GROWTH KINETICS OF THE BIOCONTROL AGENT
METSCHNIKOWIA ANDAUENSI S PBC-2 IN SUBMERGED BATCH CULTURES

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ABSTRACT

Limited success has been achieved in the large scale production of postharvest biocontrol agents. High production cost and low productivity are the key factors that hindered progress. Thus to be a practical alternative to fungicides, the production of the biocontrol agent M. andauensis PBC-2 must be optimized. M. andauensis was grown in four different media commonly described in yeast production. The highest viable cell was observed in YPD medium. The effect of pH, temperature and yeast extract and peptone concentrations was tested in 250-ml flasks. Maximum cell growth was achieved at pH 6.5 at 25 and 30 ºC nonetheless no significant differences were noted between temperatures. Yeast extract at 10 g/l and peptone at 20 g/l, were chosen for the subsequent tests. The effect of the concentration and nature of the carbon source was evaluated in YPD modified medium, replacing the usual carbon source, glucose, by sucrose or fructose. YPS with sucrose at 12.5 g/l was chosen for scale-up M. andauensis production from shake flasks to STR. After 44 h a viable population of 3.1×10^8 cfu/ml was reached, the biomass productivity and yield was 0.439 g/l/h and 1.416 g/g, respectively. In efficacy experiments on different apple cultivars, M. andauensis PBC-2 grown in YPS in STR, effectively reduced incidence and severity of P. expansum.

Keywords: culture medium, growth, batch, fermentations, carbon

1. INTRODUCTION

Synthetic fungicides are the primary mean to control postharvest pathogens (Eckert, 1990). However, the use of postharvest fungicides is being increasingly limited because of environmental and toxicological risks, and in some European countries, postharvest fungicide treatment has been banned (Nunes, 2011).

Biological control using microbial antagonists has emerged as one of the most promising alternatives to postharvest applications of chemical fungicides (Janisiewicz, 1998; Nunes et al., 2010, Nunes, 2011; Teixidó et al., 2011). The identification, development and commercialization of a biocontrol agent are a long, costly and interactive process that involves several steps (Droby et al., 1998, 2009; Nunes et al., 2009; Nunes, 2011). Production is one of the most critical steps for the success of biocontrol product. The objective is to obtain the greatest quantity of efficacious biomass in the shortest period of time (Janisiewicz, 1998; Costa et al., 2001; Nunes, 2011; Teixidó et al., 2011).

Two methods are commonly used for biomass production, liquid and solid fermentations (Fravel et al., 1999). For yeasts and bacterial strains, commercial production of biomass for preparations generally requires liquid culture production in large, industrial scale fermenters (Roberts and Lohrke, 2003). Because industry has developed equipment and methods for large-scale fermentation for the production of microbial products such as antibiotics, enzymes and organic acids, this expertise provides a starting point for production of biocontrol agents (Jackson, 1997; Fravel et al., 1999; Montesinos, 2003). However, there is a lack of scientific information because usually production and formulation are conducted in association or directly by private companies and all the research is developed under secret agreements (Vero, 2006; Teixidó et al., 2011). The first step in optimization strategy is the development of a defined or semidefined medium
which supports good culture growth of the biocontrol agent. Nutritional factors such as carbon, nitrogen sources and carbon nitrogen ratio can all have an influence on growth and biocontrol efficacy. Manso et al. (2010a) have optimized the production of the biological agent *P. agglomerans* PBC-1, in batch and fed-batch technology and obtained the highest yield with sucrose at 5 g/l. Similar results were obtained by Costa et al. (2002) on the production of another strain of *P. agglomerans*. Likewise sucrose was the preferred carbon source followed closely by fructose, on the production of *Serratia entomophila* (Visnovsky et al., 2008). On the other hand, glucose provided the maximum cell growth of *Pseudomonas* sp. M18G, a strain capable to produce PCA, a metabolite active against several soil-borne fungal pathogens (He et al., 2008).

Just like media composition, operational conditions such as aeration, agitation, pH and temperature may affect the quality and quantity of the biocontrol agent. Many microorganisms are easily produced in laboratory in liquid culture in shake flasks, but do not produce the expected quantity and quality when produced in large scale, particularly due to the gas exchange (Fravel et al., 1999). For this reason the optimization of these parameters is essential in the production procedure especially on the scale-up process. For example, the number of colony forming units of *Fusarium oxysporum* was greater when dissolved oxygen was high than when dissolved oxygen was low (Hebbar et al., 1997). Different results were reported by Verma et al. (2006) on the production of *Trichoderma viride*, the conidia concentration, entomotoxicity and inhibition index were either stable or improved at lower DO concentration (30%).

Once a defined medium has been developed which support adequate growth, nutrients are varied in a directed way and their impact on effectiveness must be assessed. Bae et al. (2007) demonstrated that culture media can alter biocontrol ability of *Burkholderia gladioli* B543 on the control of cucumber damping-off.

By comparison with antagonistic bacteria, yeasts and yeast-like microorganisms have been pursued actively in recent years since their rapid colonization skill and their activity against postharvest pathogens does not generally depend on the production of toxic metabolites, which could have a negative environmental or toxicological impact (Li and Tian, 2006; Zhang et al., 2010).

Mainly the mode of action of yeast as biocontrol agents are based on competition for nutrients, direct physical interaction with fungal hyphae and production of cell-wall lytic enzymes (Droby and Chalutz, 1994).

The yeast genus *Metschnikowia* has lately become of considerable interest as a result of increasingly frequent isolation and an increasing diversity of known habitats. Species isolated from terrestrial habitats are typically associated with flowers or fruits and transmitted to new niches by insects (Miller and Phaff, 1998). Two species of this genus *M. pulcherrima* and *M. fruticola* have been reported as effective biocontrol agents against *Botrytis cinerea*, *Penicillium expansum*, *Alternaria alternata* on apples (Piano et al., 1997; Janisiewicz et al., 2001; Spadaro et al., 2002, 2004, 2008; Conway et al., 2004), against *Penicillium digitatum* (Kinay and Yildz, 2008) and *Penicillium italicum* (Droby, 2006) on citrus, against *B. cinerea* on grapes (Schena et al., 2000; Kurtzman and Droby, 2001; Sipiczki, 2006), on strawberry (Karabulut et al., 2004) and on cherry tomato (Schena et al., 2000). *M. pulcherrima* proved effective in preventing the growth and survival of food borne human pathogens, such as *Listeria monocytogenes* or *Salmonella enterica* on fresh-cut apple tissue (Leverentz et al., 2006).

Recently, another species of this genus, *M. andauensis* PBC-2, was reported by its antagonistic ability. This yeast highlighted an excellent activity against *P. expansum*, *B. cinerea*, *Rhizopus stolonifer* on pome fruits and *P. digitatum* and *P. italicum* on citrus fruits (Manso and Nunes, 2011). The mechanisms involved in biological control ability of this new biocontrol agent are not completely known, however the dose response and effectiveness relationship as well the rapidly growth in the wound, suggests that competition for space and nutrient could have an important role in the mode of action of *M. andauensis* PBC-2 (Manso et al., 2011).

In the present paper batch experiments were carried out in shake flasks to study different standard media, temperatures, commercial carbon and nitrogen sources and it concentration on the production of the biocontrol agent *M. andauensis* PBC-2. The scale-up were made in a mechanically stirred and aerated bioreactor (STR), and the efficacy of the biomass produced was assessed on the control of *P. expansum* on pome fruits.
2. MATERIAL AND METHODS

2.1. Microorganisms

Metschnikowia andauensis PBC-2, used in this study, was originally isolated from the carposphere of ‘Bravo de Esmolfe’ apples. This strain was stored at -80 °C in liquid medium with 20 % (v/v) glycerol. It is deposited in National Collection of Yeast CulturesNCYC 3728 (UK). When required for fermentation studies, M. andauensis PBC-2 was streaked on NYDA medium (8 g/l nutrient broth, Biokar BK003HA; 6 g/l yeast extract, Biokar A1202HA; 10 g/l glucose, Riedel-de-Haën 16325; 15 g/l agar, Vaz Pereira) and incubated at 25±0.5 °C.

Penicillium expansum isolated from decayed pome fruit, were selected based on their high virulence. The fungal pathogen was maintained on PDA medium (200 g/l extract of boiled potatoes; 20 g/l glucose), incubated at 25±0.5 °C and periodically transferred through fruit.

2.2. Inoculum preparation

A 48 h preculture of M. andauensis PBC-2 was transferred from NYDA plates to 5 ml potassium phosphate buffer (pH 6.5). Fifty millilitres of liquid medium in 250 ml conical flasks were inoculated with 0.5 ml of this freshly prepared M. andauensis PBC-2 suspension. After incubation at 25±0.5 °C for 40 h on a rotary shaker-flask incubator (Pentlab, Neifo refrigerada, Portugal) at 150 rev/min until reached the exponential phase, cells were used as starter in the shake flasks and bioreactors fermentation experiments.

2.3. Batch experiments in shake flasks

In order to obtain a suitable medium and growth conditions to produce great amount of viable biomass of M. andauensis PBC-2, different assays were carried out in batch experiments in shake flasks using the methodology described below. Conical flasks were inoculated with fresh cells of M. andauensis PBC-2, prepared as described above, at an initial concentration of 10⁵ cfu/ml and incubated under orbital agitation at 150 rev/min, for 40 h. Samples were taken and viable cell concentrations were estimated by dilutions and plated on NYDA medium. Plates were incubated at 25±0.5 °C for 48 h, and the number of cfu/ml was determined. Each flask assay was conducted in three replicates and each experiment was repeated twice.

2.3.1. Standard media selection

The selection of a commercial defined or semi-defined medium which supports good culture growth of this new biocontrol agent and the appropriate temperature was the starting point in the production optimization process. The culture media NYDB (8 g/l nutrient broth; 6 g/l yeast extract; 10 g/l glucose), YM (10 g/l glucose; 5 g/l peptone, Oxoid PL0037; 3 g/l yeast extract; 3 g/l malt extract, Oxoid PL0039), YPD (10 g/l yeast extract; 20 g/l peptone; 20 g/l glucose) and PDB (24 g/l, Scharlau 02-483) were tested. Flasks were incubated at 25±0.5 °C and 30±0.5 °C.

2.3.2. Effect of carbon sources concentration

The effect of nitrogen sources concentration was tested in YPD medium, at 25±0.5 °C, the medium and temperature that previously presented the best results. Yeast extract and peptone were tested in 9 runs, combining these 2 independent variables at 3 concentrations, 0, 10 and 20 g/l. Yeast extract at 10 g/l and peptone and 20 g/l were selected for the following experiments.

2.3.3. Effect of pH and temperature

The binominal effect of temperature and initial pH was studied in YPD medium at 20, 25 and 30 °C and 4.5, 6.5 and 8.5, respectively. The pH of the media was adjusted using either citric acid or NaOH. The temperature of 25 °C and a pH at 6.5 were selected.

2.3.4. Variation in carbon source and concentration

The studies of the nature and concentration of the carbon source were performed based on YPD medium, replacing the standard carbon source and concentration by the tested variations. The initial pH was adjusted at 6.5. Glucose, fructose (Fluka Biochemistry 47740) and sucrose (Panreac 141621.1211) were tested as carbon sources at 20 g/l. The effect of carbon source concentration was studied with sucrose at 5, 12.5 and 20 g/l. Flasks were inoculated and incubated at 25±0.5 °C, during 60 h. At the beginning of the experiment and at regular intervals of 12 h, the pH was measured. Different samples were taken from the flasks to measure the Optical Density (OD540nm) in a spectrophotometer (Amershampharmacia Biotech, Ultrospec 1100pro, Sweden) and fresh weight were estimated by a fresh weight vs. optical density correlation curve.
2.4. Batch experiments in STR

Stirred tank cultures were carried out in instrumented 3 l (2.7 l working volume, 1.5 H/D ratio volume) glass bioreactor (ADI 1010/1025, Applikon Biotechnology, Netherlands) equipped with pH (AppliSens pH+ Z00103551) and dissolved oxygen probes (AppliSens, Low Drift Z010023520). A Rushton-type turbine with an impeller diameter (D) of 0.045 m and an L-shaped sparger were used. The culture was kept at 25±0.5 ºC, sparged with an initial airflow rate of 1vvm (volume of air per volume of medium) and with an agitation rate of 300 rev/min. The selected medium (YPs) was composed by yeast extract at 10 g/l, peptone at 20 g/l, sucrose at 12.5 g/l and antifoam B (Sigma A-5757) at 0.5 ml/l.

The fermentation was monitored online, using the BioXpert program version 2.1, temperature, pH and dissolved oxygen were measured by respectively probes and registered constantly. Samples were taken immediately after inoculation and at given fermentation times, to determine the optical density (OD590nm), viability population counts and sugar concentrations, as previously described. Fresh weight biomass was determinate after centrifugation at 1920×g (Universal 320, Hettich Zentrifugen, Germany), for 15 min.

2.5. HPLC analysis

Residual carbon source concentration was detected with a high-performance liquid chromatography system (Hitachi, Elite LaChrom), equipped with a refractive index (RI) detector. The column Purospher STAR NH2 (25×4.5 cm, 5 µm particle size) from Merck (Germany), was used at room temperature. The mobile phase consists in acetonitrile: water (75:25) applied at a flow rate of 1 ml/min (Peris-Tortajada, 2000). The different sugars were identified by comparison of their retention times with those of pure standards. The concentration of these compounds was calculated from standard curves of the respective sugars.

2.6. Efficacy assay

The effect of the biomass produced in STR, with the optimized medium (YPs) on controlling blue mould caused by P. expansum on pome fruit was studied. Cells were separated from supernatants by centrifugation at 5,4189×g (Beckman, Avanti J-14, Coulter, USA) for 12 min and then resuspended in sterile phosphate buffer. Cell concentrations were adjusted to 107 cfu/ml using a spectrophotometer. Tween-80. Pathogen concentration was adjusted to 105 conidia/ml using a haemocytometer.

Apples, cv. ‘Golden Delicious’, ‘Bravo de Esmolfe’, ‘Reineta’, ‘Jonagold’, ‘Red Delicious’, ‘Royal Gala’, were obtained from commercial orchards in Alcobaça, Portugal, stored at 1±0.5 ºC. Fruit were artificially wounded at the equatorial region (2 mm deep by 1 mm wide; 2 wounds per fruit).

Twenty microlitres of M. andauensis PBC-2 suspension was inoculated into the wound, followed after 2 h by the inoculation of 15 µl of a suspension of P. expansum. Each fruit with two wounds constituted a single replicate and each treatment was repeated three times. Fruit were stored at 20±1 ºC and 80±5% HR for 7 days at the end of which, incidence and severity (lesion diameter) was determined.

2.7. Statistical analysis

Statistical analysis was done using software package StatSoft Statistica (version 8, Inc.).

Statistical significance was generally judged at the level of P <0.05 for the shake flasks growth and biocontrol experiments. When the analysis of variance was statistically significant either in the shake flask growth or in the biocontrol experiments, Duncan’s multiple range test was used for the separation of means.

3. RESULTS

3.1. Standard media selection

On a first approach to find a suitable culture medium for the production of the biocontrol agent M. andauensis PBC-2, different standard media (YPD, PDB, YM and NYDB) commonly described in the production of yeasts were tested. Liquid culture media significantly affected the growth of M. andauensis PBC-2. Biomass productivity observed in YPD, YM, PDB and NYDB, at 25 ºC was 0.657, 0.497, 0.364 and 0.57875 g/l/h, respectively. At 30 ºC, biomass productivity was 0.672, 0.504, 0.399 and 0.599 g/l/h, in YPD, YM, PDB and NYDB, respectively (data not shown). The highest viable cell was observed in YPD medium, at both studied temperatures, 25 and 30 ºC (Fig. 1). In the 4 tested media, a higher temperature did not promote higher production, when compared viable cells after 40 h of incubation. YPD medium was selected for the following experiments.
**Figure 1.** Production of *M. andauensis* strain PBC-2 in different standard media, at 25 °C and 30 °C. Growth was carried out in 250 ml conical flasks with 50 ml of medium, shaken at 150 rev/min for 40 h. Columns represent the means of three replicates of two experiments and the vertical bars indicate the standard deviation. Columns with different letters are significantly different for each medium using Duncan test (P< 0.05).

**3.2. Effect of nitrogen sources concentration**

The combination of yeast extract and peptone concentration, the two nitrogen sources of YPD medium, was tested at 25 °C. After 40 h of incubation, practically no growth was observed on the repetitions without nitrogen sources, the viable population remained at a similar level to the initial moment of the experiment, 4×10^5 cfu/ml (Fig. 2), the biomass productivity and biomass yield were 0.042 g/l.h and 0.085 g/g, respectively (data not shown). The viable population with yeast extract at 10 and 20 g/l, without peptone, was 9.2x10^7 and 2.1x10^8 cfu/ml, respectively. In this experiment, the maximum population achieved was 4.2x10^8 cfu/ml with both nitrogen sources at 20 g/l, nonetheless no significant differences were observed maintaining peptone at 20 g/l and reducing yeast extract to 10 g/l, for this reason yeast extract at 10 g/l and peptone at 20 g/l, were chosen for further assays.

**3.3. Effect of pH and temperature**

Batch assays in shake flasks were performed to optimize temperature and pH variations in biocontrol agent production (Fig. 3). Viable population increase was not linear with temperature and pH increment. At pH 8.5, the viable population was about 20 times lower than the population at pH 6.5. The biomass yield and productivity were 0.308 g/l.h and 0.616 g/g, 0.311 g/l.h and 0.623 g/g, 0.289 g/l.h and 0.579 g/g, at 25, 30 and 35 °C, respectively. A poor growth was observed in the repetitions under acid rather, with a biomass yield and productivity of 0.007 g/l.h and 0.015 g/g, 0.063 g/l.h and 0.125 g/g, 0.024 g/l.h and 0.047 g/g, at 25, 30 and 35 °C, respectively. Maximum population was achieved at pH 6.5 at 25 and 30 °C, nonetheless no significant differences were noted between both temperatures. The selected pH and temperature for the subsequent tests were 25 °C and an initial pH at 6.5.

**3.4. Variation in carbon source and concentration**

Despite increase of peptone concentration promotes enhancement on the biocontrol agent growth, differences are only observed when yeast extract was added to the medium. Biomass productivity determined with 20 g/l of both nitrogen sources was 0.702 g/l.h and 0.698 g/l.h when peptone concentration was reduced to 10 g/l (data not shown). The viable population with yeast extract at 10 and 20 g/l, without peptone, was 9.2x10^7 and 2.1x10^8 cfu/ml, respectively. In this experiment, the maximum population achieved was 4.2x10^8 cfu/ml with both nitrogen sources at 20 g/l, nonetheless no significant differences were observed maintaining peptone at 20 g/l and reducing yeast extract to 10 g/l, for this reason yeast extract at 10 g/l and peptone at 20 g/l, were chosen for further assays.
fructose. The growth profiles of *M. andauensis* PBC-2 are illustrated in Figure 4.

Concerning biomass accumulation, cultures had a lag phase of about 12 h, followed by an exponential phase that lasted until 36-40 h (Fig. 4a). The highest specific growth rate was observed with sucrose, however no significant differences were registered between this carbon source and glucose (Table 1.) On the other hand, differences in biomass productivity, biomass yield and amount of biomass accumulated after 36 h, were observed among sucrose and the others two sources tested.

At the end of the experiment no difference in biomass yield was registered. In all cultures during the exponential phase, the increase of the viable population was accompanied by a pH and sugar content decrease (Fig. 4 c, d). The substrate uptake rate was quite similar in all carbon sources with no statistical differences. After 40 h of incubation, when approximately 90 % of the carbon source was consumed, the viable population reached 1.2×10⁸, 5.3×10⁸ and 1.3×10⁸ cfu/ml with glucose, sucrose and fructose, respectively (Fig. 4b). Sucrose was selected as carbon source instead of glucose in YPD medium, yielding the YPS medium.

Figure 5 depicts the growth profiles of *M. andauensis* PBC-2, in YPS medium, submitted to different sucrose concentrations 5, 12.5 and 20 g/l.

The behaviour of fresh weight biomass in all sugar concentrations was similar (Fig. 5a), a lag phase of 10-12 h followed by an exponential phase and a long stationary phase which began approximately after 37 h of incubation. The specific growth rate determined in the three concentrations did not shown significant differences (Table 2). After 11 h of incubation, the viable population raised 10-fold and passed 37 h reached 2.1×10⁸, 3.1×10⁸ and 3.0×10⁸ cfu/ml at 5, 12.5 and 20 g/l, respectively (Fig. 5b). Immediately after inoculation the sugar depletion occurred (Fig. 5d), as expected the lower sugar concentration was the first to be completely depleted. Although, biomass yield had registered differences between concentrations, the same was not observed in the biomass productivity between the two higher concentrations, leading to the selection of the intermediate sucrose concentration (12.5 g/l) for additional studies.

### 3.5. Batch experiments in STR

The production of *M. andauensis* PBC-2 was scaled-up from shake flasks to stirred tank reactor. The chosen media was YPS with sucrose at 12.5 g/l. Figure 6 shows the pH, pO2, sugar consumption, viable cells and biomass fresh weight, during the production process at 25 °C.

There was an 8 h lag phase before growth, after which an exponential growth occurred. At this time growth slowed eventually reaching a maximum population of 3.1×10⁸ cfu/ml. After 44 h the biomass productivity and yield was 0.439 g/l.h and 1.416 g/g, respectively.

On the first 12 h of *M. andauensis* PBC-2 growth oxygen was rapidly consumed, the oxygen consumption rate was 19.8 µmol O2 /l.h, reaching 0% at that moment. The increase of this parameter only occurs on the stationary phase, however never reaching the initial level, indicating that after 60 h cells maintain their viability. A rapid decrease in sugar concentration also occurred earlier in the growth curve, followed by moderate depletion.

Initial pH was 6.5 and during the first 20 h the value decreased near 5, after that period the pH tended to increase until the end of the experiment.

### 3.6. Efficacy assay

Figure 7 shows the effectiveness of *M. andauensis* PBC-2 growth in YPS in different apple cultivars to control blue mould. *P. expansum* incidence was reduced by 76, 74 and 63 % on ‘Bravo de Esmolfe’, ‘Jonagol’ and ‘Golden Delicious’ apples, respectively. Likewise, severity was also reduced in 84, 74 and 64 % (data not shown). The decay control was less expressive in the other apple cultivars nonetheless the treatment with the biocontrol agent reduced at least in a half the fungal development.

![Figure 7. Postharvest incidence of blue mould on wounded apples of different cultivars, untreated (■) or treated with *M. andauensis* PBC-2 at 10⁷ cfu/ml (●) and infected with 10⁴ conidia/ml of *P. expansum*. Incidence was assessed after 7 days at 20±1 °C. Ten fruit](image-url)
constituted a replicate and each treatment was repeated three times.

4. DISCUSSION

To be considered as a feasible alternative to fungicides, production, one of the important steps in the multifaceted development process of a biocontrol agent, must be sustainable. It is essential to know the growth profile of the microorganism as well as study the nutritional requirements and maximize production conditions. Medium constituents must satisfy basic requirements for biomass and metabolic production however it is equally important that these requirements are simple and inexpensive.

The optimized defined medium has as main advantage being standard and stable, but their cost is often high, leading to the search for alternatives, therefore defined medium often serves as a nutritional framework from which a production medium can be formulated.

In the present work culture media commonly described in yeast production, like NYDB, PDB, YM and YPD (Spadaro et al., 2002; Arroyo-López et al., 2009; Liu et al., 2009) were tested. The best performance was achieved in YPD medium at both studied temperatures (25 and 30 °C). In the study of the effect of nitrogen source the combination of yeast extract and peptone seems to be more favorable to M. andauensis PBC-2 growth than the combination of yeast extract with nutrient broth or malt extract. The lowest growth was obtained in PDB medium, which given its composition was expectable. The medium PDB and PDA (PDB with agar) are common microbiological growth media made from potato infusion and dextrose. Potato is a versatile, carbohydrate-rich food prepared, freshly harvested, it contains about 80% water and 20% dry matter. About 60–80% of the dry matter is starch (Lutaladio and Castaldi, 2009). The physiological and biochemical characterization made by NCYC (National Collection of Yeast Cultures), revealed that M. andauensis PBC-2 was unable to assimilate starch; this inability may have contributed to the poor results observed. The inability to assimilate starch found in this species seems to be a common feature of the species belongs to this genus, it was noted in M. fructicola (Kurtzman and Droby, 2001), M. reukaﬁ (Nozaki et al., 2003), M. bicuspidate (Fell and Pitt, 1969), M. shivoigae (Lachance et al., 2008), M. vanudenii (Giménez-Jurado et al., 2003), M. sinensis, M. zizyphicola and, M. shanxiensis (Xue et al., 2006).

Nitrogen source optimization was performed with the medium that shown the best results, YPD, by changing the ratio of the two nitrogen sources which constitutes this medium, yeast extract and peptone. It was found that without nitrogen source, the population remain practically identical to the initial, 4×10⁵ cfu/ml. Similar results were obtained by Spadaro et al. (2010) in the production of M. pulcherrima. It was reported that the ringer solution, used as control, allowed the initial inoculum to be kept alive. The study also reports that an increase in the initial yeast extract concentration from 5 to 30 g/l yielded a proportional increase in the biomass produced, however no significant increase in biomass was observed at 40–60 g/l of yeast extract. In our study increments in yeast extract concentration also yielded an increase in M. andauensis PBC-2 viable population. The maximum population achieved was 4.2×10⁶ cfu/ml with both nitrogen sources at 20 g/l, nonetheless no significant differences were observed maintaining peptone at 20 g/l and reducing yeast extract to 10 g/l. The slight enhance in cell concentration, promoted by using an extra 10 g/l of yeast extract would not justify the additional cost incurred. Visnovsky et al. (2008), considers a threshold for the system, they founded that increasing yeast extract from 10 to 20 g/l in batch mode did not increase yield and, when added in fed-batch mode, increase yield by only 4.5%.

Yeast extract, is a complex raw material, usually produced from baker’s or brewer’s yeast through autolysis at 50 °C in the presence of solvents or salts. Due to its low cost and rich content of various amino acids, peptides, water-soluble vitamins, growth factors, trace elements, and carbohydrates, is commonly used as a key medium component for cultivating many microorganisms (Crueger and Crueger, 1989), including the production of biocontrol agents (Costa et al., 2001; Peighami-Ashnaei et al., 2009).

The effects of temperature and pH on the production of M. andauensis PBC-2 were studied simultaneously, in YPD medium. As a yeast characteristic, the biocontrol agent PBC-2, grown in a wide range of pH values, from acid to alkaline and the maximum population was achieved at pH 6.5, at 25 and 30 °C. The nonexistence of significant difference between those temperatures revealed that there is no need of heating, which turn the process more economical.

The results of the growth experiments indicated that sucrose is a suitable carbon source for M. andauensis PBC-2 production, since shown higher biomass yield and productivity, when compared
with glucose and fructose, also assayed. These results are interesting, since sucrose is an economical carbon source, comparing with others commercial sources, moreover is usually available and is frequently found it in the composition of some by-products of food industry, which enables the optimization of a low-cost medium. The selection of sucrose as carbon source in the production of a biocontrol agent was also made by Manso et al. (2010) in the production of P. agglomerans PBC-1 and by Visnovsky et al. (2008) in the production of Serratia entomophila.

The effect of sucrose concentration on the growth of M. andauensis was tested at 5, 12.5 and 20 g/l. No differences on specific growth rate were observed among the three tested concentrations. Concerning to biomass yield and productivity, higher concentrations promoted the best results, nevertheless no significant differences were observed between the intermediate and the highest concentration, whereby, the concentration 12.5 g/l, was chosen to further studies.

Our results are in accordance with that obtained in the production of the biocontrol agent M. pulcherrima. Spadaro et al. (2010) reported that in general, 20 g/l of carbon source did not improve yeast biomass compared to 10 g/l, suggesting that a high concentration of an external carbon source is not beneficial to growth of this yeast. Only D-glucose yielded a greater biomass of M. pulcherrima when applied at 20 versus 10 g/l.

In the scale-up of M. andauensis PCB-2 production in STR a viable population of 3.1x10^8 cfu/ml was achieved after approximately 40 h of incubation, using the YPS optimized medium, with sucrose at 12.5 g/l. This result supported the possibility to produce commercial amounts of PCB-2 in a short period of time and provided a reliable basis for the fermentation scaling-up process to an industrial level. The specific growth rate determined (0.439 1/h) was comparable to this parameter observed in M. pulcherrima BIO126, grown in YEMS medium (0.45 1/l) (Spadaro et al., 2010).

On the first 12 h of M. andauensis PBC-2 growth, oxygen was rapidly consumed, accompanied by a pH decrease, which can be considered an indicator of exponential growth. The increase of dissolved oxygen only occurs on the stationary phase, however never reaching the initial level, indicating that after 70 h cells maintain their viability. The starvation of oxygen and its suitable supply must be object of interest in future studies, in order to maximize biomass production and avoid possible limitation in oxygen. Growth of P. anomala was inhibited not for carbon source depletion but by oxygen-limited conditions (Fredlund et al., 2004). Oppositely, the oxygen depletion seems not to influence the Bacillus subtilis CPA-8 growth, possibly attributing to the fact this bacterium was able to grow by a fermentation pathway in the absence or scarcity of oxygen (Yánez-Mendizábal et al., 2012).

A key factor to consider in the production of a biocontrol agent is the development of an economical culture medium that supports production of large commercial amounts of a microbial agent at a low price while maintaining biocontrol efficacy (Patiño-Vera et al., 2005; Teixidó et al., 2011). The influence of the growing medium on the biocontrol capability of M. andauensis was assayed in different apple cultivars against blue mould. In all apples P. expansum development was limited by the treatment with the biocontrol agent. The results obtained in this study showed that the changes performed in culture medium, allowed a great amount of biomass, without compromising the antagonist capability of the biocontrol agent.

Piano et al. (1997) reported that the biocontrol activity of M. pulcherrima could be strongly affected by the media and the addition of certain nutrients. In that study the presence of glucose, fructose and peptone the biocontrol ability was not significantly affected, while the addition of Czapeck substrate significantly reduced their antagonistic potential. Likewise, Spadaro et al. (2002) in an in vitro study shown, that inhibition of mycelial growth of Alternaria sp., B. cinerea, Monilia sp. and P. expansum only occurred on some substrates, probably due to the production of secondary metabolites influenced by the nutritional environment. The influence of substrate composition on the antifungal activity was demonstrated, as well, by Mezghanni et al. (2012) in medium optimization of Bacillus amyoliquefaciens.

For the development of industrial bioprocesses it is crucial to study different conditions to maximize biomass productivity and yields. In the specific case of biocontrol agent production, biomass viable count (Verma et al., 2005) and biocontrol efficacy are others objectives function of the process. The nutrients for a medium must satisfy the basic requirements for cell biomass and metabolite production (Stanbury et al., 1995). This study has demonstrated that M. andauensis PBC-2 may use different nutrients in its growth. Among the tested media, the one that provided the highest population
was the YPD medium, however when glucose, the standard carbon source of YPD medium, was replaced by sucrose, specific growth rate and biomass yield were even higher. The good results obtained with sucrose, led to the selection of this sugar as the carbon source, furthermore it was also possible to reduce its concentration from 20 to 12.5 g/l, without significantly influence the results and without affect the antagonist activity of this recent biocontrol agent. The results obtained provide evidence for low cost biomass production with food industry by-products, since those products are mainly constitute for sucrose.

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REFERENCES:


Figure 4. Growth of M. andauensis PBC-2 in YPD modified medium with different carbon sources, sucrose (squares), glucose (circles), fructose (triangles). a: fresh cell weight (FW), g/l; b: viable cells, expressed in log cfu/ml; c: pH; d: sugar depletion, g/l. Cultures were grown in 50 ml of media shaken at 150 rev/min. at 25 °C. The experiments were performed twice, in triplicates under identical conditions and the vertical bars indicate the standard deviation.
Figure 5. Growth of *M. andauensis* PBC-2 in YPS medium, with different sucrose concentrations, 5 g/l (circles), 12.5 g/l (squares), 20 g/l (triangles). a: fresh cell weight (FW), g/l; b: viable cells, expressed in log cfu/ml; c: pH; d: sugar consumption, g/l. Cultures were grown in 50 ml of media shaken at 150 rev/min. at 25 ºC. The experiments were performed twice, in triplicates under identical conditions and the vertical bars indicate the standard deviation.

Figure 6. Time course of viable cells cfu/ml (triangles), fresh cell weight (squares), pH value (diamonds), oxygen concentration (circles), consumption of sugar (times) in batch cultivation of *M. andauensis* PBC-2, performed in STR with airflow rate of 1vvm, L-shaped sparger, 300 rev/min., Rushton-type turbine, at 25 ºC, in YPS medium (sucrose 12.5 g/l, yeast extract 10 g/l, peptone 20 g/l), inoculated at initial concentration at 106 cfu/ml. The experiments were performed three times. pH, oxygen concentration were measured online; fresh cell weight, viable cells (cfu/ml) and sugars data are averages of three replicates.
Table 1. Growth parameters of *M. andauensis* PBC-2, in shake flasks experiments in YPD modified medium with different carbon sources, at 25 °C, stirring at 150 rpm.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg (1/h)</td>
<td>0.083±0.014a</td>
<td>0.098±0.011ab</td>
<td>0.107±0.008b</td>
</tr>
<tr>
<td>Pmax (g/lh)</td>
<td>0.603±0.013a</td>
<td>0.599±0.012a</td>
<td>0.669±0.007b</td>
</tr>
<tr>
<td>Rs (g/lh)</td>
<td>0.368±0.013a</td>
<td>0.360±0.030a</td>
<td>0.364±0.031a</td>
</tr>
<tr>
<td>Yx/s (g/g)</td>
<td>1.350±0.129a</td>
<td>1.401±0.234a</td>
<td>1.487±0.153a</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates. Within a row, values followed by the same letter are not statistically different according to Student–Newman–Keuls (P<0.05). µg: specific growth rate; Pmax: biomass productivity; Yx/s: biomass yield; Rs: substrate uptake rate.

Table 2. Growth parameters of *M. andauensis* PBC-2, in shake flasks experiments in YPS medium with different sucrose concentrations, at 25 °C, stirring at 150 rpm.

<table>
<thead>
<tr>
<th>Sugar concentration (g/l)</th>
<th>5</th>
<th>12.5</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg (1/h)</td>
<td>0.102±0.002a</td>
<td>0.106±0.003a</td>
<td>0.095±0.014a</td>
</tr>
<tr>
<td>Pmax (g/lh)</td>
<td>0.638±0.002a</td>
<td>0.665±0.004b</td>
<td>0.653±0.011ab</td>
</tr>
<tr>
<td>Rs (g/lh)</td>
<td>0.246±0.070a</td>
<td>0.194±0.060a</td>
<td>0.373±0.011b</td>
</tr>
<tr>
<td>Yx/s (g/g)</td>
<td>4.515±0.588a</td>
<td>2.253±0.178b</td>
<td>1.357±0.104c</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates. Within a row, values followed by the same letter are not statistically different according to Student–Newman–Keuls (P<0.05). µg: specific growth rate; Pmax: biomass productivity; Yx/s: biomass yield; Rs: substrate uptake rate.