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SOLUBILIZATION OF ROCK PHOSPHATE BY IMMOBILIZED *ASPERGILLUS NIGER*

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Abstract

Aspergillus niger, *an acid-producing filamentous fungus, was immobilized on polyurethane foam. Various amounts of foam cubes and spore suspension were tested in order to obtain an efficient immobilization process. The best combination selected for further experiments was 0.2 g polyurethane foam and 3 ml spore suspension. Immobilized cells were reused, with higher levels of acid formation being maintained for longer periods (at least 240 h) than for free cells. The highest titratable acidity, of about 315 mmole/l, was reached with 0.5 cm foam cubes after 3 × 48 h batch cultures in a laboratory shake-flask experiment. Rock phosphate (2.5 g/l and 5.0 g/l) solubilization was carried out in repeated 48 h batch fermentation using* A. niger *immobilized on polyurethane foam cubes. The greatest accumulation of soluble P, approximately 360 μg/ml, was obtained after the second batch in flasks with 5 g/l rock phosphate, but process efficiency was higher at lower concentration of the rock phosphate. After 10 days of culture a total production of 1.2–1.6 mg/ml soluble P was obtained depending on the concentration of rock phosphate in the medium.* © *1997 Elsevier Science Ltd. All rights reserved.*

Key words: Aspergillus niger, immobilized cells, rock phosphate, solubilization, repeated batch fermentations.

INTRODUCTION

Phosphorus plays a vital role in plant nutrition (Hayman, 1975) but its concentration in soil solution is only 0.05 mg/l. For this reason, the possibility of practical use of rock phosphate as a fertilizer has received significant interest in recent years. Unfortunately, rock phosphate is not plant-available in soils with pH greater than 5.5–6.0 and, even when the conditions are optimal, plant yields are, as a rule, lower than those obtained with soluble phosphate (Khasawneh & Doll, 1978). The conventional method for enhancing the rock phosphate availability is to increase its solubility by treating with inorganic acids, mainly sulphuric acid and phosphoric acid; but, from the industrial point of view, this approach is not applicable because of high capital costs (Hammond *et al.*, 1989). One very attractive approach for rock phosphate solubilization is the application of microorganisms able to excrete organic acids. It has been repeatedly shown that low-molecular-weight organic acids can strongly increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Earl *et al.*, 1979; Fox & Comerford, 1990; Gerke, 1992).

Filamentous fungi are widely used as producers of organic acids (Mattey, 1992) and, particularly, *Aspergillus niger* and some *Penicillium* species have been tested in fermentation systems or inoculated directly into soil in order to solubilize rock phosphate (Kucey, 1987; Asea & Kucey, 1988; Cerezine *et al.*, 1988; Cunningham & Kuiack, 1992; Vassilev *et al.*, 1995, 1996a). However, until recently, no reports of solubilization of rock phosphate by a microorganism in an immobilized state had been made, although the advantages of immobilized systems in fermentations are well known (Vassilev & Vassileva, 1992).

Recently we have reported the successful solubilization of inorganic phosphate by immobilized *P. variabile* P16 (Vassilev *et al.*, 1996b). The purpose of the present work was to obtain an efficient immobilization of *A. niger* and to verify the potential of further application of this system in solubilization of rock phosphate.

METHODS

Microorganism and fermentation media

The microorganism used was *Aspergillus niger* 11 (Collection of the Institute of Microbiology-Sofia), maintained on potato-dextrose-agar slants (PDA Oxoid) at 4°C by subculturing monthly. The growth medium, used for the immobilization procedure, contained (g per litre): glucose (100), NH_4NO_3 (1.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.2),

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$ZnSO_4.7H_2O$ (0.007), corn steep liquor (1.0). The production medium, used for the repeated-batch fermentations, consisted of (g per litre): glucose (100), NH_4NO_3 (0.5), $MgSO_4.7H_2O$ (0.2), $ZnSO_4.7H_2O$ (0.004). Sedimentary rock phosphate (12.8% P) was added to the production medium at concentrations of 2.5 and 5.0 g/l when necessary. The pH was adjusted to 5.0 with HCl.

Immobilization technique

The polyurethane foam (PUF) cubes (1.0 and 0.5 cm^3) used for the immobilization of *A. niger* had an average pore size of 0.6–0.8 mm. Prewashed foam material (2.0 g/l) was submerged in 100 ml growth medium in 250-ml Erlenmeyer flasks and autoclaved for 20 min at 121°C for sterilization. The carrier cubes, inoculated with 5-day-old *A. niger* spores (1.2×10^7), were cultivated for 24 h on a rotary shaker at 220 rpm. The immobilized mycelium was then separated from the liquid, washed with sterile distilled water and transferred into 100 ml fresh production medium in 250-ml Erlenmeyer flasks. The fermentation liquid was centrifuged (1600 g/10 min) and was further used for analysis after each batch cycle. Production medium was changed following the same procedure every 48 h.

Free mycelium cultivation

This was carried out in shaken 250-ml Erlenmeyer flasks with 100 ml growth medium, inoculated with 5-day-old *A. niger* spores. After 24 h, the mycelium was centrifuged (3000 *g*, 5 min), washed with sterile distilled water and transferred to 100 ml fresh production medium, which was changed every 48 h during the repeated-batch process following the same procedure. At the end of each batch cycle, the supernatant, obtained after centrifugation of the mycelium, was used further for analysis.

Fermentation

This was carried out with either free or immobilized cells of *A. niger* in shaken flasks (in triplicate) under agitation of 220 rpm at 30°C and pH 5.0.

Analytical methods

The dry weight of immobilized cells was determined by subtraction of an average predetermined dry weight of foam cubes from the weight of foam cubes plus mycelium after washing with distilled water and drying overnight at 105°C. pH was measured by glass electrode and titratable acidity—by titration of samples to pH 7.0 with 0.1 M NaOH. Dissolved phosphorus concentration in the fermentation liquid was determined by the molybdo-vanado-method described by Lachica *et al.* (1973).

RESULTS AND DISCUSSION

Aspergillus niger immobilization on PUF depended in this study upon both inoculum size and amount of PUF cubes (Table 1). An improvement of the biomass concentration in the foam cubes was achieved as the amount of the foam was decreased and this effect was more pronounced with increasing inoculum size. It was also observed that in flasks with the highest amount of both PUF and spore suspension mycelium and carrier were embedded in a whole mass. Free mycelium was detected in flasks with a lower amount of PUF and a higher inoculum size. Thus, the selected combination for further experiments was 0.2 g PUF and 3 ml of spore suspension.

Microscopic examinations showed that the filamentous growth favoured cell immobilization in such an open porous carrier, allowing strong biomass retention, although, as reported earlier, the characteristic filamentous structure was retained only by single filaments of surface-growing mycelium (Vassilev *et al.*, 1993).

Aspergillus niger immobilized on PUF was able to acidify the fermentation medium in a repeated-batch process (Table 2). However, the titratable acidity achieved by immobilized 0.5 cm cell-cubes was 34.6% higher than by larger cubes. This might have been due to an increase in the outer surface of immobilized cubes, which facilitated mass transfer. Bearing this in mind, and the observation of Robins *et al.* (1986) that in fully-loaded foam cubes only the outer 1.5 mm of the immobilized cube is well-oxygenated, we could explain the higher acidity achieved by smaller cubes in such a highly aerobic process. The registered total value of medium acidification by the smaller cube-cell system after five batches was 54% higher than that achieved by freely-suspended mycelium.

Table 1. Effect of inoculum size on dry weight of biomass immobilized on various amounts of 1 cm polyurethane foam (PUF) cubes after 24-h shake flask cultivation in glucose-based growth medium without rock phosphate

PUF (g)	Immobilized biomass (g per flask/g per g carrier)				
	Inoculum size (ml spore suspension)				
	1	2	3	4	5
0.2	0.6/3.0	0.8/4.0	1.1/5.5	1.2/6.0	1.1/5.5
0.5	0.9/1.8	1.3/2.6	1.5/3.0	1.6/3.2	1.7/3.4
0.8	0.5/0.6	0.6/0.8	0.6/0.8	0.7/0.9	0.7/0.9

Results are the average of three replicates and the standard errors were smaller than 5%.

Table 2. Medium titratable acidity values during repeated 48 h batch cultivation of free (FC) and immobilized (IC) *A. niger* in glucose-based production medium without rock phosphate

Culture	Titratable acidity (mmole/l)				
	Number of batch cultures				
	1	2	3	4	5
IC (1.0 cm^3)[a]	170±5.4	195±6.1	228±5.9	210±7.3	215±6.8
IC (0.5 cm^3)[a]	210±8.0	290±9.6	280±8.8	315±9.8	275±7.3
FC	250±7.0	270±5.8	165±3.2	106±2.5	100±2.8

[a]PUF Size.

In order to verify the possible application of immobilized PUF–*A. niger* cubes in solubilization of rock phosphate, a separate experiment was carried out using a medium supplemented with 2.5 and 5.0 g/l rock phosphate (Table 3). The results clearly demonstrate that immobilized fungus was able to overcome the neutralizing effect of rock phosphate. The greatest accumulation of soluble P, approximately 264 μg/ml and 360 μg/ml, was achieved after the second batch in flasks with 2.5 g/l, and after the third batch in 5.0 g/l-rock phosphate-flasks, which corresponded to 82.5% and 56.25% of the total amounts of P present in the rock phosphate, respectively. Despite the higher amount of soluble P (28.6%) obtained in the process with 5.0 g/l rock phosphate as compared with the experiment with 2.5 g/l rock phosphate, the efficiency was lower in the first case because the percent of solubilized P decreased in relation to total rock phosphate added. It is interesting also to compare the average diameter (*d*) of PUF cell-cubes and the immobilized dry biomass (IDB), from flasks with different amounts of rock phosphate (*d* in cm/g IDB per flask). In a medium with high concentration of rock phosphate this parameter was 1.5/2.8, compared to 1.3/2.2 and 0.9/1.9 in the cases with lower concentration of rock phosphate and rock-free medium, respectively. This increase of immobilized biomass was most likely a result of utilization of the available phosphate by the fungus. On the other hand, the direct effect of the soluble phosphorus on the fungal metabolic behaviour should be mentioned. Phosphorus is known to be essential for the growth and metabolism of *A. niger*: low levels of phosphorus positively affected citric acid production. Conversely, the presence of more than 0.07 g PO_4^-/l in a submerged fermentation process with freely-suspended cells was reported to reduce acid formation and to stimulate the mycelium growth (Martin & Steel, 1955). Bearing this in mind, we could explain the differences between rock phosphate-free and rock phosphate-supplemented experiments in relation to the growth and titratable acidity, which in turn affected the solubilization activity of the immobilized fungus. This effect was even more pronounced on increasing the concentration of the rock phosphate.

Table 3. Medium titratable acidity values and rock phosphate solubilization during repeated 48 h batch cultivation of immobilized *A. niger* in glucose-based production medium with rock phosphate

No. of batches	Titratable acidity/soluble phosphorus (mmole/l) (μg/ml)	
	Rock phosphate concentration (g)	
	2.5	5.0
1	71±1.9/210±5.7	77±1.2/290±7.6
2	85±2.6/264±6.6	73±1.4/310±8.0
3	81±2.1/256±6.8	72±1.1/360±9.7
4	80±2.2/248±6.0	71±1.1/320±6.5
5	75±0.8/260±8.4	70±2.0/312±8.9

There are several reports on rock phosphate solubilization by filamentous fungi but to our knowledge this is the first attempt to use immobilized *A. niger*. The magnitude of the results reported here could be appreciated by the fact that using immobilized cells the total amount of soluble phosphate reached 1238–1592 μg/ml in a 10-day repeated batch fermentation, while with free-fungal cultures the maximal concentration was obtained on average for the same period of time, but was about 4–5 times lower. This result could be explained by the fact that the fungus in the foam cubes did not exhibit any lag phase during the repeated-batch process. It was also evident that the transport of nutrients to the fungal mycelium widely distributed in the foam was much better than to that inside a mycelial pellet. Another advantageous characteristic of the immobilized fungal culture was the enhancement of the stability and catalytic longevity. Despite some decrease in the solubilizing activity at the end of the described process (only in the 5.0 g/l rock phosphate treatment), it was proved possible to maintain the immobilized systems for eight repeated-batch cycles, when the amount of soluble phosphorus fell to half of the registered maximum (data are not shown). In a previous study, we have observed similar high solubilizing activity of immobilized *P. variabile* but the maximum level of soluble phosphorus of 340 μg/ml (19% of the total rock phosphorus) was obtained in a medium supplemented with 14 g rock phosphate/l (Vassilev *et al.*, 1996b).

Immobilized microbial systems could be used for better understanding of the mechanisms of solubilization of insoluble phosphates. Gel-encapsulated preparations could be introduced into soils in order

to improve phosphorus plant nutrition, which is the aim of our further experiments. Thus, it can be concluded that a culture technique with immobilization is more suitable for solubilization of rock phosphate by *A. niger* than other microbially-based techniques described so far. However, a culture medium optimization, evaluation of other inexpensive carbohydrate sources and application of continuously-operating column bioreactors is needed to allow a further scale up of immobilized cell solubilization of rock phosphate.

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