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PII: S1049-9644(23)00056-7
DOI: https://doi.org/10.1016/j.biocontrol.2023.105203
Reference: YBCON 105203

To appear in: *Biological Control*

Received Date: 20 October 2022
Revised Date: 1 February 2023
Accepted Date: 21 February 2023

Please cite this article as: Gorordo, M.F., Lucca, M.E., Sangorrín, M.P., Statistical Media Optimization Using Cheese Whey Powder for Production of *Vishniacozyma victoriae* Postharvest Biocontrol Yeast in Pears, *Biological Control* (2023), doi: https://doi.org/10.1016/j.biocontrol.2023.105203

STATISTICAL MEDIA OPTIMIZATION USING CHEESE WHEY POWDER FOR PRODUCTION OF *Vishniacozyma victoriae* POSTHARVEST BIOCONTROL YEAST IN PEARS

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ABSTRACT

Vishniacozyma victoriae NPCC 1263 was selected for this work because of the active antagonistic effect over several fungi. A cheese whey powder (CWP) based medium for the maximum biomass production of biocontrol agent was statistically optimized using a central composite design based on response surface methodology (RSM). The optimization condition was found to be in g L^{-1}: CWP 80, KH_2PO_4 10, $SO_4(NH_4)_2$ 1.2 and, under optimum conditions, the biomass production in a 15-L fermenter was about 15 g L^{-1} after 74/78 h cultivation. The efficacy of *V. victoriae* NPCC 1263 for controlling postharvest decay of pear fruits was evaluated in semicommercial packing-house conditions with the application of 10^8 CFU mL^{-1} yeast cells, with $CaCl_2$ (2% w/v) addition in two treatments, fresh and lyophilized-rehydrated biomass on Packham's Triumph pear cultivars. After 150 days of storage, lyophilized-rehydrated biomass treatment reduced the incidence caused by *B. cinerea*, *P. expansum* and *Cladosporium spp*., reaching 36%, 71% and 55% of control respectively. The fresh biomass treatment reached 47% and 58% of control for *B. cinerea* and *P. expansum*, but no control for *Cladosporium spp.* was observed. The two treatment yeasts were able to control decay and colonize the surface of the fruits during the postharvest period, with an estimated increase in the population density of approximately three log units independently of the treatment applied.

KEYWORDS

Biocontrol, Cheese Whey Powder (CWP), statistical optimization, *Penicillium expansum*, *Botrytis cinerea, Vishniacozyma victoriae*.

INTRODUCCIÓN

Cheese whey (CW) is the main liquid by-product obtained from the cheese industry. Each year in Argentina, around 4,200 million liters of milk are processed, this produces 3,800 million liters of whey (Schaller, 2009). This waste causes serious pollution problems since whey is a heavy organic pollutant with high chemical (COD) and biochemical oxygen demands (BOD), with values of 80,000 ppm and 60,000 ppm respectively (Ibarruri & Hernández, 2019).

Actually, cheese whey is used for many researches for the production of baker's yeast (Ferrari et al., 2001), production of single cell protein (Bosso et al., 2019; Ghaly & Kamal, 2004), production of several microorganisms biomass and protein production

(Aouidi et al., 2010; Kanzy et al., 2015; Vamvakaki et al., 2010) and ethanol fermentation (Guimarães et al., 2010; Ozmihci & Kargi, 2007; Yamahata et al., 2020) due to its availability and high carbohydrate content. Moreover, cheese-whey powder (CWP) is a dry concentrated form of CW which maintains the quality of fresh cheese whey for a longer period of time, facilitating manipulation and transport (Bosso et al., 2020). Among the nutrients found in cheese whey, the largest portion corresponds to lactose 75% and protein 15%.

In the Upper Valley of Río Negro and Neuquén provinces (Patagonia, Argentina) the production of pome fruits (apples and pears) is one of the main economic activities, however, fungal spoilage causes significant losses during fruit storage affecting up to 25% of the total production (Nunes, 2012; Reyes-Bravo et al., 2021). *Penicillium expansum* and *Botrytis cinerea* are the most important postharvest pathogens of pome fruits worldwide (Lutz et al., 2020; Nunes, 2012; Usall et al., 2016), although other fungi, e.g. *Alternaria* sp. and *Cladosporium spp.,* are also relevant fruit pathogens (Di Canito et al., 2021; Lutz et al., 2017; Sugar & Basile, 2008).

Studies on postharvest biocontrol showed that postharvest exogenous application of biological control agent (BCA) is an appropriate and reliable approach for the management of fungal diseases during postharvest handling (Carvalho Castro et al., 2020). Yeasts are considered ideal biocontrol agents of post-harvest diseases of fruits and vegetables. They are easy to produce in large- scale using inexpensive culture media; they can colonize and survive for long periods of time on the surfaces of fruits; most genera have GRAS (Generally Recognized as Safe) status; ideally, they are effective against a wide range of pathogens in a wide range of fruits (Al-Maawali et al., 2021; Di Canito et al., 2021; Díaz et al., 2020; Freimoser et al., 2019; Lutz et al., 2020; Pretscher et al., 2018). We have recently isolated and identified the epiphytic *Vishniacozyma victoriae* NPCC 1263, during cold postharvest storage of pear fruits in Argentinian North Patagonia and we tested their efficacy for controlling the postharvest diseases of pears caused by postharvest pathogens under semi-commercial conditions (Gorordo et al., 2022; Lutz et al., 2013, 2020).

To produce BCA on a large scale, it is necessary to consider some criteria. Firstly, the production of biocontrol agents must be commercially viable and easily produced from the point of view of the technical procedures. A low-cost production medium and a high viable biomass productivity are necessary. In addition, formulated products must have adequate shelf life, without losing its biocontrol efficacy. The best

alternative is to subject the yeast to a process that allows a dry powder formulation easy to rehydrate to be obtained, facilitating storage and distribution (Di Canito et al., 2021; Liu et al., 2017; Melin et al., 2011). There are few reports about the efficacy of different freeze-dry methods and the additions of individual cryoprotectant and combination with CWP or powdered skimmed milk (PSM) for conserving viability of cells of postharvest biological control agents. A good protective medium should provide cryoprotection to the cells during the process and a good matrix to allow stability and viability. Polymers, sugars, milk and polyols have shown to provide a good protective effect during freeze-drying (Navarta et al., 2011; Niu et al., 2016).

The optimization of biomass production can be done through different strategies. Optimization processes involve three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Using the mathematical model, the levels of the factors giving maximum response can then be calculated (Vohra & Satyanarayana, 2002). This technique has been used for the commercial production of biocontrol agents using low-cost medium components (Liu et al., 2017; Wang et al., 2011), for example, through the determination of optimal values for processing parameters such as pH, culture conditions, inoculum size, concentration of carbon, nitrogen and phosphor, among others (Singh et al., 2017; Vohra & Satyanarayana, 2002).

The statistical design of experiments is an organized approach that produces more reliable information per experiment than unplanned approaches, this allows the visualization of the interactions between several experimental factors and is essential for the production of biomass, metabolite or enzyme (Armando et al., 2013; Kalil et al., 2000). This methodology involves the selection of a potentially large number of input factors (Screening Designs) and, once the factors that influence this process have been identified, they are used in the optimization of the bioprocess employing Central Composite Designs (CCD) and Response Surface Methodology (RSM) (Galvagno et al., 2011; Montgomery, 2017; Plackett & Burman, 1946).

The aim of this study was to optimize a CWP based culture medium and maximize biomass production of *V. victoriae* NPCC 1263, yeast used as BCA of postharvest pears by defining the major components of the yeast production medium and their optimal concentration through experimental statistical design. We expected to obtain a high cell concentration and to assess the viability and efficacy of lyophilized

formulations to obtain a powdered product that preserves viability and antagonistic effectiveness. After optimization of the medium and lyophilization, experiments were carried out in commercial packing lines to investigate the biocontrol efficacy of the yeast on controlling the decays caused by postharvest pathogens on pears.

MATERIAL AND METHODS

MICROORGANISM

Vishniacozyma victoriae NPCC 1263 (Gen Bank access number MN 848352) was previously isolated from Packham's Triumph pears over a storage period of six months at -1±0°C (Lutz et al., 2012). The yeasts were preserved in glycerol 20% (v/v) and stored at -20°C in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina. The culture was activated in Glucose Peptone Yeast-agar (GPY-agar: glucose 40 g L^{-1}; peptone 5 g L^{-1}; yeast extract 5 g L^{-1}; agar 20 g L^{-1}) plates for 48 h at 20°C.

OPTIMIZATION OF GROWTH PARAMETERS AND MEDIA COMPOSITION

Different initial carbon concentrations (CWP and dextrose), nitrogen ($(NH_4)_2SO_4$), phosphorus (KH_2PO_4) and magnesium ($MgSO_4$) were evaluated in single factor experiments in order to determine maximum biomass production of the yeast after 96 h of culture (Gorordo et al., 2022). These experiments allow us to determine the concentration chosen for statistical optimization. CWP was autoclaved for 20 min at 121°C and it was then filtered in aseptic conditions. The whey supernatant was recovered and used as base media.

The optimal levels of the factors for enhancing the biomass production of *V. victoriae* were determined by response surface methodology (RSM) using Central Composite Design (CCD) with five factors, CWP (X_1), dextrose (X_2), $(NH_4)_2\ SO_4$ (X_3), $MgSO_4$ (X_4) and KH_2PO_4 (X_5).

The design was generated with STATISTICA 8.0 software (Stat-Soft, 2007, Franch), each factor was studied in five different levels (-α, −1, 0, +1, +α) and their values are summarized in Table 1. Twenty seven runs were done and star points (±α) were situated at ± 2 from the center to account for rotatability, which permits to make predictions with the same precision in all directions of the fitted surface (Montgomery, 2017).

Each 250 mL Erlenmeyer flask containing 100 mL of culture medium were inoculated with 1 mL of pre-inoculum (yeast culture growth for 24 h on GPY-agar was resuspended in sterile distilled water to a final concentration of $1x10^5$ cells mL^{-1}. The flask was then incubated at 150 rpm in an orbital shaker at 20°C. Microbial growth was estimated by measuring optical density (OD) at 640 nm and the dry weight was determined once the stationary phase was reached.

The second-degree model used to fit the response to the five factors (x_i and x_j) and the effect of each was as described in Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i \cdot x_i + \sum_{j=1}^{N} \sum_{i=1}^{j-1} \beta_{ij} \cdot x_i \cdot x_j + \sum_{i=1}^{N} \beta_{ii} \cdot x_i^2 \quad (1)$$

where Y is the predicted response, β_0 is the mean/intercept term, β_i are the coefficient for the linear effect of the factors, β_{ij} are the coefficient for the interaction between factors and β_{ii} are the coefficient for the quadratic effect of the factors.

Model fitness was evaluated by the determination R^2 coefficient (percentage of variability in the response that can be explained by the model), F test and close agreement of measured *vs* predicted mean biomass. To determine the variable values where optimal growth parameters were obtained, numerical analysis was carried out to solve a partial derivatives system for the different factors that were significant for the model.

To ensure that the model is valid, a new experiment was carried out in optimized media. Fermentations were carried out at laboratory scale, two in Erlenmeyer of 250 and two in 2000 mL, containing 100 mL and 1000 mL of optimized medium respectively, cultures were incubated at 20°C with agitation at 150 rpm. Two additional batch reactor was carried out in 15 L bioreactor under the optimized conditions. Validation indexes (A, accuracy and B, bias factors) were calculated for the maximum biomass obtained in the process (Mellefont et al., 2003).

DETERMINATION OF CONSERVATION CONDITIONS AND OPTIMAL PROTECTIVE MEDIUM

In order to obtain an optimal protective medium, two different temperatures for cold conservation and three cryoprotectants in combination with two protective agents

for lyophilization was evaluated. The biomass was produced in a 250 mL Erlenmeyer flask containing 100 mL of optimized medium and once the yeast reached the stationary phase, it was recovered as described above. Serial dilutions of this suspension were made and spread plated in duplicate onto the surface of Petri plates. Plates were incubated at 20°C for 48 h and the initial number of colony forming units per milliliter (CFU mL^{-1}) was calculated. Aliquots of yeast samples (1 mL) were added to 2 mL tubes with different cryoprotectants. Viable counts were made after 30 days of cold storage at 4 and -20°C. Three cryoprotectants were evaluated, monosodium glutamate (MSG) (1% w/w), sorbitol (10%w/w) and glycerol (15% w/w).

The best cryoprotectant was then combined with 30% w/w CWP and PSM. The mixtures were frozen at -80°C for 48 h before lyophilization. The lyophilizer (Christ Beta 2-8 LD Plus) was operated at 0.18 mbar for 72 h and -37°C for primary dry and, for final dry, it was operated at 0.14 mbar for 1.5 h and -39°C. Colony forming unit per gramme was determined after lyophilization (considered as 0 time of preservation) and after 30, 60 and 90 days of storage at room temperature. Aliquots of yeast samples powder was rehydrated with sterile distilled water to match the sample volume before dehydration. The dried cells were shaken vigorously for 1 min and then allowed to rehydrate for 1 hour before viability evaluation.

GROWTH IN BATCH REACTOR

The 15 L bioreactor was loaded with 11 L of optimized culture medium previously sterilized and inoculated with 1 L of the 48 h pre-culture. This pre-culture was initiated from 100 mL of medium contained in 250 mL Erlenmeyer flasks and it was inoculated with 1 mL of a loopful of yeast cells ($1x10^5$ cell mL^{-1}). The culture was maintained for 48 h on a rotary shaker (150 rpm) at 20°C. After incubation, the full flask content was added to 900 mL of medium contained in a 2 L Erlenmeyer flask and further incubated under the same conditions.

Microbial growth was estimated by measuring optical density (OD) at 640 nm. During cultivation, pH, temperature and pO_2 were constantly registered. Once the stationary phase was reached, the biomass was recovered by centrifugation (6000 rpm, 20 min at 4 °C). The final CFU mL^{-1} was determined by plating the serial dilutions onto the surface of Petri plates. Plates were stored at 20°C for 48 h and the number of viable cells was determined.

Initial and final concentration of lactose were determined using the dinitrosalicylic acid (DNS) method. The concentration of monosaccharides was evaluated employing a commercial kit (Wiener Lab Glycemic enzymatic), this value was subtracted from the DNS value. Initial and final concentration of ammonium were determined using a commercial kit (Wiener Lab Urea color 2R) respectively.

Yeasts biomass was washed with sterile distilled water (SDW) and produced before the application in semi commercial conditions, due to the COVID pandemic in 2020 the trials were delayed by three months because of external conditions. The collected biomass in reactor 1 was refrigerated at 4°C, instead the biomass produced in reactor 2 was frozen at -80°C for 48 h with the optimal protective medium and then lyophilized to confirm the predicted result. The survival of fresh and dehydrated biomass was determined until their use in the packing lines.

PEARS HARVEST AND CONSERVATION CONDITIONS

Organic pears (*Pyrus communis*) Packham's Triumph cultivar were harvested at physiological maturity from commercial orchards located near Centenario, Neuquén province, Argentina. The fruits were stored in bins (337 Kg /each bin; approximately 1400 pears per bin) and were stored in refrigerated chambers (-1/0 ºC - 95% HR) for three months, until the biocontrol treatment was done.

BIOCONTROL EVALUATION IN SEMI-COMMERCIAL LEVEL

In order to evaluate *V. victoriae* NPCC 1263 biocontrol activity, the application was carried out in commercial packing lines located in Centenario, Neuquén, Argentina. One bin of pears per treatment was dipped into a water dunk tank and then sprinkled (through a drilled PVC tube) with 25 L of the yeast suspension (10^8 CFU mL^{-1}) supplemented with $CaCl_2$ 2% (p/v). This treatment was done in 3 runs: **Control**: water + $CaCl_2$ 2% (p/v), **FB***: V. victoriae* NPCC 1263 growth in optimized culture medium + $CaCl_2$ 2% (p/v), **LRB**: *V. victoriae* NPCC 1263 growth in optimized culture medium, lyophilized and rehydrated + $CaCl_2$ 2% (p/v).

After each treatment, the fruit was dried in a drying tunnel (room temperature) and placed on tray packs which were stacked in one-bushel boxes (0.035 m^2) with perforated polyethylene bags. Each treatment was stored in 23 cardboard boxes in a conventional atmosphere chamber at -1/0°C. The natural incidence of *P. expansum, B.*

cinerea and *Cladosporium spp.* decays in fruits (n=1400) were evaluated after 90 and 150 days for Packham's Triumph fruits. Natural incidence (%) was calculated as: (total number of fruits/ decaying fruits number)/total fruits number.

BIOLOGICAL CONTROL AGENT RECOVERY

In order to evaluate the influence of the application system (drilled PVC tube) over the BCA viability, the antagonist yeasts were recovered in two steps during the process of spraying in the packing house. 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 recipients. Aliquots of 100 µL were inoculated in plates containing GPY medium with chloramphenicol (100 mg L^{-1}). Plates were incubated at 4°C (similar conditions to the storage temperature of the fruit) for at least 15 days (Gorordo et al., 2022). To evaluate the population dynamic of the antagonist from packed fruit after the cold storage period (0, 30, 60, 90, 120 and 150 days), two fruits were put in bags with 100 mL of CWP and agitated for 2 min (Gorordo et al., 2022). The CFU mL^{-1} were quantified as describe above. Yeasts were counted and expressed as log CFU cm^{-2} per fruit (Babic et al., 2012). Experiments were conducted two times in triplicate as described in Lutz et al., (2020).

DATA ANALYSIS

The statistical analysis of the central composite design was done with STATISTICA 8.0 software in order to determine which of the factors are significant to the model. The natural incidence of each decay type was analyzed by a generalized linear model (GLM) assuming binomial distribution of the response variable, using the Statistical Analysis System INFOSTAT, version 2020 (Di Rienzo et al., 2020) The results of different treatments were compared against control treatment (water + $CaCl_2$ 2%).

RESULTS

OPTIMIZATION OF CULTURE CONDITIONS FOR BIOMASS PRODUCTION

As there are no reports on statistical optimization of cultural conditions for biomass production of *V. victoriae*, the main factors involved with strain growth were screened. Different compositions of the culture medium (CWP, dextrose, $(NH_4)_2SO_4$,

$MgSO_4$ and KH_2PO_4) were evaluated in order to delimit quantities of the different factors (based on Gorordo et al., 2022). Taking these data as a reference, a CCD design was employed to determine the optimum levels of the five factors. The 27 runs carried out in the CCD, as well as the observed and predicted results for the responses corresponding to yeast biomass production, are shown in Table 1.

After regression analysis of the data, the resulting second-order polynomial equation was given as Equation 2:

$$Y = 8.25 + 2.53X_1 - 0.83X_3^2 + 0.65X_5 \quad (2)$$

where X_1, X_3 and X_5 are the coded factor of CWP, $(NH_4)_2$ SO_4 and KH_2PO_4 respectively.

Results in this study showed that CWP and monobasic potassium phosphate have a significantly positive linear effect, meaning that maximum development was obtained in cultures with the maximum carbon and phosphate supplementation. On the other hand, ammonium sulphate had a significantly negative quadratic effect, implying that a more demanding nutritional contribution is necessary to obtain the maximum possible biomass production. Dextrose, magnesium sulfate and the interaction between factors were not significant (Table 2). In this way, the model predicted maximum biomass of *V. victoriae* (14.61 g L^{-1}) with the optimal values (coded) of the three significant variables, X_1=2, X_3=0 and X_5=2. Correspondingly, the optimum culture conditions were: CWP 80 g L^{-1}, $(NH_4)_2SO_4$ 1.2 g $L^{-1}$and KH_2PO_4 10 g L^{-1}.

The coefficient of the determination (R^2) was 0.96, indicating that 96% of the variability in the response could be explained by the model. In this case, the R^2 value was high enough to ensure a satisfactory adjustment of the proposed model. The Model F-value of 7.51 demonstrated that the model applied for each regression was significant (p-value < 005).

Figure 1 shows the three-dimensional (3D) surface graphs of each pair of the three significant factors by keeping the third one at its zero level. The maximum biomass production predicted is indicated by the surface in the response surface diagram. The biomass increased with the increase of CWP (X_1) and monobasic potassium phosphate (X_5) to its maximum point at about the maximum levels of the two substances (Figure 1A). Secondly, the biomass increased with the increase of CWP and in the middle level of ammonium sulphate (X_3) (Figure 1B) and increased with the increase of monobasic potassium phosphate and in the middle level of ammonium sulphate, it was predicted at about 1.2 g L^{-1} (Figure 1C). The response surface shows

the same results obtained with the second-order polynomial equation. Figure 1D represents the observed values of the dependent variable with respect to the ones predicted by the model.

MODEL VALIDATION

The accuracy factor (A) provides an indication of the spread of the results within the predicted values. The bias factor (B) is a measure of the relative average deviation of predicted and observed values. A bias factor of 1 and an accuracy factor of 1 represents perfect agreement between observed and predicted values (Bravo et al., 2019; Mellefont et al., 2003).

Six experiments were performed for the validation of the optimal culture medium obtained in the central composite design. The time of biomass collection was 192 h in 250 mL and 2000 mL Erlenmeyer flask, reaching a biomass production of 17.3 g/L ± 0.8 and 17.8 g/L ± 1.0 respectively. For 15 L bioreactor, the time of biomass collection was 74-78 h with a biomass production of 15.04 g/L ± 0.3. For the maximum biomass obtained in these experiments, A factor and B bias were calculated. The values obtained were 1.13 and 0.91 respectively, these results showed a 9 and 13% discrepancy when compared to the observed and the predicted values.

The values obtained in this work are very close to 1, however, a bias factor (B) < 1 indicates the model predicts biomass production lower than the one obtained in these experiments; an accuracy factor (A) > 1 indicates the average estimate between observed and predicted biomass production is less accurate.

SURVIVAL TO COLD CONSERVATION AND LYOPHILIZATION

Three cryoprotectants were evaluated at two cold storage temperatures, 4 and -20 ºC. Biomass conservation at 4°C showed low viability values with all cryoprotectants at -20°C (Table 3). Monosodium glutamate at -20ºC was chosen, showing 37.9% of biomass survival after 30 days of conservation, while glycerol and sorbitol showed 4.6% and 6.7% of viability respectively (Table 3).

In order to evaluate different protective agents, MSG was tested with CWP and PSM. The initial biomass before lyophilization was $5.74x10^{13}$ CFU and, after the process, it was $1.7x10^{11}$ using PSM + MSG and $2.4x10^{11}$ using SWP + MSG. Monosodium glutamate in combination with CWP showed significantly better yeast survival compared to its combination with SPM after 90 days of conservation (Table 4,

Figure 2). A marked difference was observed between the efficacy of the different protective agents in maintaining biomass viability throughout the storage. After 90 days of conservation at room temperature, MSG in combination with CWP reached 60% biomass viability, whereas, when using MSG in combination with SPM, the viability was 43%.

YEAST BIOMASS PRODUCTION

Biomass production was performed in two runs using the bioreactor. The yeast reached the stationary phase after 74 h of culture in reactor 1, with a total biomass production of $1.08x10^9$ CFU mL^{-1}; reactor 2 produced $3.5x10^9$ CFU mL^{-1} in the 78 h of cultivation. The pO_2 and pH profile were similar in both reactors (Figure 3 A-B). The kinetic parameters and biomass production parameters were determined (Table 5). The kinetic parameters were similar in both reactors, although within biomass production parameters, significant differences were found only in biomass yield ($Y_{X/S}$).

The biomass produced in reactor 2 was lyophilized using the combination defined above (MSG 1% w/w + CWP 30% w/w). We observed that the survival decreased in one magnitude order after 300 days of conservation (Table 6), a sufficient quantity to carry out the treatment at semi-commercial level ($9.80x10^8$ CFU g^{-1}).

SEMI-COMMERCIAL BIOASSAYS

To evaluate the effect of different conservation biomass on the biocontrol efficacy of *V. victoriae* NPCC 1263, fresh and lyophilized-rehydrated biomass (FB and LRB) were applied on Packham's Triumph fruits in the packing house. After 90 and 150 days of fruit cold storage, the biocontrol efficacy was determined.

The total incidence decay on pear fruit in control condition was 1.23% after 90 days of storage in the packing house (Figure 4A), both treatments reduced the total incidence: 6 % for FB and 52 % for LRB. The LRB treatment controlled 100% incidence caused by *B. cinerea*, 65% incidence caused by *P. expansum*, but no control for *Cladosporium spp*. was observed (Figure 4A).

After 150 days of storage, total disease incidence reached 4.06% (Figure 4B). LRB treatment reduced the incidence caused by *B. cinerea*, *P. expansum* and *Cladosporium spp*., reaching 36%, 71% and 55% of control respectively. The FB treatment reached 47% and 58% of control for *B. cinerea* and *P. expansum*, but no control for *Cladosporium spp.* was observed (Figure 4B).

YEAST COLONIZATION OF FRUIT SURFACE IN COLD STORAGE

Yeast counting and viability tests were done in tank water and in sprayed water through drilled PVC application system. The initial amounts of yeasts applied in FB and LRB treatment in the packing line were reduced by one log unit with regard to the initial number in tank water. There was no significant reduction in the viability when the yeast passes through the application system (Table 7). Before yeast application with both treatments, the pear surface contained an average of $4.77x10^4$ CFU per fruit with FB and $6.45x10^4$ CFU per fruit with LRB. Yeast cell numbers were determined on pear surface for each treatment. Exponential growth was observed in LRB during the first 30 and 150 days of cold conservation, increasing the number of cells per surface area in 2.9 log units (Figure 5). However, in FB treatment the yeast cell number grew slowly during all commercial storage and remained similar to the number of cells per surface area on last estimation at 150 days, increasing in approximately 2.5 log units.

DISCUSSION

In this work, experimental designs as a whole proved to be adequate for the optimization of biomass production intended to be used at an industrial scale, because it allowed the selection of the economically most important production factors. Response surface methodology analysis indicated that the optimized concentrations for the main substrates as well as for other operating conditions for yeast production determined in shaken-flask cultures could be applied at a larger scale.

Using the CCD and RSM analysis, it was possible to determine optimal operating conditions to obtain a high biomass concentration preventing yield loss. Both analysis retrieved important information about the culture sources evaluated (Bardhan et al., 2021; Bravo et al., 2019). The value of R^2 was 0.96, enough to ensure the validity of the model. This was proved by fitting the values of the factors in the model equation and by actually carrying out the experiments (Vohra & Satyanarayana, 2002). Following the CCD and RSM, the biomass production of *V. victoriae* NPCC 1263 was increased to 9.51 gL^{-1} in the initial cultures, 11.96 g L^{-1} in 250 mL shaken flask system and 15.3 gL^{-1} in the 15-L fermenter. Nevertheless, to date, no report in the literature has studied the nutritional requirements of this biocontrol agent, and there is no optimized medium for its growth and biomass production by statistical design of experiments.

The time of biomass collection in Erlenmeyer flask was longer than in the bioreactor, which resulted in an overall 2.2-fold increase compared with that using the original conditions in shake-flask cultivation.

As a result, the medium optimized with CWP is a suitable culture medium to produce a valuable product such as yeast biomass. CWP is an excellent source of functional proteins, peptides, lipids, vitamins, minerals, and lactose (Bosso et al., 2020; Prajapati et al., 2017; Ryan & Walsh, 2016; Vamvakaki et al., 2010). In addition, some authors demonstrated that the addition of $(NH_4)_2SO_4$ and KH_2PO_4 increased biomass yield and biomass production (Cristiani-Urbina et al., 2000; Galvagno et al., 2011; Vamvakaki et al., 2010). Our results showed that CWP (80 g L^{-1}), monobasic potassium phosphate (10 g L^{-1}) and ammonium sulphate (1.2 g L^{-1}) had a significant effect in biomass production.

The yeast biomass obtained in this work achieved $3.5x10^9$ CFU mL^{-1}, this value is similar to those described for other yeasts using cane molasses as medium: Abadias et al. (2003) and Pelinski et al. (2012) reported $8x10^8$ CFU mL^{-1} and $1.7x10^9$ CFU mL^{-1} of *Candida sake* and *Lachancea thermotolerans*, respectively. The same *V. victoriae* NPCC 1263 strain grown in molasses at 20°C developed $5.75x10^9$ CFU mL^{-1} (Lutz et al., 2020).

According to our results in bioreactors sugar concentration was not a limiting nutrient in the assayed conditions. However, CWP concentration had a significative effect on biomass production. This could be due to the CWP composition (salts, proteins and small amounts of vitamins) that could have a positive effect on the biomass production. There are few reports on the production of yeast biomass from CWP as a substrate (Barba et al., 2001; Bosso et al., 2020; Prajapati et al., 2017). The biomass yield coefficient ($Y_{X/S}$) obtained in this work (0.44-0.77 g_B/g_S) was similar or better than those reported previously in literature, with different carbon and nitrogen sources (Ariyanti & Hadiyanto, 2013; Fonseca et al., 2007; Kurniawati et al., 2022; Bosso et al., 2019; Ferrari et al., 2001; Cristiani-Urbina et al., 2000; Schnierda et al., 2014). The differences observed in the biomass yield coefficient could be due to the CWP sterilization and filtration processes that probably alter the composition of the whey referred to the availability of some micronutrients.

To use a biological agent as a commercial product it is essential to improve its formulation. This is necessary to provide the product in a suitable form, and to optimize the efficacy, stability, safety and ease of application of the product. In previous works

we observed that *V. victoriae* NPCC 1263 decreased its viability and antagonistic capacity was reduced within the days of cold storage in a liquid formulation (Gorordo et al., 2022). Dehydrated cells have the advantage of not requiring cool temperatures during storage, distribution and application, thus reducing the cost of its production. The use of cryoprotectants may have influenced the viability of dehydrated yeast, three cryoprotectants have been evaluated for their protective effects for cold conservation (Abadias et al., 2001; Costa et al., 2000; Coulibaly et al., 2010). Monosodium glutamate (1% w/w) was chosen and then evaluated in combination with CWP and PSM (30% w/w) (Pietrowski et al., 2015; Li & Tian, 2007; Navarta et al., 2011). As a result, CWP 30% was used as a protective agent as it was the main substrate growth medium and, in combination with MSG 1%, it caused a significant increase in viability along the time. This suggests the importance of the impact of the growth medium on viability throughout storage of dried yeasts (Carvalho et al., 2003; Morgan et al., 2006). A culture with more than 10^7 CFU mL^{-1} is generally recommended to ensure a sufficient amount of cells remaining after the freeze drying process, long term storage and recovery for rehydration and propagation of the strain (Morgan et al., 2006). Some authors suggest that a sufficient number can be about 0.1% of the original biomass concentration (Day & Stacey, 2007; Pietrowski et al., 2015).

To evaluate the antagonistic capability of *V. victoriae*, we applicate fresh and dehydrated biomass. In both cases, fresh and lyophilized biomass reached concentrations over the 10^8 CFU mL^{-1}, this value is similar than those reported by other authors to obtain an effective control (Janisiewicz et al., 2008; Lutz et al., 2020; Marsico et al., 2021).

The results of the antagonistic activity in semi-commercial bioassays demonstrated that *V. victoriae* was able to inhibit the growth of *B. cinerea, P. expansum* and *Cladosporium sp.* at different percentages after 150 days of cold storage. The total biocontrol of postharvest diseases was better in LRB than in FB. The LRB treatment reached 36% and 71% of control for *B. cinerea* and *P. expansum* while FB treatment reached 47% and 58% of control for the same pathogens. *Cladosporium sp.* was only controlled by the LRB treatment, reaching 54% of control. Our results suggest that both fresh biomass and lyophilized rehydrated biomass were effective in reducing significantly the postharvest diseases and that they are comparable to the results obtained in the same and other fruits (Gorordo et al., 2022; Janisiewicz et al., 2008; Long et al., 2006; Lutz et al., 2020).

Due to the COVID-19 pandemic, the semi-commercial assay in 2020 was carried out three months after the harvest and cold storage. The 2019 assay was done when the fruit had only one month of cold storage and a low physiological maturity (Gorordo et al., 2022). The mean control percentage in 2020 assay (53%) was lower than in 2019 assay (80%). The complexity of the antagonistic relationship established within the host-antagonist-pathogen system explains the differences between the levels of protection in the assays, because they are modulated by the maturity of the pear, the pathogen susceptibility and the storage time (Jijakli & Haïssam, 2011; Leyva Salas et al., 2017) . The evidence suggests that it is desirable to apply postharvest treatments as early as possible, to prevent pathogenic infection of fruit ensuring optimal protection against fruit rot (Jijakli & Haïssam, 2011; Lutz et al., 2020).

The application system is an important aspect to be considered during the evaluation of a BCA in semi-commercial scale, there are few reports about this phenomenon affecting the cell viability. Chand-Goyal & Spotts (1997) report a 10% loss in CFU mL^{-1} after passage through a controlled droplet line-spray applicator. Other works evidence that sprayed nozzles application system reduced the total number of cells in at least 2 magnitude orders (Gorordo et al., 2022; Lutz et al., 2020). This suggests that the driving pressure of nozzles during spray application possibly generated a shear force which caused the rupture of the cells, reducing their number and viability. Our results showed that the application system used in this work reduced the total number of cells only in one magnitude order, since it avoids the spray nozzles effect.

In a previous work, fresh culture of the antagonistic yeast grown in a molasses based medium, showed an exponential growth during the first 90 days over Packham's Triumph pears surface. The total cells number per square centimetre of fruit increased in approximately 3 logarithmic units. (Lutz et al., 2020). In this work, during cold storage, the yeast grew in approximately 3 log units in the same pear cultivars, the yeast biomass reached approximately 5×10^3 CFU cm^{-2} (around $8x10^5$ CFU per fruit) independently of the treatment applied (FB or LRB). The number of CFU cm^{-2} in LRB treatment increased during the cold storage but more slowly than FB treatment. (Figure 5). These results demonstrated that after application the yeasts were able to survive, grow and colonize the fruit surface at -1/0 °C. This density has shown to be enough for the protection against pathogens affecting fruits during cold storage (Lahlali & Jijakli, 2009; Lutz et al., 2020).

The LRB biomass was able to establish better on pears surface and to achieve higher percentages of control over *P. expansum* and *Cladosporium* sp. than FB treatment. Yeast cells exposed to stress develop tolerance to higher doses of the same stress, and also to stress caused by other agents, like the culture medium in which yeasts were produced, the collection moment and the down-stream processes (Estruch, 2000; Galvagno & Cerrutti, 2004). This phenomenon, known as cross-protection, suggests the existence of an integrating mechanism that senses and responds to different forms of stress (Galvagno & Cerrutti, 2004; Harish & Osherov, 2022) . In this case, the better establishment and growth of LRB biomass could be due to the osmotic shock tolerance with cold adaptation before lyophilization. Drying and subsequent rehydration subjects the microbe to high osmotic pressure gradients that can damage or kill the cells. Therefore, drying and stabilizing biological control agents require to develop cost-effective methods of drying and stabilizing to produce a product with a suitable shelf-life (Dunlap et al., 2007; Liu et al., 2011; Stefanello et al., 2019).

V. victoriae NPCC 1263 has the capability to grow using different industrial wastes and salts (Lutz et al., 2020, Gorordo et al., 2022), in both optimized and non-optimized culture medium. These results provide new information on culture medium optimization to grow yeast antagonists in order to improve biomass production and biocontrol efficacy.

Our data suggest that *V. victoriae* is an effective BCA in controlling the most common diseases affecting pear cultivars in semi-commercial assays, probably due to the competition for nutrients and space. Furthermore, these results allow us to conclude that CWP is a suitable substrate to produce biomass in a bench-scale bioreactor with considerable advantages. We have also shown that the experimental statistical design is a powerful tool to improve their use. Implications for the large-scale production of biocontrol agents must be considered. These trials meet consumer expectations in relation to organically-produced food and also encourages the development of eco-friendly approaches (Agarbati et al., 2022; Droby & Wisniewski, 2018).

ACKNOWLEDGEMENTS

We would like to thank La Deliciosa S.A. (Centenario) who kindly provided us with fruit, packing materials, staff assistance, and cold storage units to carry out this work. This work was supported by funds from ANPCyT PICT 2014-2780, Universidad Nacional del Comahue and CONICET PUE0067.

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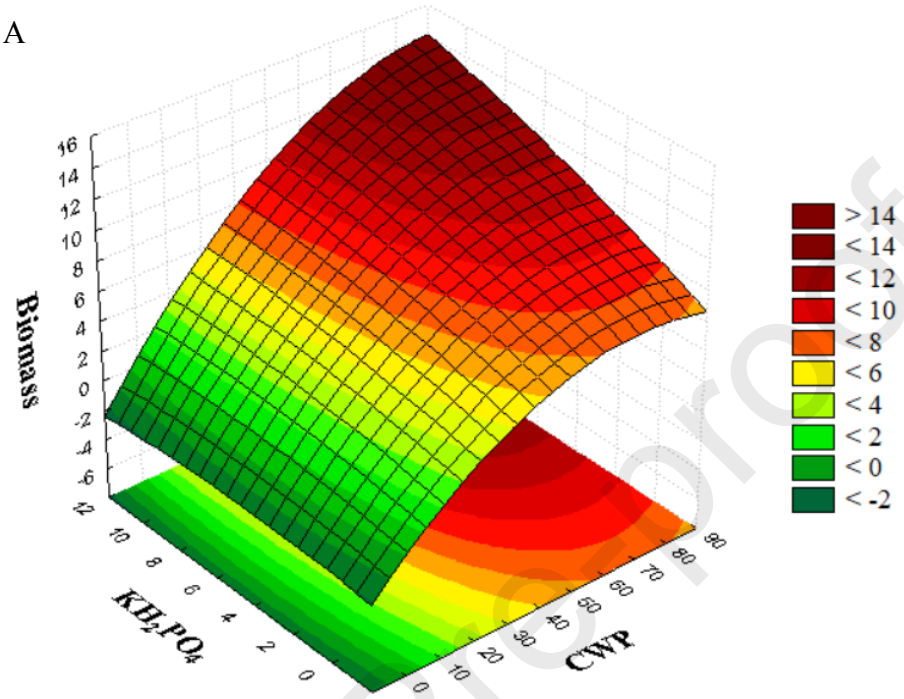

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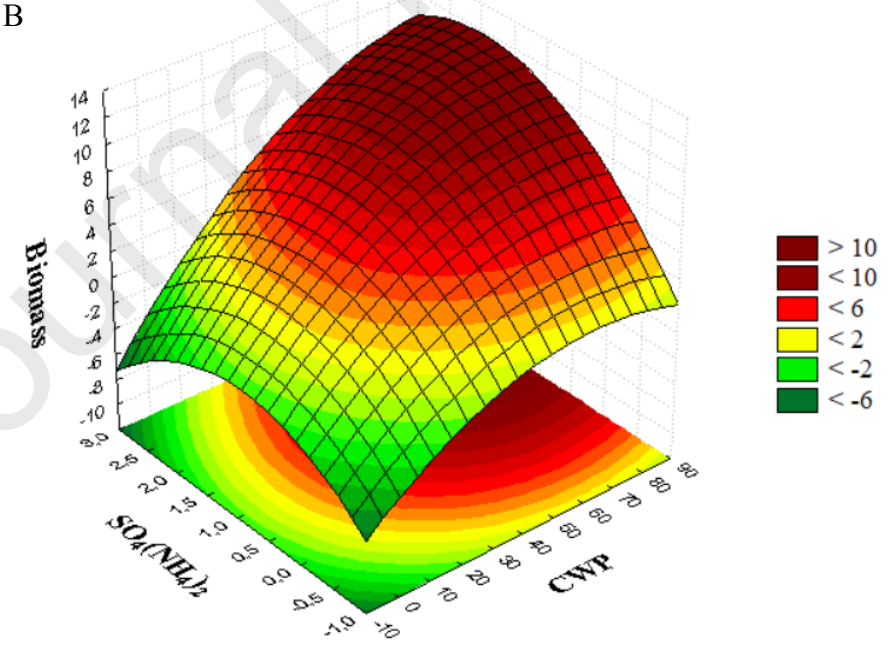

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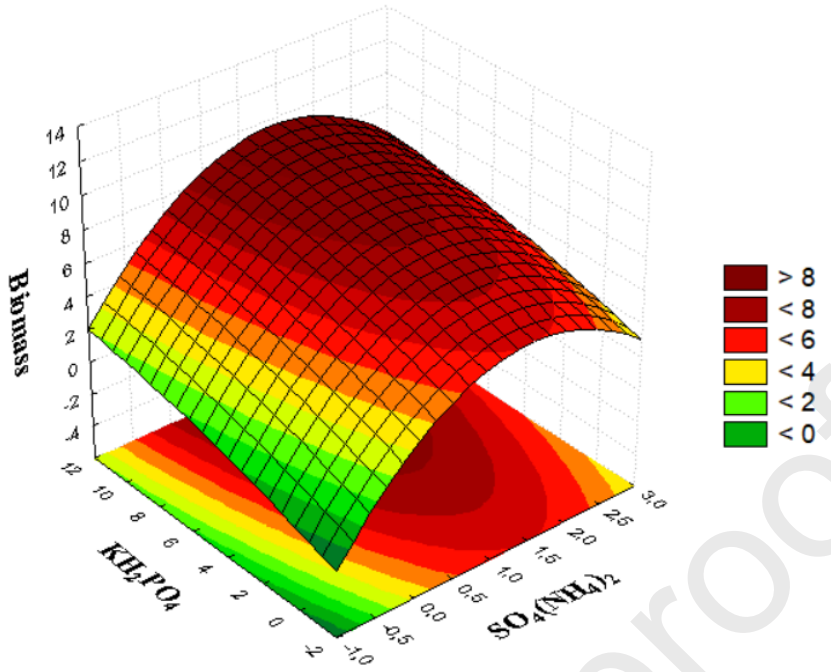

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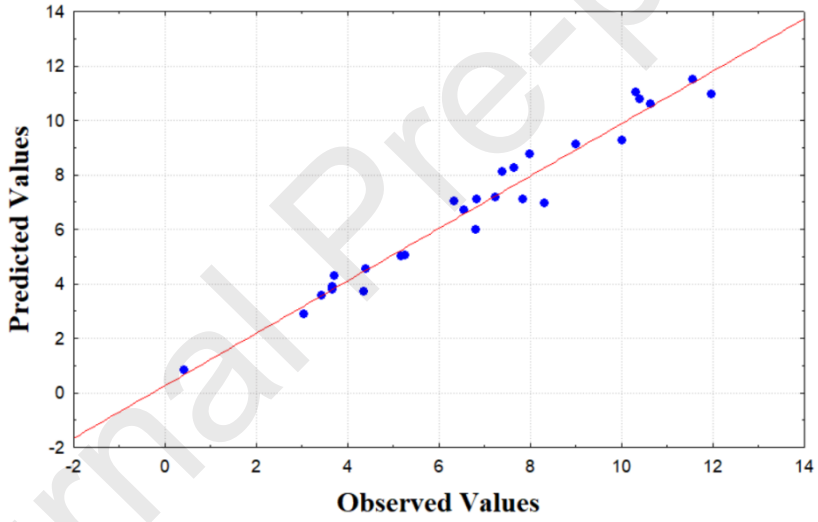


Figure 1: Fitted response surface for *Vishniacozyma victoriae* NPCC 1263 analyzing biomass production and predicted vs. observed values. Modelled CWP (g L^{-1}), $SO_4(NH_4)_2$ (g L^{-1}) and KH_2PO_4 (g L^{-1}) for maximum biomass production reached (g L^{-1}).

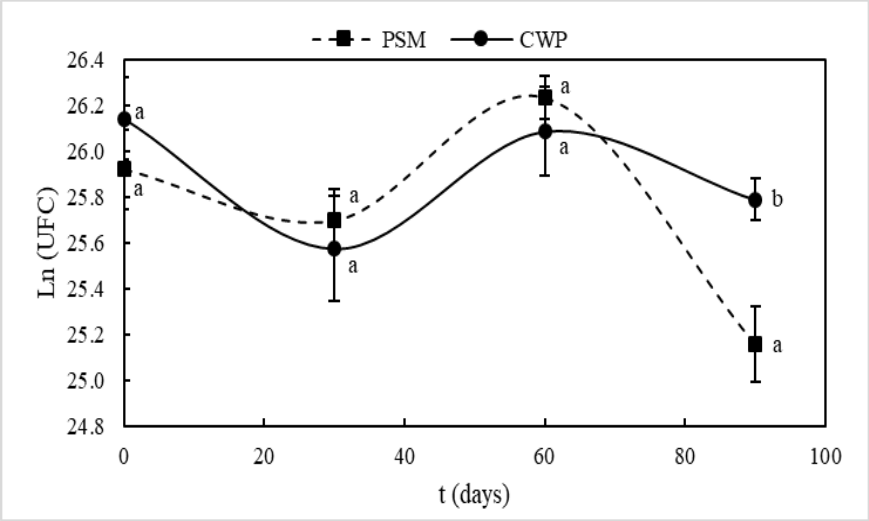
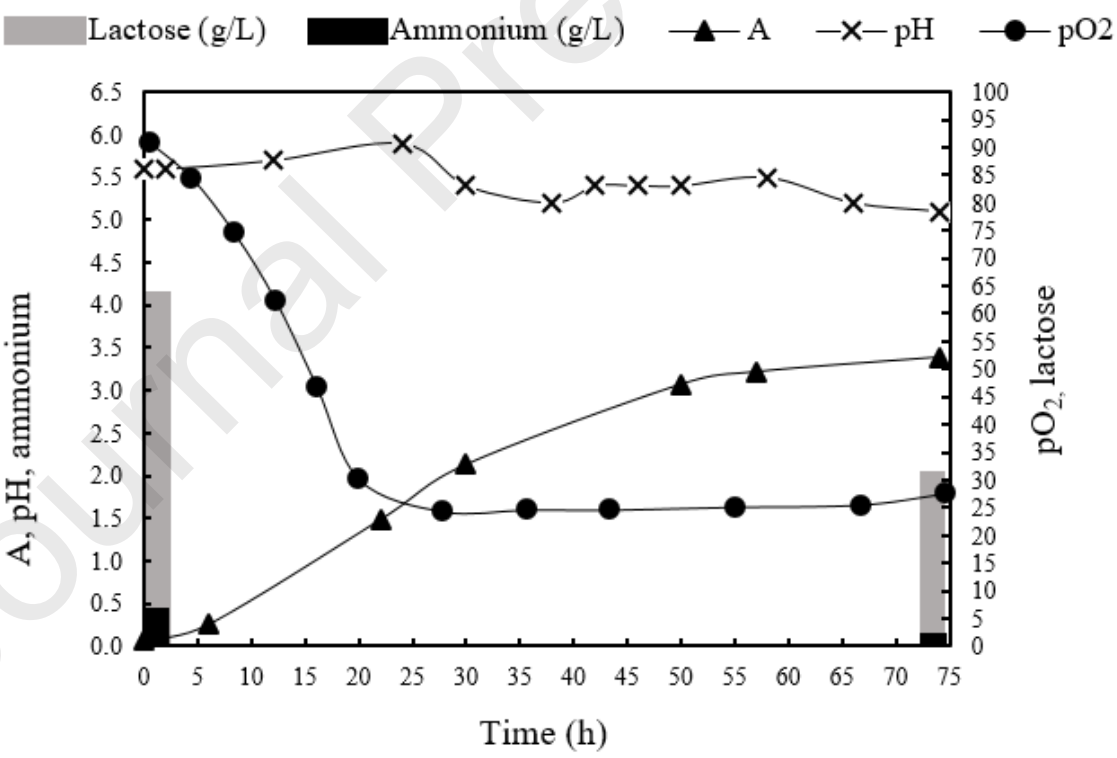


Figure 2: Cellular viability preserved by lyophilization after different periods of storage using two combinations of protective agents.

Averages between curves with different letters indicate significant differences ($p < 0.05$) in the populations.

A



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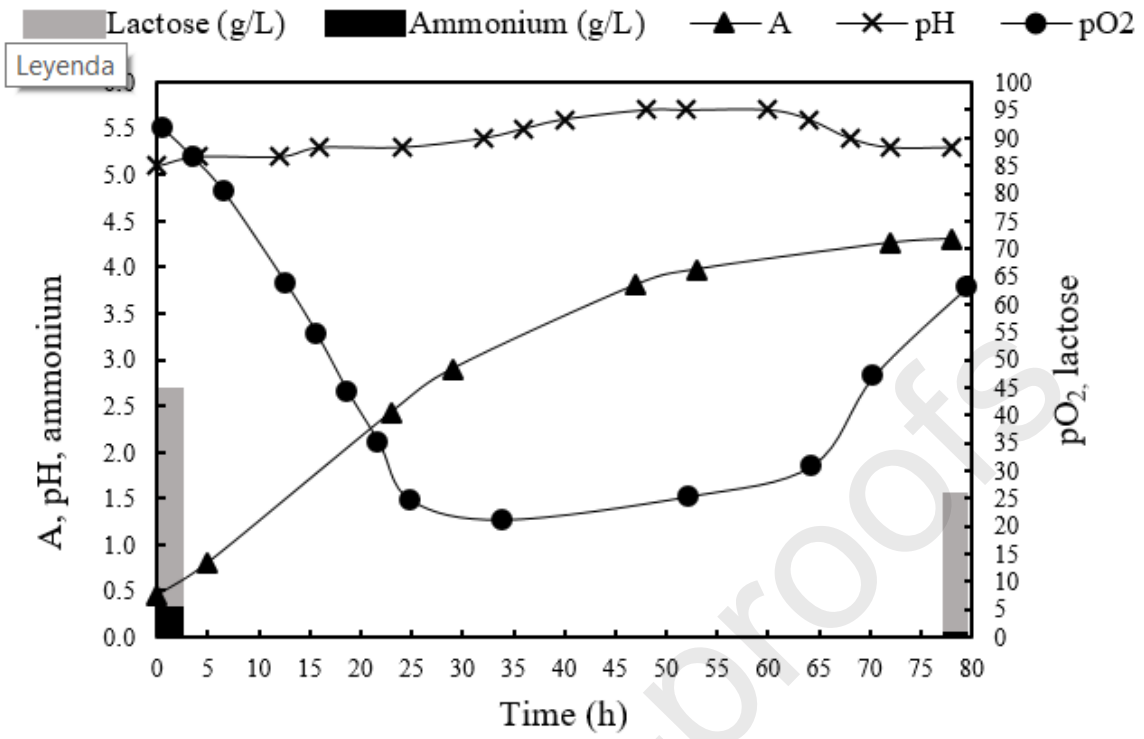

Figure 3: Growth curves of the two reactors of *Vishniacozyma victoriae* NPCC 1263 grown at 20°C on a bench-scale bioreactor (15 L). Evolution predicted by the modified Gompertz model (R^2= 0.99). (A) Reactor 1. (B) Reactor 2.
A: $Ln(DO_f/Do_i)$, DO_f: final yeast optical density, DO_i: initial yeast optical density.
pO_2: percentage of partial pressure oxygen

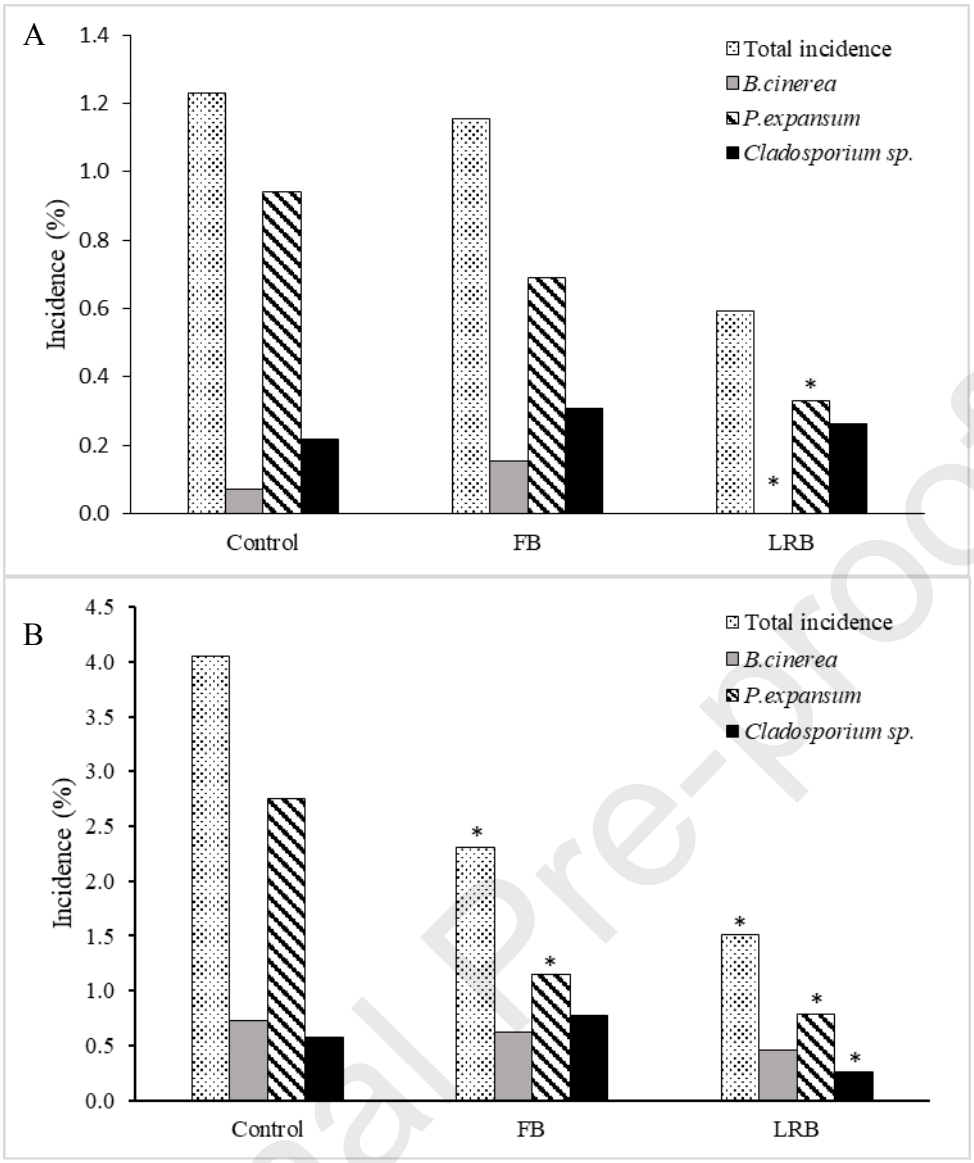

Figure 4: Effect of yeast treatments on total natural incidence after 90 (A) and 150 (B) days of storage at −1/0 °C and 95% RH in packing houses. Decays on pears evaluated: *Penicillium expansum, Botrytis cinerea* and *Cladosporium* sp. Treatment: FB: fresh biomass; LRB: lyophilized and rehydrated biomass. 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).

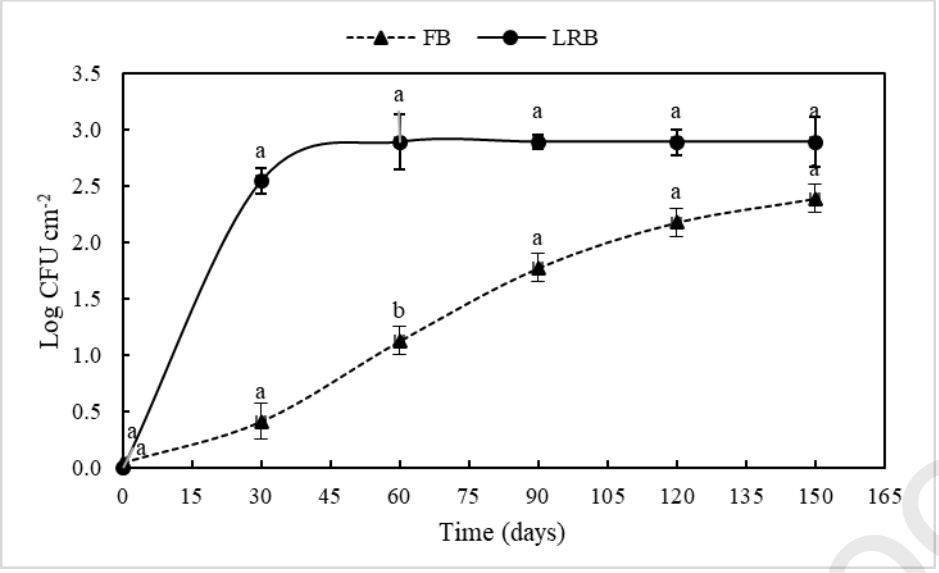

Figure 5: Yeast growing on pear surface during commercial storage (-1/0°C and 95% RH) for FB and LRB treatment, predicted by the modified Gompertz model. Different letters at specific time points indicate significant treatment differences ($p < 0.05$; Tukey test).

Table 1: Central Composite Design (CCD) and results for optimization of biomass production (X_1, CWP; X_2, dextrose; X_3, $SO_4(NH_4)_2$; X_4, $MgSO_4$; and X_5, KH_2PO_4).

	Coded level					Real level (g/L)					Biomass (g/L)		t* (h)
Run	X_1	X_2	X_3	X_4	X_5	X_1	X_2	X_3	X_4	X_5	Observed	Predicted	
1	-1	-1	-1	-1	1	20	4	0.4	0.05	7.5	5.25	5.04	189
2	-1	-1	-1	1	-1	20	4	0.4	0.25	2.5	3.67	3.78	170
3	-1	-1	1	-1	-1	20	4	2	0.05	2.5	3.05	2.88	170
4	-1	-1	1	1	1	20	4	2	0.25	7.5	3.71	4.29	161
5	-1	1	-1	-1	-1	20	20	0.4	0.05	2.5	4.36	3.72	189
6	-1	1	-1	1	1	20	20	0.4	0.25	7.5	3.44	3.56	189
7	-1	1	1	-1	1	20	20	2	0.05	7.5	5.18	5.01	137
8	-1	1	1	1	-1	20	20	2	0.25	2.5	4.41	4.56	170
9	1	-1	-1	-1	1	60	4	0.4	0.05	7.5	7.23	7.16	161
10	1	-1	-1	1	-1	60	4	0.4	0.25	2.5	6.33	7.01	137
11	1	-1	1	-1	-1	60	4	2	0.05	2.5	10.39	10.79	189
12	1	-1	1	1	1	60	4	2	0.25	7.5	7.39	8.11	161
13	1	1	-1	-1	-1	60	20	0.4	0.05	2.5	11.56	11.50	221
14	1	1	-1	1	1	60	20	0.4	0.25	7.5	6.83	7.09	137

				-	-	6	2		0.0	2.			
15	1	1	1	1	1	0	0	2	5	5	10.64	10.61	189
						6	2		0.2	7.			
16	1	1	1	1	1	0	0	2	5	5	10.32	11.05	170
	-						1	1.	0.1				
17	2	0	0	0	0	0	2	2	5	5	0.42	0.83	170
						8	1	1.	0.1				
18	2	0	0	0	0	0	2	2	5	5	11.96	10.95	170
		-				4		1.	0.1				
19	0	2	0	0	0	0	0	2	5	5	7.84	7.12	170
						4	2	1.	0.1				
20	0	2	0	0	0	0	8	2	5	5	9	9.12	161
			-			4	1		0.1				
21	0	0	2	0	0	0	2	0	5	5	3.68	3.89	189
						4	1	2.	0.1				
22	0	0	2	0	0	0	2	8	5	5	6.8	6.00	161
				-		4	1	1.					
23	0	0	0	2	0	0	2	2	0	5	7.99	8.76	137
						4	1	1.	0.3				
24	0	0	0	2	0	0	2	2	5	5	8.32	6.95	189
					-	4	1	1.	0.1				
25	0	0	0	0	2	0	2	2	5	0	6.56	6.69	161
						4	1	1.	0.1	1			
26	0	0	0	0	2	0	2	2	5	0	10.01	9.28	221
27						4	1	1.	0.1				
(C)	0	0	0	0	0	0	2	2	5	5	7.65	8.25	170

*Time at the beginning of the stationary phase.

Table 2: Regression coefficients estimated by means of the ANOVA analysis for the response (biomass production g L^{-1}), obtained in the CCD with five different factors.

	Value	SD	p-value
β_0-Mean/Intercept	**8,2500**	**1,0473**	**0,0002**
β_1-lineal CWP	**2,5292**	**0,2424**	**0,0000**
β_{11}-quadratic CWP	-0,5900	0,2969	0,0941
β_2-lineal Dextrose	0,5017	0,2424	0,0839
β_{22}-quadratic Dextrose	-0,0325	0,2969	0,9164
β_3-lineal $SO_4(NH_4)_2$	0,5275	0,2424	0,0725
β_{33}-quadratic $SO_4(NH_4)_2$	**-0,8275**	**0,2969**	**0,0317**
β_4-lineal $MgSO_4$	-0,4542	0,2424	0,1101
β_{44}-quadratic $MgSO_4$	-0,0988	0,2969	0,7507
β_5-lineal KH_2PO_4	**0,6458**	**0,2424**	**0,0373**
β_{55}-quadratic KH_2PO_4	-0,0663	0,2969	0,8308

Table 3: Survival to freezing treatment and lyophilization using three cryoprotectants at two cold storage temperatures after 30 days of cold conservation

	Viability (%)		
	MSG	Sorbitol	Glycerol
Temperature	(1% w/w)	(15%w/w)	(10% w/w)
4°C	10%	0.6%	1.2%
-20°C	37.9%	6.7%	4.6%

Table 4: Percentage of cellular viability preserved by lyophilization after different periods of storage using two combinations of protective agents.

	Viability (%)			
	MSG (1%) + CWP (30% w/w)		MSG (1%) + PSM (30% w/w)	
Time (days)	Mean	SD ±	Mean	SD ±
0	0.39[a]	0.06	0.32[a]	0.05
30	0.22[a]	0.05	0.25[a]	0.03
60	0.37[a]	0.07	0.43[a]	0.04
90	0.27[a]	0.02	0.15[b]	0.02

Averages with different letters indicate significant differences ($p < 0.05$) in the populations.
MSG: monosodium glutamate
CWP: cheese whey powder
PSM: powdered skimmed milk

Table 5: 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	CFU mL^{-1}	X ($g\ L^{-1}$)	$Y_{X/S}$ (g_B/g_S)	P_v ($g.L^{-1}.h^{-1}$)
1	3.47	0.09	3.45	$1.08x10^{9a}$	14.77^a	0.44^a	0.20^a
2	4.41	0.09	4.28	$3.50x10^{9a}$	15.30^a	0.77^b	0.20^a

μ: specific growth rate; A: $Ln(DO_f/Do_i)$, DO_f: final yeast 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 ($p < 0.05$).

Table 6: Cellular viability of *Vishniacozyma victoriae* NPCC 1263 preserved by lyophilization after different periods of storage using the selected protective medium.

Time (month)	CFU g^{-1}	SD ± (CFU g^{-1})	CV (%)
0	$2.33x10^{9a}$	$2.12x10^6$	0.09
3	$9.80x10^{8a}$	$2.76x10^7$	2.82
8	$1.50x10^{9a}$	$4.24x10^7$	2.83
10	$5.15x10^{8a}$	$7.78x10^6$	1.51

Average of three replicates. Different letters in the same row indicate significant differences ($p < 0.05$) in the populations. SD: Standard deviation. CV: Coefficient of variation.

Table 7: Effect of drilled PVC tube on the viability and number of antagonist *Vishniacozyma victoriae* on pears surface at a 'time zero'.

	Number of CFU		
	Input*	Ouput**	Pears***
FR	$3.73x10^{7a}$	$2.50x10^{6a}$	342
LRB	$1.69x10^{7a}$	$5.72x10^{6a}$	462

CFU: colony forming units on GPY with chloramphenicol.

* Input: CFU mL^{-1} in tank water.

** Output: CFU mL-1^{-1} in sprayed water through drilled PVC application system.

***Cell by cm^2 on pear surface

FR: fresh biomass.

LRB: lyophilized and rehydrated biomass.

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

Highlights

- The statistical optimization improves the cheese whey powdered-based medium.
- Fresh and lyophilized biomass controls pears decay on semi-commercial scale.
- *Vishniacozyma victoriae* was able to colonize the pear surface during cold storage.
- Is desirable to apply biomass as early as possible ensuring optimal protection.
- The use of cryoprotectants improves the viability of dehydrated yeast.

Editorial Office

We submit the manuscript as an article: “STATISTICAL MEDIA OPTIMIZATION USING CHEESE WHEY POWDER FOR PRODUCTION OF Vishniacozyma victoriae POSTHARVEST BIOCONTROL YEAST IN PEARS.” with authors M. Florencia Gorordo, M. Ester Lucca and Marcela P. Sangorrín.

The authors declare that they have no conflict of interest. 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. Dra. Sangorrín designed the study and corrected the manuscript. Dra. Lucca analysed data and contributed new methods. Ing. Gorordo has carried out the experimental work and wrote the manuscript. 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. No data have been fabricated or manipulated (including images) to support our conclusions.

We confirm compliance with the requirements given under Ethics and Conflict of Interest.

Sincerely,

Dra. Marcela Paula Sangorrín

Freimoser F, Rueda-Mejia M, Tilocca B, Migheli Q (2019) Biocontrol yeasts: mechanisms and applications. World J Microbiol Biotechnol 35:.

Lutz MC, Lopes CA, Rodriguez ME, et al (2013) Effi cacy and putative mode of action of native and commercial antagonistic yeasts against postharvest pathogens of pear. Int J

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