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N. Vassilev · M. T. Baca · M. Vassileva
I. Franco · R. Azcon

Rock phosphate solubilization by *Aspergillus niger* grown on sugar-beet waste medium

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Abstract Solubilization of rock phosphate by *Aspergillus niger* was studied in solid-state fermentation on sugar-beet waste. This combination was selected after testing three agroindustrial waste materials, namely rice hulls, sugar-beet waste and alperujo. Sugar-beet waste was the best substrate for fungal growth with 69% mineralization, followed by rice hulls and alperujo. The fungus was successfully cultivated on sugar-beet waste supplemented with 3.0 g/l rock phosphate, acidifying the medium and thus decreasing the pH to 3–3.5. Solubilization of insoluble phosphate increased during the first half of the process, reaching a maximum of 292 μg phosphate/ml, although a part of it was probably consumed by the mycelium.

Introduction

Phosphorus plays a vital role in plant nutrition (Hayman 1975) but its concentration in soil solution is approximately 0.05 mg/l (Ozanne 1980). For this reason, the possibility of using rock phosphate as a fertilizer has received significant interest in recent years. Unfortunately, rock phosphate is not available to plants in soils with pH greater than 5.5–6 and, even when conditions are optimal, yields are as a rule lower than those obtained with soluble phosphate (Khasawneh and Doll 1978). 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-mass organic acids can strongly increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Earl et al. 1979; Fox and Comerford 1990; Gerke 1992).

Filamentous fungi are widely used as producers of organic acids (Mattey 1992; Vassilev and Vassileva 1992) and particularly *Aspergillus niger* and some *Penicilium* species have been investigated in fermentation systems or inoculated directly into soil in order to solubilize rock phosphate (Kucey 1987; Asea et al. 1988; Cerezine et al. 1988; Cunningham and Kuiack 1992). Using vinasse (a residue from alcohol production) as a substrate in fungal fermentation it proved possible to solubilize 79% of the total phosphorus content of fluorapatite (Nahas et al. 1990). However, there are no other studies on the possible fermentation of agroindustrial wastes by *A. niger* in order to solubilize rock phosphate.

The objective of this study was to select the best fungus-substrate combination for further application in rock phosphate solubilization as a part of a large project for development of an efficient process for promoting plant growth.

Materials and methods

Microorganism

The strain of *Aspergillus niger* NB2 used throughout this study, selected from 20 acid-producing cultures of *Aspergillus* (Vassilev and Ganchev 1986) and further proved to produce only citric acid on complex substrates (Vassilev et al. 1986), was maintained on potato/dextrose/agar slants.

Culture media and fermentation conditions

Three agroindustrial lignocellulosic wastes, namely rice hulls, sugar-beet waste and alperujo (a solid waste obtained after olive-oil extraction processes), all ground in an electrical grinder to 1-mm fragments, were used at concentrations of 10% and 20% as substrates for static fermentation in 50 ml Czapek's solution (described in Fluka Chemica, catalogue no. 70185). The characteristics of

N. Vassilev (✉) · M. T. Baca · M. Vassileva
I. Franco · R. Azcon
Estacion Experimental del Zaidin, CSIC, Prof. Albareda 1,
E-18008-Granada, Spain.
Fax: + 58 129600

Table 1 Characteristics of rice hulls (*RH*), sugar-beet waste (*SB*) and alperujo (*ALP*). Values are means from three experiments. C_t total carbon, C_o oxidizable carbon, N_t total nitrogen

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	C_t (%)	C_o (%)	N_t (%)
RH	37	20	18	48	13	0.8
SB	29	23	5	55	16	1.7
ALP	24	8	30	54	5	1.7

lignocellulosic wastes are presented in Table 1. After sterilization at 120°C for 30 min, experiments were carried out in 250 ml conical flasks (in triplicate) inoculated with 1.2×10^7 spores/flask. Rock phosphate (flourapatite from Morocco with 12.8% P) at a concentration of 3.0 g/l was added when necessary. Experiments were performed at 30°C for 20 days with periodical shaking for 5 min/day.

Analytical methods

Mycelial growth was determined by weighing the mycelium, which was carefully separated from the fermentation medium, washed, and dried in an oven at 100°C. Medium pH was measured with a glass electrode and titratable acidity was determined by titrating each sample to pH 7.0 with 0.1 M NaOH. The weight loss of lignocellulose during the fermentation process was calculated on the basis of ash content according to Kumar and Sign (1990) and presented as a percentage of mineralization. Lignin, cellulose and hemicellulose contents were measured according to the method of Goering and Van Soest (1970). Oxidizable carbon was measured by a wet oxidation method, total carbon was determined from the ash by the Van Bemelin factor, and total nitrogen was estimated by the Kjeldal method (AOAC 1980). The citric acid content was determined by the microcolorimetric method of Taussky (1949). The residual sugar was analysed according to Miller (1959). Phosphorus content was determined by the molybdovanado method described by Lachica et al. (1973).

Results

The results of a preliminary study, involving the comparison of three lignocellulosic substrates, rice hulls, sugar-beet waste and alperujo, for fungal growth, titratable acidity and level of mineralization, are presented in Table 2.

In the present study *A. niger* grew well on all tested materials but the filamentous fungus showed different levels of growth depending on the type of lignocellulosic substrate and its concentration. Sugar-beet waste at concentrations of 10% and 20% appeared to be the best material, which provided a mycelial growth of 1.0 g/fl and 1.24 g/fl respectively, followed by alperujo and rice hulls. In all cases, it seemed that the 20% concentration of substrates provided better conditions for fungal growth than did 10%. Again, the amount of mycelial mass grown per day increased as the concentration of each substrate increased. It should be noted that the amount of biomass observed on all types of substrate was high at the end of the period studied, which could be attributed to the unique conditions provided by the microorganism/air/water interface during this kind of fermentation (Oriol et al. 1988). On the other hand, the growth and activity of *A. niger* were influenced by the composition and degree of complexity of substrates. For this reason, the percentage of mineralization was lower than 39% and 21% for rice hulls and alperujo respectively and higher than 56% for sugar-beet waste. The lowest amounts of nitrogen (0.8%) in rice hulls and oxidizable carbon (5%) in alperujo among the all tested substrates should be noted also when assessing their biodegradability.

The initial pH value of 6.5–7.0 significantly decreased to about 3 after 1 week of fermentation in flasks with rice hulls and sugar-beet waste, and to 3.5–4.0 when the substrate was alperujo, but thereafter increased slightly. The final titratable acidity detectable in the media was significantly higher (four to five times) when the substrate was sugar-beet waste than when it was rice hulls or alperujo. The maximum level of mineralization, about 69%, was achieved by *A. niger* on the 10% concentration of sugar-beet waste, which correlated with the results of a more detailed analysis of its composition, although a part of this material was degraded during the sterilization (Table 3). A definite difference was found in reducing-sugar content in the liquid phase of the sugar-beet waste medium with and without *A. niger*, ranging from 5.2 g/l and 7.2 g/l to 0.25 g/l and 0.53 g/l at 10% and 20% substrate respectively.

Bearing in mind the above results, a separate experiment was carried out with 10% sugar-beet waste supplemented with 3 g/l rock phosphate which, analysed the fungal growth, titratable acidity and soluble

Table 2 Growth, titratable acidity and mineralization after 20-day cultivation of *Aspergillus niger* on rice hulls (*RH*), sugar-beet waste (*SB*) and alperujo (*ALP*). Values are means from three experiments

Substrate/conc. (%)	Biomass (g/fl)	Yield (g/day)	pH first week/final	Titratable acidity (mmol/l)	Mineralization (%)
RH/10	0.63	0.032	3.2/4.0	11.1	38
RH/20	0.70	0.035	3.0/4.5	9.3	20
SB/10	1.00	0.051	2.7/3.0	53.0	69
SB/20	1.24	0.062	2.9/3.5	42.0	56
ALP/10	0.78	0.039	3.5/4.0	10.7	20
ALP/20	1.07	0.053	4.0/5.0	7.3	17

Table 3 Composition of sugar-beet waste suspension with and without growth of *Aspergillus niger* after a 20-day treatment. Values are ± standard deviation for three replicate cultures

Substrate/conc. (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reducing sugar (g/l)
With *A. niger*				
10	11.3 ± 0.8	3.1 ± 0.1	4.1 ± 0.8	0.25 ± 0.07
20	17.0 ± 0.6	4.2 ± 0.3	4.2 ± 0.3	0.53 ± 0.04
Without *A. niger*				
10	22.0 ± 1.2	9.1 ± 0.5	5.2 ± 0.2	5.21 ± 0.70
20	24.9 ± 1.4	13.6 ± 0.8	5.7 ± 0.1	7.15 ± 0.48

Table 4 Mycelial growth, titratable acidity and rock phosphate solubilization by *Aspergillus niger* cultivated on sugar-beet waste. Values are mean ± standard deviation for three replicate cultures

Time (days)	Biomass (g/fl)	Titratable acidity (mmol/l)	Citric acid (% of total acidity)	Phosphate conc. (μg/ml)	Soluble P/ total P (%)
3	0.57 ± 0.12	38.2 ± 1.4	100	47.4 ± 1.6	12
6	0.68 ± 0.08	60.6 ± 0.7	98	172.6 ± 0.8	44
10	0.77 ± 0.05	72.2 ± 0.6	92	292.2 ± 2.3	76
15	0.89 ± 0.05	58.8 ± 1.1	87	276.8 ± 3.1	71
20	1.20 ± 0.10	48.1 ± 0.2	71	224.0 ± 0.9	58

phosphate (Table 4). The data indicated a rapid mycelial growth at the beginning of the fermentation followed by a slow growth phase. The growth was higher at the beginning, with an average rate of 0.19 g/fl/day, compared to that up to day 15, when the average growth rate was about 0.03 g/fl/day. It increased again thereafter and the total mass of mycelium produced on the medium supplemented with rock phosphate was higher with 20% substrate than that on the medium without rock phosphate. Mycelial growth and titratable acidity production were parallel in the first half of the process on sugar-beet waste medium with rock phosphate. An increase of the titratable acidity to 72 mmol/l was observed during this period, which resulted in a solubilization of 76% of the insoluble phosphate. However, the fungus started to sporulate after this period, which was a sign of adverse conditions for acid productivity, and this caused a slow decrease in titratable acidity, which reached a level of 48 mmol/l at the end of the experiment. It is interesting to note that the amount of citric acid released by the mycelium accounted for 100% of the titratable acidity during the first days of the process, but this started to decrease after day 6 and reached a final concentration that corresponded to 71% of the titratable acidity.

Discussion

Biomass growth on the substrates tested in this study was higher in comparison with that in liquid culture conditions using the same strain of *A. niger* (Vassilev et al. 1986). Similar results have been reported by other authors applying sugar-cane bagasse (Lakshminarayana et al. 1988; Oriol et al. 1988) and vinasse (Nahas et al. 1990). The most likely explanation of *A. niger* growth on the rice hulls, sugar-beet waste and alperujo is the presence of sufficient amounts of nutrients in the initial period of the fermentation and some lignocellulitic activity, bearing in mind the ability of *A. niger* to degrade such materials (Czajkowska et al. 1988; Gomes et al. 1989). This was demonstrated particularly in the experiments with sugar-beet waste, where more easily available sugars can be consumed by *A. niger* during the first days of the fermentation without the need for lignocellulose to be broken down enzymatically (Table 3). However, the total percentage of substrate mineralization suggested some fungal activity in this direction, although probably at a low level of exoenzymes because of the low pH. The higher biomass growth on the 20% concentration of all substrates could be explained by the higher quantity of fermentable substances in comparison with cases when 10% substrate concentration was applied (Table 2). On the other hand, the insoluble and crystalline nature of cellulose, associated with a higher content of lignin in the experiments with rice hulls and alperujo (Table 1), decreased the available surface area, which led in turn to a mycelial growth lower than that on sugar-beet waste. Especially in the case of rice hull fermentation, the lower content of oxidizable carbon in comparison with that of sugar beet might cause additional problems as well as the presence of polyphenolic compounds in alperujo (Juven and Henis 1970).

Among the three lignocellulosic materials tested in this work, sugar-beet waste proved to be the best substrate for growth and acid production by *A. niger*. Although the process was directed towards biomass growth, the level of acidity obtained by *A. niger* on sugar-beet waste was sufficient to overcome the neutralizing effect of the rock phosphate (Table 4). The results also showed that the process of solubilization

increased after the active growth phase. However, as the amount of fungal biomass continued to increase slowly, the phosphate determined in the solution probably corresponded to that which was not consumed by the mycelium. This speculation should not be surprising bearing in mind that, when trace metals are not limiting, the additional phosphate results in prolongation of mycelial growth and changes in the fungal metabolism (Martin and Steel 1955; Khan et al. 1970). It was evident that the presence of rock phosphate added directly to the fermentation medium affected the behaviour of *A. niger*, particularly its growth and citric acid production. The latter accounted for about 2/3 of the titratable acidity at the end of the process and further work should be performed to study the changes in fungal metabolism and the nature of acidic metabolites other than the main acid released by the mycelium. The decrease in both titratable acidity (about 25 mmol/l) and phosphorus concentration in the solution (about 70 μg/ml at the end of the process) was most likely a result of citric acid utilization by the fungus under conditions of nutrient depletion, which also caused a sporulation of the fungal culture.

Although a high percentage of mineralization of sugar-beet waste was achieved, a careful assessment should be done in order to find a better compromise between the level of degradation and the concentration of soluble phosphate before the resulting system is applied in soil. The type of application of *A. niger* is also significant – inoculation directly into a soil/sugar-beet waste/rock phosphate mixture in order to ensure a sequential release of soluble phosphate, or following preliminary solubilization by a fermentation process, as described in this work.

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