cleaved with great difficulty and the stability of their aromatic structure resulting in their accumulation in nature (Copley, 2000; Fetzner and Lingens, 1994).

4-chlorophenol (4-CP) as one of the derivatives of phenol, is a toxic and recalcitrant compound which is formed from wastewater chlorination in pulp

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mills, biological breakdown of chlorophenoxyacetic acid herbicides and anaerobic degradation of more highly chlorinated phenols (Westerberg *et al.*, 2000; Haggblom, 1990). In sites where 4-chlorophenol has accumulated to an elevated level, its toxicity is expressed by the inhibition of growth of organisms utilizing it as carbon and energy sources. The biodegradation of 4-chlorophenol is usually limited to relatively low concentrations in the range of 5 - 100 mg/l.

Several genera of bacterial strains have been reported to degrade 4-CP under aerobic conditions. Principal members of the genera that degrade 4-CP include *Arthrobacter chlorophenolicus* A6, *Arthrobacter ureafaciens* CPR706, *Comamonas testosteroni* CPW301 and *Alcaligenes* sp. A 7-2 (Backman and Jansson, 2004; Bae *et al.*, 1996a; 1996b; Balfanz and Rehm, 1991). The extent of microbial utilization of 4-CP is related to the ability of the microorganisms to adapt to the new environment. This adaptation is also directly proportional to the synthesis of new enzymes that are capable of transforming toxic compounds to innocuous end products of CO_2, cell mass and water (Agarry and Durojaiye, 2008). In polluted sites, one pollutant does not occur in such impacted sites rather mixed pollutants do exist comprising both inorganic and organic pollutants. The most frequent inorganic pollutants encountered in soil and industrial wastewaters as well as aquatic sediments are heavy metal ions such as Zinc (Zn), Copper (Cu), Cadmium (Cd), Lead (Pb), Nickel (Ni), Chromium (Cr), etc. In industrial wastewaters, phenol and its derivatives are the main organic pollutants encountered. The occurrence of different pollutants in impacted sites is a vital problem because the degradation of one component can be inhibited by other compounds in the mixture (Sandrin and Maier, 2003). Heavy metals occur naturally in all soils in minute quantities, but can accumulate to higher concentrations in agricultural soils and sediments as a result of continuous discharges from various sources, such as fertilizer applications, organic supplements, atmospheric deposition and deposition of urban and industrial wastes in the environments (Ahialey *et al.*, 2014). However, some heavy metals such as Zinc (Zn), Copper (Cu), Nickel (Ni), Cobalt (Co), etc are essential or beneficial micronutrients for microorganisms at lower concentrations though at higher concentration, these metals have toxic effect on microbial communities (Ahemad, 2012).

Zinc as one of the micronutrients is a component of enzymes and acts as stabilizer of membrane and various macromolecules at low concentrations. At high concentrations, Zn is a potent inhibitor of respiratory electron transport system of bacteria (Choudhury and Srivastava, 2001). Despite its toxicity to microorganisms at high concentrations, Zn plays an important role in the development, growth and stimulation of metabolic activity in all living systems at low concentrations. However, only a few studies have been done on detoxification of a heavy metal and phenolic compounds mainly by native microbial cells. Only microbial consortia have been employed for simultaneous detoxification of phenolic and heavy metal compounds impacted media (Silva *et al.*, 2007). No report in which only one indigenous bacterial strain tolerant to high concentrations of 4-CP and heavy metal compounds has been employed for simultaneous microbial detoxification. If a single potent native strain is available, Tripathi *et al.*, (2011) reported that its nutritional requirement, growth and maintenance would be more convenient to manage than bacterial consortium.

The present study is thus aimed at finding a bacterial isolate from sediment of Okrika River that can tolerate high concentrations of 4-Chlorophenol and Zinc in order to determine its capability to detoxification phenolic compounds and heavy metals of impacted media.

Materials and methods

Reagents and Culture conditions.

All the items used were purchased from sigma chemical Co (St. Louis, Mo, USA) and all the preparation were done in deionized distilled water. A stock solution (100 mg/l) of 4-chlorophenol (4-CP) was prepared in Bushnell Haas (BH) broth medium of pH 7.2 consisting of (g/l): NH_4NO_3, 1.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $CaCl_2.2H_2O$, 0.02; $MgSO_4.7H_2O$, 0.2; $FeCl_3.6H_2O$, 0.085 (Atlas, 1994). The medium was contained in 250 ml Erlenmeyer flask and stored in the dark until use. Petri dish plates containing the same BH medium, requisite concentrations of 4-CP was used followed by addition of 1.5 % agar.

Enrichment and isolation of 4-CP degrading bacteria.

Sediment sample was collected from effluent discharge site of a petroleum refinery plant at Port Harcourt, Rivers State, Nigeria, located along the bank of Okrika River. The site has a history of contamination with organic compounds (Otokunefor and Obiukwu, 2005). Ten grams (10 g) of the sample was added to BH (100 ml) medium with 4-CP (20 mg/l) as sole source of carbon and energy and incubated at 28 ± 2°C for 30 days in a rotary shaker at 120 rpm. This was followed by repeated transfers (at four weeks intervals) of the suspensions into fresh medium with increased doses of 4-CP upto a concentration of 100 mg/l in 16 weeks. 4-CP degrading bacteria were isolated from the enrichment culture on BH agar plates containing 1.5 mM of 4-CP (BH-4CP) by spreading 0.1 ml of decimally diluted (10^4) of the culture on the surface of the plates. Thereafter, the plates were incubated at 28 ± 2°C for 72 h. Colonies that developed on the BH-4-CP agar were isolated and purified by streaking on freshly prepared nutrient agar medium and then stored on agar slants at 4°C for further studies.

Assay for adhesion of the test isolate to soil

The adhesion of the test isolates to soil was determined using the method as adopted by Huysman and Verstraete (1993b) and modified by Mehmannavaz *et al.*, (2011). Cell culture grown overnight in nutrient broth was harvested, washed twice in physiological saline (150 mM NaCl) and resuspended in the same medium to an optical density (OD_{540nm}) of 0.7. Ten mililitre of the cell suspension was added to 1.0 g of sterilized garden soil samples (Silt, 2.6%; Clay, 17.2%; Sand, 76.3%; organic matter, 78.6% and pH, 6.7) in a 20 ml screw capped glass test tube, vortexed for 60 s and allowed to settle for 15 min. Two mililitre of the aqueous layer from the top was collected and the OD_{540nm} was measured. Control consisting of 10 ml of

NaCl (150 mM) and 1.0 g of soil sample in same glass tube was used for the adjustment of the ODs that was obtained. The adhesion of the test isolates to the soil was calculated using the expression (Mehmannava *et al.*, 2001):

$$\%\ Adhesion = \left\{ \frac{[OD_I - (OD_F - OD_C)]}{OD_I} \right\} \times 100$$

Where OD_I, OD_F and OD_C are the OD_{540nm} of the initial, final and control suspensions of the test isolate respectively. Thereafter, those isolates that showed adhesion > 40 % were selected for further studies.

Screening of the isolates on different concentrations of 4-CP and Zinc

The test isolates were screened by cross streaking against the 4-CP and Zn gradient on gradient plates in order to determine their optimum tolerance to the two pollutants. This indicates the utilization (4-CP) and detoxification (Zn ion) of the pollutants respectively. In brief, BH agar medium amended with different concentrations of 4-CP ranging from 50 – 500 µg/ml was prepared while nutrient agar (NA) medium supplemented with different concentrations of Zn ion (NA-Zn) as $ZnSO_4.5H_2O$ ranging from 50 – 400 µg/ml was also prepared. The isolates were grown to exponential phase in nutrient broth medium. The cells were pelleted, washed twice with sterile phosphate buffer and the optical density ($OD_{540\ nm}$) was adjusted to 0.6 in the same medium. Then one tenth millilitre (0.1 ml) of decimally diluted standardized culture was spread on the surface of both NA-Zn agar and BH-4CP agar plates. The NA-Zn and BH-4-CP agar plates were incubated for 24 and 72 h at 28 ± 2°C respectively. Controls consist of NA and BH agar culture media with neither of the toxicants. Thereafter, the isolate that showed efficient optimum tolerance to both toxicants at higher concentrations was finally selected for further studies. The isolate was tentatively characterized using a battery of biochemical tests and identified to the generic level following the schemes of Holt *et al.*, (1994).

Growth on agar medium containing 4-CP and Zn^{2+} mixture

The test isolates were tested for growth in the presence of increasing doses of 4-CP while Zn ion was kept at constant concentration in BH agar medium plates as described here. Briefly, the plates were amended with varying concentrations of 4-CP (50 – 400 µg/ml) and 200 µg/ml of Zn^{2+} (4CP-Zn). Control medium with inoculum but devoid of chemical mixtures was set up. The plates were incubated at 28 ± 2°C for 72 h after which the colonies that developed were enumerated and expressed as colony forming unit per milliliter (CFU/ml).

Tolerance to other heavy metals

The test bacterium that shows a high tolerance to 4-CP and Zn was further studied for tolerance to other heavy metals. A 24 h old culture of the bacterial isolate was inoculated aseptically on nutrient agar plates supplemented separately with other heavy metals of copper (Cu), lead (Pb), cobalt (Co), Cadmium (Cd), nickel (Ni) and mercury (Hg). The heavy metal ions were used as Copper sulphate, Lead acetate, Cobalt nitrate, Cadmium Chloride, Nickel chloride, Mercuric chloride respectively along with 4-CP

(400 μg/ml) and Zinc (200 μg/ml). A control was set up as stated above. The plates were incubated at 28 ± 2°C for 48 h after which colonies that developed were visually observed, enumerated and expressed as cfu/ml.

Growth Study of Selected Bacterium

The isolate selected was subjected to further study in BH broth medium supplemented with 4-CP (400 μg/ml) and Zn^{2+} (200 μg/ml) individually and combined respectively. This was done by inoculating the medium contained in 250 ml Erlenmeyer flask with 2% (v/v) standardized culture (OD_{540nm} 0.6). Culture flask devoid of any of the chemicals was set up as control. The flasks were incubated on a rotary shaker (120 rpm) at 28 ± 2°C for 120 h. Growth of the culture was monitored by measuring the culture absorbance at 540 nm at 24 h intervals. 4-CP concentration in the sample was measured using a 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992) while the zinc ion concentration in the culture medium was not determined.

Statistical Analysis

All experiments were performed in duplicate. The statistical calculation was done according to the standard method of Steel and Torrie (1992).

Results and discussion

Table 1 depicts the physicochemical constituents of the sediment from which the isolates were obtained. Phenol, oil and grease as well as zinc, copper and lead were found to be higher than the recommended permissible limits as prescribed by the federal environmental protection agency (FEPA) [Otokunefor and Obiukwu, 2005]. This is as a result of continuous discharge of wastewaters into Okrika River by the petroleum refinery and industrial complexes sited along the bank of the river. However, other constituents of the sediment sample were within the recommended permissible limits of FEPA. Le Dreau *et al.*, (1997) had in their work observed that around a petroleum refinery site in the Gulf of Fos (South France), sediments are always highly contaminated as the hydrocarbons tend to sediment out into the final receptable site which is the River sediment. This suggested that Effluent from the petroleum refinery and other industrial plants are poorly purified before release into the environments. This is in agreement with Tripathi *et al.*, (2011) and Otokunefor and Obiukwu (2005) who reported ineffective treatment of both tannery and petroleum refinery effluent samples respectively. The continuous discharge of pollutant-impacted effluents by these industries into the aquatic ecosystems resulted in the accumulation of pollutants in the water body that eventually ended up in sediments hence sediment pollution.

After acclimation for 16 weeks, strains of bacterial species were isolated from the mixed culture in 4-CP amended BH medium. The isolation of 4-chlorophenol tolerant bacteria from the sediment sample revealed that the sample is good source for isolation of organic (phenolic compounds) utilizing microbial cells. The ability of individual bacterial strains to adhere to soil was tested. The results indicated that the adhesion properties of the isolates vary ranging from 20 – 50 % (Table 2). The result is similar to that of Mehmannavaz *et al.* (2001) who tested cell surface properties of rhizobial strains isolated from soils contaminated with hydrocarbons. Adhesion as one of the factors affecting biodegradation has influence on vertical transport, distribution and survival of microorganisms in soil environment. High adhesion is connected with low mobility of strains in environment and is useful attribute during bioaugmentation.

Out of the three isolates that showed adhesion > 40 %, only one isolate exhibited high tolerance to 4-CP and zinc ion in BH and nutrient agar media respectively (Figure 1). The test isolate was identified as *Bacillus* sp. SD12 and hence was selected for further detailed studies. The organism was observed to grow on BH and nutrient agar media amended with various

Table 1: Physicochemical properties of sediment sample

Parameter	Value
pH	6.90
Electrical Conductivity (μscm^{-1})	619
Oil and grease (mg/kg)	103
Phenol (mg/kg)	20
Copper (mg/kg)	2.05
Cobalt (mg/kg)	<0.01
Cadmium (mg/kg)	<0.01
Lead (mg/kg)	5.03
Mercury (mg/kg)	<0.01
Nickel (mg/kg)	<0.01
Zinc (mg/kg)	9.04
Sediment texture (%)	
Silt	0.80
Clay	0.24
Sandy	98.96

Table 2: Adhesion of sediment bacteria to soil particles

Organism	% Adhesion
SD1	21.5
SD2	20.7
SD3	31.7
SD4	36.9
SD5	23.8
SD6	41.6
SD7	39.2
SD8	22.9
SD9	45.5
SD10	34.8
SD11	37.9

SD12	47.4
SD13	35.9
SD14	42.7
SD15	29.6

concentrations of 4-CP (0 – 500 µg/ml) and Zinc ion (0 - 400 µg/ml) respectively. The growth on the two media progressively decreases as the concentrations of chemicals increases. *Bacillus* species has been known to tolerate zinc stress at higher concentrations (Ahemad, 2012; Rathnayake *et al.*, 2009; Silva *et al.*, 2007; Choudhury and Srivastava, 2001). *Bacilus* species have been reported (Vijayagopal and Viruthagiri, 2005) to utilize phenol and other phenolic compounds as sources of carbon and energy for growth. *Bacillus subtilis* OS1 isolated from petroleum contaminated soil was observed to grow on mineral salt agar medium supplemented with 100 µg/ml of 4-CP (El-Sayed *et al.*, 2009). Bhatt and Vyas (2014) in their work reported that *Bacillus subtilis* sp. RR-1 tolerated zinc stress as high as 100 µg/ml. However, this value obtained by the authors is less than the highest concentration tolerated by the test isolate in this work. The high tolerance of 4-CP and Zn by the test isolate may be as result of high contamination of Okrika river sediment with organic and inorganic laden wastewater. Figure 2 depicts the growth of the test isolate on BH agar medium amended with increasing concentrations of 4-CP at a constant concentration (200 µg/ml) of Zinc ion. The growth of the organism on 4CP-Zn agar medium was observed to be highest at 50 µg/ml and lowest at 400 µg/ml with growth values of 6.4 x 10^6 and 3.1 x 10^6 CFU/ml respectively. Growth of *Bacillus* sp. SD12 progressively decreased as the concentration of 4-CP increases at constant concentration (200 µg/ml). This indicated that 50 µg/ml of 4-CP and Zinc (200 µg/ml) stimulated the growth of the test organism while other concentrations were toxic.

The 4-CP and Zinc tolerant isolate was also tested for tolerance to other heavy metals based on the presence of some of these metals commonly found in the environments (Figure 3). The results obtained indicate that the growth of the isolate was more in media containing Copper, Cobalt and Nickel ions than in media containing Cadmium, Mercury and Lead ions. This may due to biological role of Cu, Co and Ni in microbial processes and the highest growth was observed in Nickel (5.1 x 10^6 CFU/ml). The lower cell count that was observed in Cd, Hg and Pb may be as a result of their toxicity as well as non-biological role in microbial processes (Rathnayake *et al.*, 2009). The least growth among them was observed in medium containing mercury ion (0.2 x 10^6 CFU/ml).

The growth response of bacterial isolate in the presence of 4-CP and/or Zn was investigated in BH broth medium and the results obtained are presented in Figure 4. The highest growth response

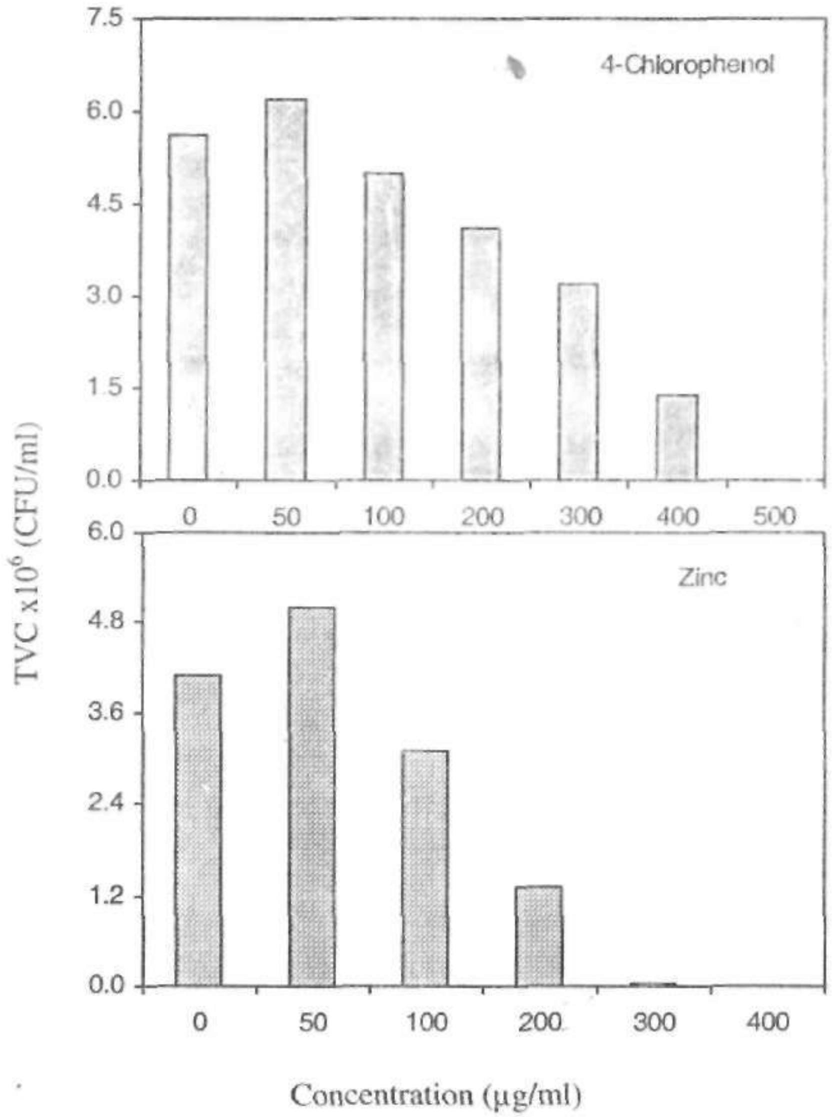

Figure 1: Growth profile of Bacillus sp. SD12 on BH and nutrient agar media amended with different concentrations of 4-CP and Zinc respectively.

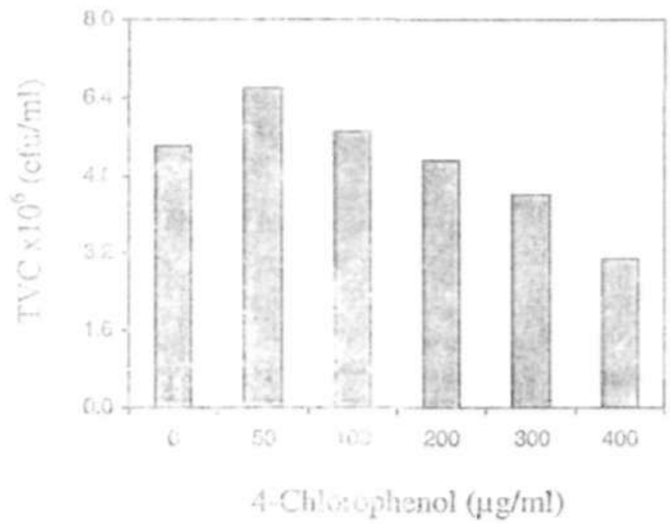

Figure 2: Growth response of Bacillus sp. SD12 in the presence of increasing doses of 4-CP and 200 µg/ml of Zn ion

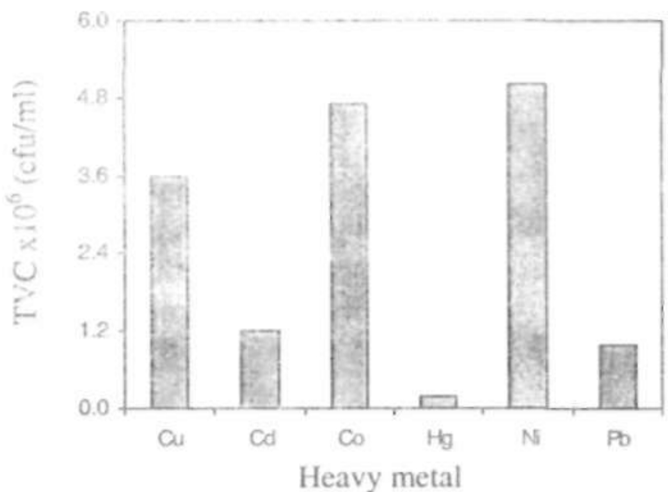

Figure 3: Growth response of Bacillus sp. SD12 in presence of both 4-CP and Zinc along with other heavy metals

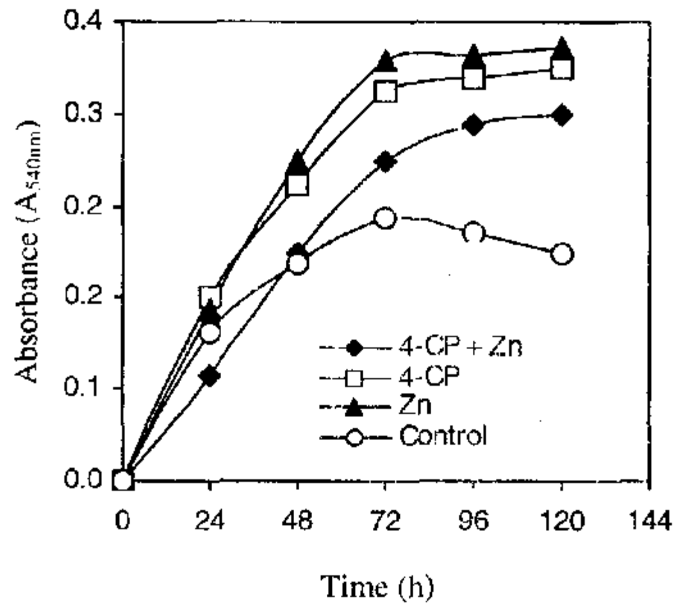

Figure 4: Growth response of Bacillus sp. SD12 in individually and combined presence of 4-CP and Zinc respectively.

was observed to be highest in medium containing Zn ion alone with OD_{540nm} value of 0.36 while least growth response was observed in medium containing mixtures of 4-CP and Zn ions with OD_{540nm} value of 0.29 at 120 h. At 72 h of incubation, growth response was observed to be slow in Zinc and 4-CP when alone and same slow growth response pattern was exhibited at 96 h medium containing mixtures of 4-CP and Zn. The growth response in the control medium devoid of any of the toxicants (Zn and 4-CP alone or their mixtures) was highest at 72 h with growth value of 0.23 OD_{540nm} and thereafter slowed down progressively. The low growth response may due to lack of any of the toxicants. Figure 5 shows the percentage degradation of 4-CP in media containing 4-CP alone and in combination with Zn. The highest percentage of 4-CP degraded was obtained 4-CP alone. This may be due to lack of combined toxicity of 4-CP and Zn.

In conclusion, the test isolate in this study exhibited high tolerance to 4-CP and Zn in both agar and broth medium. This shows a positive sign for application of this strain in the treatment of industrial effluents. Further detailed studies on simultaneous biodetoxification of phenols and heavy metals are underway.

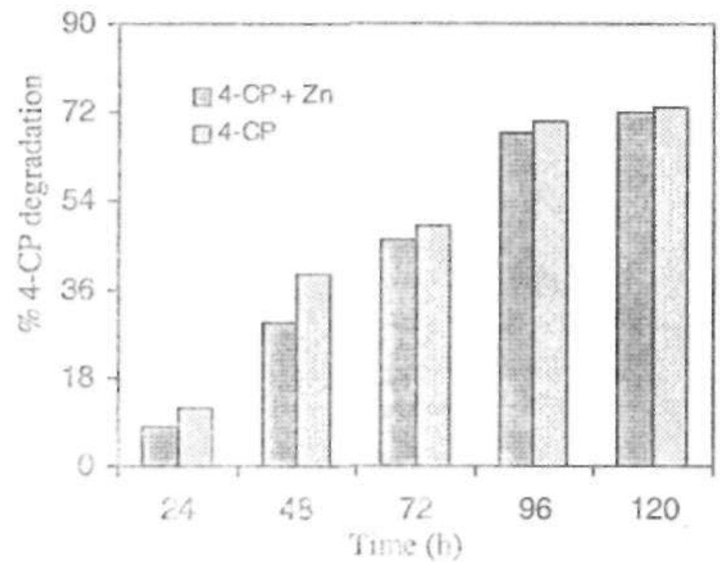

Figure 5: Time course variation of 4-CP utilization in BH medium containing 4-CP alone and mixtures of 4-CP and Zn ion

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