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Short communication

# Solubilization of animal bonechar by a filamentous fungus employed in solid state fermentation

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ABSTRACT

Experts are concerned by the scarcity of rock phosphate and the vulnerability of the modern agricultural systems which is highly dependent on the existing fertilizer industry based exclusively of this natural, finite, non-renewable resource. In this work, the filamentous fungus *Aspergillus terreus*, which produce itaconic acid, was used to solubilize animal bonechar (HABO, a derivate from meat industry) in conditions of solid-state fermentation. By-products of the sugar and olive oil production industry (sugar beet press-mud and dry olive residues) containing lignocellulosic mass were used as substrates. The effect of humidity, inoculum size, nitrogen source, and phosphate on itaconic acid production and HABO solubilization was studied at optimal other conditions. Results showed that 70% humidity, 1.5 g meat and bone meal, 1 ml initial inoculum size, and 20 g HABO $kg^{-1}$ dry substrate remarkably enhanced the itaconic acid production which reached 44.0 g/kg dry substrate. At these conditions, the amount of soluble P in the medium reached 30.9 mg/flask which corresponded to 50% yield of soluble P vs. total P in the supplied insoluble phosphate. Glycerol was applied in the medium for inoculum production and further in the solid-state fermentation process it was introduced as a part of the medium and as a moistening agent.

The results reported here should be evaluated bearing in mind the problem of phosphate fertilizers, P plant nutrition, and existing phosphate bearing resources. On the other hand, due to the fact that solid (waste) particles serve simultaneously as a support and source of nutrients for cell growth, this laboratory scheme can be used in processes of production and formulation of soil microbial inoculants.



## 1. Introduction

The problem of phosphate fertilizers, P plant nutrition, and existing phosphate bearing resources can be considered in two main aspects. The first one is related to the scarcity of rock phosphate (RP) and the vulnerability of the modern agricultural systems which is highly dependent on the existing fertilizer industry based exclusively of this natural, finite, non-renewable resource (Neset and Cordell, 2012). The second is that the actual chemical production of P fertilizers based on the use of high-grade (>32–35% P content) phosphate ore is an inefficient, high energy consumption process, which generates residual materials harmful to environment (Goldstein and Rogers, 1999). Direct use of insoluble inorganic phosphates is possible and would minimize pollution and decrease the costs of chemical phosphate fertilizer production but the solubilizing efficiency in soil conditions is low (Sharpley, 1995). A biological approach for liberating phosphate from P-bearing materials by organic acid producing microorganisms was proposed as a less expensive and lower-energy technique compared with the conventional chemical techniques (Sahu and Jana, 2000; Xiao et al., 2008). Particularly attractive are the microbially-mediated processes based on the use of low-grade (<32–35% P content) RP and alternative P sources (Vassilev et al., 2009b, 2013). Using this biotechnological scheme based on by-products of the sugar and olive oil production industry (Vassilev et al., 2009a), solubilization of insoluble inorganic phosphates was investigated during the last 20 years in conditions of solid-state fermentations (SSF) (Vassilev and Vassileva, 2003). SSF offers unique

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substrate-air-microorganism interrelations and a number of advantages over submerged batch cultivation. The latter include low energy requirements, low water content (easy downstream processing), and low volume of wastes (Pandey et al., 2000). SSF was widely used for production of different organic acids (Pandey et al., 2000) but the main body of research was dedicated on the production of citric acid (Vandenberghe et al., 2000).

In this work, *Aspergillus terreus*, which produces itaconic acid, was used to solubilize animal bonechar (a derivate from meat industry) in conditions of SSF. Until recently, waste materials from meat production have been used as high-protein components of animal feed. However, as a result of the bovine spongiform encephalopathy crisis the use of these animal wastes is strictly controlled and the main disposal alternative is incineration. Combustion material from meat and bone meal is characterized by high calcium and phosphorus content (up to 47%) (Deydier et al., 2005). During the last years, a steady increased interest is observed on the possible use of animal bone char as a source of phosphate (Conesa et al., 2003; Warren et al., 2009).

## 2. Materials and methods

### 2.1. Microorganism and culture media

The filamentous fungus *A. terreus* (CECT 20365-Spanish National Microorganism Collection) was used throughout this study. It was maintained on potato-dextrose agar slants at a temperature of 4 °C and transferred every 3 months. Sugar beet wastes (sugar beet press mud) were obtained from the local fabric for sugar production (Azucarera Jerez de la Frontera, Spain). Dry olive wastes were kindly provided by "COLGRA", Spain. Portions of 10 g of the solid wastes (dry olive wastes:sugar beet wastes, 1:5, w/w), ground to pass a 1-mm-pore screen, were placed in 250-ml Erlenmeyer flasks and mixed with medium strength Czapek-Dox mineral salt solution at a ratio of 0.5:1 (solid particles:liquid phase, w/v) dissolved in glycerol (8.0% water solution). All flasks were sterilized by autoclaving at 120 °C for 30 min and inoculated with *A. terreus*. Hydroxiapatite of animal bone origin (HABO, animal-bone char: 60 mesh; 31% phosphate), kindly provided by BES Ltd, Scotland, was added at a concentration of 20 g/kg dry substrate (0.2 g/flask).

### 2.2. Fermentation process

Fermentation experiments were carried out at 30 °C for 5 days. Four different experiments were carried out to optimize initial humidity, nitrogen source, inoculums size, and the effect of P source, respectively. In the first experiment, humidity levels were adjusted to 50, 60, and 70% and fermentations were carried out at 30 °C and initial pH 5.5 using as a nitrogen source ammonium sulphate. SSFs in the second experiment were performed at the same temperature, pH and at optimal humidity level and applying different nitrogen sources. In the third experiment, the inoculums size was the variable parameter applied at 1, 2, 4, and 6 ml. The last experiment was carried out to compare the effect of $KH_2PO_4$ and HABO on the investigated parameters. $KH_2PO_4$ was added to the medium as follows: 0.5 g $l^{-1}$ in treatments SB/DOW (-HABO); 1 g $l^{-1}$ in treatments SB/DOW + $KH_2PO_4$ (-HABO); 0.1 g $l^{-1}$ in treatments SB/DOW + HABO.

In all experiments, the sterilized (121 °C, 30 min) flasks were inoculated (carefully spread over the surface of the medium) with homogenized 30-h fungal culture which was previously grown on medium for inoculum production, at a rate depending on the treatment. Free space in flasks was flushed with pure oxygen (100 ml $min^{-1}$, 1 h) every 24 h. All experiments were carried out in triplicate.

### 2.3. Effect of water extracts of the substrates/final fermentation products on seed germination

The phytotoxicity bioassay was based on the method described by Zucconi et al. (1981). Healthy seeds of *Trifolium repens* were surface sterilized with $HgCl_2$ for 2 min and rinsed 5 times with sterile distilled water. After drying, the seeds were imbibed for 4 h/25 °C in water extracts (1:5) prepared from the substrates (microbially treated or not agro-industrial wastes ± HABO) and the final fermentation products. They were further transferred in Petri dishes (9 cm; 25 seeds/dish) lined with double layer filter paper, moistened with 2.5 ml of the same extract, and incubated at in the dark at 25 °C. Distilled water was used as a control. A seed with at least 1 mm of protruded radicle was accepted as germinated. Germination index was calculated using the formula $GI = (G/G_o) \times (L/L_o) \times 100$ where $G_o$ and $L_o$ are respectively the germination percentage and radicle growth of the control. All experiments on seed germination were performed in quintuplicate.

### 2.4. Analytical methods

Mycelial mass weight was determined as described by Shakurai et al. (1977) and Papagianni et al. (1999). Itaconic acid was determined by the method of Hartford (1962). The concentration of soluble phosphate was determined using vanadate–molybdate reagent (Sigma–Aldrich Cat. No 94686). 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. Samples of flasks were processed by analyzing 3 flasks/treatment. The means were analyzed using Duncan's multi-range test.

## 3. Results and discussion

The optimal environment for SSF process includes a number of variable parameters such as solid substrate and medium mineral components selection, initial pH, temperature, moisture, inoculum size, and time length. In this work, initial pH value was not experimented as it would affect the solubilization of HABO artificially. Temperature was also fixed at 30 °C as it is recommended by the Culture Collection experts. Therefore, the effect of humidity, inoculum size, nitrogen source, and phosphate on itaconic acid production and HABO solubilization was studied at optimal other conditions.

### 3.1. Effect of humidity level

Initial humidity is one of the most important parameters in SSF processes by fungal microorganisms (Krishna, 2005). Low level of humidity is known to provoke a decrease in the fungal acid productivity (Shankaranand and Lonsane, 1994) and metabolic activity most likely because of the reduced solubility and acquisition of nutrients by fungal biomass (Ramesh and Lonsane, 1990). To study the effect of humidity, three initial moisture levels were experimented without changing the concentration of the medium components (Table 1). The results showed that the increase of the initial moisture from 50% to 70% greatly enhanced the acid production with a maximum of 26.1 g itaconic acid per g of dry substrate. Addition of HABO to the medium at equal other conditions resulted in significantly higher itaconic acid production and yield of 0.308 g $kg^{-1}$ $h^{-1}$. Solubilization of HABO was also affected due