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Biocontrol Efficacy of the *Vishniacozyma Victoriae* in Semi-Commercial Assays for the Control of Postharvest Fungal Diseases of Organic Pears

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Abstract

Vishniacozyma victoriae NPCC 1263 was selected for this work because of the active antagonistic effect over several fungi in postharvest organic pears. Yeast biomass production was carried out in a 15 L stirred-tank bioreactor with 12 L of working volume at 20 °C and 300 rpm and 0.64 vvm of aeration. The selected production medium was based on cheese whey powder and salts. The present study aims to evaluate the possibility of using an inexpensive growth substrate for production of added value products (yeast biomass), this innovation also requires evaluate biocontrol efficacy of yeast against fungal diseases of pears in semi-commercial assays. The yeast biomass was collected, cold stored for 60 days (Treatment 1) and 15 days (Treatment 2) and sprayed on the pears in semi-commercial level testing assays. After 180 days of postharvest conservation, significant reduction of fungal infection by *Penicillium expansum, Botrytis cinerea* and *Cladosporium sp.* was observed. The Treatment 1 reduced total diseases incidence by 71%, instead Treatment 2 reduced it by 92%. The effect of spray application on the yeast viability with different cold storage was tested. These work provides information on the bench-scale bioreactor yeast production using a new low-cost medium, viability and biocontrol efficacy of *Vishniacozyma victoriae* in controlling common diseases affecting pears in semi-commercial assays.

Introduction

Postharvest disease causes several losses in the fruit and vegetable market segments around the world [1, 2]. It can reach up to 30% in tropical and developing countries [3, 4]. *Penicillium expansum* and *Botrytis cinerea* are the most important postharvest pathogens of apple and pear fruits worldwide [1, 2, 5, 6]. *Alternaria* sp. and *Cladosporium* sp. fungi are also reported as fruit pathogens [7, 8]. Biological control agents (BCA) have been proposed as an effective and eco-friendly alternative described in scientific publications and a few yeast-based biocontrol products are registered and marketed as plant protection products [9–11]. Among the various BCA, the use of yeast may offer a distinct advantage, as they are environmentally friendly, possess large stress tolerance, strong antagonistic activity against fungal pathogens and there is a well-developed system for culturing and handling them [12–14].

Studies on postharvest biocontrol showed that exogenous application of BCAs is an suitable and safe approach for the management of fungal diseases during fruit postharvest [15]. *Vishniacozyma victoriae* NPCC 1263 yeast was previously isolated by our group from the Upper Valley of Río Negro and Neuquén provinces (Patagonia, Argentina) from pear fruits during cold postharvest storage [5], was selected for this work because of the active antagonistic effect over several fungi [16], and tested their efficacy for controlling the postharvest diseases of pears under semi-commercial conditions [16, 17].

V. victoriae has been isolated from cold natural habitats [18, 19] and although the ability of yeast to survive and grow at low temperatures has attracted considerable attention, the mechanisms underlying this phenomenon have not yet been fully described [14, 20]. Furthermore, this yeast strain exhibited a variety of different mechanisms including: germination of pathogen inhibition, biofilm formation, secretion of killer toxins, colonization of pear wound, competition for

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nutrient and secretion of hydrolytic enzymes (protease, chitinase and glucanase) in experiments were carried out at the temperature of postharvest storage (0 ± 1 °C) [16, 17].

Commercial production of selected antagonist yeast to be used in semi-commercial scale need to produce sufficient biomass using a low-cost culture medium [15, 21, 22]. Whey, a dairy industry by-product is composed of lactose, proteins, lipids and mineral salts. It represents an environmental problem because of its high biological oxygen demand (BOD) [23]. In order to solve the environmental problem, food company have been looking for alternatives for its recycle. There are many reports in the scientific publications on the use of cheese whey for the development of microorganism biomass for different biotechnological applications [24–26]. The yeast *V. victoriae* is able to grow using lactose [27]. However, there are no reports of the use of cheese whey as a substrate for biomass production of this species. The objective of the present study was to evaluate the ability of *V. victoriae* to grow in a medium based with cheese whey and to determine the effect on subsequent biocontrol efficacy in semi-commercial assays. In our previous report the yeast had grown only in a sugarcane molasses medium [17].

Many commercial factors restrict the development and commercialization of biocontrol products, including the maintenance of viable cell in the formulation, biocontrol efficacy on a commercial scale and compatibility of formulated products with the existing application equipment [28]. Both liquid and dry products can be prepared; however, high cell mortality due to dehydration and rehydration processes is one of the disadvantage of dry formulations [29]. Liquid formulation is therefore a mild process, but cell viability loss was observed after storage for six months at 4 °C [30, 31].

In this work, *V. victoriae* NPCC 1263 was efficiently propagated in batch fermenter with a low-cost production medium with cheese whey as the main substrate and it was employed as BCA in semi-commercial level testing assays in post-harvested pears. In order to evaluate the yeasts biocontrol efficacy against pear pathogens in semi-commercial packing houses after postharvest storage, the selected yeast was used in liquid formulation, after different time ranges of cold preservation.

Materials and Methods

Yeast Strain with Antagonism Capacity

Vishniacozyma victoriae NPCC 1263 (Gen Bank access number MN 848352) was previously isolated from Packham's Triumph pears over a storage period of 6 months at 0 ± 1 °C [5]. The yeasts were stored at − 20 °C (glycerol 20% v/v) in the North Patagonian culture collection (NPCC), Argentina. The culture was activated in GPY-agar (40 g glucose L^{-1}; 5 g peptone L^{-1}; 5 g yeast extract L^{-1}; 20 g agar L^{-1}) plates for 48 h at 20 °C and incubated at 20 °C during 24 h.

Biomass Production

Cheese whey powder (CWP) was used as the main substrate for the propagation of the *V. victoriae*. Among the nutrients found in the dry cheese whey, the largest portion corresponds to lactose 75% and protein 15%. CWP solution (60 g L^{-1}) was autoclaved at 121 °C for 20 min for sterilization, the solids were removed by filtration for deproteinization and the supernatant was kept in a refrigerator at 4 °C to avoid any decomposition. The growth medium used for cultivation of inoculum culture consisted of 60 g CWP L^{-1}, 2 g $SO_4(NH_4)_2$ L^{-1}, 5 g KH_2PO_4 L^{-1} and 0.25 g $MgSO_4$ L^{-1} [32].

Fresh cultures grown of *V. victoriae* was made in GPY-agar. For this, 100 ml of CWP medium contained in 250 ml Erlenmeyer flasks was inoculated with 1 ml of active culture of yeast cells (1×10^5 cell mL^{-1}). The culture was maintained for 48 h on a rotary shaker (150 rpm) at 20 °C. After incubation, 900 mL of CWP medium contained in 2000 mL Erlenmeyer flasks was inoculated with the 100 mL culture and further incubated under the same conditions.

The 15 L bioreactor was loaded with 11 L of CWP medium previously sterilized for 40 min at 121 °C and inoculated with 1 L of the 48 h preculture described above. Yeast biomass production was carried out at 20 °C and 300 rpm and 0.64 vvm of aeration. Yeast growth was estimated by measuring optical density (OD 640 nm). The biomass was collected by centrifugation (4500 g, 10 min at 4 °C) after the stationary phase was reached (96 h), washed twice with sterile distilled water and stored at 4 °C until their use in the packing lines. Two reactor runs (R1 and R2) were carried out under the same working conditions.

Growth parameters; maximum specific growth rate (μ), maximum growth (A) and lag phase (λ) were calculated to the reparametrized Gompertz equation [33].

Pears Harvest and Conservation Conditions

Organic pears Beurre d'Anjou cultivar (*Pyrus communis*, family: *Rosaceae*) were harvested at commercial maturity. The fruits were stored in bins (270 kg /each bin; approximately 2000 pears per bin) and were stored in refrigerated chambers (– 1/0 ºC 95% HR) until the biocontrol treatment was done.

Postharvest Treatment at Semi-Commercial Level

The treatment with *V. victoriae* NPCC 1263 was carried out in commercial packing lines located in Centenario, Neuquén, Argentina. One bin of pears per treatment was dipped into a water tank and then sprayed with 25 L of the yeast suspension (~ 10^8 cell mL^{-1}) supplemented with $CaCl_2$ 2% (p/v) [20]. This treatment was performed in 3 runs: Control: water + $CaCl_2$ 2% (p/v), T1*: V. victoriae* NPCC 1263 biomass grown in R1 after 60 days of cold storage + $CaCl_2$ 2% (p/v), T2: *V. victoriae* NPCC 1263 biomass grown in R2 after 15 days of cold storage + $CaCl_2$ 2% (p/v).

After each treatment, the fruits were stored in 36 one-bushel boxes (0.035 m^2) with perforated polyethylene bags in a conventional atmosphere chamber at − 1/0 °C. The percentage natural incidence (%) disease in pears was evaluated after 120 and 180 days.

Biological Control Agent (BCA) Recovery

In order to evaluate the influence of the application system over the BCA viability, the antagonist yeast must be recovered during specific phases of the process [17].

The first sample was collected from the 25 L yeast tank; the second sample was collected from the output of the pulverization tubes. In both cases 5 mL samples were collected in 10 mL sterile tube. Plates GPY (Glucose Yeast Peptone) medium with chloramphenicol were incubated at 4 °C for at least 15 days. Yeast were counted and expressed as colony forming unit per millilitre (CFU mL^{-1}). Experiment was conducted twice by triplicate.

For the recovery of BCA from packed fruit at 'time zero' after yeast application, two fruits were put in bags with 100 mL of CWP and manually moved for 2 min [17]. Aliquots were plated on GPY-agar medium with chloramphenicol (100 mg L^{-1}), and incubated at 4 °C for at least 20 days. Yeasts were counted and expressed as cell/cm^2 by fruit [34]. All the assays were done twice by triplicate.

Data Analysis

The natural incidence of each pathogen was analysed by a generalized linear model (GLM), using the statistical analysis system INFOSTAT, 2020 version. The incidence of different treatments was compared against control treatment ($CaCl_2$ 2%). The effect of the application system on yeast viability (CFU mL^{-1}) was analysed by Tukey Test ($p < 0.05$).

Results

Biomass production for use in semi-commercial conditions was performed in batch fermenter and the collected biomass was refrigerated at 4 °C until the application assay. This yeast reached the stationary phase after 70 h of culture (Fig. 1), with a total biomass production of 5.78×10^8 CFU mL^{-1} for R1 and 7.46×10^8 CFU mL^{-1} for R2 (Table 1)**.** Yeast biomass was produced at different times and stored at 4 ºC in liquid formulation until its use in the packing lines.

To evaluate the effect of physiological conditioning of having grown in cheese whey and cold conservation of fresh yeast biomass (liquid formulation) on the biocontrol efficacy of *V. victoriae* NPCC 1263, the yeasts with different cold storage times (T1 and T2) were applied on Beurre d'Anjou fruits in packing house. After 45 and 90 days of fruit cold storage no disease was observed. In control conditions, the total decay on pear fruit was 0.31% (87% *B. cinerea)* after 120 days of storage in packing house (Fig. 2A). After 180 days of storage, total disease incidence in control fruit reached the 1.56% (Fig. 2B), that is to say, 0.98% (*B. cinerea*), 0.27% (*P. expansum)* and 0.31% (*Cladosporium sp*.).

After 120 days of storage, significant reduction in disease by both *B. cinerea* and *Cladosporium sp.* was observed for two treatments (Fig. 2A). Both T1 and T2 treatment showed control over total incidence after this first evaluation period, 63 and 90%, respectively.

After 180 days of cold storage, *B. cinerea* disease incidence in control fruit remained unchanged at 0.98%, while *P. expansum and Cladosporium sp*. disease incidence was of 0.27 and 0.31%. *V. victoriae* NPCC 1263 in the T1 treatment

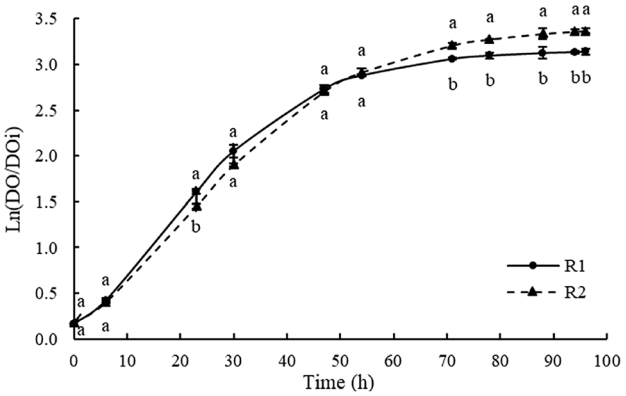

Fig. 1 Growth curves of the two reactor of *Vishniacozyma victoriae* NPCC 1263 grown on a bench-scale bioreactor (15 L). Growth curves were fitted using the Gompertz modified equation. The bars represent standard deviations of the means. Values with the same letter at each particular time of assays are not significantly different at $p = 0.05$ (Tukey test)

Table 1 Kinetic parameters of growth curves obtained and biomass production parameters for two reactors of *Vishniacozyma victoriae* NPCC 1263

Reactor	Kinetic parameters				Biomass production parameters			
	Lag phase [h]	μ [h^{-1}]	A	t [h]	CFU ml^{-1}	X [g/L]	$Y_{X/S}$ [gX/gS]	P_v [$g.L^{-1}.h^{-1}$]
R1	1.28	0.07	3.16	96	5.78E+08[a]	3.65[a]	0.86[a]	0.04[a]
R2	1.56	0.07	3.42	97	7.46E+08[a]	4.62[b]	0.92[a]	0.05[a]

μ specific growth rate, A Ln(DO_f/Do_i), DO_f: final yeas optical density, DO_i initial yeast optical density, X final biomass concentration, $Y_{X/S}$ biomass yield expressed as g biomass (dry weight/g consumed substrate, P_v volumetric productivity

Values within a column followed by the same letter are not significantly different according to Tukey test ($p < 0.05$)

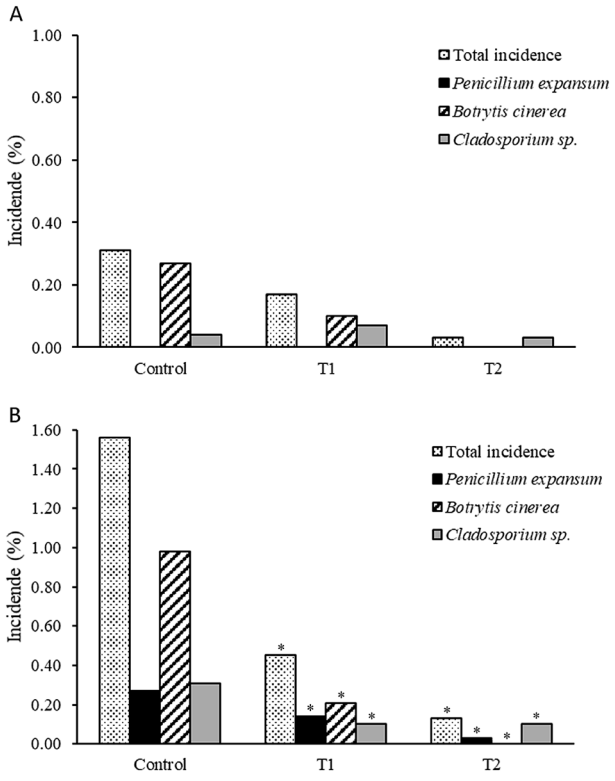

Fig. 2 Effect of *Vishniacozyma victoriae* NPCC 1263 treatments on total natural incidence after 120 (**A**) and 180 (**B**) days of storage at − 1/0 °C and 95% RH in packing houses. Decays on pears evaluated: *Penicillium expansum, Botrytis cinerea* and *Cladosporium* sp. Treatment: T1: biomass with 60 days of conservation; T2: biomass with 15 days of conservation. Asterisks (*) indicate significant differences between treatments and control (water + $CaCl_2$ 2%) according to the generalized linear model of binomial distribution ($p < 0.01$; GLM)

reduced total disease by 71% (78% *B. cinerea*, 68% *Cladosporium sp.* and 48% *P. expansum*), instead T2 treatment reduced it by 92% (100% *B. cinerea* and 80% *P. expansum* and *Cladosporium sp*).

Yeast counting and viability test were done in tank water, in sprayed water through nozzles and surface pears for each of the applied treatments. The initial amount of yeasts applied in T1 treatment in the packing line (approximately 6.15×10^7 CFU mL^{-1}) was reduced by two log units with regard to the initial number, whereas the yeasts in T2 treatment with less conservation time, the reduction was approximately one log unit, since this initially presented 6.63×10^8 CFU mL^{-1} (Table 2).

Table 2 Effect of spray nozzles on the viability and number of antagonist *Vishniacozyma victoriae* on pears surface at a 'time zero'

	Number of CFU		
	Input*	Output**	Pears***
T1	6.15E+07a	4.50E+05b	276
T2	7.50E+07a	5.66E+06b	569

CFU colony forming units on GPY with chloramphenicol, *T1* biomass with 60 days of conservation, *T2* biomass with 15 days of conservation, P_v volumetric productivity

*Input: CFU mL^{-1} in tank water, **Output: CFU/mL^{-1} in sprayed water through nozzles, ***Cell by cm^2 on pear surface

Values within a column followed by the same letter are not significantly different according to Tukey test ($p < 0.05$)

Yeast numbers on pear surface were determined previously for each treatment (Table 2). After yeast application, the pears surface with T2 treatment contained an average of 5.69×10^2 CFU cm^{-2}, which represented 7.28×10^4 cell per fruit approximately, evidencing twice as many cells as those present on the surface of pears treated with T1 (Table 2).

Discussion

It has been frequently reported the performance of the BCA under laboratory conditions, the achievement of BCA-based products has been narrow and only a few products have reached phase of commercialization [12, 35]. The determination of their efficacy in pilot semi-commercial and large-scale tests is very important, considering all the factors intervening in the true postharvest fruit decay [1, 9]. Scaling-up the assays requires important amounts of yeast biomass which must be produced using cost-effective substrates for microbial production to procure high yields, provided they do not impact viability and performance of

the BCA [17, 36]. To obtain effective control, it is usually necessary to spray the fruits with a suspension yeast of 10^7–10^9 CFU mL^{-1} [17, 37–41].

The low-cost biomass production medium employed in this work (Cheese whey powder, CWP) permitted the necessary amount of yeasts for scale-up biocontrol testing against mold infections in pear fruits. Elevated biomass production was archive in this work using 12 L bioreactors, with yields reaching 7.46×10^8 CFU mL^{-1}. These yields were similar than those described for other yeasts using cane molasses as medium: Abadias et al. [30] and Pelinski et al. [42] reported 8×10^8 CFU mL^{-1} and 1.7×10^9 CFU mL^{-1} of *Candida sake* and *Lachancea thermotolerans*, respectively. The same *V. victoriae* NPCC 1263 strain grown in molasses at 20 °C developed 5.75×10^9 CFU mL^{-1} [17]. No significant differences were observed between R1 and R2 in the kinetic and biomass production parameters: CFU, $Y_{X/S}$ and Pv, except in the final biomass concentration (X).

Biomass yield ($Y_{X/S}$) obtained were in accordance with those reported previously [25, 43, 44] and are enough to perform the semi-commercial level testing assays in postharvested pears. The yields achieved in our work (0.92 g_X/g_S), using cheese whey and salts as substrate, were similar or higher than the 0.123 g_X/g_S and 0.78 g_X/g_S biomass yield of *Kluyveromyces marxianus* and *Kluyveromyces fragilis*, respectively [26, 45]. Ferrari et al. [46] evaluated the partial interchange of sugarcane molasses by cheese whey in the production of yeast *Saccharomyces cerevisiae*, reaching a biomass yield of 0.45 g_B/g_S. However, there are few reports on the production of yeast biomass from CWP as a substrate [24, 47].

The production of fresh yeast implies the cold conservation of the yeast biomass until the moment of application in the packaging lines. In this work was evaluated the yeast antagonistic capacity previously conserved under different time conservation (T1 and T2). The total control capacity of postharvest diseases was 92% for T2 and 71% for T1 compared with the results obtained with the control treatment after 180 days of postharvest conservation. These data showed that longer storage of fresh yeast before its application, decreases its antagonistic capacity, since the initial number of cells applied on pear surface in T1 and T2 was very similar but the observed effects were quite different. It might be considered that cold storage can affect the physiological state of the yeast [10, 35].

Biocontrol results presented in this work corresponded to the processing of 270 kg of commercial-quality, organic healthy pear fruit for each treatment. The fruit volume involved in the tests allowed us to evaluate the ability of *V. victoriae* to reduce the incidence of postharvest decays spontaneously induced on pear fruits by fungal inocula. In both treatment whit yeast was maintained pear quality, such as toughness, sugar content, acidity and colour (data not shown), after its application. This suggests that the yeast would serve as an effective biological control in postharvest pears under actual commercial processing conditions (organic production).

Semi-commercial assay of large amounts of fruit should be carried out in packinghouses using the application system. Driving pressure of the pumps and nozzles during spray application possibly generated a shear force which caused the rupture of the cells, reducing their number and viability [17]. However, this aspect has not been generally evaluated in reports employing spray application [39, 48]. In this work, we observed that the passage of cells through the spray peaks lowers the viability of the yeasts and the effect is higher in the yeasts with a longer storage period. The yeasts conserved for 2 months at 4 °C reduced their number in two orders of magnitude and those conserved for 15 days reduced only one order of magnitude with respect to the initial number (~ 10^7 CFU mL^{-1}). In the future, the application of yeasts will be sought by means of a system that avoids the impact caused by spray peaks, with the aim of increasing the number of cells on the treated fruit.

Finally, the yeast number after the application was evaluated on the pears surface and twice as many yeasts were found in treatment T2 compared to treatment T1 (6×10^2 CFU cm^{-2}). This low number of cells at time zero has been shown to be sufficient to achieve high levels of total disease control. Previous result demonstrated that *V. victoriae* is adapted to cold storage as it effectively colonized the pear surface (2.5×10^8 CFU cm^{-2}), increasing its population four-fold during the postharvest conservation period [17]. The results suggest that the yeast fresh application on pears is effective for the control of postharvest decay; however, it decreases its viability when going through the spray peaks, and its antagonistic capacity is reduced with the days of cold storage. Driving pressure of the pumps and the nozzles during spray application could damage the cells by the action of shear forces, reducing their number and viability [17, 27]. Previous studies indicated that a shelf life of several months could be obtained without a significant loss in viability or efficacy of ACB [17]. Oxidative stress is one of the major factors leading to a decrease in the viability of yeast cells stored in a liquid formulation [49]. Further studies to identify the factors responsible for antagonistic activity and survival during storage are expected to lay the foundation for the future development of an organic pear protection product in postharvest.

Conclusion

Overall, this work reports highly advantageous storage properties of *V. victoriae* and encouraging results for the possible development of a novel postharvest organic pear biocontrol

system. Although these are important first steps towards an application, there is ample room for making the production, storage, and application of *V. victoriae* more reliable. Better understanding the factors responsible for survival of *V. victoriae* during production, in the field, and during storage is required in order to develop a reliable and efficacious protection product that is based on this yeast as the active ingredient.

V. victoriae NPCC 1263 produced in a bench-scale facility using a low-cost production medium, was effective in controlling three common diseases affecting pears in semi-commercial conditions. The relevant reduction in the pear decay achieved by the selected yeast would entail a considerable decrease in postharvest economic losses in organic pear production in the Patagonian region.

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Author Contribution DS designed the study and corrected the manuscript. DL analysed data and contributed new methods. IG has carried out the experimental work and wrote the manuscript.

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Data Availability No data have been fabricated or manipulated (including images) to support our conclusions.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The manuscript has neither been published or it is currently under consideration for publication either in whole or in part, by any other journal. The manuscript has not been submitted to more than one journal for simultaneous consideration.

Informed Consent Informed consent was obtained from all individual participants included in the study. Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities—tacitly or explicitly—at the PROBIEN institute where the work has been carried out, before the work is submitted.

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