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Effect of Additional Carbon Sources on Biodegradation of Phenol

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Phenol is a common constituent of effluents discharged from industries such as petroleum refineries and coking plants, which has been found to affect aquatic life, causing ecological imbalance. It is lethal to fish even at relatively low concentrations of 5 to 25 mgL^{-1} (Saha et al., 1999). Phenol also imparts objectionable tastes to municipal drinking water at far lower concentrations. As a result, phenol-containing effluents have to be properly treated prior to discharge.

Conventional methods of treatment for phenolic wastes have been largely chemical or physical, but these processes have led to secondary effluent problems. Biological treatment for the bulk removal of these pollutants is therefore generally preferred. Biological degradation of phenol has been extensively studied using pure and mixed cultures. Several studies have been carried out with the bacterium *Pseudomonas putida* in pure cultures (Allsop et al. 1993; Wang and Loh, 1999), in which phenol is degraded via the meta-pathway (Sala-Trepat et al. 1972). However, it has been found that these bacteria suffer from substrate inhibition, whereby growth (and consequently phenol degradation) is inhibited at high phenol concentrations (Hill and Robinson 1975; Wang and Loh 1999).

Various methods have been proposed to overcome substrate inhibition in order to treat high-strength phenolic wastewater. These include adapting the cells to higher concentrations of phenol (Masque et al. 1987), immobilization of the cells (Keweloh et al. 1989; Chung et al. 1998; Loh et al. 2000) and using genetically engineered microorganisms (GEMs) (Soda et al. 1998). The method demonstrated by Masque and his co-workers required very long lag times; as high as 20 days of adaptation was required to degrade 1000 mgl^{-1} phenol. There is much controversy over whether to use natural or genetically engineered microorganisms in bioremediation. Government agencies, very often, are unwilling to release GEMs into the environment due to the potential unforseen ecological impact (Caplan 1993). Immobilization in various media, including hydrogels, activated carbon and hollow fiber membranes, have been shown to be a promising technique in overcoming substrate inhibition.

Another possible method of increasing the tolerance of the cells to substrate inhibition is to supplement the growth medium with conventional carbon sources, such as yeast extract or glucose. Armenante et al. (1995) have shown that the

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presence of yeast extract in the growth medium enhanced the degradation rate of chlorobenzoic acids. Based on these results and those of cell immobilisation, it may be advantageous if both techniques can be employed simultaneously for enhancing the substrate inhibition tolerance of the bacteria. This study seeks to optimize the biodegradation rate of phenol by *P. putida* by supplementing the growth medium with either yeast extract or glucose, and to study the effect of adding yeast extract or glucose on the substrate inhibition tolerance of the bacteria.

MATERIALS AND METHODS

Pseudomonas putida ATCC 49451 was used throughout this work. The cells were acclimated in a medium containing 200 mgL^{-1} of phenol. Acclimated cells in the late exponential growth phase were harvested as inoculum for the experiments. The late exponential growth phase was evident from the change in medium colour from colorless to a distinctive yellowish-green as well as an optical density (OD) of 0.35-0.40 absorbance units. All batch cultures were performed in 500ml Erlenmeyer flasks with cotton plug at 50% medium volume. The composition of the mineral salt medium has been reported elsewhere (Loh and Wang 1998). 10mL of the trace mineral solution was added per liter medium. Phenol was prepared in 1N NaOH. When yeast extract was augmented to the medium, $(NH_4)_2SO_4$ was omitted. When glucose was added, the medium was slightly modified to contain 1.74 gL^{-1} K_2HPO_4 and 0.24 gL^{-1} KH_2PO_4. This modification provided additional pH buffering capacity (in the pH range 7.2 - 7.4) required in the presence of glucose (Loh and Wang 1998). Phenol, yeast extract and glucose were added in amounts as desired in each of the experiments.

2 mL of activated *P. putida* was inoculated into 250 mL of culture medium. After inoculation, the cells were grown in the flasks on a New Brunswick rotary shaker at 30°C and 200 rpm. Samples were withdrawn periodically for analysis. Cell density, medium pH and concentration of phenol were monitored.A 6mL sample was taken each time for determination of pH and biomass. Biomass concentration was determined by measuring the optical density (OD) at 600 nm with deionized water as a reference.

Phenol concentration was determined by first acidifying 3 mL of the sample to pH 2 with 6 N sulphuric acid to quench the biodegradation reaction. This was followed by extraction of phenol with an equal volume of methylene chloride containing 100 mgL^{-1} *o*-cresol as internal standard. A 2 µL extract was then analysed using a capillary gas chromatograph (GC) equipped with a split injector and flame ionization detector. The carrier as used was nitrogen. The injector and detector temperatures were 225°C and 300° C, respectively. The sample split ratio was 20:1. The oven temperature profile started with maintaining at 100°C for 1 min before ramping at 10°C/min to 130°C, after which the program was halted. From our experimental experience and that provided by the vendor, the sensitivity of the GC is within 1 mg/l, and the experimental error in duplicates of the experiments is less than 5%.

RESULTS AND DISCUSSION

It has previously been established that substrate inhibition is severe for *P. putida* ATCC 49451 at phenol concentrations above 1000 mgL^{-1} (Chung et al., 1998). At this concentration, neither cell growth nor phenol degradation occurred to any measurable extent. In order to investigate the effect of additional carbon supplementation on the degradation of phenol at high concentrations, yeast extract or glucose was added in various concentrations initially to 750 mgL^{-1} phenol media. For both yeast extract and glucose, concentrations were varied from 0.2 gL^{-1} to 4 gL^{-1}.

Figure la shows the growth curves at the various concentrations of yeast extract supplemented. The baseline study (with phenol only) is also included for comparison. In all cases, cell growth followed the typical batch growth curve with a lag phase. an exponential growth phase and a stationary phase. The specific growth rate (h^{-1}) at each yeast extract concentration was obtained from a semi-log plot during the exponential growth phase. These were found to be almost constant at an average of 0.230 ± 0.010 h^{-1}. Compared to the specific growth rate of the cells fed on phenol alone ($\mu_{max} = 0.222$ h^{-1}), supplementation of yeast extract has neither positive nor adverse effects on specific growth rate. The anomaly in growth observed at 4 gL^{-1} yeast extract added is attributed to the slightly higher inoculum size.

Figure lb shows the phenol degradation profiles for the different amounts of yeast extract added. In all cases, phenol was completely degraded, albeit a longer lag time was experienced at high concentrations of yeast extract. The average phenol degradation was calculated based on the slope of the period of rapid disappearance of phenol and Figure 2 presents the data obtained. The presence of yeast extract was found to improve the phenol degradation rate when supplemented at concentrations from 0.2 gL^{-1} to about 2 gL^{-1}, but deteriorated it with further increase to 4 gL^{-1}. The presence of a maximum point indicates that there is indeed an optimal amount of yeast extract to be supplemented for optimal rate of phenol degradation. Topp and his co-workers (1988) also discussed the existence of an optimum amount of carbon to be supplemented for the biodegradation of pentachlorophenol.

The reason for the enhanced degradation rate at less than 2 gL^{-1} can be attributed to the attenuation of phenol toxicity by yeast extract and the buildup of more cell mass formed as a result of the additional carbon source. This is similar to that reported in earlier work by Loh and Wang (1998). It could also be that the presence of yeast extract enhanced the affinity of *P. putida* for phenol (Armenante et al. 1995) and also favoured the activity of the enzymes needed to degrade phenol. Beyond the 1.5 gL^{-1} optima, the degradation rate fell, and was only 64.2 $mgL^{-1}h^{-1}$, which was 13 % lower than that without yeast extract augmentation. Grady (1984) has suggested that the presence of very high concentrations of yeast extract can impede the activity of enzymes needed to degrade phenol.

Based on these results, the optimum amount of yeast extract to be supplemented was determined as 1.5 gL^{-1} per 750 mgL^{-1} of phenol. This ratio was subsequently used for experiments on degradation of phenol at higher concentrations.

Similar to yeast extract, the concentration of glucose in the culture medium was varied from 0.6 gL^{-1} to 4.0 gL^{-1} with initial phenol concentration of 750 mgL^{-1} in all the experiments. In all cases (Figure 3) phenol was completely degraded (Figure 3(a)) and the growth of *P. putida* (Figure 3(b)) was in accordance with the typical microbial batch growth curve. Again, the addition of glucose to the culture medium did not affect the specific growth rate and the average value for all the experiments was calculated to be 0.226 ± 0.004 h^{-1}. This is similar to that when the cells were fed on phenol alone.

The average degradation rate of phenol with glucose supplemented is plotted in Figure 4. As can be seen, an optimum concentration of glucose supplementation exists. Below 1.0 gL^{-1}, phenol removal rate increased with the amount of glucose supplemented. This is again due to the production of additional cell mass which enhanced the rate of phenol removal. However, the improvement in the phenol removal rate is only marginal at 13%.

When glucose was added in concentrations exceeding 1.0 gL^{-1}, the degradation rate of phenol decreased with increasing glucose concentration and dropped below the degradation rate achieved in the absence of glucose. This occurred despite an increase in the cell mass. This may be a result of catabolite repression by glucose, which has been observed by some researchers (Papanastasiou 1982) that the presence of glucose can inhibit the utilisation of the target substrate. Satsangee and Ghosh (1990) have also reported the interference in phenol uptake by glucose.

In another study (Rozich and Colvin 1985), it was found that presence of glucose attenuated the rate of phenol removal by phenol acclimated cells. It was proposed that the presence of a more metabolisable carbon source permitted more rapid growth and the activity of the phenol degradation pathway was suppressed in order to quicken biomass acclimation to glucose as the alternate carbon source. Based on these results, the optimum glucose concentration was found to be 0.8 gL^{-1} of glucose per 750 mgL^{-1} phenol.

Following the determination of the optimal ratio of carbon supplementation to phenol concentration, studies were carried out to test the tolerance of the organisms to high phenol concentrations in the presence of yeast extract and glucose. Both yeast extract and glucose were supplemented in the ratio as determined previously. Cells were grown at 1000 mgL^{-1} phenol, supplemented with 2 gL^{-1} yeast extract, and in the case of glucose supplemented experiments, at 1000 and 1200 mgL^{-1} phenol supplemented with 1.1 and 1.3 gL^{-1}, respectively of glucose. Figures 5 and 6 display the results obtained, respectively, for yeast extract and glucose. Cell growth (not shown) and complete removal of phenol were observed.

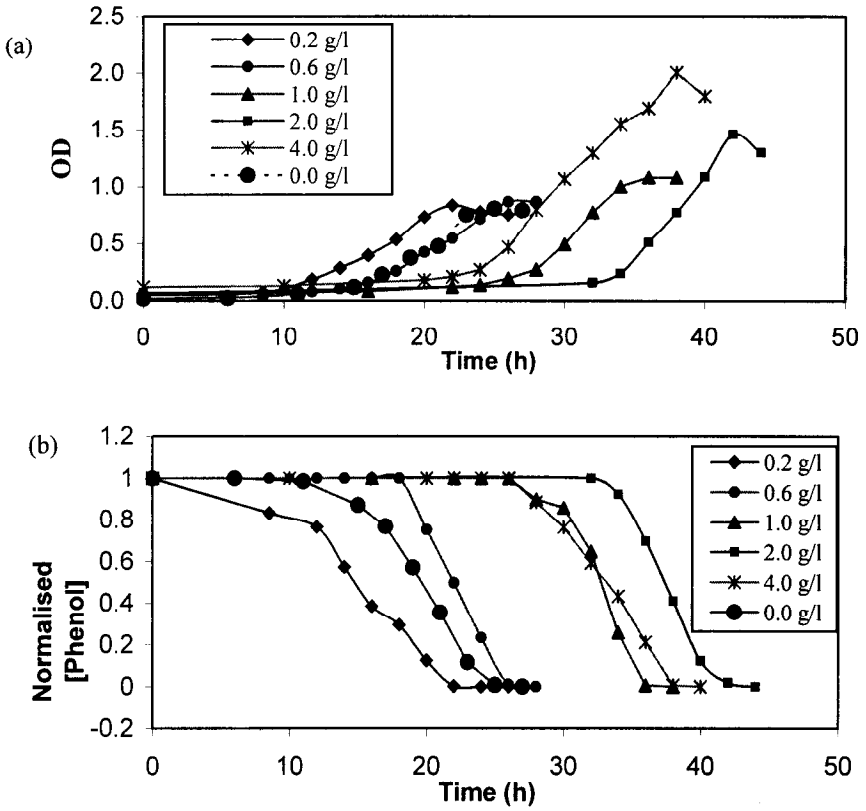

Figure 1. Effects of supplemented yeast extract on (a) cell growth, and (b) phenol degradation at 750 mgL^{-1} initial phenol concentration.

1000 mgL^{-1} phenol was degraded at a rate of 56.1 $mgL^{-1}h^{-1}$ with yeast extract added. This is slower than the degradation of 750 mgL^{-1} of phenol at a rate of 73.7 $mgL^{-1}h^{-1}$. This is due to the slower growth of the cells in 1000 mgL^{-1} of phenol attributed to the substrate inhibition effect. Nevertheless, these results show that the cells can tolerate a much higher phenol concentration when grown in the presence of yeast extract.

In the case of glucose supplementation, phenol was completely degraded at both 1000 and 1200 mgL^{-1}, although a very long lag time (about 4 and 10 days, respectively) was experienced. The degradation rates were obtained as 61.0 $mgL^{-1}h^{-1}$ and 52.4 $mgL^{-1}h^{-1}$. While cells were not able to grow and degrade phenol as sole carbon source at 1000 mgL^{-1}, the presence of glucose in the culture medium increased the tolerance of the organisms to high phenol concentrations by providing a good source of readily metabolisable carbon to support cell growth. In summary, these results show that *P. putida* is able to tolerate higher levels of phenol when supplemented with glucose or yeast extract as additional carbon sources, at an optimum ratio of 1.5 gL^{-1} and 0.8 gL^{-1}, respectively, per 750 mgL^{-1} phenol.

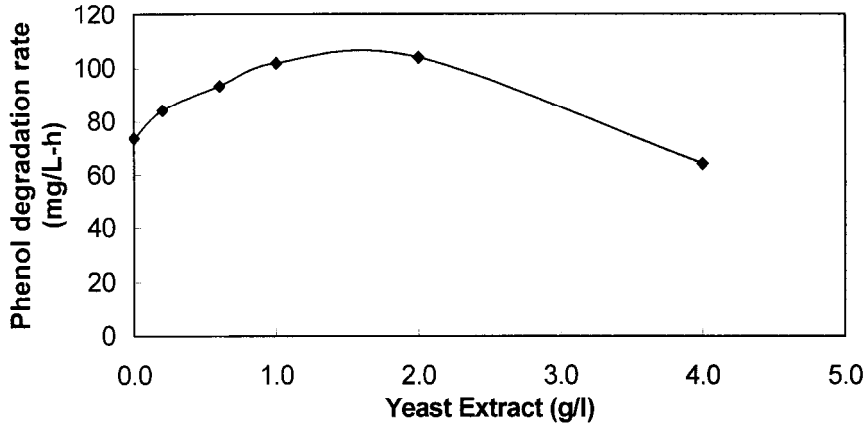

Figure 2. Effects of supplemented yeast extract on average phenol degradation rate at 750 mgL^{-1} initial phenol concentration.

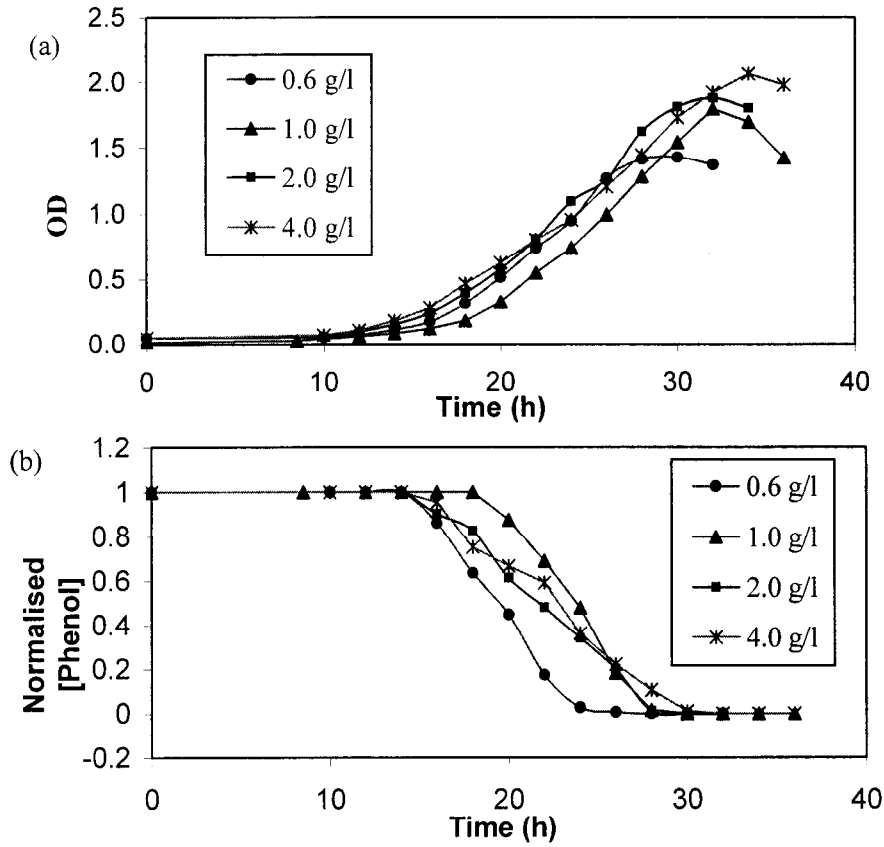

Figure 3. Effects of supplemented glucose on (a) cell growth and (b) phenol degradation at 750 mgL^{-1} initial phenol concentration.

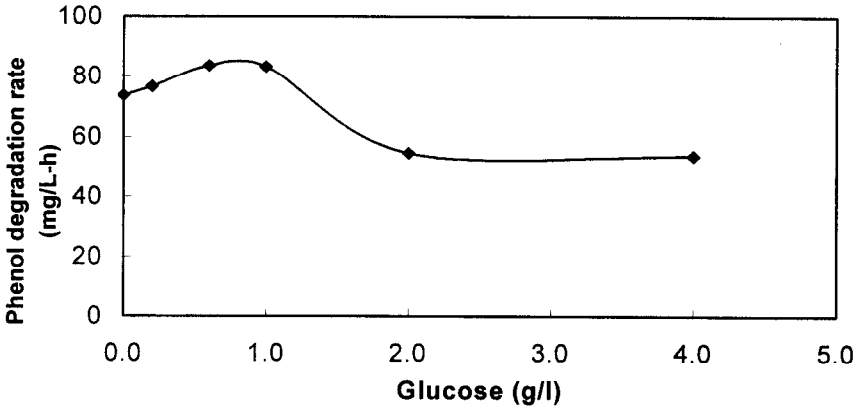

Figure 4. Effects of supplemented glucose on average phenol degradation rate at 750 mgL^{-1} initial phenol concentration.

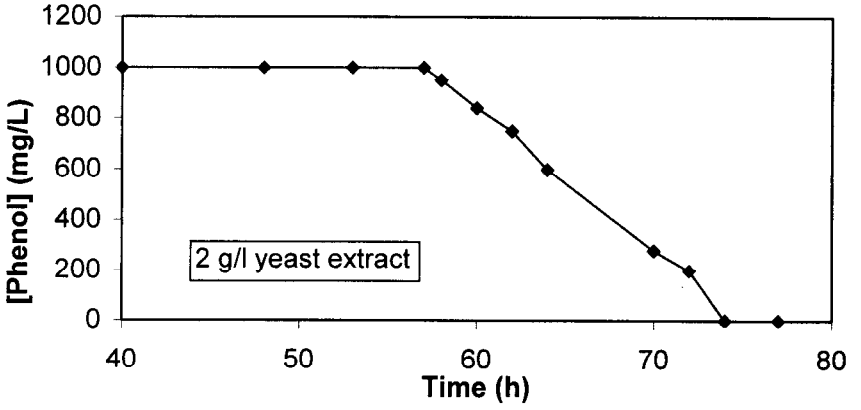

Figure 5. Degradation of 1000 mgL^{-1} phenol supplemented with 2 gL^{-1} yeast extract.

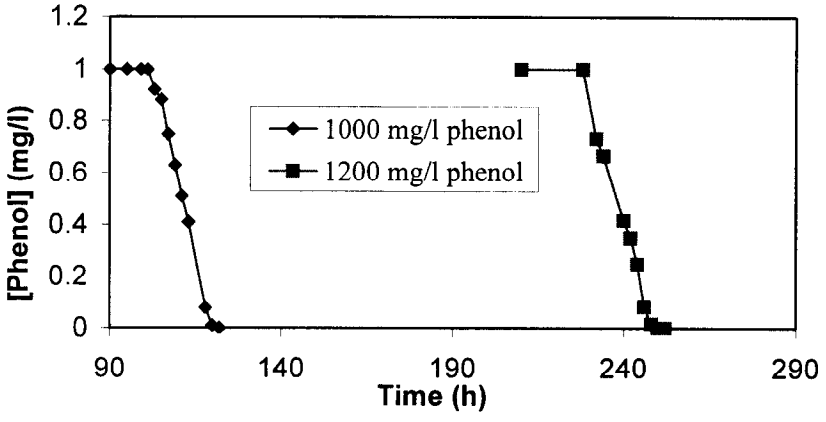

Figure 6. Degradation of high phenol concentrations supplemented with glucose.

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