Production of chitinase by free and immobilized cells of *Penicillium janthinellum*. A comparison between two biotechnological schemes for inoculant formulation

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Introduction

Development of an efficient inoculant delivery strategy involves a number of critical stages such as strain selection, selection of a carrier and mass multiplication, and formulation of the inoculant. Recent advances in agro-biotechnology and particularly in formulation and application of plant-beneficial microorganisms, have led to isolation of wide number of microbial cultures with multifunctional properties including nitrogen-fixing, phosphate-solubilizing, plant-growth promoting, and biocontrol activities (Vassilev et al., 2006). A successful inoculant should overcome the various biotechnological operations during production, preserving its viability over long periods of storage, while maintaining its functional characteristics (Xavier et al., 2004)). In addition, one and the same microorganism should be formulated in different forms depending on different climates, soil-types, and final user preferences. For large-scale production of microbial inoculants, it is important to select inexpensive and easily available sources of nutrients for culture medium.

During the last years, we tried to follow all of the above considerations and requirements – to select plant beneficial microorganisms with multifunctional properties, develop laboratory technologies for mass production based on agro-industrial wastes, and prepare various formulation procedures employing different biotechnological models.

The aim of this work is to test two different schemes for *Penicillium janthinellum* cultivation aimed at formulation of an efficient biocontrol inoculant for its further introduction into soil-plant systems. Entrapment of fungal cells in a water-soluble polymer was performed as such formulations are now widely used in agro-industries (Vassilev et al., 2001). Another series of experiments was carried out in solid-state mode of fermentation (SSF) on dry olive wastes (DOW) as a substrate. The latter was selected as it is economically cheap and available in large quantities in the Mediterranean area.

Materials and Methods

Microorganis: *Penicillium janthinellum* was maintained on malt extract agar plates at 4^{o} C and sub-cultured every month. It was incubated at 28^{o} C for 7 days before use to prepare the inoculum in fermentation experiments. The standard liquid medium contained colloidal chitin, 15 g/l, and corn steep liquor, 0.5 g/l.

Solid-state fermentation*:* The basal medium composition included (per flask) 15 g of solid dry olive wastes (DOW), previously dried in a 60^{o} C oven and then ground by mechanical blender to pass a 2-mm pore screen, and 0.5 g chitin. 20 ml of Czapek-Dox broth containing additionally 0.5 g/l Tween 80 and 0.5 g/l corn steep liquor were added to each Erlenmeyer flask of 300 ml. The initial pH was adjusted to 5.0 before autoclaving. The sterilized (121^{o}C, 30 min) flasks were inoculated with homogenized 30-h *P. janthinellum* culture, previously grown on standard medium for inoculum production, at a rate of 5 ml per flask. SSFs were performed at 28^{o} C for 20 days.

Experiment with gel-entrapped fungus: Gel-entrapment of the fungal biomass was performed as described by Loomis et al. (1997). Homogenized 30-h mycelium was mixed in 2% sodium alginate enriched with 2.5 g of DOW powder obtained during the preparation of the medium for the SSF experiments. In some experiments, 0.1 g chitin/100 ml alginate was supplemented. Medium solution contained the same components as the standard medium without DOW. Alginate beads (1.5 mm/d; 5 ml) were introduced into 50 ml medium/300-ml Erlenmeyer flask and medium was changed every 30 h to carry out at least 3 repeated batches. Repeated-batch process conditions were as follows: pH, 4.8-5.0; temperature, 28^{o}C; total time period of 90h where samples were analyzed each 30 h (time period of one single batch). In all experiments insoluble inorganic rock phosphate (1 mm mesh Morocco fluorapatite; 12.8 % P; 0.3 g/flask) sterilized separately was additionally included into the media to test a possible P-solubilising activity of both systems (SSF and gel-entrapped *P. janthinellum*)

Soil-plant test: Surface sterilized (70% ethanol, 2 min; 0.5% sodium hypochlorite, 2 min; sterile distilled water, rinsed 4-5 times) seeds of tomato (*Lycopersicon esculentum*) were sown in sterilized soil. Two weeks after germination the seedlings were transplanted in pots (three seedlings per pot) containing 300 g of amended or non-amended soil according to the treatments i-iii. The treatments (five replications each) used in this experiment were as follows: (i) (C) control, soil without amendments; (ii) Control + DOW/*P. janthinellum*; (iii) Control + *P. janthinellum* entrapped in alginate. All treatments were enriched with rock phosphate. When necessary, soil was inoculated with *Fusarium oxysporum* f. sp. *lycopersici*.

Analytical methods: Samples of flasks were processed by analyzing 3 flasks for each time point. Phosphorus content was determined by the molybdo-vanado method described by Lachica et al. (1973). Chitinolytic activity activity was measured as described by Molano et al. (1977) modified by Ulhoa & Peberdy (1992). The assay mixture contained 1 mL of 0.5 % pure chitin from crab shell (Sigma) suspended in 50 mM acetate buffer (pH=5.2) and 1 mL of crude enzyme preparation. The reaction mixture was incubated for 7 h at 37 °C by shaking, then the reaction was stopped by centrifugation (5000 rpm for 10 min) and 1 mL of dinitrosalicylate (DNS) reagent was added. All experiments were carried out in triplicate.

Plants were harvested after 7 weeks and rhizosphere soil samples (closely associated with the plant roots) were collected, stored at 4^{o} C, and subsequently assayed. Ten grams of air-dried rhizosphere soil were shaken for 1h in a 250-ml Erlenmeyer flask containing 100 ml of 0.2% agar-water. One ml of the suspension from each sample (100-fold dilution) was spread on Petri dishes containing selective medium. The plates were further incubated for 7 days and the number of fungal colony-forming units per gram soil was determined by colony counting.

Results and Discussion

The results of this study showed that DOW-based medium provided necessary nutrients for chitinase production by fungal culture of *P. janthinellum* in conditions of SSF (Table 1). Optimizing several process parameters (data not shown in this work) such as initial solid:liquid ratio, temperature and pH, and inoculums size resulted in enhanced chitinolytic activity. The latter was significantly improved in media supplemented with chitin although chitinases are known to be produced constitutively. The maximum chitinase yield (Table 1) reached 1612.9 U per kg initial dry matter (DOW+chitin) in the presence of insoluble inorganic phosphate source (rock phosphate, RP). Solubilization of RP added to the medium was achieved which was probably due to proton release by *P. janthinellum*. In a recent study, employing *Phanerochate chrysosporium* in almost equal

conditions RP solubilisation was performed through the production of various carboxylic acids which serve as organic chelators thus stabilizing the formation of Mn^{3+} (Vassilev et al, 2007). However, in this case pH increased from 4.8-5.0 to 6.4-6.6 which explains the lowering of RP solubilisation rate from 49.7 % to 0% at the end of the fermentation experiment.

Treatment	Time (h)	Chitinase (total per flask)	(per kg IDM)	P_{sol} (mg/flask)	% solubilization of total
- RP	50	6.0±1.1	387.1	+	ND
	100	13.5±1.3	871.0	+	ND
	144	17.3±2.1	1096.5	-	ND
+RP	50	8.7±1.0	561.3	18.9±0.2	49.7
	100	20.0±2.3	1290.3	9.8±1.0	25.8
	144	25.5±0.8	1612.9	+	ND

T 28°C; solid:liquid 1:1.5; initial pH 4.8-5.0; +, traces; IDM – initial dry matter

Table 1. Chitinase production and RP solubilization by *Penicillium janthinellum* on dry olive wastes enriched with chitin in conditions of solid-state fermentation

Similar fungal metabolic behaviour was observed when *P. janthinellum* was entrapped in alginate enriched with both DOW powder and chitin from crab shell (Table 2). In this case, however, the chitinase activity was sufficiently lower than the maximum values obtained in the SSF experiment independently of the presence of RP. It is also interesting to note the steady level of soluble P in the medium solution during the whole experiment. Although the absence of agro-waste in the medium, the DOW powder and chitin, used as fillers in the alginate beads, stimulated the enzyme synthesis compared to combinations with only one or none of the fillers (data not shown).

Treatment	Batch No	Chitinase (U/l)	P_{sol} (mg/flask)	% solubilization of total
- RP	1	70±2.4	+	ND
	2	229±22	+	ND
	3	551±31	+	ND
+RP	1	82±11	15.8±1.0	41.5
	2	394±37	12.3±0.5	32.4
	3	785±40	10.2±0.3	26.8

T 28°C; pH 4.8-5.0; 1 batch, 30h; +, traces

Table 2. Chitinase production and RP solubilization by alginate-entrapped *P. janthinellum*

Fresh preparations of SSF-grown and alginate-entrapped *P. janthinellum* were efficient antifungal agents as proved in a microcosm soil-plant experiment inoculated with the phytopathogen fungus *Fusarium oxisporum* (Table 3). The higher biocontrol activity of alginate-entrapped *P. janthinellum* can be explained by the absence of DOW in its formulation which probably served as a substrate for the pathogen fungus thus ensuring advantageous growth conditions.

Treatment +*F. oxysporum*	Days/severity of symptoms 10	20	30	45	*F. oxysporum* (log CFU/g soil)
Control	A	C	D	E	3.83
+DOW/Pj	A	A/B	A/B	C	2.84
+Alg/Pj	A	A	A/B	B	2.37

A, plant without symptoms; B, lower leaves yellow; C, lower leaves dead and some upper leaves wilted; D, lower leaves dead and upper leaves wilted; E, dead plant

Table 3. Observations of disease severity and number of *Fusarium oxysporum* colony- forming units (CFU) in the rhizosphere of tomato. Soil inoculated or not (Control) with *Penicillium janthinellum* grown in conditions of solid-state fermentation on dry olive wastes (DOW/Pj) or entrapped in alginate (Alg/Pj)

Conclusions

Both biotechnological approaches can be used in production and formulation of biocontrol products and efficiently used in suppression of *Fusarium oxisporum*. Compared to SSF, gel-entrapped culture demonstrated lower chitinolytic activity per unit ((U/IDM vs. U/l), it was more active against the pathogenic fungus in soil-plant conditions. Further studies are necessary to test the efficiency of these products after storage to determine their final economic parameters.

Acknowledgements

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