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Lanthanum accumulation from acidic solutions using a *Citrobacter* sp. immobilized in a flow-through bioreactor

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SUMMARY

A biotechnological process for the uptake of metals from solution was evaluated at low pH. Metal uptake is mediated by the enzymatic liberation of phosphate by immobilized cells of a *Citrobacter*; insoluble metal phosphate is retained at the cell surface. Lanthanum uptake was abolished at pH 4, but the activity of the mediating phosphatase was reduced by only 50% at this pH. At pH 5 phosphate release was largely unaffected as compared to that at pH 7, but La removal was reduced. Growth of the strain under conditions which gave a four-fold increase in phosphatase activity gave only a two-fold increase in metal removal at pH 5 by immobilized cells. The precipitated species was identified as $LaPO_4$; the poor removal seen at low pH was attributed to inefficient desolubilization of lanthanum phosphate. In addition, some contributory biochemical factors were identified. The apparent K_m ($K_{m\ app}$) of the whole-cell enzyme for *p*-nitrophenyl phosphate was little-affected by the pH, but the $K_{m\ app}$ for glycerol 2-phosphate was increased at pH 5. This influences the activity of the bioreactor at high flow rates and is reflected in the gradient seen on plots of the ln flow rate-activity relationship, in accordance with a Michaelis–Menten description of the bioreactor. It is concluded that although reduced metal uptake at low pH is mainly attributable to chemical effects, these can be exacerbated according to the choice of substrate supporting the desolubilization reaction: *p*-nitrophenyl phosphate is the better substrate for efficient metal desolubilization at high flow rates at low pH values.

INTRODUCTION

Many metal-bearing liquid industrial wastes are produced at low pH. For example, metal ores are often treated with mineral acids for metal extraction, while acid dissolution is commonly used in metal and resource recycling, e.g. during the reprocessing of spent nuclear fuels [11]. Acidic conditions can arise naturally, for example where biological leaching is used to extract metals from low grade ores [2,19]. Complete neutralization prior to discharge may be uneconomic; furthermore at neutral pH many metals form insoluble hydroxylated species giving particulate or colloidal suspensions which require settling-out. Continuous production processes often generate large volumes of waste daily; on-site storage facilities for settlement may be impractical and 'end of pipe' filtration technology may form an attractive alternative.

Microorganisms and microbial products have been utilized for the removal of soluble heavy metal species from solution [4,12,13]; in some cases metal biosorption onto biomass has been observed at low pH [3,22,27–29]. Notably, Gadd and White [5] demonstrated biosorptive uptake of thorium at pH 1–2; however other authors observed loss of biomass integrity upon treatment with mineral acids [19,26].

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Although metal accumulation by growing cells is well documented [12], microbial growth and metabolism generally occur only within physiologically-permissive limits of pH. This can be overcome to some extent by identification of possible enzyme step(s) responsible for metal bioaccumulation. If continued cell viability and metabolism are not required for metal uptake, the limits of pH tolerance may be expanded to those of the appropriate enzyme(s) and may permit metal uptake under harsher conditions.

This study applies this concept to metal bioaccumulation in a flow-through column system utilizing immobilized cells of a *Citrobacter* sp., upon which metal is desolubilized as the metal phosphate and retained on the biomass, with treated solution flowing to waste [10]. Metal desolubilization (uptake onto the cells) is mediated by the activity of an acid-type phosphatase induced during heavy metal-free pre-growth. The extant enzyme continues to function subsquently in growth-decoupled immobilized cells in the liberation of inorganic phosphate from a suitable organic phosphate 'donor' molecule incorporated into the flow; with divalent cations e.g. Cd^{2+}, Pb^{2+} and UO_2^{2+}, metal and phosphate are precipitated stoichiometrically to high cellular loads [10,12].

Early studies suggested that reduction in metal removal efficiency at pH 5 was attributable to increased metal solubility at low pH [10]. The present study investigates the extent to which this can be compensated by increased phosphate production by the cells, and attempts to describe the data in terms of the kinetics of the immobilized whole-cell enzyme. This work formed part of a wider investigation into the feasibility

of using this approach in the treatment of wastes produced from the nuclear fuel cycle. Such wastes are contaminated with various nuclides, for example ^{241}americium; a previous study has shown that this isotope can be removed from solution by the immobilized cells [17,25]. For demonstration in model systems lanthanum provides a stable, non-radioactive model metal whose chemical behaviour resembles that of Am; La was accordingly used in the present study to investigate the effects of reduced pH on metal bioaccumulation by the immobilized cells.

MATERIALS AND METHODS

Organisms and growth conditions

Citrobacter sp. strains N14 and dc5c were as described previously [10,15]. Batches (31) were routinely grown to the late logarithmic-early stationary phase of growth in tris-buffered minimal medium (30 °C) containing glycerol (3 g L^{-1}) and glycerol 2-phosphate (0.67 g L^{-1}) as the respective carbon and phosphorus sources [7,15]. Carbon-limiting (0.6 g L^{-1} of glycerol and 0.67 g L^{-1} of glycerol 2-phosphate) continuous cultures were grown in a New Brunswick (NJ, USA) 'Bioflo' apparatus as described previously [7], substituting 10 mM MES (2-[*N*-Morpholino]ethanesulfonic acid)-NaOH buffer and 8.5 g L^{-1} NaCl for the tris buffer previously employed. The pH of the cultures was progressively reduced from c. 6.6 to 5.6 during each run, with fine adjustments made as necessary using an autotitrator and dropwise feed of NaOH or HCl (2 M). The chemostats were run at D = 0.26 h^{-1}, corresponding to 0.5 μ_{max} at neutral pH, where μ_{max} corresponds to a doubling time of 1.5 h at 30 °C. No attempt was made to establish the μ_{max} under pH stress since the chemostats were here used as a mutant selection procedure with no attempt to achieve steady-state (see text).

Assay of phosphatase activity

Samples were removed from the batch cultures or chemostat outflows, washed twice in isotonic saline (8.5 g L^{-1} of NaCl) and resuspended at c. 2–3 × 10^9 cells ml^{-1} in saline. Phosphatase activity was determined by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate as described previously [1,8,15] in buffers appropriate to the pH under test: pH 6–7: 200 mM MOPS (3-*N*-Morpholino)propanesulfonic acid)-NaOH; pH 4–6: 200 mM MES-NaOH; pH 3–4; 200 mM sodium acetate-acetic acid. The ratio of cell suspension to buffer in the assay was 1:9 vol:vol. The use of two buffers at the overlap pH values allowed for corrections due to buffer effects on the enzyme. The use of acetate buffer at pH 4 gave a specific activity of 22.9 ± 0.7% higher than the corresponding result with MES buffer; corrections were made as appropriate. No discrepancy was seen between MES and MOPS buffers at pH 6. Phosphatase specific activity (unit) is expressed as nmol of product released min^{-1} mg^{-1} of bacterial protein, with protein assayed by the method of Lowry and related to the culture optical density at 600 nm [8]. Where comparisons were made between batches the activity at pH 7 was taken as 100% and other results were expressed relative to this control value.

Cell immobilization, and use of immobilized cell columns for product release

Five grams (wet weight) of cells from each 31 batch was harvested, washed and immobilized in polyacrylamide gel (PAG) as described previously [1]. Each immobilization provided material for five replicate columns. The shredded polyacrylamide gel was divided into five equal parts by weight; each was washed in saline to remove unpolymerized gel components and packed into glass columns of working volume c. 25 ml. The columns were challenged at varying flow rates at 30 °C with 5 mM citrate buffer (pH 7, 5 or 4) supplemented with substrate (5 mM *p*-nitrophenyl phosphate). Liberated *p*-nitrophenol was estimated in the outflow solution in alkaline solution at 410 nm versus *p*-nitrophenol standards prepared in the same solution [1,7].

Heavy metal accumulation by the immobilized cells

For metal uptake experiments the substrate (phosphate donor) was 5 mM *p*-nitrophenyl phosphate or glycerol 2-phosphate (sodium salt) prepared in citrate buffer (concentration as specified in individual experiments), in the presence of 1 mM uranyl or lanthanum nitrate. The columns were run at varying flow rates with determination of metal in the inflow and outflow solutions using arsenazo III [24]: the column inflow or output solution was diluted 20-fold (U) or 10-fold (La) in the citrate buffer-substrate mixture (final volume 2 ml). This was mixed with 0.3 ml of 0.75 M HCl and 0.1 ml of arsenazo III solution, with visualization of the metal-arsenazo III complex at 652 nm. The arsenazo III solution was prepared by adding 0.038 g of arsenazo III to 25 ml of distilled water and mixing for at least 1 h. The solution was filtered through a Whatman no. 1 filter paper to give a saturated solution that could be stored for up to one month before use. Metal uptake by the column was expressed as a percentage of the input metal removed.

Identification of the accumulated lanthanum deposit

After prolonged exposure to La^{3+} in the buffered continuous flow solution the deposited metal was evident as a white granular encrustation on the gelled material. The precipitate was scraped from the gel, washed in water, dried and examined under an electron microscope (Jeol Ltd, Welwyn Garden City, Herts, UK) using energy dispersive X-ray microanalysis (EDAX) for qualitative element determinations as described previously [14,16]. A reference crystal of $LaPO_4$ was obtained from the Clarendon Laboratory, University of Oxford, but could not be easily examined under EDAX due to surface charging, even following carbon coating of the sample. Instead quantitative elemental analyses were done using proton induced X-ray emission (PIXE), using the Oxford Scanning Proton Microprobe [6,9,30] in the Department of Nuclear Physics, University of Oxford. Elemental maps were obtained of 2 mm^2 specimens mounted upon a hollow aluminium target within the proton beam. Matrix major element composition and thickness, which are needed to calculate PIXE corrections, were determined using simultaneously-determined Rutherford Back Scattering (RBS) spectra. The intrinsic accuracy of PIXE using the RBS correction was demonstrated by comparison of

data obtained by PIXE with that determined by other methods [23].

Determination of an apparent K_m ($K_{m\,app}$) *value for the immobilized cells*

For determination of the $K_{m\,app}$ of the immobilized cells the gelled material was divided into aliquots of 1 g (50 aliquots per immobilization using 5 g wet weight of cells). Each was suspended in 30 ml of 5 mM citrate buffer/50 mM MOPS buffer (pH 7) or citrate/MES buffer (pH 5) and supplemented with varying concentrations of either *p*-nitrophenyl phosphate or glycerol 2-phosphate at 30 °C. The initial rate of product release (nmol min^{-1}) was used to calculate the $K_{m\,app}$ using a double reciprocal (Lineweaver–Burk) plot. For *p*-nitrophenol release from *p*-nitrophenyl phosphate timed samples were quenched in 0.2 M NaOH, which also visualized the yellow colour at 410 nm. For phosphate release from glycerol 2-phosphate the inorganic phosphate was determined in samples quenched in H_2SO_4 (2.5 M) and assayed by a modification of the method of Pierpoint [20].

RESULTS AND DISCUSSION

Heavy metal accumulation by polyacrylamide gel (PAG)-immobilized cells

Columns of PAG-immobilized cells were challenged with flows containing 1 mM uranyl ion (UO_2^{2+}) or lanthanum (La^{3+}) in citrate-buffered solution (Fig. 1). Citrate was incorporated as a chelating ligand throughout to suppress the formation of hydroxylated metal species and ensure a known speciation. Although the *Citrobacter* sp. is capable of growth in the metal mixture, growth was very slow and no detectable citrate utilization occurred within the timescale of the experiments (M.R. Tolley, P. Yong and L.E. Macaskie, unpublished).

The activity of the columns can be expressed in terms of the $FA_{1/2}$ value, which is defined as that flow rate at which 50% of the input metal is removed. Initial tests to compare the removal of UO_2^{2+} and La^{3+} (Fig. 1(A)) established that for a given batch of cells the $FA_{1/2}$ values were 1.2 and 5.2 ml min^{-1}, respectively. The greater efficiency of lanthanum removal was anticipated, as the solubility product of the metal phosphate is eleven orders of magnitude smaller for lanthanum than for uranyl phosphate (K_{sp} values for HUO_2PO_4 and $LaPO_4$ are $10^{-10.7}$ and $10^{-22.3}$, respectively [18,21]. Given the more efficient precipitation of lanthanum phosphate, La^{3+} was chosen as the metal species upon which to do subsequent tests.

The $FA_{1/2}$ value is related to the specific activity of the culture at harvest. As shown in Fig. 1(B) the ratio of the $FA_{1/2}$ values for two batches (I and II) was 8.0/5.2 (1.54:1) as compared to a ratio of specific activities of 325/206 (1.58:1). The correlation between phosphatase activity and metal uptake was observed up to a specific activity of approx 800 units; above this value the rate of metal precipitation becomes limited by chemical and biomineralization factors [17].

In order to confirm that lanthanum removal was phosphatase-mediated, the columns were challenged with substrate-unsupplemented flows. The percentage of input metal removed at non-limiting flow rates (1.48 and 2.93 ml min^{-1}) was

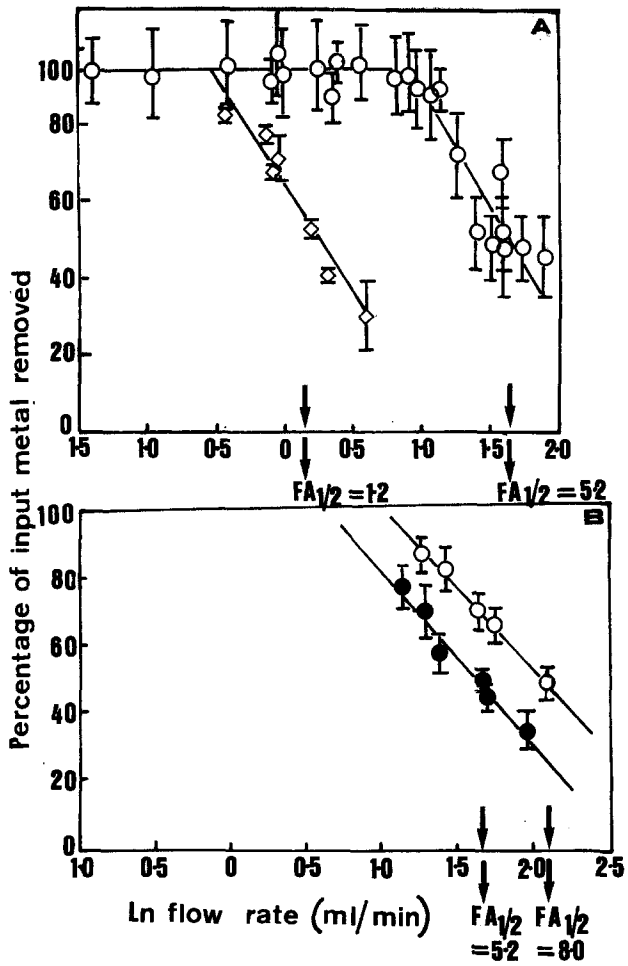

Fig. 1. Removal of lanthanum and uranyl ion by immobilized cells of *Citrobacter* sp. (A) Batch-grown cells, harvested and immobilized as described, were challenged with a flow (2 mM citrate buffer pH 6.9/5 mM glycerol 2-phosphate) supplemented with La^{3+} or UO_2^{2+} (1 mM final concentration) at varying flow rates, as shown. Data are means ± standard errors (triplicate determinations) for cells challenged with: (○), La^{3+} and (◇), uranyl ion. The $FA_{1/2}$ value (that flow rate giving 50% removal of metal from the flow: ml min^{-1}) is arrowed. (B) As in legend to 1(A). Here two batches of cells were used (the phosphatase specific activity of batches I and II was 325 and 206 units, respectively). Data shown are for lanthanum removal by the two batches. The $FA_{1/2}$ values (arrowed) were 8.0 ml min^{-1} and 5.2 ml min^{-1}, respectively.

13.6 ± 9.3% and 3.1 ± 6.9%, confirming that substrate was necessary for significant La removal. Substrate-containing solutions were also passed through columns containing cell-free PAG. No La removal was seen, demonstrating the requirement for cells within the gel.

Analysis of the accumulated metal precipitate

After continuous exposure of the PAG-immobilized cells to the flow for several days a white encrustation was seen. Samples of the encrustation were removed for analysis. In accordance with previous data using UO_2^{2+} [16], a mass of electron dense material was evident (Fig. 2(A)), which had a granular appearance (Fig. 2(B)). Analysis of the precipitate by

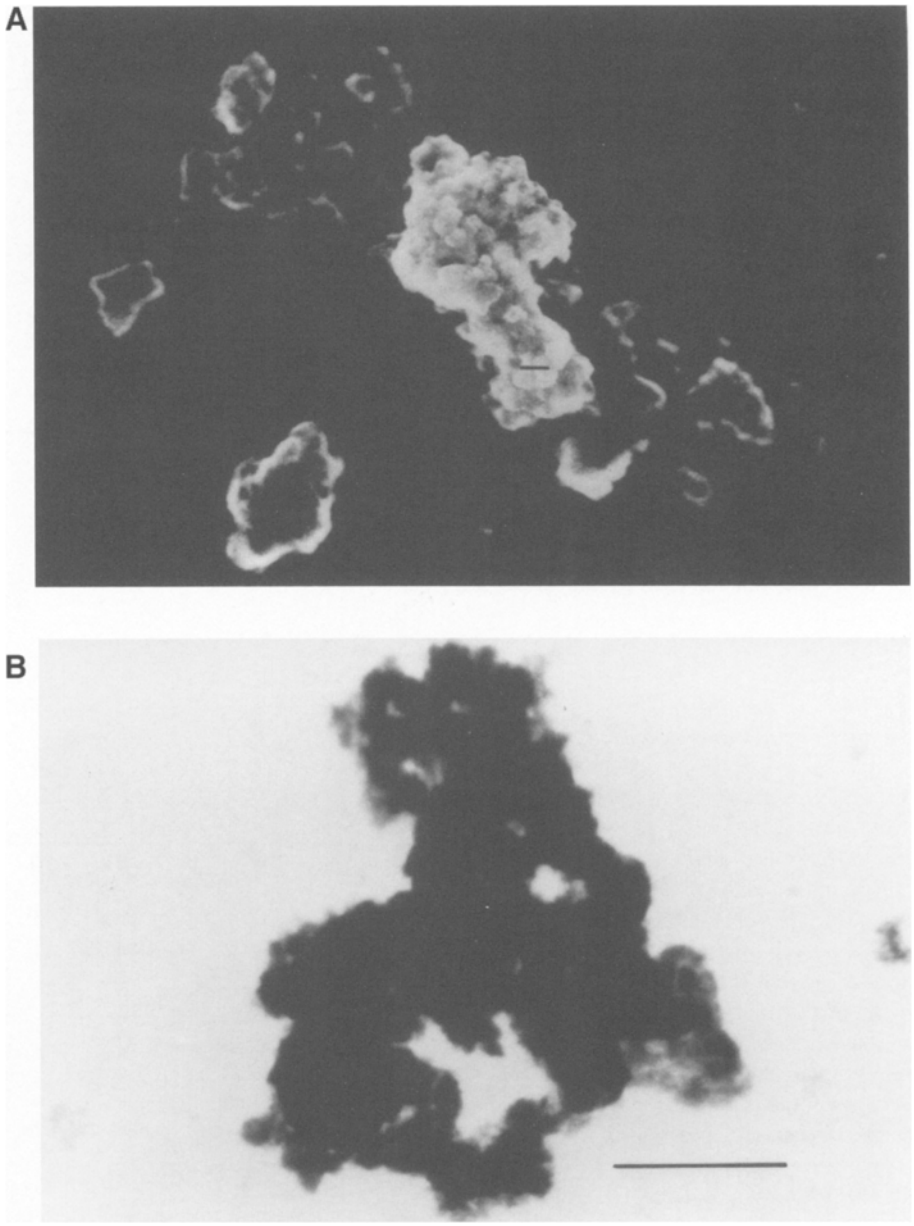

Fig. 2. Scanning electron microscopy (SEM, A) and scanning transmission electron microscopy (STEM, B) of the accumulated precipitate removed from the gel. Bars: 1 μm.

PIXE showed the predominance of lanthanum and phosphate, with an absence of other heavy metals and only low concentrations of other elements (probably attributable to those carried over in the immobilized cells and PAG) (Fig. 3). EDAX analysis of the precipitate confirmed co-deposition of metal and phosphate (Fig. 4): quantitative elemental analysis using PIXE in conjunction with RBS correction showed that the molar ratio of lanthanum to phosphate precipitated was 1:1 (Table 1). The accumulated material was taken to be $LaPO_4$ in accordance with the 1:1 metal to phosphate ratios seen with $PbHPO_4$, $CdHPO_4$ and HUO_2PO_4 previously [14,16].

The effect of pH on the phosphatase activity of free and immobilized cells

Preliminary experiments established an inhibitory effect of low pH on phosphatase activity using batch-grown cells resuspended and assayed at different pH values. In accordance with previous work [7] whole-cell phosphatase activity was constant at pH 5–7, with 50–60% retention of activity at pH 4 (Fig. 5). Below this pH the activity decreased sharply; little was apparent at pH 3. The pH-activity profile was conserved in the phosphatase-overproducing mutant dc5c.

Product release by immobilized cells in a plug flow reactor such as the flow-through columns employed here, can be described by an integrated form of the Michaelis–Menten equation which relates the input substrate concentration, the flow rate (inversely related to the residence time, t), enzyme activity, total biomass loading and bioreactor efficiency (proportion of substrate converted to product at a given residence time [1,10,12]). Flow rate-activity plots (ln flow rate versus the percentage of substrate cleaved) can be linearized for convenience; the deviation from a straight line is well

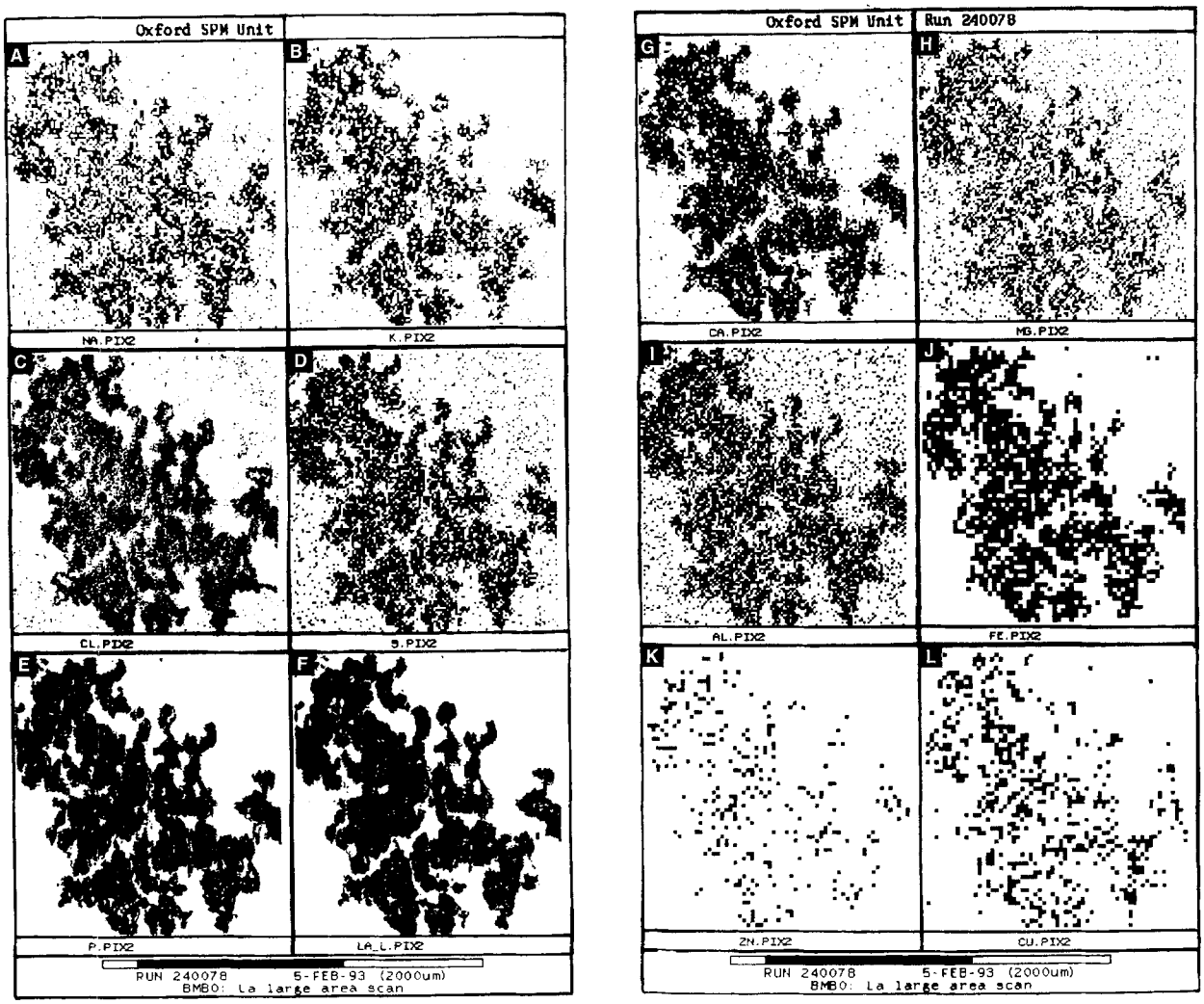

Fig. 3. Proton induced X-ray emission analysis (PIXE) of 2 mm^2 area scans giving elemental maps of the distribution of Na (A), K (B), Cl (C), S (D), P (E), La (F), Ca (G), Mg (H), Al (I), Fe (J), Zn (K) and Cu (L). Dark: areas of increased element concentration (relative values). RBS corrections were done on selected specimen microareas to give absolute elemental concentrations (see Materials and Methods).

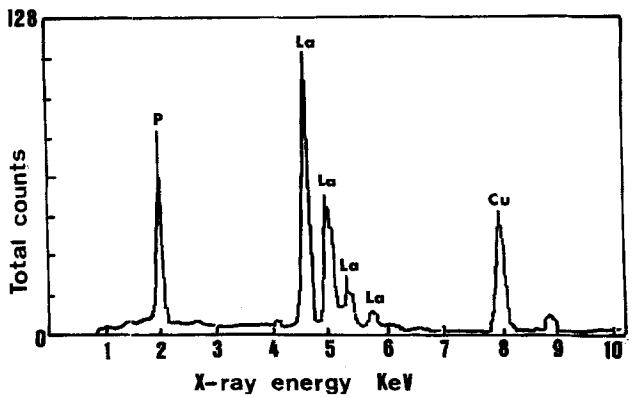

Fig. 4. Energy dispersive X-ray analysis (EDAX) of the accumulated precipitate. Precipitate removed from the gel (Fig. 1) was examined under the electron microscope with analysis of specimen microareas to show co-deposition of La and P (see Materials and Methods).

TABLE 1

Concentrations of lanthanum and phosphorus in the accumulated metal precipitate

Determination	La*	P*	La/P ratio
1	12.7	13.5	0.94
2	13.5	21.4	0.63
3	15.2	15.0	1.01
4	14.1	18.3	0.77
5	15.3	15.0	1.02
6	13.4	9.3	1.44

A suspension of the precipitate from a *Citrobacter* column that had been extensively challenged with La^{3+} was prepared as described in the text. Specimen microareas were analysed for lanthanum and phosphorus by PIXE with the RBS correction to give absolute elemental concentrations. *Elemental concentrations are expressed as mol of element per L of precipitate. The mean La/P ratio was 0.97 ± 0.28.

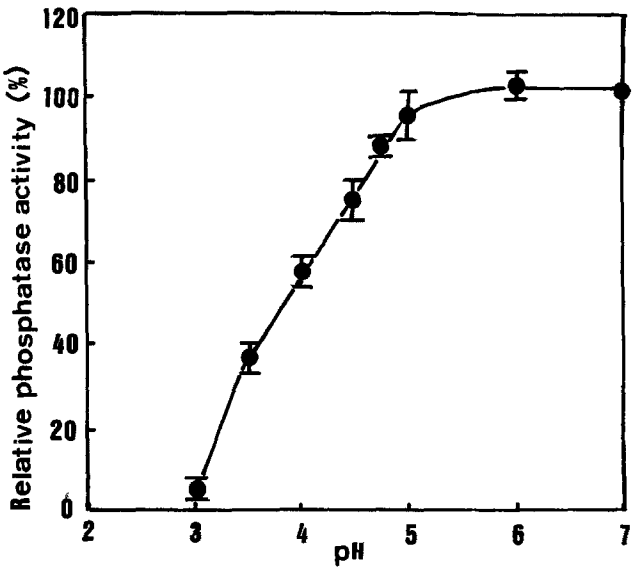

Fig. 5. pH-activity profile of whole-cell *Citrobacter* phosphatase. Batch-grown cells were harvested as described and assayed in buffer as appropriate to the pH under test (see text). Data are pooled (mean ± standard errors) from three batches of strain N14. Strain dc5c gave identical results. For each batch the specific activity at pH 7 was afforded a value of 100% and the activities at other pH values were expressed relative to this.

within the experimental error (correlation coefficient is typically >0.9). A typical flow rate-activity relationship is shown in Fig. 6; bioreactor activity can be defined in terms of the $FA_{1/2}$ value, or that flow rate giving 50% conversion of substrate to product, or 50% removal of the presented metal from the flow. Representative data are shown in Fig. 6(A), where the phosphatase specific activity of the cells before immobilization was 367 units, corresponding to a $FA_{1/2}$ value (for product released, i.e. that flow rate at which 50% of the input substrate is cleaved) of 276 ml h^{-1} for the immobilized cells. As predicted from the data of Fig. 5, immobilized cells challenged at pH 5 gave *p*-nitrophenol release identical to those challenged at pH 7 (see later). Product liberation was reduced at pH 4; for the batch illustrated in Fig. 6 this represented a $FA_{1/2}$ value of 86 ml h^{-1}, or 31% of the activity at pH 7 (Fig. 6(B)). For two other batches of immobilized cells the respective values were 40.3% and 47.9%; the mean ± standard error was calculated at 39.7 ± 4.9%, which is not significantly different from the data obtained with free cells (Fig. 5). Preliminary tests established that metal uptake by the immobilized cells was negligible at pH 4.

Selection of populations of Citrobacter *sp. growing at low pH*

The above data show that bioreactor performance and metal accumulation were reduced at pH 4 as compared to pH 7; attempts were made to improve the amount of product liberated at low pH to compensate for these effects. Initially, attempts were made to select for cells having enhanced phosphatase activity at low pH, or a pH activity profile showing a shift towards a lower pH optimum. Phosphatase production, regulated by the carbon supply, was maximal under carbon-

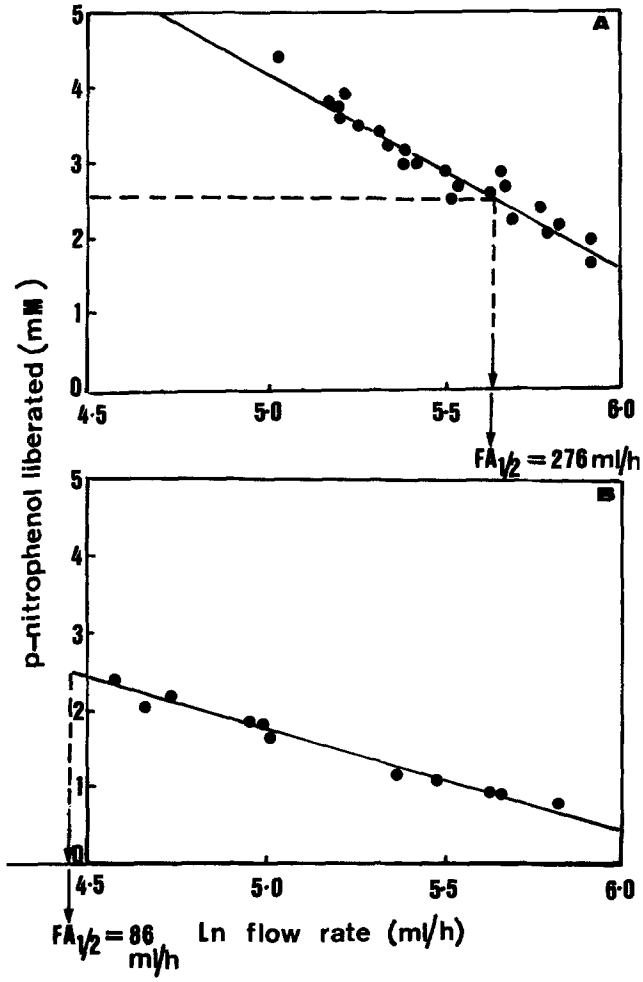

Fig. 6. *p*-Nitrophenol release by polyacrylamide gel-immobilized cells of *Citrobacter* sp. strain N14 challenged with citrate buffered flows (5 mM citrate, pH 7) containing 5 mM *p*-nitrophenyl phosphate. The $FA_{1/2}$ value is defined as that flow rate (ml h^{-1}) giving 50% conversion of substrate to product (see text). Data are pooled from several experiments using a batch of phosphatase specific activity of 367 units. (A) *p*-Nitrophenol release at pH 7. (B) *p*-Nitrophenol release at pH 4.

limited continuous culture [7,10]. Continuous cultures were run for extended periods at progressively low pH. Growth of the cells at low pH (pH 5.7) resulted in a concomitant loss of phosphatase production (Fig. 7), and no shift in the pH-activity profile. This approach was not considered further.

Use of the phosphatase overproducing mutant dc5c

Isolation of the phosphatase overproducing mutant dc5c was described previously [15]. This strain had a specific activity of 670 units with a $FA_{1/2}$ value for PAG-immobilized cells of 578 ml h^{-1} (Fig. 8(A)). The increased activity was reflected in a better bioreactor performance at pH 4; here a $FA_{1/2}$ value of 277 ml h^{-1} (Fig. 8(B)) compensated for the pH-inhibitory effect seen in Fig. 6(B), and restored product release to a level equivalent to that of non pH-stressed cells of strain N14. Strain dc5c was used in subsequent experiments.

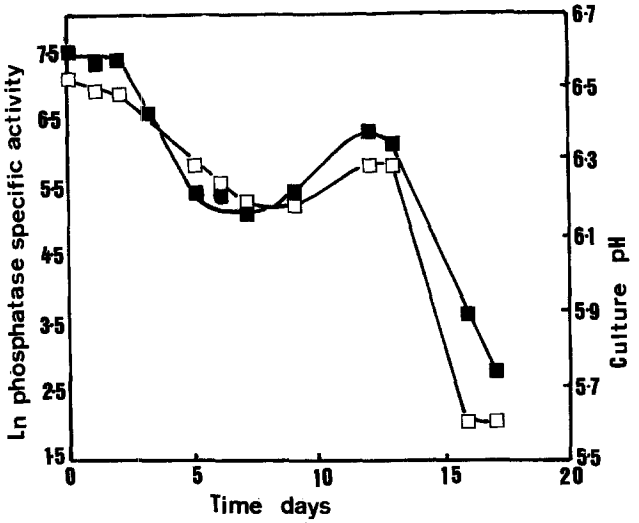

Fig. 7. Phosphatase activity of *Citrobacter* sp. grown in continuous culture at a progressively low pH. The cells were grown under carbon limitation at D = 0.26 h^{-1} as described, with the pH maintained as shown using an autotitrator. □: Culture pH. ■: Phosphatase specific activity.

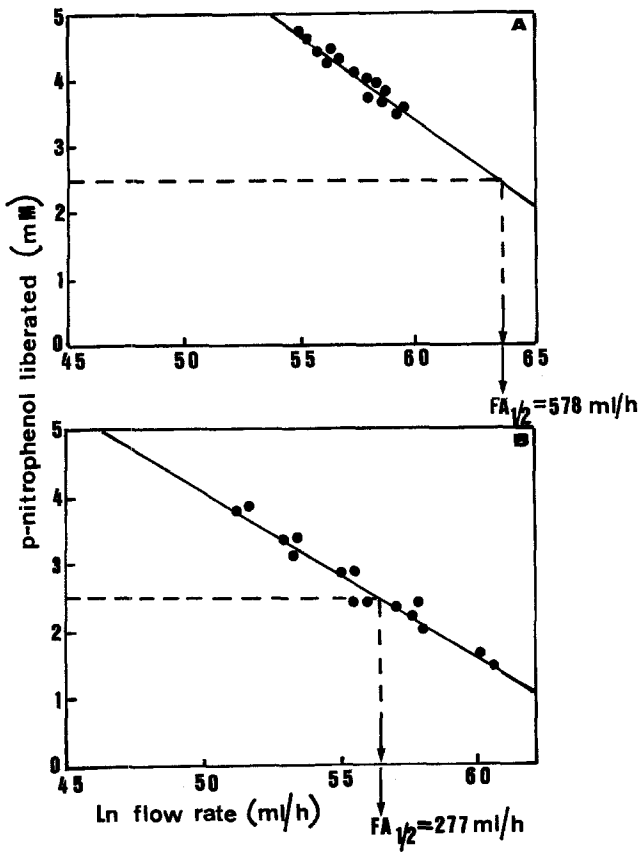

Fig. 8. *p*-Nitrophenol release by immobilized cells of strain dc5c. The phosphatase specific activity was 670 units. Legend as in legend to Fig. 6.

Product release and lanthanum removal by immobilized cells at neutral and acid pH

In spite of the increased activity of strain dc5c (above) preliminary tests showed that metal removal at pH 4 was still negligible; clearly factors other than the amount of phosphate released were influencing metal removal. These were investigated further at pH 5, at which pH *p*-nitrophenol release was identical to that at pH 7 (Fig. 9; c.f. Fig. 5). The flow rate was fixed at 300 ml h^{-1}, at which 3.6 mM *p*-nitrophenol was present in the column outflow, corresponding to 72% cleavage of the input substrate (Fig. 9). At pH 7 at this flow rate 83.5% of the input lanthanum was removed; the ratio of substrate liberated/lanthanum removed was thus 4.3:1 (the removal of La was 0.835 mmol L^{-1} from an input concentration of 1 mmol L^{-1}). A similar calculation was done using the data obtained at pH 5. Here the efficiency of La removal was 55% (Fig. 9) and the corresponding ratio of product released/lathanum removed was 6.5:1. This means that more phosphate is required to precipitate lanthanum at low pH, i.e. it is the metal precipitation reaction rather than enzyme activity *per se* which limits the efficiency of metal removal from acid solution. The experiments were repeated using glycerol 2-phosphate as the phosphate donor and substrate for phosphate release. As before, metal removal was reduced at pH 5 (Fig. 10); the $FA_{1/2}$ values for strain dc5c at pH 5 and 7 were 70 ml h^{-1} and 300 ml h^{-1}, respectively. It had been shown previously that growth of the *Citrobacter* sp. under carbon limitation, either in batch [10] or continuous culture [7,10] (see also Fig. 7) can increase the phosphatase activity to in excess of 2000 units. A culture prepared thus with a specific activity of 2268 units was tested in the flow-through system at the two pH values. As shown in Fig. 10 this increase in activity compensated to some extent for the pH-inhibitory effect; here the respective $FA_{1/2}$ values at pH 5 and 7 were

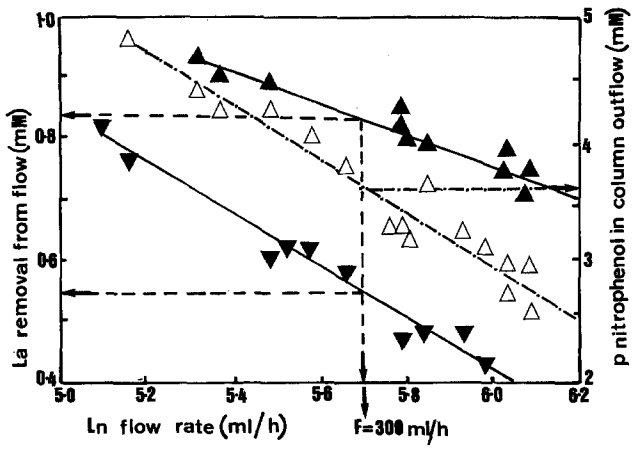

Fig. 9. *p*-Nitrophenol release and lanthanum accumulation by immobilized cells of strain dc5c (specific activity as in legend to Fig. 8) challenged at pH 5 and pH 7. The immobilized cells were challenged with citrate-buffered flow (5 mM citrate; pH 5 or pH 7) containing 5 mM *p*-nitrophenyl phosphate with or without lanthanum nitrate (1 mM). *p*-Nitrophenol release was identical at both input pH values and the data were pooled (△). ▲: La removal at pH 7. ▼: La removal at pH 5.

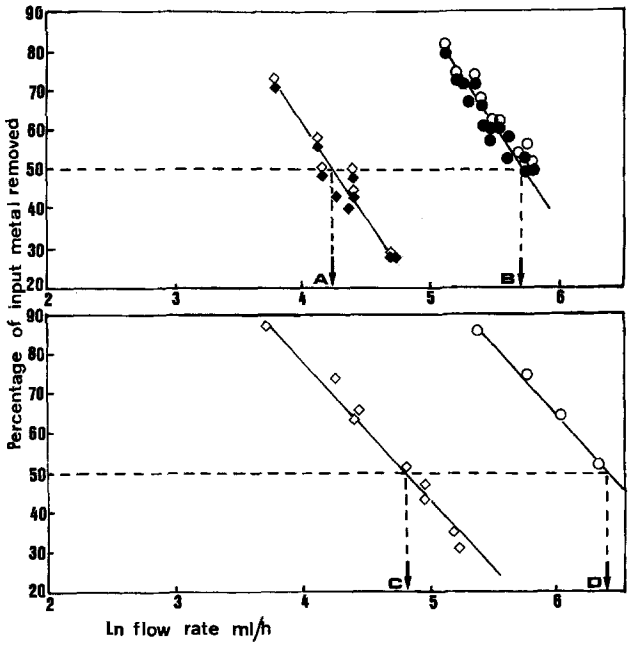

Fig. 10. Lanthanum accumulation by PAG-immobilized cells of strain dc5c (top: specific activity as in legend to Fig. 8) and strain N14 grown in carbon-limiting continuous culture to give a phosphatase specific activity of 2268 units (bottom). For high activity preparations the citrate buffering was insufficient, and MOPS buffer or MES buffer (50 mM) were incorporated as appropriate at pH 7 (●,○) or 5 (◆,◇), respectively.

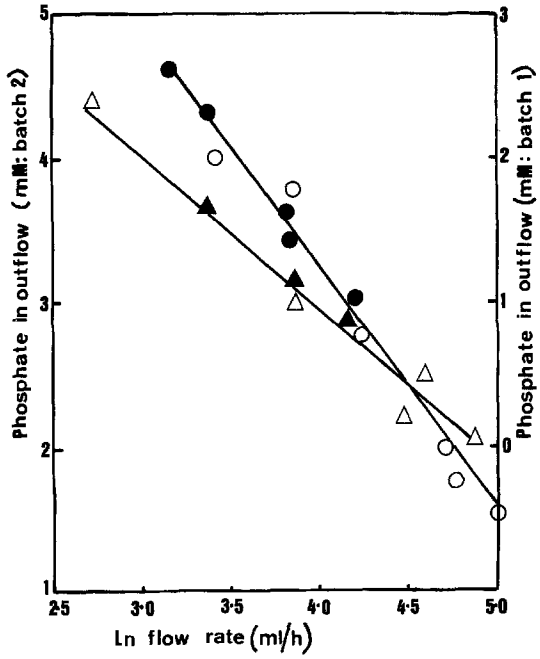

Fig. 11. Phosphate release from glycerol 2-phosphate (heavy metal-unsupplemented) flows at pH 5 and 7. Legend as in legend to Fig. 10, except that here batches of strain N14 were used. Filled and open symbols denote batches I and II; the data are superimposed for interpretation. ●,○: Phosphate release at pH 7. ▲,△: Phosphate release at pH 5.

120 ml h^{-1} and 600 ml h^{-1}, respectively. In this example an increase in phosphatase specific activity of approx. 4-fold resulted in an increase in bioreactor performance of only approx. 2-fold (cf. earlier). This is consistent with earlier findings that very high phosphatase activities are not reflected in an increased efficiency of metal removal (chemical and precipitation effects become rate-limiting [17]). However these data show that some bioreactor activity can be restored at low pH by the use of high-activity cells.

The effect of substrate on the pH-inhibitory effect

Inspection of the data of Figs 9 and 10 reveals an inconsistency. For cells of the same phosphatase specific activity the $FA_{1/2}$ values for metal removal at pH 5 are 330 ml h^{-1} and 70 ml h^{-1} as supported by the cleavage of *p*-nitrophenyl phosphate and glycerol 2-phosphate, respectively. The effect of pH on the utilization of glycerol 2-phosphate was investigated further (Fig. 11). Whereas product release from *p*-nitrophenyl phosphate was pH-independent (see above), a different flow-rate activity relationship was seen for glycerol 2-phosphate cleavage (metal-unsupplemented cells), depending on the pH. The threshold flow rate (that flow rate at which activity started to be lost) was lower at pH 5, but the flow rate-dependent loss in activity was proportionally less at pH 5 than at pH 7; product liberation was sustained better with high flow rate at the higher pH. These data can be interpreted in terms of the integrated form of the Michaelis–Menten equation that may be used to describe bioreactor activity [1,10,12]. Here,

$$t = 1/F, = \frac{S_0X + K_m \cdot \ln(1/1-X)}{E_0K_3} \quad \text{(i)}$$

where t = substrate residence time in the column (min or h), F = flow rate through the column (ml min^{-1} or ml h^{-1}), S_0 = initial substrate concentration, X = fraction of the substrate converted at a given flow rate, K_m = the Michaelis constant of the enzyme as operating in the immobilized cells (i.e. the apparent K_m: $K_m = K_{m\,app}$), K_3 = an intrinsic kinetic constant of the enzyme and E_0 = the enzyme concentration. The actual E_0 is not known; this is related to the phosphatase activity per cell, the total number of cells per bioreactor and the substrate diffusional threshold into the gel (which determines the number of substrate-interactive cells). Rearranging, and taking logs of both sides:

$$\ln F = \ln(E_0 \cdot K_3 - \ln[S_0 \cdot X + K_m \cdot \ln(1/1-X)] \quad \text{(ii)}$$

$$= \ln(E_0 \cdot K_3) - \ln[S_0 \cdot X - K_m \cdot \ln(1 - X)] \quad \text{(iii)}$$

which relates ln (flow rate) to the fraction of substrate converted. Inspection of Eqn (iii) shows that the gradient is not dependent upon E_0. Instead, varying E_0 has the effect of 'shifting' the curve along the X axis; this is shown experimentally by the use of strains of different phosphatase specific activity (Figs 6 and 8). K_m, however, affects the gradient of the $\ln F/X$ relationship. Thus it can be predicted that the K_m for *p*-nitrophenyl phosphate should be largely pH-independent, while that for glycerol 2-phosphate may depend on the pH of the solution. This prediction was tested by determination of the $K_{m\,app}$

value for the two substrates at pH 5 and 7 (Table 2). It was found that at pH 7 the $K_{m\,app}$ for *p*-nitrophenyl phosphate was approximately half of that observed for glycerol 2-phosphate, i.e. the latter is intrinsically a less good substrate for efficient phosphate release at limiting flow rates. Importantly, while the $K_{m\,app}$ for *p*-nitrophenyl phosphate was approximately doubled by a drop in pH from 7 to 5, the $K_{m\,app}$ for glycerol 2-phosphate was increased approximately four-fold. This means that the affinity of the enzyme for glycerol 2-phosphate would be markedly reduced at the lower pH value.

These experiments were done in metal-unsupplemented solutions. The presence of the metal might introduce another complicating factor if the degree of metal binding to the substrate was not the same for both substrates. Incorporation of citrate would have been expected to have held the metal in the metal citrate complex, since the complexing ability of citrate is very high. However, nothing is known of the binding constants of lanthanum to *p*-nitrophenyl phosphate and glycerol 2-phosphate (i.e. to what extent the free substrate concentration is metal-dependent); this may mean that in addition to the simple pH effect on the $K_{m\,app}$, the amount of free substrate available for interaction with the enzyme could be reduced in the presence of metal. These compounded effects could well explain the discrepancy seen between metal uptake supported by the two substrates at the two pH values. Further studies to develop a working model are clearly required in order to predict the anticipated pH effects in a given metal and substrate system. The use of *p*-nitrophenyl phosphate or glycerol 2-phosphate as substrates for industrial-scale metal waste decontamination would be uneconomic and efforts are currently in progress to find, and evaluate, more appropriate phosphatase substrates. The present study illustrates the importance of obtaining appropriate kinetic data under the anticipated flow conditions.

This work defines the lowest operating pH of the *Citrobacter* system as above 4.0. Many industrial wastes are produced at pH values below this, and neutralization would be required. It should be stressed that the limitation at pH 4–5 is not that of enzyme activity *per se*, but is attributable to the solution chemistry and desolubilization of the metal phosphate at low pH. Similar problems would be encountered in chemical precipitation processes which would also lack the nucleation foci which the bacterial cells provide.

TABLE 2

Apparent K_m($K_{m\,app}$) for *p*-nitrophenyl phosphate and glycerol 2-phosphate at pH 5 and 7

Substrate	$K_{m\,app}$ at pH 7	$K_{m\,app}$ at pH 5
p-Nitrophenyl phosphate	1.36, 1.10	2.93, 2.50
Glycerol 2-phosphate	2.22 ± 0.15 (3)	8.53 ± 0.23 (3)

PAG-immobilized cells (see Materials and Methods) were suspended in batch medium supplemented with substrate at varying concentrations. $K_{m\,app}$ values were calculated from the initial rates of product release using a Lineweaver–Burk double reciprocal plot. Data for product release from glycerol 2-phosphate are means ± standard errors from three batches of cells. For *p*-nitrophenyl phosphate two batches were used; both results are shown.

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