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Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes

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Abstract

Dry olive waste (DOW), an agro-industrial material derived from the two-phase-manufacture of olive oil, was used as a substrate for phytase production by *Aspergillus niger*. A series of experiments were performed in conditions of solid-state fermentation in the presence of rock phosphate. Both enzyme production and phosphate solubilization depended on water medium content, type of nitrogen source, inoculum size and the presence and initial concentration of phosphate in the medium. It was found that at optimized process conditions (moisture 70%; corn steep liquor as a nitrogen source; inoculum size of 3–4 ml; presence of slow release phosphate), the filamentous fungal culture was able to produce 58 U phytase/g dry substrate and 31 mg soluble phosphate per flask.

Keywords: *Aspergillus niger*; Agro-waste; Phytase; Phosphate solubilization; Fermentation

1. Introduction

The recycling of agro-industrial residues to replenish soils with nutrients, otherwise provided by chemical fertilizers, is an important part of sustainable agriculture (Tengerby and Szakacs, 1998; Saber, 2001). Organic matter affects positively soil structure and water holding capacity thus reducing the process of anaerobiosis and stimulating soil microbial activity (Swift, 2001). These effects, in turn, result in higher soil fertility and resistance against phytopathogens. It is also known that microbially treated organic matter is a stable product which introduced into soil ensures a long-term accumulation of organic matter derivates (Thermorshuizen et al., 2004).

Agro-industrial wastes that are abundant at the local level can be treated microbiologically and converted to value-added final products. Particularly in the Mediterranean region, residues and by-products derived from the olive oil production industry are abundant sources of organic matter with high fertilizing value (Alburquerque et al., 2004). However, as the content of phosphate (P) in olive mill residues is low, enrichment with soluble P is needed before application in soil–plants systems. Bearing in mind that the use of soluble P fertilizers is not recommended in organic agriculture, microbially processed rock phosphate (RP) could be a valuable supplement to olive mill residues. Fermentation processes aimed at transformation of agro-industrial residues have recently been reported as an efficient tool

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for solubilization of insoluble inorganic P (Vassilev and Vassileva, 2003). Olive mill wastewaters and dry olive cake obtained from the three-phase production scheme have been used as substrates for solid and submerged fermentation processes with free and immobilized cells of P-solubilizing microorganisms (Vassilev et al., 1997, 1998, 2006; Cereti et al., 2004). However, the ability of these microorganisms to produce phytase and, potentially, solubilize organic P sources has not been tested. On the other hand, to save water and energy resources and avoid wastewater generation, a relatively novel, two-phase system is now widely used in Spain. The by-product derived from this eco-technology, called alpeorujo, is normally subjected to additional extraction thus forming extracted dry olive wastes (DOW).

In this study, experiments were carried out to assess the feasibility of using DOW in solid-state fermentations with *Aspergillus niger* and examine fungal phytase production in the presence and absence of RP.

2. Materials and methods

2.1. Microorganism

A. niger NB2 used throughout this study, was maintained on potato-dextrose agar, stored at 4 °C and renewed every 3 months. This filamentous fungus was isolated in our laboratory and previously proved to grow and produce citric acid on natural complex substrates (Vassilev et al., 1986).

2.2. Culture media and fermentation conditions

Czapek-Dox liquid medium was used for inoculum production. The sterilized (121 °C/30 min) medium was inoculated with 1 ml spore (2.3×10^6 spores) suspension. Biomass for inoculum preparation was achieved by shake-flask cultivation of *A. niger* at 250 rpm and 30 °C for 70 h.

Experiments for phytase production/RP solubilization were conducted in 250 ml Erlenmeyer flasks. Fermentation medium contained 15 g of DOW/flask, obtained from a local (Granada province, Spain) olive oil production fabric, which was supplemented with medium strength Czapek's mineral solution where sodium nitrate and potassium sulphate were replaced by 0.5% ammonium sulphate and 0.3% KH_2PO_4. Other nitrogen sources were also tested during the experiment. In some treatments, additional KH_2PO_4 was added to the medium at a P concentration equal that of RP containing treatments in order to study the effect of soluble P on phytase production. The solid waste was previously ground to pass a 2 mm pore screen. Liquid mineral solution was used to adjust the initial moisture content at 50–75% prior to sterilization. Medium pH was adjusted to 5.3–5.5. After sterilization at 120 °C for 30 min, flasks were supplemented or not with 0.3 g inorganic insoluble P (Morocco RP; 12.8% P; 1 mm mesh) sterilized separately. The flasks were inoculated with homogenized 70 h *A. niger* culture, previously grown on medium for inoculum production, at a rate of 3 ml per flask. However, 2–6 ml of the seed culture was also tested in experiments aimed at inoculum volume optimization.

Solid-state fermentations were performed at 30 °C for 120 h. All experiments were carried out in triplicate. The means were analyzed using Duncan's multi-range test.

2.3. Analytical methods

Analyses of fermentation products were performed after homogenization of 4 g sample in 96 ml distilled water (shaking the suspension at 250 rpm for 100 min). The resulting material was centrifuged and the clear supernatant was analyzed.

Mycelial mass weight was determined as described by Shakurai et al. (1977) and Papagianni et al. (1999). Weight loss of lignocellulose after the fermentation process was calculated on ash content basis according to Kumar and Sign (1990) and presented as a percentage of mineralization. P content was determined by the Molybdo–Vanado method described by Lachica et al. (1973). Citric acid content was determined by a spectrophotometric method using pyridine and acetic anhydride reagents (Marrier and Boulet, 1958). The activity of phytase was determined according to Engelen et al. (1994). One unit of enzyme activity was defined as the amount of phytase that released 1 μmol of inorganic P per 1 min and expressed as units per gram dry substrate (U/g DS).

3. Results and discussion

Both phytase production and inorganic P solubilization were found to be dependent upon solid-state fermentation parameters such as water medium content (moisture), type of the nitrogen source, inoculum size and the presence of P in the culture media.

3.1. Effect of moisture level

The water content of a solid-state medium is a key factor that strongly affects microbial development and metabolic activity (Hesseltine, 1972; Nagel et al., 2001). The effect of different moisture levels on the phytase pro-