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Testing a *Pseudomonas putida* strain for 4-chlorophenol degradation in the presence of glucose

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ABSTRACT

One of the most studied process for the decomposition of phenolic compounds involves the use of different microbial species or consortia. The main drawback of biological methods is the possible inhibitory effect of high concentrations of phenolic compounds impacting on the microorganisms. To overcome these difficulty bacteria can be acclimated to higher concentrations of phenols and the use of supplemental carbon sources. In this work a *Pseudomonas putida* strain isolated from a waste treatment plant receiving phenolic waste was tested for 4-chlorophenol removal using 1% (w/v) glucose as a co-substrate. Total removal of 4-chlorophenol concentrations of 50, 150 and 200 mg l^{-1} were obtained in approximately 27, 53 and 93 h respectively. For a higher 4-chlorophenol concentration of 250 mg l^{-1}, only 22% of degradation was obtained suggesting that at this high concentration, the 4-chlorophenol exerts an inhibitory effect on the bacteria.

Keywords: 4-Chlorophenol; *Pseudomonas putida*; Wastewater; Biodegradation; Acclimation; Glucose

1. Introduction

The massive industrialization of current society has lead to increasing discharges of toxic compounds in the environment. Phenol and its chlorinated derivatives are among the most dangerous pollutants. Chlorophenols can be released into the environment following their use as agricultural chemicals, pharmaceuticals, biocides and dyes, while the main uses of 4-chlorophenol are for the extraction of sulphur and nitrogen from coal, as an intermediate in the synthesis of dyes and drugs, as a denaturant in alcohol or a solvent in the refining of oils [1].

In Europe, the presence of chlorophenols in surface waters is regulated by the European Decree 2008/105/EC and in the USA by United States Environmental Protection Agency (US EPA) Clean Water Act (CWA). Among the monochlorophenols, 4-chlorophenol which has been classified by the US EPA as priority pollutant in the aquatic environment as it is much more toxic than either 2- or 3-chlorophenol.

The concentrations of chlorophenols in contaminated wastewater typically vary from 150 μg l^{-1} to 200 mg l^{-1} [2,3]. Since they are widely distributed and highly toxic, more attention has been paid to their particular removal with the adaptation of different methods. Conventional physical [4,5] and chemical techniques [6] can effectively

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remove chlorophenols. However, these techniques are often very complex and expensive [7,8]. Microorganisms and soluble and/or immobilized enzymes have proved to be cost-effective alternatives [9–11]. Thus, pure bacteria, yeasts and fungi capable of utilizing phenolic compounds found in soil and water are frequently utilised in biodegradation processes.

Some of the microorganisms that have been used to remove phenolic compounds include *Pseudomonas putida* [12,13], *P. fluorescens* [14,15], *P. testosterona*, *P. aeruginosa* and *Agrobacterium radiobacter* [16] and *P. pictorum* [17], *P. pickettii* [18,19], *Pseudomonas* sp. [20], *Phanerochaete chrysosporium*, *Aspergillus níger*, *Aspergillus terreus Geotrichum candidum* [21,22] and mixed consortia [23–25]. *Pseudomonas* appears to be the most common bacterial genus implicated in the degradation of phenolic compounds.

The ability of microorganisms to utilise phenolic compounds as carbon sources, is central to a high degree of elimination, although the high microbial biomass which is produced may be considered a disadvantage. Additionally, high substrate concentrations can cause catabolic inhibition. One of the methods used to overcome microorganism inhibition caused by high levels of phenolic compounds is the addition of conventional carbon substrates such as glucose, sodium glutamate and yeast extract in the growth medium [26,27]. In the presence of these carbon sources microorganisms increase their growth and can accelerate the biodegradation of chlorophenols. It has been reported that the degradation of 2-chlorophenol was enhanced in the presence of concentrations of glucose up to 1% (w/v) [28]. The addition of glucose to the medium as a co-substrate has also been shown to attain a higher 4-chlorophenol biodegradation [29].

In this work, a *Pseudomonas putida* isolate from a waste treatment plant receiving phenolic waste has been used to study 4-clorophenol removal in the presence of glucose as an external carbon source.

2. Materials and methods

2.1. Microorganism

The bacterial strain of *Pseudomonas putida* was originally isolated from an activated sludge plant treating phenolic waste from a coke plant [30].

2.2. Chemicals and media

4-Chlorophenol (99%) was purchased from Sigma-Aldrich Co. The composition of the minimal medium [31] to cultivate *Pseudomonas putida* was: K_2HPO_4, 4.36 g l^{-1}; Na_2HPO_4, 3.45 g l^{-1}; NH_4Cl, 1.0 g l^{-1}; $MgSO_4.6H_2O$, 0.912 g l^{-1}; trace solution, 1 ml l^{-1}. The pH of the medium was adjusted to 7.0 with 2M NaOH. The composition of the trace salt solution was: $CaCl_2.2H_2O$, 47.7 g l^{-1}; $FeSO_4.7H_2O$, 3.7 g l^{-1}; $CoCl_2.6H_2O$, 3.7 g l^{-1}; $MnCl_2.4H_2O$, 1 g l^{-1}; $Na_2MoO_4.2H_2O$, 0.2 g l^{-1}.

2.3. Preparation of the inoculums for the biodegradation studies

P. putida was maintained on LB agar at 4°C. Prior to the experiments *P. putida* was grown overnight in LB broth at 28°C and 150 rpm. After that, the bacteria were centrifuged at 5,000 rpm for 10 min and washed twice with 0.01 M sodium phosphate buffer.

All the experiments were done in 500 ml conical flasks containing a 5% (v/v) bacterial inoculum, 1% of glucose and 4-chlorophenol concentrations between 50 and 250 mg l^{-1} in sterile mineral salts medium [31] to a final volume of 200 ml. After inoculation, flasks were incubated in an orbital shaker at 150 rpm and 28°C. Samples were aseptically removed at regular intervals and analyzed for growth, 4-chlorophenol removal and glucose utilization.

2.4. Optical density measurement

Growth of *P. putida* was followed by measurement of the optical density at 600 nm using a Pye Unicam Pu 8600 UV/Vis spectrophotometer.

2.5. 4-Chlorophenol determination

4-Chlorophenol concentrations were measured by a colorimetric method [32] using solutions of $K_3[Fe(CN)_6]$, (83.4 mM in 0.25 M $NaHCO_3$) and 4-aminoantipiryne (AAP; 20.8 mM in 0.25 M $NaHCO_3$ solution). Aliquots of the sample (0.8 ml) were placed in a spectrophotometer cuvette (1 ml) together with 0.1 ml of ferricyanide solution and 0.1 ml of AAP solution. After a few minutes to allow the colour to develop fully, absorbance was measured at 505 nm against a blank. Absorbance values were transformed to 4-chlorophenol concentrations using a calibration curve.

2.6. Glucose determination

Glucose concentrations were determined by the dinitrosalicylate (DNS) colorimetric method [33]. One millilitre of sample at the suitable concentration was placed together with 1 ml of DNS reagent. The mixture was stirred and heated in a water bath at 100°C for 10 min. The mixture was then cooled and 10 ml of water were added. After 15 min absorbance was read at 540 nm. The glucose concentrations were determined from the calibration curve of corresponding known standard concentrations.

2.7. Data analysis

All the results presented are the mean of quadruplicate treatments. The standard deviation of the whole set of experiments was 9%.

3. Results and discussion

Fig. 1 shows the results for 4-chlorophenol removal from initial 4-chlorophenol concentrations of 50, 100, and 150 mg l^{-1} and 5% of bacterial inocula.

Fig. 1 shows no degradation of 4-chlorophenol at any assayed initial 4-chlorophenol concentration. This result is in agreement with the study by Lu et al. that microorganisms need phenol substrate in the growth medium to induce enzymes required for degradation of 4-chlorophenol [16]. The main disadvantage of this treatment is the incorporation in the media of additional pollutants. It has also been reported [22] that the capacities of the bacteria can be enhanced by acclimation. No 4-chlorophenol removal was observed with unacclimated activated sludge, whereas 300 mg l^{-1} of 4-chlorophenol were almost removed with the same acclimated activated sludge.

Others (*see* Ref. [29]) have proved that in the presence of an external carbon source such as glucose, 4-chlorophenol concentrations up to 200 mg l^{-1} were able to be transformed by *Pseudomonas putida* ATCC 49451 in the absence of added phenol although they reported longer lag time compared to using phenol as the co-growth substrate. Similarly Fakruddin and Quilty [28] show that the degradation of 2-chlorophenol was enhanced in the presence of concentrations of glucose up to 1%. The present results also agree with the study by Tarighian et al. [34] where it is reported that 4-chlorophenol was not consumed as a sole substrate by *Pseudomonas putida* but as a co-metabolite with either glucose or phenol acting as the primary growth substrate.

In order to test the capacity of our bacteria to degrade 4-chlorophenol, experiments were done in the presence of 1% glucose without acclimation of the bacteria. Fig. 2 shows the results for 4-chlorophenol removal at initial 4-chlorophenol concentrations of 50, 150, 200 and 250 mg l^{-1} with a 5% bacterial inocula in the presence of 1% glucose.

The data show that *Pseudomonas putida* is able to completely remove 4-chlorophenol concentrations of 50 mg l^{-1} in 27 h of batch treatment. For initial 4-chlorophenol concentration of 150 mg l^{-1} the bacteria achieved a 93% removal in 51 h. At a higher initial concentration of 4-chlorophenol, *P. putida* totally degraded 200 mg l^{-1} following 93 h incubation. However, with initial concentrations of 250 mg l^{-1} the bacteria only achieved a 22% 4-chlorophenol degradation after 120 h which can be attributed to the inhibitory effects of high concentrations of chlorophenols/phenols to growth.

It has been reported that a pure strain of *Pseudomonas putida* was able to degrade 30 mg l^{-1} of 4-chlorophenol in 50 h with glucose as the growth co-substrate [23]. The present results suggest that *P. putida* strain was able to remove higher 4-chlorophenol concentration in shorter times, which can be attributed to the fact that our adapted bacterium was isolated from a waste plant treatment plant receiving phenolic waste.

Pseudomonas pickettii was able to removed 100 mg l^{-1} of 4-chlorophenol in 40 h of incubation [18]. The same authors [19] reported that the addition to the culture medium of a vitamin solution containing biotin, folic acid, pyridoxine hydrochloride, riboßavin, thiamine hydrochloride, niacin, pantothenic acid,

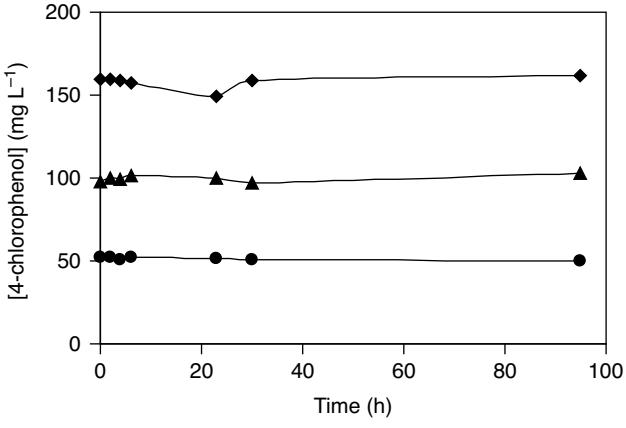

Fig. 1. Temporal variation of 4-chlorophenol concentration for different initial 4-chlorophenol concentrations: (●) 50, (▲) 100 and (◆) 150 and 5% bacterial inocula.

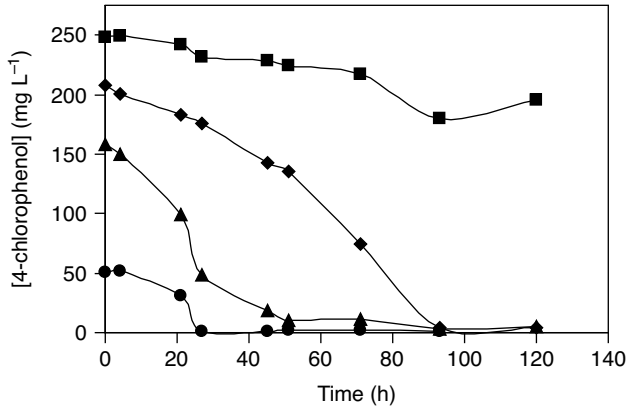

Fig. 2. Temporal variation of 4-chlorophenol concentration for different initial 4-chlorophenol concentrations: (●) 50, (▲) 150, (◆) 200 and (■) 250 mg l^{-1}, 5% bacterial inocula and 1% glucose.

cyanocobalamin, *p*-aminobenzoic acid, and thioctic acid improve 4-chlorophenol degradation by 11%–16%. More efficient degradation with a *Pseudomonas putida* strain CP1 was reported [35] where the removal of 100, 200 and 300 mg l^{-1} of 4-chlorophenol in 18, 36 and 52 h, respectively. In this study the bacteria were maintained on 200 ppm chlorophenol agar which suggests that the adaptation of the bacteria is very important and can lead to higher tolerance degrees of the bacteria to high 4-chlorophenol concentrations.

Fig. 3 shows the results of bacterial growth at 4-chlorophenol initial concentrations of 50, 150, 200 and 250 mg l^{-1} with 5% bacterial inocula and in the presence of 1% glucose.

Fig. 3 shows that with the initial 4-chlorophenol concentration of 200 mg l^{-1} *P. putida* was able to completely remove 4-chlorophenol after 93 h. It is interesting to note that after the incubation period, bacterial growth was optimal. Even with longer times and with no 4-chlorophenol remaining in the medium, the bacteria continued growing utilising the available glucose.

From Fig. 3 it is evident that with the lowest 4-chlorophenol concentrations (50 and 100 mg l^{-1}) there is no lag time in the growth of the bacteria, whereas with that for 200 mg l^{-1} of 4-chlorophenol the bacteria required a 40 h lag period before exhibiting growth and more than 70 h for 4-chlorophenol concentration of 250 mg l^{-1}.

Taking into account that the IC_{50} of this bacterial strain [36] for an incubation period of 30 min in 4-chlorophenol was 216 mg l^{-1} it is not unreasonable to expect 50% of inhibition of the growth of this bacterial strain close to this 4-chlorophenol concentration.

Fig. 4 shows the results of glucose removal at initial 4-chlorophenol concentrations of 50, 150, 200 and 250 mg l^{-1} with 5% of bacterial inocula and in the presence of 1% glucose. It can be seen that all the glucose was utilized by the bacteria in the presence of the two lowest 4-chlorophenol concentrations, whereas for 200 mg l^{-1} all the 4-chlorophenol was removed and there was still available glucose in the medium. For 250 mg l^{-1} most of the initial glucose is not used for the bacteria due to the previously described inhibition of the microorganism.

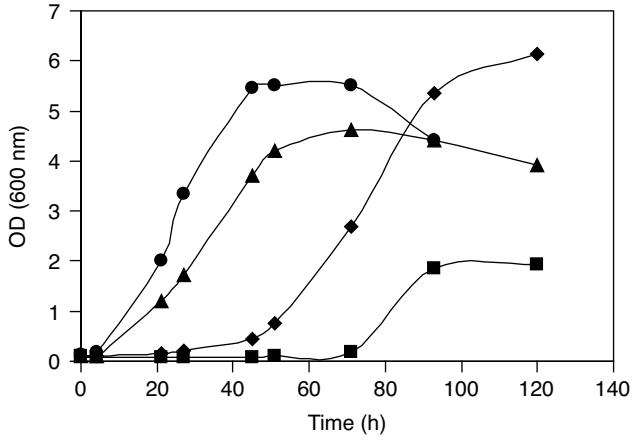

Fig. 3. Temporal variation of *Pseudomonas putida* population for different initial 4-chlorophenol concentrations: (●) 50, (▲) 150, (◆) 200 and (■) 250 mg l^{-1}.

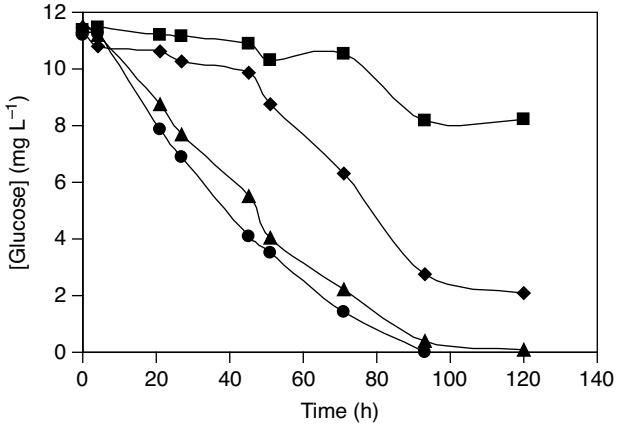

Fig. 4. Temporal variation of glucose concentration for different initial 4-chlorophenol concentrations: (●) 50, (▲) 150, (◆) 200 and (■) 250 mg l^{-1}.

4. Conclusions

Pseudomonas putida was not able to metabolize 50, 100 or 150 mg l^{-1} of 4-chlorophenol as a sole substrate source. With the addition of 1% (w/v) of glucose, 4-chlorophenol concentrations of 50, 150 and 200 mg l^{-1} were removed in 27, 53 and 93 h respectively. Higher 4-chlorophenol concentrations inhibited the growth of the bacteria.

The strain of *Pseudomonas putida* that was isolated from a treatment plants receiving high concentrations of phenolics from a coke manufacturing plant has proved to be suitable for the removal of 4-chlophenol compound in the range of concentrations typically present in wastewaters. The organism was adapted to growth in high concentrations of phenolics.

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