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Biodegradation of cyanides, cyanates and thiocyanates to ammonia and carbon dioxide by immobilized cells of *Pseudomonas putida*

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Pseudomonas putida **utilizes cyanide as the sole source of carbon and nitrogen. Agar, alginate, and carrageenan were screened as the encapsulating matrices for *P. putida*. Alginate-immobilized cells of *P. putida* degraded sodium cyanide (NaCN) more efficiently than non-immobilized cells or cells immobilized in agar or carrageenan. The end products of biodegradation of cyanide were identified as ammonia (NH_3) and carbon dioxide (CO_2). These products changed the medium pH. In bioreactors, the rate of cyanide degradation increased with an increase in the rate of aeration. Maximum utilization of cyanide was observed at 200 ml min^{-1} of aeration. Immobilized cells of *P. putida* degraded cyanides, cyanates and thiocyanates to NH_3 and CO_2. Use of Na[^{14}C]-CN showed that 70% of carbon of Na[^{14}C]-CN was converted into $^{14}CO_2$ and only 10% was associated with the cell biomass. The substrate-dependent kinetics indicated that the K_m and V_{max} values of *P. putida* for the substrate, NaCN were 14 mM and 29 nmol of oxygen consumed mg protein^{-1} min^{-1} respectively.**

Keywords: biodegradation; cyanides; cyanates; thiocyanates; immobilization; bioreactor; alginate beads; *Pseudomonas putida*

Introduction

Industries dealing with metal plating and finishing, production of synthetic fibers, and the mining and extracting of metals generate large quantities of cyanide-containing wastes [20,28,35]. Cyanide is a potent inhibitor of cellular metabolism [4,20,21,33]. Hence, cyanide in industrial waste waters must be treated or reduced to the lowest levels possible before it can be discharged [35]. Many chemical processes currently used to detoxify cyanide-contaminated industrial wastewater suffer from major drawbacks especially the uncontrolled formation of other toxic and biologically persistent chemical compounds. The effluents thus produced require additional treatment before they can be discharged [20,34].

Biological treatment provides an alternative method of choice without creating or adding new chemicals into the environment. A few microorganisms are known to possess enzymes that are capable of converting cyanides into compounds which may serve as C and N sources [17,23,28]. Various microbial systems for the treatment of cyanides in wastewaters have been reported earlier [20,21,29,34,35].

The application of immobilized cells to the treatment of wastewater offers the possibility of degrading higher concentrations of toxic pollutants. The scientific and technical aspects involved in using immobilized microbial cells in biodegradation of pollutants have been extensively reviewed with a particular focus on cells encapsulated in biopolymer gels [8]. Immobilized cells are cells that are entrapped within or associated with an insoluble matrix. Immobilization may be considered to be the physical separation, during continuous operation, of the catalyst (cell, cell fraction or enzyme) from the solvent in such a way that substrate and product molecules may readily exchange between phases [9,19]. Under many conditions, immobilized cells have advantages over either free cells or immobilized enzymes [5,10,13,16]. Immobilization reduces loss of cells from the reactor system, and allows a high cell density to be maintained at any given flow rate [19]. Also, catalytic stability is often improved upon immobilization. Industrial wastewaters, in particular, contain antimicrobial substances in sufficiently high concentrations such that microbial activities can be inhibited [15]. Under these conditions, the application of immobilized cell technology can be regarded as a strategy for bacterial survival.

The present study was therefore aimed at exploring the biodegradation of cyanides, cyanates, and thiocyanates by immobilized cells of *P. putida* in the bioreactor.

Materials and methods

Chemicals and medium

All chemicals used in the study were ACS reagent grade or 98–99% purity and were purchased from Sigma Chemical Co, St Louis, MO, USA. Sodium [^{14}C] cyanide (specific activity, 3.0 mCi mmol^{-1}) was purchased from Pathfinder Laboratories, St Louis, MO, USA. Aquasol-2 was purchased from DuPont, NEN Research Products, Boston, MA, USA. The sterile phosphate buffer medium (pH 7.0) used for the isolation of cyanide-utilizing microorganisms contained (in g L^{-1}): K_2HPO_4, 4.3; KH_2PO_4 3.4; and $MgCl_26H_2O$, 0.3. The medium was amended with 0.5 ml

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of a trace element solution containing the following (in mg L^{-1}): $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot H_2O$, 2.6 and $Na_2MoO_4 \cdot 2H_2O$, 6.0.

Isolation and identification of the bacterium

The bacterium used in this study was originally isolated from an industrial site by cyanide enrichment techniques [3]. The microorganism was identified and confirmed as *Pseudomonas putida* based on the classification scheme described previously [27] and tests mentioned by Smibert and Krieg [32].

Optimal growth conditions

The optimal pH and temperature for growth of the microorganism were determined by measuring growth in phosphate buffer medium containing 4 mM NaCN after 72 h of incubation. Temperature response was tested over the range of 5–55°C. The response to pH was tested over the range of 3.0–9.0.

Immobilization of P. putida

Bacterial cells were cultured in the phosphate buffer medium (10 L) containing 4 mM NaCN as the sole source of C and N. After 72 h of incubation, the cells were harvested by centrifugation at $15000 \times g$ for 15 min at 5°C and cell paste (300–400 mg wet weight) was collected for immobilization. The bacterial cells were immobilized by encapsulating in different matrices (agar, alginate or carrageenan), as described in our previously published paper [9].

Experimental design

Screening of different encapsulating matrices was carried out using 250-ml EPA soil biometer flasks filled with 50 ml of 0.85% NaCl supplemented with 4 mM NaCN and 50 ml bead volume of agar/alginate/carrageenan. In the case of the flasks containing non-immobilized cells, 1 ml of the cell suspension ($A_{660} = 1.0$) of *P. putida* was used as the inoculum. The rate of cyanide degradation was monitored for 120 h by analyzing pH, NH_3, CO_2 and cyanide in the samples.

The oxidation of cyanide by immobilized cells of *P. putida* was studied by measuring O_2 uptake with a Gilson 5/6 Oxygraph (Gilson Medical Electronics, Middleton, WI, USA). One milliliter of beads (100 μg cell protein) was added to the cell chamber followed by 1.0 ml of substrate (cyanide, cyanate or thiocyanate). The suspension was then gently stirred and equilibrated at 30°C for 20 min before readings were recorded. The O_2 uptake over the range of 10–100 mM of substrate was recorded for 10 min. The data were corrected for endogenous respiration.

Batch reactor experiments

The effect of aeration on biodegradation of cyanide was studied in an 800-ml, air-uplift-type fluidized bed reactor containing equal volumes (375 ml each) of alginate-immobilized cells of *P. putida* and 0.85% NaCl solution supplemented with 4 mM NaCN. The beads were aerated with different flow rates (50, 100, 150 and 200 ml min^{-1}) of CO_2 and NH_3-free air. Influent air was passed through 0.5 M KOH and 0.5 M boric acid to remove CO_2 and NH_3 respectively. Samples of effluent air trapped in 0.5 M KOH and 0.5 M boric acid, and the medium were collected at regular intervals to determine CO_2, NH_3 and cyanide. The degradative capability of immobilized cells of *P. putida* towards free, metal-complexed cyanides, and other CN^- containing compounds such as cyanates and thiocyanates (100 ppm each) was tested by monitoring the levels of pH, CO_2 and total NH_3 (dissolved and gaseous) from the reactor.

Biodegradation of radiolabeled Na[^{14}C]-CN

The mineralization of radiolabeled Na[^{14}C]-CN to $^{14}CO_2$ was determined in the bioreactor containing equal volumes (375 ml each) of immobilized cells of *P. putida* and 0.85% NaCl solution supplemented with non-radiolabeled NaCN (4 mM) and Na[^{14}C]-CN (30000 cpm ml^{-1}: specific activity 3 mCi $mmol^{-1}$). $^{14}CO_2$ evolved during cyanide degradation was trapped in 1 N NaOH. Samples (5.0 ml) of NaOH were taken at regular intervals, and $^{14}CO_2$ was determined using a liquid scintillation counter (model LS 6800, Beckman Instruments, Irvine, CA, USA) after the addition of 5.0 ml of Aquasol-2 scintillation fluid. Samples (0.25 ml) were collected from the reactor and the amount of Na[^{14}C]-CN left/unutilized in the medium was determined after adding 9.75 ml of the scintillation fluid. The radioactivity associated with the beads/cells was determined at the end of the experiment by suspending the beads (150–200 mg) in 5.0 ml of 0.2 M phosphate buffer followed by the addition of 5.0 ml of scintillation fluid to the sample.

For control experiments, reactors containing beads without cells and beads containing inactive cells were used. Beads containing inactive cells were amended with 10 ppm $HgCl_2$ for the prevention of any microbial growth.

Analytical methods

The growth of bacteria was measured as optical density (OD) using a Spectronic 21 spectrophotometer (Milton Ray, Analytical Products Division, Rochester, NY, USA). The OD was measured at 546 nm, wavelength, 10 nm spectral slitwidth and 10 mm cuvette. Growth medium without cells was used as a blank. Dissolved NH_3 was determined colorimetrically by Berthelot's procedure as described by Kaplan [18]. The gaseous NH_3 was determined by back titration of boric acid (known concentration) with 0.5 M KOH. The gaseous CO_2 was first dissolved in a known volume of 0.5 M KOH and back titrated for free KOH using 0.5 M HCl. Cyanide was measured colorimetrically by the method of Lambert *et al* [24]. Reactors containing beads without *P. putida* and beads with inactive cells served as controls in all the experiments. Beads containing inactive cells were amended with 10 ppm $HgCl_2$ for the prevention of any microbial growth. Experiments were repeated twice with triplicate samples and the mean values are presented. The *t*-test values were statistically significant at $P < 0.01$.

Results

Isolation and identification of bacteria

The *P. putida* isolate capable of utilizing cyanide as the sole source of carbon, nitrogen and energy was isolated originally from the industrially contaminated soil. The organism is rod-shaped, Gram-negative, motile and non-

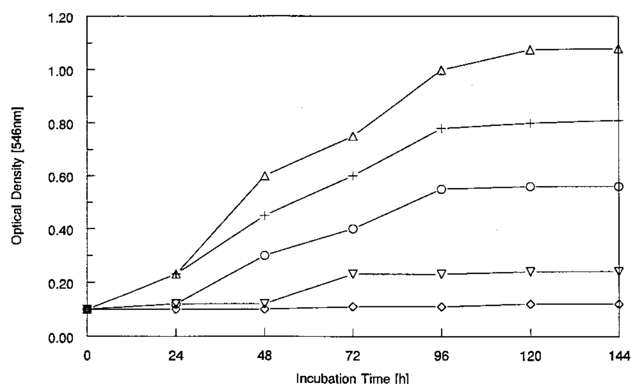

Figure 1 Growth of *Pseudomonas putida* on sodium cyanide in the phosphate buffer medium at 25°C. —+— 2 mM; —Δ— 4 mM; —○— 6 mM; —▽— 8 mM; —◇— 10 mM.

spore forming, and was originally identified and confirmed as *P. putida*. The bacterium grew as fluorescent colonies. Oxidase, catalase, and arginine dihydrolase reactions were positive. Growth was observed on sodium benzoate, McConkey, and glucose plates but not on xylose and maltose. The isolate failed to hydrolyze gelatin. The maximum growth of the bacterial isolate was observed after 72 h of incubation at 25°C and pH 7.5. No growth was observed at temperatures of 10 and 55°C and pH < 5.0 and > 10.0 [9].

Growth of non-immobilized P. putida on cyanide

The growth of *P. putida* at different concentrations of cyanide is shown in Figure 1. The maximum cell density was recorded at a concentration of 4 mM CN^-, whereas 8 mM cyanide was inhibitory. Concentrations above 6 mM induced a longer lag phase in growth and no growth was recorded at 10 mM cyanide.

Immobilization of P. putida

The typical yield following the immobilization of cells was about 0.6–1.0 g of beads per ml of cells in agar, alginate or carrageenan suspension. The beads had a mean diameter of 1–2 mm with average wet and dry weights of 12–13 and 0.6–0.7 mg respectively. At the time of immobilization, each bead contained approximately $1–2 \times 10^8$ viable cells of *P. putida* as determined by the pour plate method for disrupted beads [26].

Screening of encapsulating matrices

Table 1 presents the data on cyanide degradation by non-immobilized cells and by immobilized cells in agar, alginate or carregeenan. The changes in pH, NH_3, CO_2 and CN^- concentration during cyanide mineralization, in general, were more pronounced in the samples containing alginate beads as compared to agar or carrageenan beads or non-immobilized cells of *P. putida*, thereby indicating that the degradation of cyanide was carried out more effectively with the alginate beads of *P. putida*. In contrast, no changes in pH, NH_3 and CO_2 production and CN^- concentration were observed in the controls — beads without cells and beads with inactive cells.

Screening of substrates

Table 2 presents data on the degradation of various cyanides, cyanates, and thiocyanates by immobilized cells of *P. putida* to NH_3 and CO_2. Among the free cyanides, sodium cyanide and potassium cyanide were degraded most efficiently followed by zinc cyanide and copper cyanide. Both potassium ferricyanide and sodium nitroferricyanide were degraded less effectively than potassium ferrocyanide. In the case of other CN^- containing compounds such as cyanates and thiocyanates, sodium cyanate was more readily degraded by immobilized cells of *P. putida* than potassium cyanate and the maximum and minimum biodegradation was observed for ammonium thiocyanate and cobalt thiocyanate respectively.

Optimal conditions

The maximum biodegradation of CN^- by alginate-immobilized cells of *P. putida* was observed at pH 7.5 and 25°C. There was more production of CO_2 and NH_3 noticed when a higher concentration of CN^- was degraded by immobilized *P. putida* (data not shown). No CN^- degradation was observed when 10 mM of CN^- was used. Both CO_2 and NH_3 production and CN^- utilization by alginate-immobilized cells increased with an increase in incubation period (data not shown).

Effect of aeration on degradation of cyanide

The rate of aeration influenced CN^- degradation by alginate-immobilized cells of *P. putida* in an air-uplift fluidized bed reactor. The production of NH_3 and CO_2 and the degradation of CN^- increased with an increase in the aeration rate. Maximum changes in CN^- concentration (mM L^{-1}) as well as NH_3 and CO_2 production were observed at 200 ml min^{-1} of aeration (CN^-: 0.11; NH_3: 3.17; CO_2: 11.8). The degradation of cyanide after 120 h of incubation was greater at an aeration rate of 200 ml min^{-1} than at 150 ml min^{-1} (CN^-: 1.23; NH_3: 2.71; CO_2: 10.3),

Table 1 Changes in carbon and nitrogen during degradation of cyanide (NaCN) by non-immobilized and immobilized cells of *P. putida* after 120 h of incubation

Nature of cells	Final pH	% Change over carbon at 0 h (% carbon released)	% Change over nitrogen at 0 h (% nitrogen released)	% Cyanide utilised
Non-immobilized cells	8.5	72.22 ± 0.02	71.34 ± 0.02	79.75 ± 0.01
Immobilized cells				
Alginate	8.3	76.39 + 0.02	96.43 + 0.02	98.00 + 0.01
Agar	8.1	69.44 ± 0.02	86.90 ± 0.02	67.73 ± 0.01
Carrageenan	7.8	68.06 + 0.02	75.00 + 0.02	59.50 + 0.01

Initial values pH 7.5, Cyanide 4.00 mM. Carbon released was measured as total CO_2; nitrogen released was measured as total NH_3.

Table 2 Changes in pH, carbon and nitrogen during degradation of cyanides, cyanates, and thiocyanates by immobilized cells of *P. putida* at 25°C and pH 7.5 after 120 h of incubation

Substrate (100 ppm)	pH	% Carbon released	% Change over nitrogen at 0 h (% Nitrogen released)
Free cyanides			
Sodium cyanide	7.93	65.88 ± 0.02	64.71 ± 0.02
Potassium cyanide	7.65	81.04 ± 0.02	79.23 ± 0.02
Copper cyanide	7.75	25.35 ± 0.02	20.45 ± 0.02
Silver cyanide	7.71	47.20 + 0.02	50.96 + 0.02
Zinc cyanide	7.75	29.59 + 0.02	33.57 + 0.02
Complex cyanides			
Sodium nitroferricyanide	8.05	37.37 ± 0.02	53.69 ± 0.02
Potassium ferricyanide (Potassium hexacynoferrate (III))	7.72	36.88 ± 0.02	71.90 ± 0.02
Potassium ferrocyanide (Potassium hexacynoferrate (II))	7.77	27.46 ± 0.02	94.44 ± 0.02
Cyanates			
Sodium cyanate	7.51	21.18 + 0.02	23.80 + 0.02
Potassium cyanate	7.85	11.20 ± 0.02	12.58 ± 0.02
Thiocyanates			
Ammonium thiocyanate	8.74	99.82 ± 0.02	99.75 ± 0.02
Cobalt thiocyanate	8.34	12.08 ± 0.02	15.76 ± 0.02
Methyl isothiocyanate	8.44	55.71 ± 0.02	45.52 ± 0.02
Potassium thiocyanate	7.73	74.06 ± 0.02	73.01 ± 0.02
Sodium thiocyanate	7.63	86.27 + 0.02	86.84 + 0.02

100 ml min^{-1} (CN^-: 2.15; NH_3: 2.47; CO_2: 7.55) and 50 ml min^{-1} (CN^-: 2.31; NH_3: 2.17; CO_2: 5.26) (data not shown). Insignificant evaporation of cyanide due to the aeration was observed in the control reactors.

Biodegradation of Na[^{14}C]-CN

Figure 2 shows $^{1}CO_2$ production during mineralization of Na[^{14}C]-CN by alginate-immobilized cells of *P. putida*. Seventy per cent of carbon of Na[^{14}C]-CN was evolved as $^{14}CO_2$ after 120 h of incubation. Nearly 12% of Na[^{14}C]-CN was not utilized by alginate-immobilized cells of *P. putida* and 10% radioactivity was associated with the beads. Less than 1% of cyanide volatilized during mineralization of Na[^{14}C]-CN was found in NaOH of the control bioreactors.

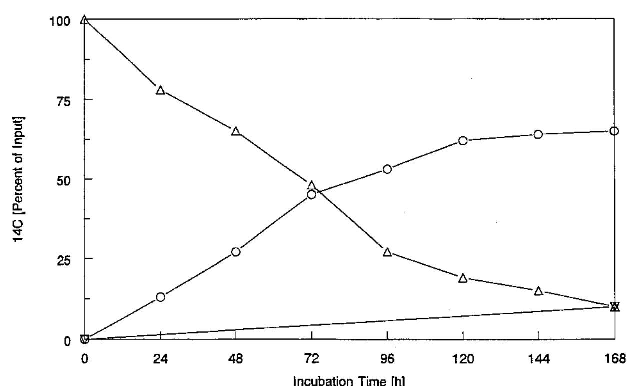

Figure 2 Productin of $^{14}CO_2$ — ❍ — radioactivity unutilized/left in the medium — Δ —; and radioactivity bound to the cells/beads — ∇ — during the mineralization of [Na[^{14}C]-CN] by alginate-immobilized cells of *Pseudomonas putida* at 25°C, 7.5 pH, 200 ml min^{-1} of aeration.

Kinetics of cyanide oxidation

The present study illustrates that immobilized cells of *P. putida* were able to oxidize cyanide thereby indicating that the cyanide is bioxidizable. The K_m and V_{max} values were found to be 14.3 mM and 28.6 nmol of oxygen mg^{-1} cell protein min^{-1} respectively (data not shown).

Discussion

Biotreatment can be considered as an efficient mechanism for the removal of chemicals released into aquatic and terrestrial environments [31]. Non-immobilized and immobilized cells of various bacterial species degrade and detoxify a wide range of toxic compounds [14,15,17,23,26,34]. A variety of enzymatic pathways for cyanide degradation has been reported. These hydrolytic pathways lead to production of formamide or formate plus ammonia [6,14,15,26,34], or the direct formation of bicarbonate plus ammonia via cyanide oxidase or by a dioxygenase [14,15,21,30].

It is evident from our study that non-immobilized and immobilized cells of *P. putida* were able to degrade cyanide to NH_3 and CO_2. The time-dependent increase in pH of the medium during the degradation of cyanides, cyanates, and thiocyanates by immobilized *P. putida* could be attributed to the accumulation of NH_3 formed due the cleavage of the CN^- group. The decrease in pH of the medium after 120 h of incubation could be attributed to the neutralizing effect of carboxylic acid forming during degradation. It is unlikely that the accumulation of formate may be responsible for the change in pH [23,25]. An increase in the levels of CO_2 and NH_3 with a decrease in cyanide concentration evidently indicates that immobilized *P. putida* could degrade cyanide into NH_3 and CO_2 by following one of the enzymatic path-

ways mentioned above. Similar conversion of cyanide into CO_2 and NH_3 has been reported under both aerobic and anaerobic conditions [11,14,17,23,25,34]. The data also suggest that higher concentrations of cyanide (up to 120 mM) can be degraded by immobilized cells than with non-immobilized cells of *P. putida* which could degrade only up to 4 mM [3].

The time-dependent production of CO_2 and NH_3 during utilization of CN^- containing compounds — free, metal complexed cyanides, cyanates, and thiocyanates — indicate that immobilized cells of *P. putida* could convert these compounds into NH_3 and CO_2. The degradation of cyanates and thiocyanates to NH_3 by the microbial cultures has been reported earlier [2,7,22]. This demonstrates that immobilized cells of *P. putida* are equipped with the necessary enzymatic mechanisms not only for the metabolism of cyanides, but also of cyanates and thiocyanates. It also leads to further investigation into the specific enzymatic mechanism(s) involved in the metabolism of cyanates and thiocyanates by *P. putida*.

Increased production of NH_3 and CO_2 with a simultaneous decrease in cyanide concentration was more pronounced in the medium containing alginate beads as compared to other beads. This could be attributed to the rapid exchange of substrate (CN^-) and products (CO_2 and NH_3) through these alginate beads. The alginate-immobilized cells exhibited higher catalytic stability as compared to agar and carrageenan beads. The lower rate of degradation of cyanide by agar/carrageenan beads of *P. putida* might be either due to the reduced diffusion of CN^- or O_2 into the beads or the assimilation of products (NH_3, etc,) by the microorganisms. This suggests that alginate is the most suitable matrix for immobilizing the bacterial cells to achieve higher degradation of CN^-.

One potential disadvantage of immobilization is the increased resistance of substrate and products to diffusion through immobilization matrices. Owing to the low solubility of oxygen in water and the high local cell density, oxygen transfer often becomes the rate-limiting factor in the performance of aerobic immobilized cell systems [1,26]. Thus, when aerobic cells are used, aeration techniques become a very important consideration in bioreactor design. This is true in the present study, as the rate of CN^- degradation was dependent on the rate of aeration. The low rate of NH and CO_2 production at 50 and 100 ml min^{-1} aeration suggests the oxygen limitation of the entrapped cells in the alginate immobilization system. The rate of CN^- degradation increased with an increase in the rate of aeration. This indicates the possible diffusion of O_2 through the outer cell matrix layers. The high local cell density within the matrix will also limit O_2 diffusion [27]. This suggests that the utilization of O_2 and the enzymes involved in the degradation of cyanide would compete for it, especially at lower rates of aeration. Under limited availability of O_2 involved in the cyanide pathway, endogenous cellular respiration might deplete O_2 concentration within the alginate matrix, thus lowering the activity of the cyanide-transforming enzymes. The present study thus suggests that the degradation of cyanides, cyanates, and thiocyanates into NH_3 and CO_2 may be carried out effectively with immobilized cells of *P. putida*.

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