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Immobilization of Moderately Halophilic *Bacillus* sp. 2BSG-PDA-16 cells: A Promising Tool for Effective Degradation of Phenol

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Abstract

Phenolic compounds are hazardous pollutants known to be toxic at low concentration. Removal of phenols from industrial wastewater streams before their discharge into receiving water bodies is thus obligatory. Numerous phenol-degrading non-halophilic bacterial isolates have been described, but detailed information regarding phenol degradation by halophiles is rather sparse. The present study aims at evaluating the capacity of phenol degradation by new species of *Bacillus*. Halophilic bacteria were isolated from mangrove region on the western coast of the Red Sea of Egypt. Bacterial isolate showed the highest potentiality of phenol degradation was identified using 16S rRNA sequencing method as *Bacillus* sp. 2BSG-PDA-16. The optimum conditions for achieving high phenol degradation were 300 mg/l phenol concentration, 1% glucose, peptone, 5% NaCl, pH7, 30°C and 6% (v/v) inoculum size. Upon applied all these conditions, the phenol degradation reached 85.6 % removal. Calcium alginate was employed to immobilize the cells. Compared to free cells, the immobilized cells were not only able to tolerate high concentration of phenol but also are able to degrade it completely in a shorter time. Free cells degrade 600 mg/l in 10 days, whereas the immobilized cells could completely degrade 800 mg/l in 8 days. It could be concluded that this moderately halophilic *Bacillus* sp. 2BSG-PDA-16 might be useful for the degradation of phenol, particularly in saline environments.

Keywords. Biodegradation, Phenol, Halophilic bacteria, Immobilizations.

1. Introduction

Several industries including olive oil mills, pickled vegetables, fish processing, meat canning, dairy products, tanning process and oil refining process generate wastewaters containing high salt content and organic contaminants (Loh *et al*., 2000). Phenol and other phenolic compounds are common organic contaminants found in saline wastewaters formed by some of these industries (Gerrard *et al*., 2006). Presence of these recalcitrant compounds in the environment possess significant health risks to humans. The substituted phenolic compounds are carcinogenic and toxic environmental pollutants which are massively discharged into the environment from anthropogenic activities (Murthy and Gayathri, 2017). Phenol causes irritation of eye, swelling and finally blindness. Moreover, exposure to high concentration of phenol causes hepatic damage, paralysis, cancer and nervous disorders (Juang and Tsai, 2006; Indu Nair *et al*., 2008). In respect to phenol effect on prokaryotes, it is toxic to microorganisms even at low concentration (Yang and Lee, 2007).

Numerous methods have been developed to treat phenols in wastewater including membrane separation (Kujawski et *al*., 2004), adsorption (Roostaei and Tezel, 2004), oxidation (Idris and Saed, 2002) and extraction by liquid membrane (Lin *et al*., 2007). However, these physicochemical methods have their own limitations viz. reaction inefficiency, high energy consumption, production of sludge containing iron, and insufficient capacity (Chen *et al*., 2007). Biodegradation, as a technology for decontaminating of phenols, is gaining great attention due to its eco-friendly characteristics and cost-effectiveness (Adav *et al*., 2007; Catia *et al*., 2010; Ravikumar *et al*., 2011). Among the various practiced approaches for its removal, bacterial utilization gets attraction due to its eco-friendly and cost effective nature (Ahmad *et al*., 2016). However, biodegradation processes are difficult to perform under saline conditions (Kafilzadeh *et al*., 2010). One alternative is the use of efficient halophilic organisms which are adapted to live in such saline conditions.

The aim of the present study is to evaluate phenol degradation efficiency of ability of *Bacillus* sp. 82BSG-PDA-16 isolated from saline environment.

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Degradation of phenol was optimized with respect to various nutritional environmental parameters. Batch experiments were carried out in order to obtain the maximum phenol biodegradation rates by analyzing the influence of the immobilization in sodium- alginate gel beads on biodegradation performance.

2. Materials and Methods

2.1. Isolation of Phenol Degrading Halophilic Bacteria

Halophilic bacterial strains were isolated from soil and water samples collected from mangrove region on the western coast of the Red Sea, Egypt. Water (5 mL) and soil (3 g) samples were mixed in 10 mL sterile nutrient broth containing peptone (3 g/L) and beef extract (5 g/L) and incubated at 30°C on a shaking incubator at 150 rpm for 24 h. Bacterial cultures were isolated by repeated culturing in mineral salt medium (MSM) containing (g/L) KH_2PO_4, 0.5; K_2HPO_4, 1.5; NaCl, 10; Mg SO_4. $7H_2O$, 0.5; NH_4Cl, 1; $FeSO_4$. $7H_2O$, 0.01 and $CaCl_2$. $2H_2O$, 0.01 in a 250 mL conical flask (Bai *et al*., 2007). Phenol was filter sterilized separately using 0.2 µm nitrocellulose membrane filter and added to the sterilized medium after cooling to room temperature at a concentration of 100 mg/L. The isolates were incubated at 30°C with shaking at 150 rpm. After five cycles of culturing, serial dilutions of the cultures were prepared and streaked onto plates of mineral salt agar medium supplemented with phenol and incubated at 30°C for 72 h. Isolates exhibiting distinct colonies were further purified by repeated sub-culturing into solidified basal salt medium.

2.2. Identification of Phenol-Degrading Bacterium

Bacterial isolate showing the highest phenol degradation was tested for species identity using the 16S rRNA sequencing method (Rochelle *et al*., 1995). The gene sequencing was done at Macrogen (South Korea). DNA sequences were aligned using Gene Mapper v4.1 & Data Collection v 3.1 Communication Patch1. To extract the genomic DNA, bacterial colonies are picked with a sterilized toothpick, and suspended in 0.5 ml of sterilized saline, then centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA), incubated at 56 ^oC for 30 min and then heated to 100 ^oC for 10 min. After heating, the supernatant can be used for the PCR reaction. Bacterial 16S rRNAs were amplified by using the following universal bacterial 16S rRNA primers: forward primer 27F (50-AGAGTTTGATCMTGGCTCAG-30) and reverse primer 1792 R (50-TACGGYTACCTTGTTACGACTT- 30). Polymerase chain reaction was performed using kits with Ampli Taq DNA polymerase (FS enzyme; Applied Biosystems). One microlitre of template DNA was added to 20 µL of PCR reaction solution. Amplification was performed using 35 cycles at 94 ^oC for 45 s, 55 ^oC for 60 s, and 72 ^oC for 60 s. The PCR amplicon was purified using a Montage PCR clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers 518 F (50-CCA GCA GCC GCG GTA ATA Cg-30) and 800R (50-TAC CAG GGT ATC TAA TCC-30). Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Sequence analysis was performed with sequences in the National Center for Biotechnology Information (NCBI), USA database using Basic Local Alignment Search Tool for Nucleotides (BLASTN) (Altschul *et al*., 1997).

2.3. Biodegradation of Phenol

A loopful of pure bacterial strain was inoculated into Luria–Bertani (LB) medium and incubated at 30^oC for 24 h. Phenol degradation experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL MSM amended with various concentrations of phenol and inoculated with 6 % (v/v) inoculums of the prepared seed cultures. The flasks were incubated at 30 ^oC on a rotary shaker (150 rpm) for 72 h. The culture suspension was centrifuged at 10,000xg for 20 min for removal of the biomass. The biodegradation of phenol was assessed spectrophotometrically by the method of Yang and Humphrey (1975). Briefly, 50 mL of diluted sample were added to 0.3 ml of 2% aqueous 4-amino- antipyrine solution and 1 mL of 2N NH_4OH. After mixing the content thoroughly, 1mL of 2 % K_3FeCN_6 is added. Absorbance of red color produced is measured at 510 nm and the percentage degradation of phenol was calculated by the following equation:

Phenol degradation (%) = OD (control) – OD (sample)/ OD (control) x 100. Where uninoculated flasks were prepared in a parallel method and used as controls.

2.4. Cell Growth

Cell growth was monitored by measuring the optical density at 600 nm using spectrophotometer (UV-VIS Double Beam PC, Labomed INC). To measure biomass concentration, ten milliliter culture medium was centrifuged at 10,000 xg, 4°C for 20 min and the cell pellets were washed with 10 mL distilled water. The cell pellet was harvested by centrifugation and dried at 105°C for 48 h, or till constant weight was obtained. Cell mass concentration was determined by the standard calibration curve between OD 600 and cell dry weight.

2.5. Optimization of Phenol Degradation

In order to optimize the nutritional and environmental factors affecting phenol degradation by *Bacillus* sp. 2BSG-PDA-16, the following variables were assayed: phenol (100-800 mg/L), additional carbon sources (glucose, sucrose, maltose, cellulose, fructose, lactose, starch), different nitrogen sources (peptone, yeast extract, urea, ammonium chloride, ammonium sulphate, sodium nitrate, leucine, asparagine, proline), NaCl (1-15 %), pH (5-9) and temperature (20- 40°C) and inoculum size (2-12 % (v/v). All assays were performed in triplicate. Uninoculated controls were prepared parallel in all experiments. Following incubation, samples were collected and analyzed for growth and phenol degradation. After determination the optimum for each parameter, an experiment was performed by applying all the optimized nutritional and environmental factors.

2.6. Immobilization Protocol

The microbe of choice was encapsulated in calcium-alginate beads. Liquid cultures were centrifuged in a 50 ml

centrifuge tube (10,000 xg) at 4°C for 20 min and the supernatant was discarded. The pellet was resuspended with a previously autoclaved solution of sodium alginate to a final concentration of 4% (w/v) and 10% (v/v) bacterial biomass. The alginate-bacterial mixture was added drop wise with sterile syringe (20 mL) fitted with a wide bore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (3% (w/v), adjusted to pH 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4°C before being harvested by filtration (Abd-elhaleem *et al*., 2003).

2.7. Batch Degradation Studies

Batch experiments on phenol degradation using free cells were performed in 250mL Erlenmeyer flasks containing 100 mL of growth medium. Phenol concentration was varied in the range of 100 to 800 mg/L. Freshly grown seed culture was inoculated to media. Inocula concentration was 6 % (v/v).

For biodegradation experiment with Ca-alginate immobilized cells, 5 g (wet weight) of beads were placed in 250 mL flasks containing 100 mL mineral salts medium and phenol was added at concentrations ranging from 100 to 800 mg/L. The flasks were placed in a rotary shaker at 150 rpm at 30°C. Every 24 h, samples were collected, centrifuged and analyzed for biomass and remaining phenol concentrations.

2.8. Long-Term Degradation Studies

To determine the long-term stability of phenol degradation by immobilized cells , the system was used for repeated batch degradation. After each cycle of incubation period (72 h), the spent medium was decanted and beads were washed with sterile water and transferred into a fresh sterile mineral salts medium (100 mL) containing 500 mg/L phenol . The degradation process was carried out under identical conditions and the spent medium was analyzed for the residual substrate.

2.9. Statistical Analysis

All experiments were done in triplicate, and the results were presented as mean± standard deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p< 0.05$.

3. Results and Discussion

In the present study, twenty-three morphologically different halophilic bacterial species capable of phenol degradation were isolated using enrichment culture technique. The most potent strain was identified using16S rRNA gene sequence analysis. The sequence alignment using BLASTN software for the comparison up to 1,500 bp of the analysis gave a high homology of 98.8 % to *Bacillus* sp. 2BSG-PDA-16. The strain was selected for further studies because of its high phenol-degrading rate (up to 800 mg / L within 72 h). This might be due to the production of different enzymes including oxygenases, hydroxylases, etc by this strain. Moreover, the enzymes responsible for the oxidizing of the phenolic compounds were very effectively (Indu Nair *et al*., 2008).

Parameters, such as 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. Microorganisms can be detrimentally affected if the initial concentration of the pollutant is very high (Nair *et al*., 2008). Hence, in order to know the tolerance levels of *Bacillus* sp. 2BSG-PDA-16, different concentrations of phenol were tried. The highest phenol degradation activity (37.3 %) was recorded at a concentration of 300 mg/l (Figure 1). As phenol concentration was increased from 400 to 800 mg/L, there was a decrease in cell density and this had an impact on phenol degradation. The results, thus, indicated that the higher concentrations of phenol were having a negative effect on the cells. Annadurai *et al.* (2002) confirmed that *Pseudomonas putida* suffered from substrate inhibition, whereby growth and consequently phenol degradation was inhibited at high phenol concentrations.

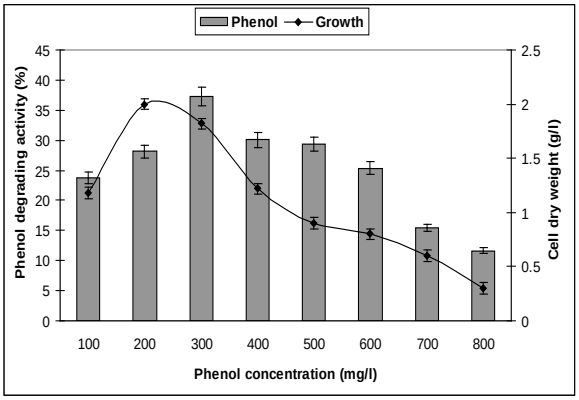

Figure 1. Effect of phenol concentrations on growth and phenol degrading activity of *Bacillus* sp. 2BSG-PDA-16. Results are means of three independent determinations and error bars represent the standard deviation

Various methods have been proposed to overcome substrate inhibition in order to treat high strength phenolic wastewater. These include selection and adaption of the cells to higher phenol concentration (Masque *et al*., 1987), immobilization of the cells (Loh *et al*., 2000) and using genetically engineered microorganisms (Soda *et al*., 1998). Another possible method increasing the tolerance of the cells to substrate inhibition is to supplement the growth medium with conventional carbon sources, such as glucose. In the present study, the phenol removal efficiency was investigated by the addition of various carbon sources and results cited in Figure 2 shows maximum degradation of phenol with the addition of 1 % glucose (62.07 %). This might be due to the fact that glucose acts as a growth substrate in presence of phenol due to its simple structure as compared to phenol. The influences of supplementary conventional carbon source on enhancing the biotransformation rates of phenol as the primary substrate has been studied by medium augmentation with conventional carbon sources (Loh and Wang, 1998).

Effect of different nitrogen sources on the removal capacity of phenol by the selected strain was tested and the results suggested that peptone supported the best degradation of phenol (80.18 %).

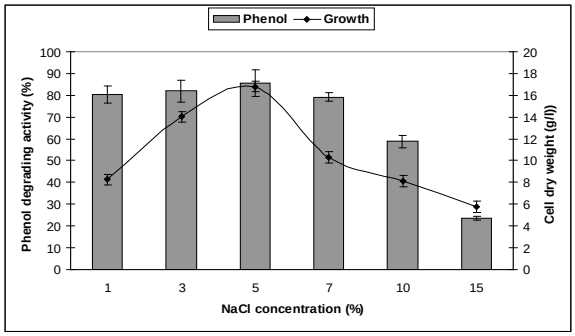

Figure 2. Effect of additional carbon sources on growth and phenol degrading activity of *Bacillus* sp. 2BSG-PDA-16. Control : MSM containing 300 mg/L phenol without any sugar addition. Results are means of three independent determinations and error bars represent the standard deviation

A considerable increase in the bacterial biomass (9.67 g/L) was recorded in the presence of peptone (Figure 3). Effect of different concentrations of NaCl on the biodegradation of phenol was also carried out. The results revealed that using 3% NaCl, the degradation efficiency of phenol by *Bacillus* sp. 2BSG-PDA-16 reached 82 % and the efficiency reached maximum up to 85.6 % at 5% NaCl. Using NaCl concentration of 7 %, 10 % and 15 %, the degradation efficiency decreased to 79.1 %, 58.74 % and 23.6 %, respectively (Figure 4). Thus, the selected bacterial strain was moderately halophilic in nature.

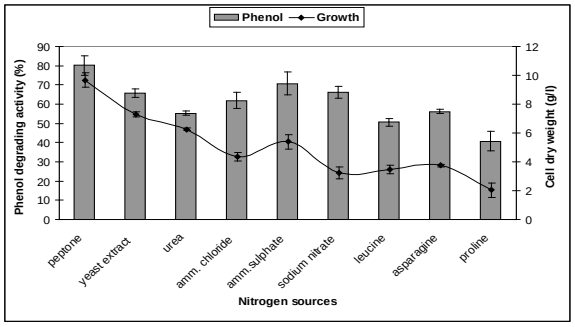

Figure 3. Effect of nitrogen sources on growth and phenol degrading activity of *Bacillus* sp. 2BSG-PDA-16. Results are means of three independent determinations and error bars represent the standard deviation

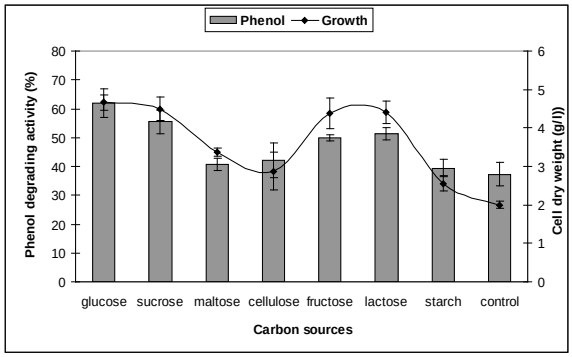

Figure 4. Effect of NaCl concentrations on growth and phenol degrading activity of *Bacillus* sp. 2BSG-PDA-16. Results are means of three independent determinations and error bars represent the standard deviation

Providing good environmental condition is one of the important parameter for bacterial bioremediation (Shweta and Handayuthapani, 2013). pH significantly affects the biochemical reactions required for phenol degradation.

Bacillus sp. 2BSG-PDA-16 showed the maximum degradation of phenol at pH 7 (data not shown). Similarly, the optimum pH for phenol degradation is 7.0 for *Pseudomonas putida* NICM 2174 (Annadurai *et al*., 2000). Below and above this optimal initial pH values, the growth rate and phenol degradation decreased gradually because the acids and bases which can easily entered in to the cell which affect the metabolic pathway and denature the proteins finally leads to lethality (Annadurai *et al*., 2000a). Furthermore, temperature plays an important role than nutrient availability in the degradation of organic pollutants. To assess the influence of the temperature changes, phenol degradation efficiency was compared under a temperature range from 20 to 40 °C with maximum activity attained at 30°C. Similar results were recorded by Ramzan and Rehman (2016) who reported that *Stenotrophomonas maltophilia* and *Bacillus subtilis* showed maximum survival and phenol degradation in the presence of phenol up to 300 µg/mL at 30°C and pH 7. Further increase in temperature resulted in marginal reduction in phenol degradation activity (data not shown). Higher temperatures seemed to have a negative effect on the activity of the bacteria and hence hindered its biodegradation capabilities. It may have a detrimental effect on the bacterial enzymes, which is the main step in the biological degradation process. Annadurai *et al*. (1999) described that when the temperature increased from 30°C to 34°C no phenol degradation was observed due to cell decay, which shows that the phenol degradation is a temperature dependent process.

On studying the effect of initial inoculum concentration on phenol degradation, results reveled that an inoculum concentration of 6 % showed the maximum phenol degradation reached 85.6 % and decreased afterwards. An experiment was performed by applying all the optimized nutritional and environmental factors and confirmed that the strain could efficiently degrade phenol.

The efficiency of the phenol degradation could be further enhanced by the process of cell immobilization (Annadurai *et al*., 2000a). Immobilized microbial cells create opportunities in a wide range of sectors including environmental pollution control. Compared with conventional suspension system, the immobilized microorganism technology offer a multitude of advantages, such as high biomass, high metabolic activity and strong resistance to toxic chemicals (Liu *et al*., 2012; Martins *et al*., 2013). Moreover, immobilized microorganisms could be cost effective since they can be used several times without significant loss of activity (Devi and Sridhar, 2000). Therefore, immobilized microorganism technology has been explored as promising for wastewater treatment (Ahmad *et al*., 2012). Calcium-alginate has been widely employed for immobilization of enzymes or whole cells since it is less toxic than synthetic polymers, easily gelled under mild conditions and inexpensive (Wang *et al*., 2007).

The cell immobilization emerged as an alternative for enzyme immobilization. Immobilization of cells containing specific enzymes has further advantages, such as the elimination of long and expensive procedures for enzymes separation and purification and its vitality in expanding their application by enabling easy separation and purification of products from reaction mixtures and

efficient recovery of catalyst (Junter and Jouene, 2004). In comparison with immobilized enzymes, immobilized cells provide new possibilities since they can be used as natural, water-insoluble carriers of required enzyme activities (Stolarzewicz *et al*., 2011).

Results illustrated in Figure 5 reveal that within the time period examined, the immobilized cells showed higher phenol degradation rate with all phenol concentrations tested. In encapsulated cell culture, the carrier material act as a protective cover against toxicity of phenol by forming networks of the beads, a diffusion barrier for phenol is build up which is not present in free cell culture (Chen *et al*., 2002). Moreover, the immobilization of whole cells tends to improve the stability of enzymes by retaining them in their natural surroundings during immobilization and subsequent continuous operation.

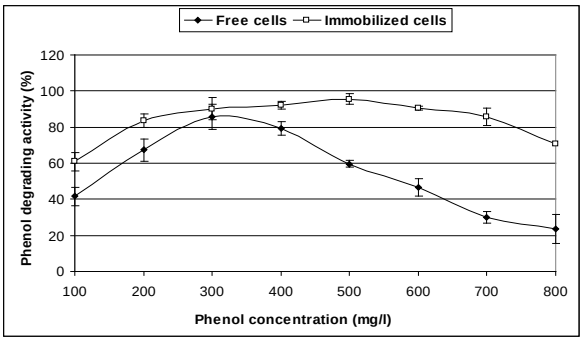

Figure 5. Effect of initial phenol concentrations on biodegradation rate by free and immobilized cells of *Bacillus* sp. 2BSG-PDA-16. Results are means of three independent determinations and error bars represent the standard deviation

Phenol biodegradation rate depends on the period in which the culture was adapted to phenol. The profiles of phenol degradation illustrated in Figure 6 show that as initial phenol concentration increased, appropriate removal efficiencies were obtained in a prolonged durations; time interval for degradation of low phenol concentrations e.g. 100 and 200 mg/Lwas at 5 days. As phenol concentration increased to 300, 400 and 500 mg/L, the degradation period was slightly prolonged. Complete removal of 300,400 and 500 mg/L phenol was achieved in 7 and 9 days, respectively. Initial phenol concentration of 600 mg/L was entirely degraded in 10 days. Further increase in phenol concentration resulted in a lower removal efficiency; for instance 62.53 % for 800 mg/L phenol in 11 days (Figure 6).

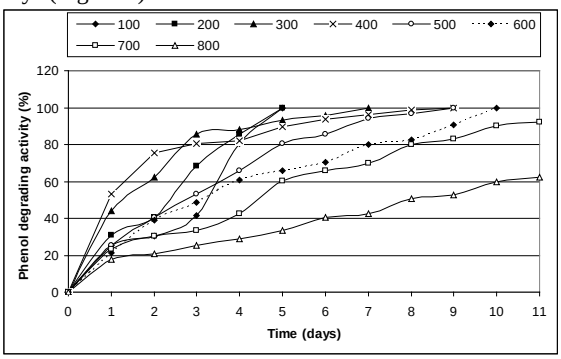

Figure 6. Time profiles of phenol biodegradation by free cells of *Bacillus* sp. 2BSG-PDA-16. Error bars represent the standard deviation

The cells immobilized in the Ca-alginate beads were not only able to tolerate high concentration of phenol but were also able to degrade it completely in a very short span of time. Figure 7 depicts the phenol degradation profiles of immobilized cells for initial phenol concentrations of 100-800 mg/L. It was observed that phenol concentrations of 200, 300 and 400 mg/L were completely degraded in 6 days. Furthermore, complete degradation of 500, 600 and 700 mg/L in 7 days and of 800 mg/L in 8 days. It seems that the tolerance of immobilized organisms against substrate inhibition has increased. In consequence, their capacity for phenol uptake was satisfactorily improved.

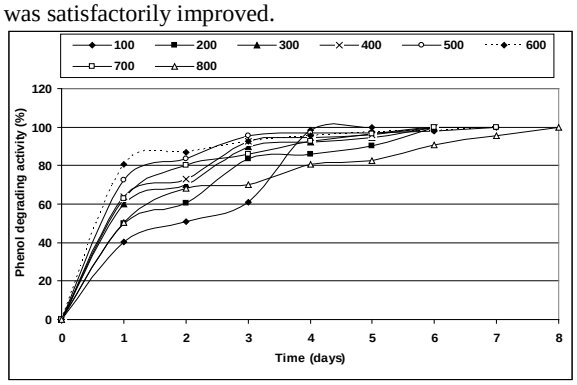

Figure 7. Time profiles of phenol biodegradation by immobilized cells of *Bacillus* sp. 2BSG-PDA-16. Error bars represent the standard deviation

It has been shown by several workers that immobilized microorganisms are better protected against phenolic compounds than are free cells. The advantages of immobilized cells in comparison with suspended ones include the retention in the reactor of higher concentrations of microorganism, protection of cells against toxic substances and prevention of suspended bacterial biomass in the effluent. Immobilization appeared as a promising procedure in overcoming substrate inhibition of phenol concentrations greater than 1000 mg/L (Loh *et al*., 2000).

Immobilized cells of *Pseudomonas putida* have been used successfully to degrade phenol at concentrations ranging from 100 to 1200 ppm in membrane and airlift bioreactors operated in batch and continuous mode (Muftah *et al*., 2009). In addition, cells of *Pseudomonas aeruginosa* adsorbed on diatomaceous earth pellets (celite R-635) and packed in column bioreactors were used to degrade phenol up to 1200 ppm in inorganic defined medium (Durham *et al*., 1994). *Alcaligenes latus* cells immobilized in polyurethane foam showed 100% degradation up to 350 ppm (1.05 mM) and 57% degradation at 500 ppm (1.5 mM). Degradation rate of Ca-alginate immobilized cells was less as compared to that of polyurethane foam immobilized cells (Usha *et al*., 2010).

In addition to this, stability during long-term operation is important for practical application of the immobilized cell system. In order to determine if there was deactivation of cells after repeated use, the immobilized cells were tested in twelve consecutive phenol degradation processes. The results showed that phenol degrading ability only decreased slightly after the immobilized cells were reused in seven cycles (Figure 8), demonstrating that the calcium alginate retained high mechanical strength. The reuse of immobilized cells might be advantageous because it can decrease waste of cells, save time, and cut down

cultivation cost. This facilitates the development of semicontinuous and continuous fermentation processes and leads to simplification of the separation of products from the fermentation broth .

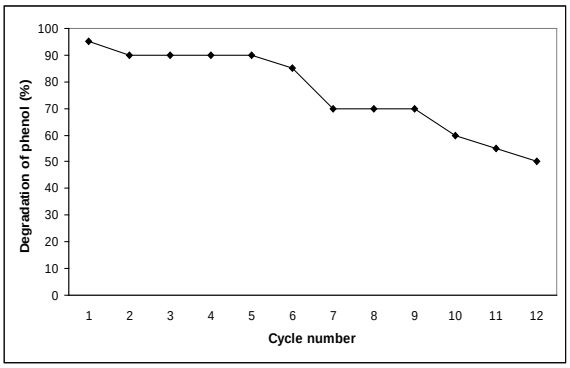

Figure 8. Repeated batch degradation of phenol by immobilized cells of *Bacillus* sp. 2BSG-PDA-16

The present findings show that immobilized cells in calcium alginate are promising for application in bio-degradation schemes in order to degrade phenol and possibly other related aromatic compounds at high concentrations in industry generated wastewater which leads to a reduction in time for complete phenol removal in relation to free cells.

4. Conclusion

The present study demonstrates that the isolated moderately halophilic *Bacillus* sp. 2BSG-PDA-16 is a potential candidate for the treatment of industrial saline wastewater contaminated with phenolic wastes. Immobilized cells in calcium alginate are promising for application in bio-degradation schemes in order to degrade phenol and possibly other related aromatic compounds at high concentrations in industry generated wastewater which leads to a reduction in time for complete phenol removal in relation to free cells.

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