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MARIA VASSILEVA , [Luis F del Moral Garrido](#) , [Vanessa Martos](#) , [Ana Isabel Garcia Lopez](#) , Giuseppe Falvo D'Urso Labate , Elena Flor-Peregrin , Antonia Reyes Requena , [Nikolay Vassilev](#) *

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Communication

The Parameters of a Fungal Fermentation Facilitate Its Formulation for Further Application in Plant-Soil Conditions

Maria Vassileva ¹, Luis F del Moral Garrido ², Vanessa Martos ², Ana Isabel Garcia Lopez ¹, Giuseppe Falvo D'Urso Labate ³, Elena Flor-Peregrin ¹, Antonia Reyes Requena ¹ and Nikolay Vassilev ^{1,*}

¹ Department of Chemical Engineering, University of Granada, Spain

² Department of Physiology Vegetal, University of Granada, Spain

³ EKROME srl, Italy

* Correspondence: nbvass@yahoo.com

Abstract: Sustainable crop production includes methods of growing food in a responsible manner avoiding application and dependence on chemically produced fertilizers and pesticides. The latter means development of approaches that lead to environmentally mild inputs based on the production-consumption-recycling principle. Biofertilizers are an important tool to achieve sustainable crop production. In this work, we report the results of experiments on growth and spore/mycelium production of plant growth promoting *A. niger* applying standard nutritional medium (potato-dextrose broth, PDB) enriched with 3% insoluble phosphate (20 to 200 mesh hydroxyapatite of animal-bone origin, HABO) and 0 to 80 g/L glycerol (a by-product of biodiesel production). Results showed the ability of *A. niger* to acidify the medium with the highest titratable acidity of 28.9 mmol/l (at 5% of glycerol) and solubilize animal bone char under these conditions. As a second stage of the experimental work, the resulting final products were used to formulate gel-based inoculant. Both, the spores and mycelium produced during the fermentation process were further used as a base for formulation to make the biofertilizer production the key in the Sustainable Agriculture. Storage of the resulting products reported here was facilitated by the presence of glycerol in the formulation system.

Keywords: plant growth promoting microorganisms; fermentation; formulation; seedlings

Introduction

Microorganisms beneficial to plant growth and health are now widely recommended as potential alternatives to chemical fertilizers and pesticides in sustainable agriculture [1]. During the last years, a large number of microorganisms have been isolated and characterized morphologically, physiologically and biochemically. Their potential as biofertilizers and biocontrol agents has been proved in controlled and natural conditions. Since the early studies in the field of microbial inoculants, the emphasis of the scientific activity during the last 15-20 years is on developing environmentally friendly and efficient microbial fermentations on cheap and assessable substrates and formulation techniques adapted to these processes. **Production, formulation, and application of biofertilizers** are the most important steps in exploitation of specific beneficial microbial strains alone or in combination with phytostimulants in enhancing nutrient availability, improving soil properties, and increasing crop yield. In general, the results confirmed the beneficial effect of the selected microorganisms on plant growth and health, which is highly dependent on the formulation procedure and the number of microorganisms in one biopreparation [2]. The main roles of the formulation of inoculants are to ensure friendly micro-environment for the microorganisms, simultaneously protecting the cell viability from different physical and chemical agents during

storage over a prolonged period and to help them in the competition with native soil microflora or reduce the negative effect of the micro-fauna after introduction into soil-plant systems while interacting with plants and soil microbiome [3]. Before formulation, spores and biomass of the plant-beneficial microorganisms must be produced, which, after formulation, should be delivered depending on the carrier, as liquids (sprays, drenches, and root dips) or as dry formulations applied at the appropriate time [4]. Independently of its form (liquid or solid) the formulated product should fulfil a number of requirements, the most important of which are to ensure high plant root (or rhizosphere) colonizing efficiency, sufficiently high competitiveness in conditions of biotic and abiotic harsh environment, simultaneously increasing plant health and nutrition [3]. One of the most promising formulation techniques is the encapsulation in macro- and micro-beads of (mainly natural) polysaccharides, which guarantees a continuous release of gel-embedded cells/spores into soil-plant systems [5]. It was proved that a simple entrapment of plant beneficial microorganisms in polysaccharide gel particles is not sufficient to ensure and desired advantages and agronomic efficiency of the formulated products [6]. Double/multiple inoculants combined with bio-stimulants and other additives (smart bio-formulates) should be developed to compete with the traditional chemical fertilizers [3].

The aim of this work was to study the behavior of a citric acid producing strain *Aspergillus niger* in the presence of glycerol as a medium component and animal bonechar as an insoluble phosphate source. Further formulation of the filamentous fungus was carried out and its storage for different time periods was tested.

Materials and Methods

Microorganism and Culture Medium

- The filamentous fungus *Aspergillus niger* NB2 (Collection of the Department of Chemical Engineering, University of Granada) was used throughout this study. It was maintained on potato-dextrose agar slants at a temperature of 4°C and transferred every 3 months.
- The culture medium for growth and immobilization was Potato-Dextrose Broth supplemented with microbial protein (MP; from mechanically destroyed yeast culture), 5%.
- The medium for acid production and P-solubilization was PDB-MP supplemented with different amounts of glycerol (0-80.0 g.L⁻¹) and animal bonechar, of 3.0 g.L⁻¹. The initial pH of the medium was 6.5 and was left unadjusted. The medium was autoclaved at 120°C for 20 min. Animal bonechar (80, 50, and 20 mesh, 31% phosphate), kindly provided by BES Ltd, Scotland, was sterilized separately.

Entrapment of *A. niger* in gel-based carrier:

Fungal mycelium and spores were obtained after the end of the fermentation in PDB/MP and glycerol (from 0 g.L⁻¹, control, to 80 g.L⁻¹).

Sodium alginate solution (4%, w/v) was prepared by continuously stirring at 70° C, autoclaved at 120 °C for 20 minutes, cooled down to environmental (lab) temperature and further mixed (1:1) with a fungal suspension (1.4x10⁸ colony-forming units, CFU mL⁻¹) previously blended together with the spent liquid medium containing the rest of the un-consumed glycerol. The alginate-fungal suspension mixture ± 3% animal bonechar was dropped slowly by **droplet generator-syringe device with a speed of 15-20 ml per hour** into a gently stirred 0.5M CaCl₂.2H₂O solution under a magnetic stirrer. The Ca-alginate beads were recovered after 30 min and washed in a sterile dH₂O water to remove excess calcium ion and un-entrapped cells before introduction into the fermentation flasks. Drying of beads was carried out in an oven for 30h at 30°C to reach a water activity close to 0.2-0.3.

Experimental procedure: The experiments were performed with free and gel-entrapped *A. niger* mycelium/spores.

- **Experiments with free *A. niger*** were performed in Erlenmeyer flasks (250 mL) containing 100 mL of sterilized medium supplemented or not with animal bonechar and inoculated with 1x10⁷ CFU mL⁻¹ (collected from 6-day-old *A. niger* grown on potato-dextrose agar; 1 ml/flask). An orbital shaker at 250 rev min⁻¹ 30° C was used to carry out the fermentations at 30°C. Each single

batch with free *A. niger* for organic acid production and animal bonechar solubilization was carried out for 120 h.

- **Experiments with gel-entrapped cells** were performed under conditions of repeated-batch fermentation as follows: each single-batch cycle was carried out for 48 h and then immobilized cells in alginate were transferred (after careful washing with sterile dH₂O) to fresh medium. 25 Ca-alginate beads were used per flask with a total number of 1x10⁸ CFU.

Analytical methods: **Biomass** was determined both in immobilized particles and as mycelium, freely suspended in the fermentation broth. Determination of fungal survival after immobilization or drying was determined as described by Mater et al. after dissolution of the Ca-alginate beads using 25 fresh or dried beads in 0.2 M citrate buffer (chelating the Ca²⁺ of the Ca-alginate) agitated at 80-100 rpm by a magnetic stirrer. Dissolved fungal mass was diluted and incubated on PDA to determine the CFU. The free biomass was determined by vacuum filtration of the samples, washed with dH₂O, and dried at 70°C to a constant weight.

Titrateable acidity was determined by titrating each sample to pH 7.0 with 0.1 M NaOH.

The concentration of **soluble phosphate** was determined by the molybdo-vanado method using vanadate-molybdate reagent (Sigma-Aldrich Cat. No 94686). All experiments were performed in triplicate (three flasks per treatment) and values were analysed and presented as means ± standard deviation.

Results and Discussion

Aspergillus niger grew well in the presence of glycerol in the medium (Table 1). However, the biomass concentration decreased, which was more pronounced at 8% glycerol while at 3% and 5% glycerol the biomass growth had similar profile with final values of 4.39 g/L and 4.34 g/L, respectively.

Table 1. Biomass accumulation and citric acid production by *A. niger* on potato-dextrose broth enriched with glycerol.

Medium Composition	Time-course fermentation	Biomass	Titrateable acidity
(PDB/MP)	(h)	(g.L ⁻¹)	(mmol.L ⁻¹)
+ Gly (%)			
0 (PDB)	40	1,80+0.06	11.0+0.5
	80	2,55+0.04	14.9+0.4
	120	5.32+0.03	21.0+0.6
3	40	1.87+0.05	18.0+0.1
	80	3.91+0.05	22.0+0.3
	120	4.39+0.02	26.0+0.7
5	40	1.71+0.06	13.1+0.2
	80	3.60+0.01	20.8+0.1
	120	4.34+0.04	28.9+0.4
8	40	1.22+0.02	9.2+0.3
	80	1.70+0.07	10.5+0.4
	120	2.20+0.07	12.0+0.2

Glycerol did not inhibit the citric acid formation expressed as titrateable acidity. Higher concentrations of citric acid were registered at 3% and 5% of glycerol compared to the control. Under these conditions, the amount of citric acid produced per gram biomass (*Y_{sp}*: yield of product in terms of biomass or g product g⁻¹ biomass) at the end of the process increased with increasing the glycerol concentration from 0% glycerol to 8% (0% - 4.2; 3% - 5.68; 5% - 6.66; 8% - 8.6) but in the last case this increase was due to the lower biomass growth compared to other treatment. The morphology of the

fungal mycelium was found to be affected by the added glycerol and its concentration. In general, filamentous fungi have the tendency to develop different mycelium morphologies, such as suspended mycelium or pellets which depend on shear forces and determine fungal metabolic response and productivity [8]. When PDB was used without glycerol, filamentous mycelium was formed which, along the fermentation process, increased to the highest biomass concentration compared to all other treatment. When glycerol concentration was in the range of 30–50 g.L⁻¹, uniform but small (<1 mm on average) pellets were observed. As the glycerol concentration increased to 80 g.L⁻¹, the pellets formed clumps and the respective biomass decreased. It is widely accepted that *A. niger* produces a high citric acid yield when the culture grows in the form of small pellets [9]. On the other hand, when the glucose concentration was high in the medium (20% in PDB), particularly in the initial part of the process, glycerol was not consumed. After depletion of the glucose the glycerol consumption continued the acid production. As the biomass in the presence of glycerol, independently of its concentration, was lower, the metabolism of the fungus was oriented towards acid accumulation, which resulted in higher acidity registered in the fermentation medium. The consumed glycerol increased with increasing its initial concentration starting from 4.31 g.L⁻¹ and 5.88 g.L⁻¹, (3% and 5% glycerol, respectively) but decreased at 8% glycerol to 3.01 g.L⁻¹. Although the results of 3% and 5% glycerol were very similar, 5% initial glycerol was selected for further experiments as in this case the acid production was a bit higher while the residual glycerol was higher (data not shown) compared to all other treatments.

The addition of different size HABO particles to the medium resulted in different response of the *A. niger* (Table 2).

Table 2. Solubilization of different particle size of HABO (3g.L⁻¹) by freely suspended *A. niger* in a single 120-h batch fermentation process and 5% glycerol in the medium.

HABO (mesh)	Biomass (g.L ⁻¹)	Titrateable Acidity (mmol.L ⁻¹)	P _{soluble} (mg.L ⁻¹)
20	5.68±0.20	30.9±0.1	411±21
60	5.39±0.11	32.1±0.3	489±12
100	4.15±0.13	22.4±0.2	280±9
120	3.46±0.14	18.7±0.2	199±4

In general, the presence of insoluble phosphate and its further solubilization due to the medium acidification and pH lowering, affected the biomass growth pattern. Normally, the acid-producing microorganisms increase their biomass growth in the presence of phosphate [10]. The higher fungal growth was also noted in this experiment but in treatments with higher HABO particles size (20 and 60 mesh). Powdered phosphate source did not positively affect biomass growth and acid-production. The results could be explained by an increase of the medium viscosity particularly in the last two particle sizes (100 and 200 mesh) when, in addition, these small particles adhered to the fungal biomass thus limiting its activity [11]. Therefore, in our experiments, the close relation between the type, size, and concentration of the added substances and the microbial biomass growth and activity was observed.

The next step was to use the fermentation specificity of our study and prepare a possible formulated product. The P-solubilizing capacity of two types of alginate beads was studied in repeated-batch fermentation of five batch cycles containing HABO in the gel structure or the same amount of HABO particles were added to the PDB. In both cases, alginate beads contained the fungal biomass and spent fermentation liquid with residual glycerol.

Table 3. Gel-entrapped *Aspergillus niger* based solubilization of HABO.(3 g.L⁻¹, 60 mesh) supplemented to the PDB/MP medium (A) or introduced in the alginate beads (B), in conditions of repeated batch cultivation.

Batch No.	Titrateable acidity (mmol.L ⁻¹)		P soluble (mg.L ⁻¹)	
	A	B	A	B
1	28.1±1.4	31.1±1.4	314±21	348±11

2	33.5+1.1	35.8+1.1	466+13	488+10
3	43.2+1.0	47.1+1.0	512+17	582+17
4	40.1+2.0	44.1+2.0	514+19	564+12
5	39.8+1.4	45.9+1.4	510+12	570+14
Overall	Total-Per Batch	Total-Per Batch	Total-Per Batch	Total-Per Batch
	184.7-36.94	204 - 40.8	2316- 463 % of total 49,8%	2552- 510,4 (+10%) 54,9%

particularly after the third batch cycle, which was well pronounced in the treatment with alginate-bead-entrapped HABO compared to the treatment with freely suspended HABO. Similarly, the P-solubilizing activity was higher when the P source was entrapped in the polysaccharide carrier thus producing 10 % more soluble total P solubilized by the gel-entrapped fungus. Therefore, the gel entrapped system containing both the fungus and the insoluble phosphate could be used as a formulated product and further tested in soil-plant system. The reason for the higher efficacy of this gel-entrapped system is the presence of glycerol (presented in the spent fermentation liquid) and HABO particles in the carrier. In the case of freely suspended HABO particles, abrasion effect of the solid P-source was observed on the outer surface of the gel-beads, which probably could affect the morphological structure of the fungal filaments developed out of the beads with a concomitant change in the metabolic (acid-producing) activity. Based on the above results, further studies were carried out to determine the viability of the entrapped fungus in dried alginate beads enriched with HABO. The CFU counts as determined at different stages of the experiment resulted as follows:

- a) Initial CFU in the ALG/MP/±HABO mixture1.4 x 10⁸
- b) CFU in wet gel beads after entrapment and bead solidification1.0 x 10⁸
- c) CFU in dried beads..... 1.3 x 10⁷
- d) CFU after a 90-d storage:
 - a. at 4° C..... 1.5 x 10⁶
 - b. at 24° C.....1.6 x 10⁸

The resulting free and gel-formulated products were tested in substrate-tomato system for 25 days (data not shown here).

Conclusions

The results of this study confirmed the possibility of applying glycerol as a substrate as shown before [12]. The inclusion of an insoluble P source in the fermentation system showed the capability of free and gel-entrapped acid-producing fungal culture to solubilized it and even at higher level when the P-bearing material was included into the immobilized system. The formulation of a mixture containing the acid-producing microorganism, spent fermentation broth with residual glycerol, and P-bearing particles was active after a 3-month period with higher number of viable cells after storage at ambient temperature. Dried gel-microorganism formulation after 3-month-storage at room temperature could be used as an efficient inoculate at least in tomato growth. Further studies are needed to show the efficacy of the formulated product and its effect on plant growth and on field soil-plant microbiome compared to other unformulated, including cell-free liquid formulation or microbial dual-based products [13–15].

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