UNIVERSIDADE DO ALGARVE

ISOLAMENTO E PRODUÇÃO DE AGENTES DE CONTROLO BIOLÓGICO DAS PRINCIPAIS DOENÇAS DE PÓS-COLHEITA, EM CITRINOS E POMÓIDEAS

Teresa Cláudia Marchão Manso

Tese para obtenção do grau de Doutor em Ciências Agrárias

Trabalho efectuado sob a orientação de:

Maria Emília Lima Costa

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída

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RESUMO

Potenciais agentes de biocontrolo foram isolados a partir da microbiota epifítica de folhas e frutos, de citrinos e pomóideas, de diferentes pomares, durante diferentes campanhas e de distintas condições de armazenamento.

A atividade antagonista de 1465 microrganismos isolados foi testada em ensaios *in vivo* em pomóideas face a *Penicillium expansum* (10^4 esporos/ml) e em citrinos face a *P. digitatum* (10^5 esporos/ml) em frutos feridos e inoculados artificialmente. Aproximadamente 7,6% dos isolados reduziu a severidade (diâmetro da podridão) e a incidência (% podres) em mais de 25%, menos de 3% reduziu ambos os parâmetros em mais de 50%, mas apenas 4 microrganismos preencheram os critérios de seleção, redução da incidência e severidade em mais de 75%. Dos 4 e pelos resultados obtidos em ensaios de seleção secundária e de determinação da concentração mínima eficaz, destacaram-se e selecionaram-se 2 microrganismos, uma bactéria isolada de laranjas 'Valencia late' e identificada como pertencente ao grupo das Enterobactérias, *Pantoea agglomerans* PCB-1 e uma levedura isolada a partir da superfície de maças 'Bravo de Esmolfe' identificada como *Metschnikowia andauensis* PBC-2, com o objetivo de usar dois modelos distintos de agentes de biocontrol, uma bactéria e uma levedura.

P. agglomerans é um agente de biocontrolo já conhecido. A estirpe PBC-1 isolada neste trabalho mostrou ter elevada eficácia face aos principais agentes patogénicos na pós-colheita de citrinos e pomóideas. *M. andauensis* é uma levedura recentemente descoberta e a estirpe PBC-2 diz respeito à primeira referência desta espécie como agente de biocontrolo, a qual foi recentemente objeto de concessão de Patente de Invenção Nacional nº 105210, como uma nova estirpe desta espécie para uso como agente de biocontrolo das doenças de pós-colheita de frutos.

A concentração mínima eficaz dos antagonistas revelou estar dentro dos limites para o seu desenvolvimento comercial. *M. andauensis* PBC-2 quando aplicada à concentração de 5×10^6 ufc/ml, permitiu uma redução da incidência e da severidade de 62 e 70%, respetivamente, e quando aplicada a 1×10^7 ufc/ml, uma redução de 90% de incidência e de 95% de severidade. *P. agglomerans* PBC-1 aplicada a 1×10^8 ufc/ml em maçãs e em citrinos no controlo de *P. expansum* e *P. digitatum*, respetivamente, propiciaram uma redução significativa de cerca de 86% de cada um dos agentes patogénicos.

O espectro de ação de *M. andauensis* PBC-2 foi avaliado, verificando-se o controlo efetivo face a *Rhizopus stolonifer*, *P. expansum* e *Botritys cinerea*, em pera 'Rocha' e em diferentes cultivares de maçã e contra *P. digitatum* e *Penicillium italicum* em clementinas e laranjas de diferentes cultivares.

Durante 4 épocas, a eficácia de *M. andauensis* PBC-2, foi avaliada e comparada com o fungicida sintético mais usado comercialmente, Imazalil, em ensaios semicomerciais. Os resultados assemelharam-se aos obtidos com o fungicida, a redução da incidência do bolor azul foi de 90% em maças armazenadas durante 3 meses a 1 ± 0.5 ºC, seguido de 7 dias à temperatura ambiente, para simular o tempo de prateleira.

Em ensaios do estudo da dinâmica populacional, verificou-se que o agente de biocontrolo *M. andauensis* PBC-2 tem uma excelente capacidade colonizadora e que consegue crescer e sobreviver nas feridas, mas também na superfície dos frutos, armazenados à temperatura ambiente e em condições de frio. Pelo contrário, *M. andauensis* PBC- não apresentou capacidade de sobrevivência no suco gástrico simulado, começando a população a diminuir imediatamente após exposição e passadas 48 h não restava população viável.

Diferentes meios de cultura usualmente descritos na produção de leveduras foram testados na produção de *M. andauensis* PBC-2. Aquele que apresentou a maior população viável ao fim de 40 h de incubação foi o meio YPD, entretanto escolhido para estudos posteriores. O pH mais favorável ao crescimento foi de 6,5, não se observando diferenças significativas entre os crescimentos a 25 e 30 ºC. Estudou-se ainda o efeito da concentração das duas fontes de azoto do meio YPD e elegeu-se a combinação de 10 g/l de extrato de levedura e 20 g/l de peptona.

Nos estudos de produção de biomassa dos dois potenciais agentes de biocontrolo, analisou-se o efeito da sacarose, frutose e glucose, como fontes de carbono e otimizou-se a concentração destes açúcares no crescimento em Erlenmeyer. Após 20 h de incubação a população viável de *P. agglomerans* PBC-1 atingiu 3.9×10^9, 1.5×10^9, 3.9×10^9 ufc/ml, respetivamente. No caso de *M. andauensis* PBC-2, foi obtida a população de 1.2×10^8, 5.3×10^8 e $1{,}3\times10^8$ ufc/ml, com glucose, sacarose e frutose, após 40 h. Os resultados permitem concluir que os dois agentes têm capacidade de metabolizar os açúcares testados, contudo e atendendo à produtividade de biomassa, rendimento, disponibilidade e custo, optou-se por usar a sacarose como fonte de carbono nos restantes ensaios, nomeadamente na transição de Erlenmeyer para reator biológico. Na produção de *P. agglomerans* PBC-1 escolheu-se o meio SAC (5 g/l sacarose e 5 g/l extrato de levedura). O meio YPS (12.5g/l sacarose, 10 g/l extrato de levedura, 20 g/l peptona) foi usado no aumento de escala de *M. andauensis* PBC-2.

A otimização da produção em reator biológico de *P. agglomerans* PCB-1 foi realizada submetendo o microrganismo a diferentes condições hidrodinâmicas, testando-se arejamentos, dois tipos de turbina (hélice; rusthon) e dispersores (poroso, em L). Foram igualmente estudados, o efeito da concentração inicial do inoculo e a adição programada da fonte de carbono. Embora tenham sido testadas diferentes variações, o perfil dos diferentes parâmetros analisados foi idêntico, a população máxima viável foi de $3\text{-}5\times10^9$ ufc/ml. A diferença mais notória foi observada na fermentação com concentração inicial de 10^7 cfu/ml, que permitiu encurtar em cinco horas a fase lag, o que pode significar uma redução de tempo de fermentação, e consequentemente uma redução de custos.

Visando a produção de biomassa a baixo custo, fator importante na implementação de um sistema de controlo biológico, estudou-se a possibilidade de utilizar subprodutos e resíduos da indústria alimentar no crescimento dos agentes de biocontrolo. Subprodutos da indústria de alfarroba e subprodutos e resíduos da indústria de sumos de citrinos foram utilizados na produção de *P. agglomerans* PBC-1 e de *M. andauensis* PBC-2, respetivamente. Desta forma, para além de uma redução dos custos de produção, pretendeu-se a valorização de um subproduto (no caso de alfarroba e do bagaço de citrinos) e mitigar os efeitos nefastos de um resíduo (licor), com elevada carga poluente, que gera graves problemas ambientais e que pode ditar o encerramento desta unidade industrial, com efeitos devastadores para a economia local.

Realizaram-se extrações de subprodutos da indústria de alfarroba, a diferentes razões sólido/líquido, tempos e temperaturas, de forma a maximizar a extração de açúcares. A potencialidade de utilizar o extrato de açúcares obtido, na produção de *P. agglomerans* PBC-1 foi avaliada, em ensaios de crescimento em Erlenmeyer, com consequente transição para reator mecanicamente agitado. Os perfis de crescimento da cultura crescida com subprodutos da indústria de alfarroba assemelharam-se aos observados com sacarose como fonte de carbono. A biomassa viável produzida com subprodutos da indústria de alfarroba, foi de $4\text{-}7\times10^9$ ufc/ml, o que permite concluir que é uma alternativa viável à produção deste microrganismo.

A produção de *M. andauensis* PBC-2 com subprodutos da indústria de sumos de citrinos, foi estudada em Erlenmeyer tendo como objetivo conhecer os perfis de crescimento do microrganismo, bem como, estudar a melhor combinação desta fonte de carbono e sua concentração. O bagaço de citrinos e um resíduo líquido, que se denominou de licor, foram testados com resultados comparáveis à produção obtida com meio usado como standard, (YPS), sem comprometer a atividade antagonista do agente de biocontrolo. Posteriormente,

foi realizada a produção em reator mecanicamente agitado, escolhendo-se para tal o meio YL (10 g/l extrato de levedura e licor à concentração de açúcares de 12.5 g/l). Os parâmetros de crescimento da cultura foram semelhantes aos obtidos com a fonte de carbono comercial. Após aproximadamente 40 h de incubação, a população viável de *M. andauensis* PCB-2 atingiu 3.1×10^8 ufc/ml. A produtividade de biomassa e rendimento foi de 0.435 g/l.h e 1.502 g/g, respetivamente, comparável a produtividade de biomassa (0.432 g/l.h) e rendimento (1.4416 g/g) observado no meio YPS.

Os resultados obtidos, são uma base sólida para o aumento de escala a um nível laboratorial e semi-industrial, permitiram concluir que é exequível produzir *M. andauensis* a baixo custo e representam uma possível alternativa para um resíduo.

Em estudos dos possíveis modos de ação, de *M. andauensis* PBC-2, conclui-se que, este agente de biocontrolo, não tem como modos de ação a produção de antibióticos ou de voláteis, uma vez que, não se verificou inibição do crescimento dos agentes patogénicos. A competição por ferro e a produção de enzimas líticas por *M. andauensis* PBC-2 foi estudada em meios com diferentes concentrações de ferro e em um meio de cultura, com paredes celulares de fungos, como única fonte de carbono. Os resultados obtidos neste estudo sugerem que a produção e secreção de enzimas líticas não é o principal ou o mais importante modo de ação do agente de controlo biológico PBC-2, uma vez que a produção de quitinase observada ao 5 e 7º dia de incubação foi muito baixa, e não foi observada a produção de β-1,3-glucanases e proteases.

Palavras chaves: agentes de biocontrolo; pós-colheita; citrinos; pomóideas; produção de biomassa; reatores biológicos

ABSTRACT

Biological control of postharvest diseases of fruit using microorganisms has emerged as an alternative to human health and environmental problems associated with the use of synthetic fungicides. This thesis has two main objectives: obtain biocontrol agents (BCAs) of the major postharvest diseases of citrus and pome fruit and find a low cost culture medium that allows high biomass productivity, and yield, viable and effective. Citrus and pome fruit and leaves microbiota were screened for *in vivo* antagonistic activity of *Penicillium digitatum* and *Penicillium expansum*. The bacterium *Pantoea agglomerans* PBC-1 and the yeast *M. andauensis* PBC-2, described for the first time as a BCA, were selected. *P. agglomerans* (1×10^8 cfu/ml) reduced 86% of *P. expansum* and *P. digitatum*, *M. andauensis* (1×10^7 cfu/ml) reduced incidence and severity by 90 and 95%, respectively. To optimize the production medium, microorganism's ability to use different carbon sources was studied. Sucrose was selected for STR production of both BCAs. Once the medium and growth of STR conditions of both BCAs were optimized, to reduce costs food industry by-products as carbon sources were used in the production of both BCAs. Carob by-products plus yeast extracts was used for *P. agglomerans* STR production, achieving a biomass productivity of 0.163 (g/l.h) and yield of 0.119 (g/g) with inoculum of 10^6 cfu/ml, and 1.527 (g/l.h) and 1.468 (g/g) with inoculum of 10^7 cfu/ml, both providing a population of 9×10^9 cfu/ml. Citrus industry wastes (liquor) plus yeast extract was used for *M. andauensis* STR production. After 40 h the viable population was 3.4×10^8 cfu/ml, biomass productivity and yield were 0.435 g/l.h and 1.502 g/g, respectively. In semi-commercial trials during four seasons, *M. andauensis* effectiveness to control *P. expansum* was comparable with the fungicide imazalil. Production of lytic enzymes and iron competition are not the mode of action of *M. andauensis*.

Keywords: biocontrol agents; postharvest; citrus; pome fruits; biomass; production; STR

ORGANIZAÇÃO DA TESE

A tese está organizada em 8 partes distintas: introdução geral, 6 capítulos cada um em forma de artigos onde se descreve o trabalho de investigação realizado e discussão geral e conclusões. Dos 6 artigos apresentados, 3 são artigos publicados em revistas científicas indexadas ao SCI, 1 como capítulo de um livro e os restantes 2 encontram-se submetidos revistas científicas indexadas ao SCI.

O diagrama de fluxo, abaixo apresentado, representa esquematicamente o programa de desenvolvimento de um agente de biocontrolo, com correspondência aos capítulos desta tese.

No primeiro capítulo é introduzido o contexto e âmbito do trabalho, com a respetiva pesquisa bibliográfica.

No segundo capítulo descreve-se o que num processo de desenvolvimento de um agente de biocontrolo, seria a primeira etapa, o isolamento massivo de microrganismos e a avaliação da capacidade antagonista de cada um dos isolados, com vista à seleção de um potencial agente de biocontrolo.

Encontrado o potencial agente de biocontrolo e paralelamente à realização de outros testes, são iniciados os ensaios que visam identificar e satisfazer as necessidades nutricionais e as melhores condições de produção de biomassa. Nos capítulos 3 e 4, são estudadas as condições de produção em Erlenmeyer,e consequente transição para reator 2-L, dos agentes de biocontrolo *M. andauensis* PBC-2 e *P. agglomerans* PBC-1, respetivamente.

No capítulo 5, expõe-se o processo de otimização da extração de açúcares de subprodutos da indústria de alfarroba e a utilização destes como fonte de carbono alternativa no crescimento de *P. agglomerans* PBC-1, com o intuito de reduzir os custos de produção.

No capítulo 6, descrevem-se os ensaios de crescimento do agente de biocontrolo *M. andauensis* PBC-2, com subprodutos da indústria de sumos de citrinos, como fonte alternativa de carbono.

O estudo dos possíveis modos de ação do agente de biocontrolo *M. andauensis* PBC-2, estão descritos no sétimo capítulo.

Por fim, são apresentadas as conclusões gerais deste trabalho conjuntamente com algumas perspetivas futuras.

THESIS ORGANIZATION

The thesis is organized in 8 distinct parts: a general introduction, 6 chapters each one structured as papers, describing the research work, a general discussion and conclusions. Of the 6 chapters, 3 are papers published in SCI indexed journals, one as a book chapter and 2 are submitted to journals indexed to SCI.

The flow diagram, presented below, depicts the development program of a biocontrol agent, with correspondence to the chapters of this thesis.

The first chapter presents the context and scope of work. The quality concepts, the postharvest of fruit, some of the major pathogens of citrus and pome fruits and their control, are covered in this chapter. It presents biological control as an alternative to synthetic fungicides, as well as the different steps of development program of BCA, since isolation to commercialization, with particular emphasis on production.

The second chapter describes what in a BCA development program would be the first step, the massive isolation of microorganisms and screening the antagonistic ability of each isolate to control pathogenic agents.

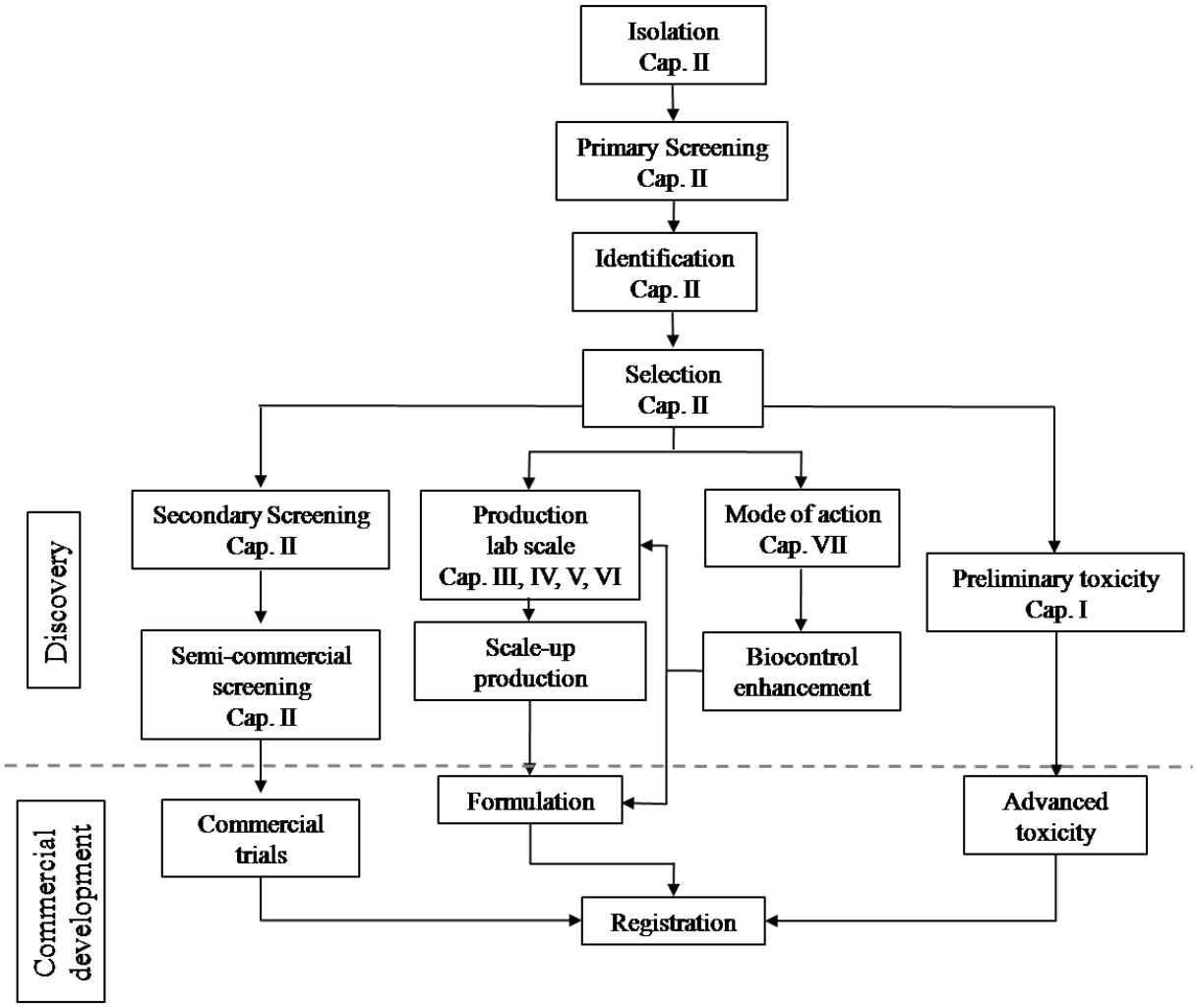
Selected the potential BCA and in parallel to other tests, are initiated trials that aim to identify and satisfy the nutritional requirements and the best conditions for biomass production. In chapters 3 and 4, are studied the conditions of production in shake flasks and consequent transition to STR 2-L of biocontrol agents *M. andauensis* PBC-2 and *P. agglomerans* PBC-1, respectively.

The chapter 5 describes the optimization process of sugars extraction from carob industry byproducts and the use of the extracted sugars as alternative carbon source on the production of *P. agglomerans* PBC-1, in order to reduce production costs.

In Chapter 6, describes the growth assays of biocontrol agent *M. andauensis* PBC-2, with waste and byproducts of the citrus juice industry.

The study of the possible modes of action of the biocontrol agent *M. andauensis* PBC-2, are described in the 7th chapter.

Finally, it is present a general discussion, conclusions of this work with some future prospects.



Flow diagram of development of a postharvest biocontrol agent, with corresponding chapters of this thesis

CONTENTS

LIST OF FIGURES

ACRONYMS AND SYMBOLS

cfuCFU - Colony forms unit

CJA - Citrus juice agar

FAO - Food and Agriculture Organization

FCT.- Fundação para a Ciência e a Tecnologia

HCl - chloride acid

HPLC - High-performance liquid chromatography

$K_L a$ - Volumetric mass transfer coefficient

LBG or CBG - Locust bean gum

NaCl - sodium chloride

NaOCl - sodium hypochlorite

NaOH - sodium hydroxide

NCYC - National Collection of Yeast Cultures

NYDA - Nutrient broth, yeast extract, dextrose, agar

NYDB - Nutrient broth, yeast extract, dextrose, agar OD - optical density

OUR - oxigen uptake rate,

OUR- oxygen transfer rate

PDA - Potato dextrose broth

PDO - Protected Designation of Origin (Denominação e Origem Protegida)

P_{max} - biomass productivity

ppm

rev/min or rpm - Revolutions per minute

RH - relative Humidity

RML - Maximum Residue Limit

R_s - substrate uptake rate

STR - Stirred tankque reactor

TCA - Trichloroacetic acid

U - enzymatic unit

Ufc – unidades formadoras de colónias

UK - United Kingdom

USA - United States of America

vvm – air flow rate

WHO - World Health Organization

X_{max} - maximum biomass

YES - yeast extract; sucrose; agar

YNB - yeast nitrogen base medium

YPD - yeast extract, peptone, dextrose

YPDA - yeast extract, peptone, dextrose agar

YPS - yeast extract, peptone, sucrose

$Y_{x/s}$ - biomass yield

μg - specific growth rate

μl – microlitre

Chapter 1

Introduction

INTRODUCTION

The benefits of an adequate intake of fruits and vegetables, is now a scientific evidence.

A poor diet in fruit and vegetables is associated with an increased risk of diseases, particularly cardiovascular (Crowe *et al.*, 2011) and carcinogenic diseases. An inadequate intake of fruit and vegetables, only in the European Union, is responsible for 1 million deaths per year, (Pomerleau *et al*., 2006).

Fruit are an important source of many essential nutrients, such as vitamins, folic acid, antioxidants, including, carotenoids, polyphenols and anthocyanins, which help in combating free radicals produced by the organism (Paliyath *et al.*, 2008).

1. Concept of Quality

The growing concerned of the consumer with issues related to food, health and environment, led to a growing demand for quality fresh produce.

Quality is a subjective concept, which varies with the type of consumers, depending for example with nationality, age and eating habits, what may be relevant for some, for others it may not be (Silva & Morais, 2000; Kader, 2008). However, there is a consensus, that factors such as visual appearance (size, shape, aspect, color, brightness, defects), texture (firmness, integrity of the tissue), flavor (aroma and taste), absence of defects, nutritional value, and more recently safety (chemical and microbiological contamination), are part of a set of attributes which define the quality (Kader, 2002; Berdegué *et al.*, 2005; Lairon 2010).

After harvesting and along the supply chain until arrive to the consumer (Fig. 1), fruit remain metabolically active and lose quality. The normal physiological processes, loss of intrinsic resistance, biological factors, such as pests and diseases, physiological disorders, together with any mechanical damage, are responsible for the high perishability of these products.

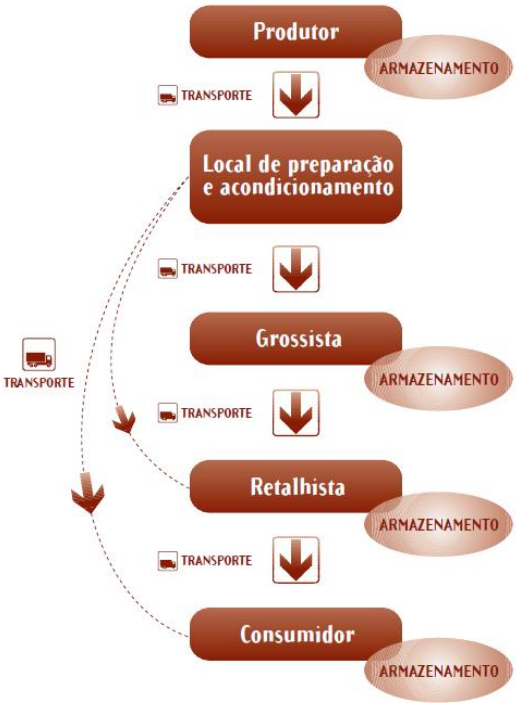


Figure 1.1. General supply chain flow for fruits and vegetables (adapted from Silva & Morais, 2000.

Postharvest losses of fruit and vegetables are significant, depending on the species, harvest, transport and storage, they may represent about 20-25% of total production in industrialized countries and more than 50% in developing countries, in which handling and storage techniques are not optimal (Harvey, 1978; Eckert & Ogawa, 1988; El-Ghaouth *et al*., 2004; Spadaro & Gullino, 2004; Droby, 2006; Nunes *et al*., 2009; Teixidó *et al*., 2011; Nunes, 2012).

In 1952, Stevens & Stevens reported that of all losses caused by plant diseases, those that occur in postharvest are the most costly in terms of money and human resources.

2. Postharvest Pathogens

The chemical composition of fruit, rich in nutrients and with high water content, makes them susceptible to be attack by fungal and bacterial pathogens (Droby *et al*. 1992).

Fungi are the main postharvest agents of decay in fruit, causing major economic losses, either when they are consumed immediately after harvest or when they are storage in cold. The main fungi responsible for the losses in pome and citrus fruit belong to the genera *Penicillium*, *Botrytis*, and *Rhizopus*.

a. *Penicillium*

Penicillium digitatum Sacc. causing green mould and *Penicillium italicum* Wehmer causing blue mould, are wound pathogens, and the most common and devastating postharvest pathogens of citrus growing countries. They can infect the fruit in the field, in the packinghouse, in transportation and in the market. Both pathogens are specific of all citrus varieties, however *P. digitatum* is more widespread and the most economically important in Mediterranean countries, California and all production areas with low summer rainfall (Eckert & Eaks, 1989). In other areas such as China, the largest world producer of citrus fruit, *P. italicum* (Fig. 2a) can cause losses of 30-50% (Long *et al*., 2005).

Conidia of both fungi are present during the season in the atmosphere of citrus growing areas, particularly in packinghouses, in their equipment and in their surroundings (Barkai-Goland, 2001). The fungus reproduces very rapidly and if there are inappropriate packinghouses sanitation measures, the inocula level in packinghouses may gradually increase during the season (Palou *et al*., 2001a).

P. digitatum is considered a strict wound pathogen, which means that despite being present on the surface of the fruit, the spores do not germinate in the absence of a wound in the flavedo. This fungus grows more rapidly at moderate temperatures so it predominates during short-term transit and storage. *P. italicum* may sometimes develop in storage as hyper-parasite over the green mold decay. *P. italicum* is also considered a strict wound pathogen, but unlike *P. digitatum* is able to spread by contact between infected and sound fruit, due to galacturonic acid and the enzymes released during the infection process that damage healthy tissues allowing the invasion of the hyphae (Whiteside *et al.*, 1993).

The optimum temperature for both fungi is 20-27 ºC, within which the fruit may rot within a few days and the growth rate decreases at 4.5-10 ºC but is still capable of growing. At early stage both fungi cause a soft rot of the peel, following with a development of a white mycelium and later starts the sporulation from the center of the colony. The sporulation part becomes olive green in the case of *P. digitatum* and blue in the case of *P. italicum*.

In pome fruit *Penicillium expansum* Link, responsible for blue mold, is the most destructive disease of apples and pears postharvest, having come to cause 80-90% of the damages. It is important in all producing countries worldwide: Italy, France, Spain,

Portugal, Greece, Germany, UK, Poland, countries of North and Central America, Chile, Israel, India and Australia (Viñas & Usall, 2000).

Germinating conidia invade the fruit through wounds or damages occurred during harvesting and handling. Penetration can also occurred through lenticels, mainly in over mature fruit. The affected areas are soft, watery, with a color that can vary in different brown shades, depending on the variety, the contours are well defined, and the affected tissue separates easily from the healthy parts (Rosenberger, 2002; Mondino *et al*., 2009). The affected area is covered with a mycelium whitish, recovering gradually with a blue-green sporulation (Fig. 2b). The fungus can spread during long months of storage, by contact between infected and sound fruit.

Besides the postharvest losses due to deterioration of the fruit, *P. expansum* is also responsible for the production of a carcinogenic mycotoxin, patulin (Ritieni, 2003; Sant'Ana *et al*., 2008). In pome fruit usually stored for many months in cold chambers with controlled atmosphere, this toxin can rise to unacceptable levels in fruit destined for processing and may also result in undesirable flavors (Nuovo & Wilson, 1973; Mattheis & Roberts, 1992).

b. *Botrytis*

Botrytis cinerea Pers.: Fr, caused the commonly called gray mold and is another pathogen responsible for postharvest losses in several fruit. Its importance is due to its faster development than other rots at refrigeration temperatures, being the most important disease in pears (Rosenberger, 2002; Montesinos, 2000). The fungus survives as sclerotia in the soil and plant debris. The process of infection can occur through wounds and direct penetration through the epidermis. The fruit rot is dry and firm in the beginning, but become soft as the rot advances. Under humid conditions abundant gray-brown conidia are produced, and sclerotia may also be formed (Fig. 2d) The fungus spread very rapidly during storage by contact between infected and sound fruit, forming nests of decaying fruit, is one characteristic of this disease, since the simple contact with an infected fruit is sufficient to cause secondary infections of fruit and generate groups joined by mycelium.

c. *Rhizopus*

Rhizopus stolonifer (Ehrenberg: Fries) Vuillemin, are the cause of *Rhizopus* rot, although is a more serious postharvest disease of stone fruit, in pome fruit could cause

severe losses, especially in pears. Originates primary in the field, appearing later during cold storage. The symptoms are a brown rot, which develops rapidly decomposing and liquefying the internal part of the fruit, so it calls the soft rot. In the infected tissue can be observed a mass of a white filamentous mycelium, which become black (Fig. 2c) (Rosenberger, 2002; Mondino *et al*., 2009). The decay can expand and affect other fruit since there is a production of an exudate acid, having a sour odor, which contains enzymes that serve to colonize other fruit by the fungus.

Figure 1.2. Some major post-harvest fungi of citrus and pome fruits a) *Penicillium italicum*; b) *Penicillium expansum*; c) *Rhizopus stolonifer*; d) *Botrytis cinerea.*

3. Control of postharvest diseases

The development of postharvest diseases, as well as the severity of the losses depends mainly of the handling during and after harvest, storage conditions and the presence and contact of the pathogens with the fruit. Therefore, the adoption of preventive measures can reduce some of these problems. There should not be collect in rainy and high humidity days; immediately after harvest, the fruit must be cooled quickly and be assured a good cold chain management; cross-contamination should be prevent through the creation of separate areas: clean and dirty area; boxes, bins, cold chambers, packaging material should be disinfected regularly.

The physiological age of the host, may be also important. Fruit in an advanced state of maturity are more susceptible to decay, has less resistance, less firmness and more likely to be injured (Sanhueza, 2004). Currently, the control of these diseases is based on the use of synthetic fungicides, usually applied in drencher in aqueous spray or combined with waxes (Droby, 2006). These products have a curative effect against pre-existing or established infections, a preventive effect against potential infections, high persistence, easy application and relatively low cost (Palou *et al*., 2008). In contrast the

disadvantages of these products arose as a result of its excessive, poorly controlled and continuous use, seriously compromising its effectiveness and use. An example is the development and spread of resistant strains of the pathogens, the development of iatrogenic diseases and increase of residues in fruit (Janisiewicz, 1991).

In the last years, there has been a growing concern with health and an increased awareness of the need to preserve natural resources and the environment, both for current and future generations. In this context, the objectives of agricultural production is to offer to the consumers products with guaranteed quality and safety, produced by methods that ensure the preservation of natural resources and respect the environment The concept of quality has changed, to the product appearance (color, shape, size), now joins the aspects related to production and marketing. In quality control of the products, besides the usual microbiological analyzes the analysis of residues are also common. The control of this parameter is made with reference to the Maximum Residue Limit (MRL), which consists of the maximum permitted concentration of a pesticide on the inside and on the surface of food or feed. In the European Union, Regulation (EC) n. 396/2005 of the European Parliament and of the Council of 23 February 2005 has set these values.

In 1963, a commission established by the FAO (United Nations Food and Agriculture Organization) and WHO (World Health Organization), produced the *Codex Alimentarius*, a code that compiles a set of standards, guidelines relating to foods, food production and food security, designed to protect the health of consumers and ensure fairness in international food trade. It is recognized by the Commercial Trade Organization as reference for resolving disputes over food safety and consumer protection (http://www.codexalimentarius.org/ (04/11/2011).

The search for methods of disease control, alternatives to synthetic products represents itself not as an option but as an urgent need to find a solution.

According to the nature of the methods, these can be classified into physical, chemical and biological, their action can be observed directly on the pathogen, or may cause effects on the host itself.

4. Methods of control alternative to fungicides

a. Physical methods

As examples of physical methods can be enumerate the following: heat treatments such as heat applied in air (curing) (Plaza *et al*., 2003; Nunes *et al*., 2007; Palou *et al.*, 2009)

or applied in water (Schirra & D'Hallewin, 1997; Porat *et al*., 2000; Torres *et al*., 2007); cold temperature (low temperature will reduce the disease development, as well as the metabolic activity of the host, delaying senescence); controlled and/or modified atmosphere (Ahmadi *et al*., 1999; Tian *et al*., 2001); UV-C illumination (Chalutz *et al*., 1992; Stevens *et al*., 1998; Shama, 2007; Nunes *et al*., 2010a), ionizing radiation such gamma radiation (Gherbawy, 1998) and the use of edible films (Cagri *et al*., 2006).

b. Chemical methods

Chemical methods alternative to conventional fungicides are chemical based on the application of natural or synthetic minimum toxicological risk, as some food additives or substances classified by the FDA (United States Food and Drug Administration) as GRAS (Generally Regarded As Safe) (Nunes, 2010b). Examples: peracetic (PAA) (Mari *et al*., 1999;Mari, 2007), ozone (Rice *et al*., 1981), sodium bicarbonate (Torres *et al*., 2007; Palou *et al*., 2009), potassium sorbate (Salazar *et al*., 2008), chlorine dioxide (ClO_2) electrolyzed water (Graça *et al*., 2011 a,b) , natural compounds from animals, plants or microorganisms (Mari *et al*., 2003; Obagwu & Korsten, 2003; Sanzani *et al*., 2010), as oils (Abdollahi *et al*., 2011; de Rodriguez *et al*., 2011; Marandi *et al*., 2011), volatile compounds and phenolic compounds, antibiotics, chitosan and its derivatives (Hirano, 1999; Devlieghere *et al*., 2004).

c. Biological methods

Over the past 25 years biological control of postharvest diseases using microbial antagonists has emerged as an effective strategy to control the major postharvest decays of fruits and has attracted much interest and effort by several research groups all over the world.

The pioneering studies of the interactions between pathogens and other microorganisms date back to 1920, with the introduction of antagonists in forest soils to control damping-off in pines (Hartley, 1921 in Boland, 1990). In 1977, Trosmo & Dennis, applied this knowledge to postharvest control of strawberry decay, using different species of *Trichoderma* spp. Later, in 1984, the use of *Bacillus subtilis* to control, *Monilia fruticola* in different stone fruit and to control different decay in citrus was published by Wilson & Pursey and Singh & Deverall, respectively. The results of these studies encouraged many others, and during the last three decades programs to develop biocontrol agents in postharvest fruit have been conducted all over the world,

but particularly in Europe (mainly Belgium, Italy, Spain and Sweden), USA, Israel, South Africa and more recently in China (Janisiewicz, 2010; Nunes *et al.*, 2009; Sharma 2009; Teixidó *et al.*, 2011, Nunes *et al.*, 2012).

Many bacteria and yeast have shown their potential in the control of different pathogens in different fruits and vegetables and in varying conditions. Table 1 presents formulated biocontrol agents targeting post-harvest diseases already on the market.

Table 1. Formulated biocontrol agents targeting post-harvest diseases already on the market (adaptated from Sharma 2009; Teixidó *et al*., 2011)

Product	**Microbial agent**	**Fruit/vegetables**	**Pathogen**	**Manufacturer/ distributor**
Biosave 10LP	*Pseudomonas syringae* (strain 10 LP)	Citrus fruit, Cherries, Pome fruit, Potatoes	*P. digitatum*, *P. italicum*, *Botrytis cinerea*, *P. expansum*, *Mucor piriformis*, *Geotrichum candidum*	JET Harvest Solutions
Biosave 110	*Pseudomonas syringae* (strain 110)	Pome fruit, Potatoes, Sweet potatoes	*Rhizopus stolonifer*, *Mucor piriformis*, *P. expansum*, *F. sambucinum*	JET Harvest Solutions
Shermer	*Metschnikowia fruticola*	Citrus fruit, Pome fruit, Strawberries, Stone fruits, grapes, Sweet potatoes	*P. digitatum*, *P. italicum*, *Botrytis cinerea*, *P. expansum*, *Rhizopus stolonifer*, *Aspergillus niger*	Bayer cropscience
Pantovital	*Pantoea agglomerans* (strain CPA-2)	Citrus fruit, Pome fruits	*P. digitatum*, *P. italicum*, *Botrytis cinerea*, *P. expansum*, *Rhizopus stolonifer*	BioDURCAL S.L
Boni Protect	*Aureobasidium pullulans*	Pome fruits	*Botrytis cinerea*, *P. expansum*, *Monilinia frutigena*	BioFerm GmbH
Candifruit	*Candida sake* (strain CPA-1)	Pome fruits	*Botrytis cinerea*, *P. expansum*, *Rhizopus stolonifer*	SPICAM-INAGRA S.A.
Aspire	*Candida oleophila* (strain 1–182)	Pome fruit, Citrus fruit	*P. digitatum*, *P. expansum*, *Botrytis cinerea*	Ecogen, Inc.
Serenade	*Bacillus subtilis*	Pome fruits, Grapes and	*Powdery mildew, late blight, brown rot*	Agro Quess Inc.

		Vegetables	*and fire blight*	
Yield plus	*Cryptoccocus albidus*	Pome fruits	*B. cinerea, P. expansum*	Anchor Yeast

d. Integration of different methods

The level of control of pathogen development by non-fungicidal methods, it is sometimes insufficient, especially when compared to control levels obtained with the conventional fungicide, therefore one approach to use these methods as an is an integrated strategy, taking advantage of the additive or synergistic effects of different treatments in order to overcome the performance and improve the efficacy of each one, such as the combination of biocontrol with physical (Plaza *et al* ., 2003) or chemical methods (Janisiewicz, 1998; Spadaro *et al*. 2004; Manso *et al.*, 2007;Torres *et al*. 2007), or either the combination with other biocontrol agent (Nunes *et al*., 2002; 2005; Conway *et al*., 2007) or even with low doses of synthetic fungicides (Qin & Tian, 2004; Lima *et al*., 2008). As a result of this integrated approach of biocontrol systems in the development of a second generation of products such "Biocoat" whose main components are *Candida saitoana and* chitosan also with *C. saitoana* and lysozyme (Wisniewski *et al.*, 20079

5. Biological control in postharvest

Postharvest environment represents a particular advantage to develop biological control when compared with field environment: storage in environmental conditions, such as humidity and temperature, which may be managed in order to favor the survival of the antagonist; the harvested products have a higher added value, which determines the feasibility of more elaborate control methods, that would not be economically viable in the field; less interference from other factors and microorganisms; the biomass to protect is concentrated, so in a single application using existing equipment, can directly treat any wounds made during harvesting and handling (Wilson & Wisniewski, 1991, Janisiewicz, 1990; Janisiewicz & Korsetn, 2002; Wisniewski *et al*., 2007). Although the postharvest environment presents a uniqueness biocontrol system, Chalutz and Droby (1989) suggest that specific difficulties should be taken in account: the required high level of disease control (95-98%) in postharvest, food safety requirements with the application of live microorganisms in food and the relatively small potential market for the use of postharvest biofungicides.

a. Definition of biological control of plant diseases

In the last years a broader definition of biological control was adopted by plant pathologists. Instead of the definition based in entomology, which involves the control of one organism by another, biocontrol is defined know as the control of a plant disease by a biological process or the product of a biological process (Wisniewski *et al*., 2007). This process can be influenced at different levels: the pathogen, the micro-environment, and the host (Droby *et al*., 2009). In accordance with a broader definition of biological control, in addition to the use of an organism, managing a plant disease could involve the use of a biological process or the product of a biological process. Therefore with this definition new approaches have been started, developing biocontrol systems in order to overcome the existing limitations. Main focus is to promote a high and constant efficacy and make possible the control of previously established and latent infections. It is possible to remark: (i) antagonistic mixture; (ii) manipulation of nutritional environment; (iii) pre-harvest application; (iv) genetic manipulation of antagonists; (v) physiological improvements, and (vi) integration with other methods. These approaches are expected to overcome the problems of the "first generation" of biocontrol agents, such high efficacy variability, reduced spectrum of activity, and preventive action only.

b. Desirable characteristics of a biocontrol agent

In 1989, Wilson & Wisniewski, synthesized the desirable characteristics of a biological control agent, and since these features have served as guidelines for biological control development programs and publications (Janisiewicz & Korsten, 2002; Droby *et al*., 2009; Nunes *et al*., 2009; Sharma *et al*., 2009; Teixidó *et al*., 2011; Nunes, 2012). A biocontrol agent should be: genetically stable; effective at low concentrations against a wide range of pathogens and commodities; ability to survive under adverse environmental conditions; simple and inexpensive nutritional requirements; inexpensive to produce and formulate with long shelf-life; easy to dispense; compatible with commercial processing practices; resistant to most common pesticides; non pathogenic for the human health and host; can effectively produce a formulation with extended validity, incapable of producing secondary metabolites toxic to people and animals.

c. Development of a biocontrol agent

An antagonist to becoming an economically feasible as biocontrol agent of postharvest diseases required intense study and involvement of multidisciplinary teams, as plant pathologists, microbiologists, chemists, biotechnologists, whose main objectives are to demonstrate the effectiveness of the microorganism in disease control and the safety for the host, human and environment (Costa *et al*., 2002; Garmendia & Vero, 2006). Furthermore, it is also advisable a close cooperation with the industry, since often it is responsible for the production and product formulation, to ensure their viability (Janisiewicz & Korsten, 2002).

In Figure 3 is shown a diagram of the steps that may be involved in the development of a biocontrol agent. Knowing each of the elements that comprise the system to protect, as the pathogen, the host, the environment is crucial and must precede the development program of the biocontrol agent (Nunes, 2012).

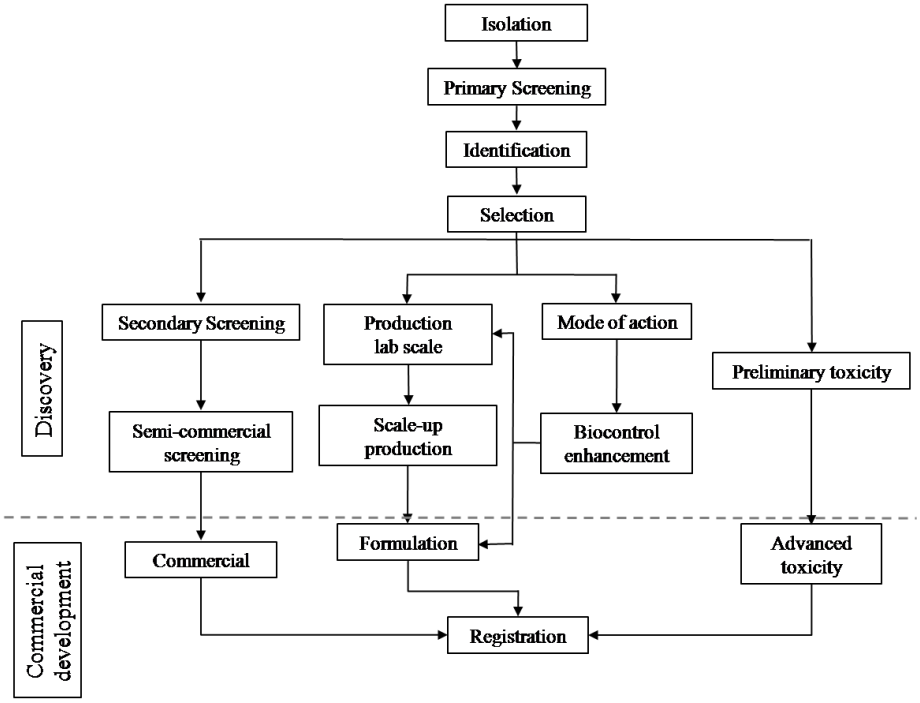


Figure 1.3. Schematic representation of the development of a biological control agent (adapted from Nunes *et al*., 2009).

5.3.1 Isolation and screenings

The first step of a biological control program involves the isolation, identification and evaluation effectiveness of the microorganism to control pathogens. It is assumed that

the microorganisms naturally present in the plant to protect or in the surrounding area, show advantages over other (Chalutz & Wilson, 1990; Wilson & Wisnieski, 1994), and it is accepted that antagonists should be isolated from the environment where they be applied (Janisiewicz & Korsten, 2002; Teixidó *et al*., 2011). Nevertheless there are those who argue that there is no relationship between efficacy and origin of isolation (Jijakli *et al*., 1999). In control of postharvest diseases, the isolation of the microbiota focus mainly on fruit and/or vegetables (Fig. 4a and 4b) before harvest and during storage (Janisiewicz, 1988; 1991), favoring the antagonists that are well adapted to survive and colonize the wound and the surface of the host, and thus increasing chances of the product being better accepted by the public.

After the massive collection of the microorganisms, follows the evaluation of its antagonist capacity *in vitro* or *in vivo* assays. The *in vitro* assays select antagonists producing antifungal substances and often do not reflect the activity of *in vivo* control (Boland, 1990; Janisiewicz, 1998). For example, from approximately 2200 microorganisms, 16% showed antagonistic activity *in vitro* and only 4.7% in *in vivo* tests (Woodhead *et al*. 1990). *In vivo* tests, although laborious, allow selecting microorganisms with modes of action different from antibiosis and for being more embracive increases the chances of finding a good biocontrol agent. The methodology adopted by different research groups for microorganism selection, though with slight modifications, is based on the methods described by Janisiewicz in 1987 and by Wilson in 1993 and involves the application of washing solutions of the fruit surface in artificial fruit wounds, followed by the application of the pathogen (Fig. 4d and 4e). Since the technique used, affects the type and characteristics of the biocontrol agents, microorganisms isolated with these procedures exhibit rapid growth and ability to colonize wounds, which are rich in nutrients, giving a protective character and not curative, making them ineffective in latent infections (Droby *et al*., 1989; El-Ghaouth *et al*., 2000), whereby the method has recently have some controversy (Droby *et al*., 2009; Janisiewicz, 2011) yet still commonly used.

The screenings tests, classified into primary screenings has the goal of determine the ability of the microorganism in reducing the development of the pathogen and secondary screenings of determine the minimum effective concentration (MEC) of the antagonist (Janisiewicz, 1998; Nunes *et al*., 2001). Simultaneously is made the identification of the microorganism, in order to exclude the possibility of be a pathogen

or taxonomically related, avoiding future problems with the registration necessary for commercialization.

To find a biocontrol agent, numerous microorganisms must be collected and tested, since only a small fraction of them meet the stringent selection criteria.

In most cases, the effectiveness of the antagonist depends on its concentration in relation to the pathogen (Karabulut *et al*., 2004; Zhang *et al*., 2009). It is important that the concentration of the pathogen used in screening assays reflect the actual concentrations observed in packinghouses, moreover, the concentration of the antagonist should be reasonable for their commercial development. From a commercial point of view, the threshold for bacteria is 10^9 cfu/mL and for yeasts is 5×10^7 cfu/mL (Janisiewicz, 1988).

The next steps involve studies of the modes of action of the antagonist, conduct efficacy studies with different hosts and pathogens, assess their ability of adaptation to environmental conditions where they have to act, particularly cold conditions and even controlled or modified atmosphere, and its compatibility with other treatments and additives used in packinghouses

Figure 1.4. Isolation of microorganisms in: a) fruit; b) leaves; c) diversity of microorganisms found; d) screening assay: pathogen inoculation in wound; e) inoculation of potential biocontrol agent; f) drying time between applications.

5.3.2. Mode of action of biocontrol agents

The mechanism by which antagonists inhibit or delay the development of the pathogen is not easy to determine. However, this knowledge is useful for the development of the system, the optimization of timing and method of application, enhancement of the control activity, the commercial registration (Droby & Chalutz, 1994) and even to isolate more effective antagonists (Wisniewski & Wilson, 1992).

A successful biocontrol agent generally has a multiplicity of mechanisms of action that work together to prevent the development of disease. Is assume that are involved interactions between the host, the pathogen, the antagonist and the environment, including processes such as antibiosis or production of inhibitory substances, competition for space and/or nutrients, direct parasitism, and induction of resistance to the host (Baker, 1991; Droby & Chalutz, 1994).

Competition for space and/or nutrients, it has been suggested by many researchers as the mode of action of many antagonists effective in controlling postharvest diseases (Piano *et al*., 1997; Poppe et al., 2003; Spadaro *et al.*, 2002; Bencheqroun *et al.*, 2007). For this mode of action, the antagonist has an indirect action on the pathogen, since both compete for the same resources. The antagonist, being able to uptake more quickly and efficiently nutrients and colonize the space needed for its development, reduces the availability of these resources to other microorganisms, establishing itself as dominant population. This mechanism of action is particularly effective in the control of postharvest diseases, in so far as antagonists are applied directly to the wound, the main point of entry of the pathogen, (Janisiewicz *et al*., 1992). A key factor to the existence of competition is the scarcity of a necessary element for the development of the microorganisms, so that the antagonist exercises its competitive advantage. Therefore this is used in studies of mode of action *in vitro* and *in vivo*, which evaluates the biocontrol activity under conditions of scarcity and abundance of an element. Examples, such *in vitro* evaluation of competition for iron (essential for fungal growth) in which it was found that certain microorganisms produce siderophores, molecules with low molecular weight, capable to chelate and transport iron to the interior of its cells (Calvente *et al*., 1999; Sipiczki, 2006; Saravanakumar *et al*., 2008; Vero *et al*., 2009), as well as assays of competition for glucose established by Filonow et al. (1996) and Filonow (1998) which showed the largest capacity of the biocontrol agents to use the carbon sources.

The production of inhibitory substances is another important mechanism by which the biocontrol agent can suppress the development of the pathogen. The nature of the inhibitory substances produced by the antagonist may be varied, such as antibiotics, volatile compounds, toxins, enzymes capable of degrading pathogen. The biocontrol agents most referenced by antibiosis belong to the genus *Bacillus*, such as *B. subtilis* (Pusey & Wilson 1984; Stein, 2005), *B. amyloliquefacien* (Arguelles-Arias, 2009; Arrebola *et al*., 2010), *B. licheniformis* (Jamalizadeh *et al*., 2008), and the genus *Pseudomonas* such as *P. syringae* (Smilanick & Denis-Arrue, 1992; Stockwell & Stack, 2007) *P. cepacia*, (Janisiewicz & Roitman, 1988), *P. fluorescens* (Cabrefiga *et al*., 2007), *P. corrugata* (Smilanick *et al*., 1993). However, it is possible to find references to other genera of microorganisms, with equally interesting results, such as *Serratia plymuthica* (Meziane *et al*., 2006), *Brevibacillus brevi* (Edwards & Seddon, 2001), *Brevibacillus laterosporus* (Saikia *et al*., 2011). There is however some concern in developing a product based on antibiosis as a mode of action and due to the introduction of an antibiotic into food and possibility of emergence of resistance not only to pathogens affecting the host, but especially humans. Thus, the use of microorganisms that produce volatiles inhibitory has a greater acceptance since direct contact with the food does not exist, furthermore has the advantage that it can be integrated into the usual postharvest technologies using the exits equipment, as is the case in citrus degreening (Mercier & Smilanick, 2005). *Muscodor albus* is one of biocontrol agents, used in fumigation to control pathogens in citrus (Mercier & Smilanick, 2005), apples (Mercier & Jiménez, 2004; Ramin *et al*., 2007), in peaches, in grapes (Mercier *et al*., 2005; Gabler *et al*., 2010) and *Bacillus subtilis* (Chen *et al*., 2008; Arrebola *et al*., 2010), *Candida intermedia* in controlling *Botritys* in strawberries (Huang *et al*., 2011) and *Streptomyces globisporus* in tomatoes (Li *et al*., 2012).

Direct interaction of the biocontrol agent with the pathogen is another mode of action described by Wisniewski *et al*. (1991) and by Arras *et al*. (1998). They found that cells of *Pichia guilliermondii* showed the ability to attach to the hyphae of *B. cinerea*, causing deformations in the attach site, verifying that there has been degradation of the cell wall of *B. cinerea*. Similar results in hyphae of *B. cinerea* were observed with *Candida sai0tana* by El-Ghaouth *et al*. (1998; 2003) and with *Metschnikowia pulcherrima* by Piano *et al*. (1997). Apart from the direct interaction Zhang *et al*. (2011) concluded that *Pichia guilliermondii* in the control of *B. cinerea* on apples also presented as modes of action competition for nutrients, production of lytic enzymes and

induction of resistance. The production of lytic enzymes such as proteases, glucanases and chitinases by the antagonist, help in the degradation of the pathogen cell wall (Haran *et al*., 1996; Sharma *et al*., 2009). Grevesse *et al*. (2003) suggest the involvement of exo-β-1,3-glucanase in the antagonistic activity of *P. anomala* in the control of *Botritys* sp. in apples. Production of lytic enzymes has also been demonstrated in *Candida oleophila* (Segal *et al*., 2002; Bar-Shimon *et al*., 2004), *Rhodotorula glutinis* and *C. laurentii* (Castoria *et al*., 1997).

Another proposed mode of action is the induction of resistance. After the detection of a pathogen or its metabolites, the host prepares defense systems to protect themselves from infection (Rodov *et al*., 1994; Fajardo *et al*., 1998; Porat *et al*., 1999; Droby *et al*., 2002). These responses include strengthening the cell wall by deposition of lignin, callose and glycoproteins rich in hydroxyproline, phytoalexin accumulation, synthesis of inhibitors of proteinases and lytic enzymes (El-Ghaouth *et al*., 2004), and the production of reactive oxygen species (Torres , 2010; Jamalizadeh *et al*., 2011). The induction of resistance as a mode of action is due to the ability of the antagonist to induce defense responses by the host. Treatment with *Aureobasidium pullulans* in apples wounds leads to the induction of resistance by increasing the production and activity of β-1,3-glucanase (Castoria *et al*., 2001), chitinase and peroxidase (Ippolito *et al*., 2000). The yeast *C. oleophila* when applied to superficial wounds of grapefruit induces resistance to *P. digitatum* by the production of ethylene, biosynthesis of phytoalexins and accumulation of chitinase and β-1,3-glucanase enzymes (Droby *et al*., 2002). The ability of *C. oleophila* and *M. fructicola* to induce oxidative defense responses in citrus and apples was reported by Macarisin *et al*. (2010) and by Torres *et al*. (2011) with *Pantoea agglomerams* CPA-2 in citrus fruit.,

6. Production of a biocontrol agent

Despite the effort, the progress in this field of research and the many references of new biocontrol agents, the use of these products represents a small portion of the potential market. The lack of consistency of control levels, compared to the usual fungicides (Droby, 2006; Heidari & Pessarakli, 2010), as well as the problems in the production and formulation of the product on a large scale are some obstacles to commercialization of biocontrol agents (Slininger *et al*., 1996; Nunes, 2012). Normally, these two important steps in the development of a biocontrol agent are over, in combination or

exclusively by private companies, which are covered by confidentiality industrial (Vero 2006; Teixidó *et al*., 2011)

Considering that biological control used large quantities of biomass and the feasibility of this alternative also depends on their costs (Visnovsky *et al*., 2008), is fundamental to develop a culture medium capable of producing large amounts of effective cells in the shortest period of time and at low cost (Janisiewicz, 1998; Costa *et al*., 2001), as well as the optimization of the production conditions (Lumsden & Lewis, 1989; Fravel, 2005; Patiño-Vera *et al*., 2005).

There are several factors involved in the optimization of the production conditions of biomass, such as temperature, pH, nature and concentration of nutrients stirring and oxygen (Doran, 1995; Gogate *et al*., 2000). Generally, these conditions are first tested on a small scale in Erlenmeyer flasks of 250-1000 mL, to determine parameters such as the specific growth rate, productivity and yield and thus know the growth profile of the microorganism (Doran, 1995).

Immediately after inoculation of the culture medium, the lag phase starts and persists until the cells have acclimated to their new environment, in which there is no visible growth. The duration of this phase depends on the physiological state, size of the inoculum and the conditions of growth (Madigan *et al.*, 1997). There follows a phase of acceleration and an exponential growth phase. With the nutrients in excess, except the limitant nutrient, the microorganisms have conditions to their multiplication, the population grows exponentially and the specific growth rate is maximal (Pelczar, 1993). Subsequently, there is a decrease in the specific growth rate due to the decrease in concentration limitant nutrient and/or increasing the concentration of products of metabolism in the culture medium, is a deceleration phase, preceded by the stationary phase. The stationary phase is characterized by a population with constant biomass and statistically similar number of viable cells; there is a diminished of nutrients concentration required for growth and eventually, an accumulation of inhibitory products resulting from cellular metabolism. Finally, there is a decline in the number of viable cells, as more pronounced with time, is a phase of irreversible loss, the death phase.

In a biotechnological process, whose aim is the production of biomass, is supposed to decrease the lag phase and increase the exponential phase, as well as trying to get the highest possible population, which depends on the operating conditions and the composition of the culture medium.

6.1. Factors affecting microbial growth

A number of factors influence all these phases of growth or death. These factors can be broken down into intrinsic and extrinsic factors.

a. Extrinsic factors

Extrinsic factors are related to the environment, among which include temperature, humidity and oxygen tension.

i. Temperature

The temperature has a great influence on the microbial growth and survival of the microorganism. This is not surprising since all the process of growth is dependent on chemical reactions that are affected by temperature (Pelczar, 1993). For each microorganism, there is a minimum temperature below which there is no growth; an optimum temperature, which allows chemical and enzymatic reactions occur at a maximum rate, and a maximum temperature (close to the optimum temperature), above which growth it is not possible due to protein denaturation and collapse of the cytoplasmic membrane. These three temperatures are called the cardinal temperatures and are a characteristic of each type of microorganism, so it is possible to distinguish four groups of organisms: psychrophiles, which grow at low temperatures; mesophiles that grow at moderate temperatures, thermophiles which grow at high temperatures and hyperthermophiles which grow to a very high temperatures (Madigan *et al.*, 1997).

ii. Oxygen

Based on their response to oxygen gas, microorganisms can be divided into aerobes (requiring oxygen and can grow in a normal atmosphere with 21% oxygen), facultative organisms (may grow in the presence or absence of oxygen, without oxygen obtain energy by fermentation), anaerobes (oxygen is the presence of toxic) and microaerobes (grow at oxygen concentrations between 1 and 15%).

iii. Relative Humidity

The relative humidity surrounding the microorganism is interrelated to the water activity. In fact, the relative humidity is essentially a measure of water activity in the gas phase surrounding the microorganism.

b. Intrinsic factors

Intrinsic factors are understood to those requirements which by nature are attributed to the microorganisms. These factors include pH, water activity, redox potential, nutrients

i. pH

Each microorganism has a pH range within its growth is possible and usually also has an optimum pH. Microorganisms able to live in highly acidic environments are called acidophilus, are examples the filamentous fungi and yeasts that growth is normal at pH 5 or less, there are some that can grow to a pH near of 2. In contrast, organisms with optimum pH between 10-11, are called alkaliphiles (Madigan *et al.*, 1997). Most of the bacteria have the minimum pH of 4 and the maximum of 9, and the optimal between 6 and 8. To grow in acidic or basic environment, the microorganism must be able to maintain their intracellular pH near neutrality, regardless of the external pH, which is achieved by the ability of the cell to expel or capture hydrogen ions.

ii. Water availability

Water availability depends not only on its presence but also on the concentration of solutes dissolved in it. In physical terms the availability of water is expressed as water activity a_w and is the ratio of the vapor pressure of the solution dissolved in water and the vapor pressure of pure water. Values range between 0 and 1, with 0 being the value of pure water.

Most microorganisms are unable to grow in an environment with low water activity, there is still the xerophiles (very dry medium), the osmophiles (medium with high sugar concentration), the halophiles (requires medium with 1-15% of salt), extreme halophiles (requires medium with 15-30% of salt). When the medium has low water activity, the microorganism could die, may simply enter in latency, or/and as a strategy increase the internal concentration of solutes and thus obtain water from the exterior, such is achieved by pumping inorganic ions from the environment to the inside or the synthesis of compatible organic solutes such as sugars, alcohols, amino acids (Madigan *et al.*, 1997).

In addition, besides a direct affect in the growth, water activity also has influence in other important growth factors such as pH and temperature. In media with low water activity the pH range favorable to growth narrows and the minimum temperature is higher (Mossel *et al*., 1995)

iii. Redox Potential

The redox potential is a measure of oxidation degree, that is the intrinsic tendency to oxidize and concentration of oxidizing substances and reducing the pH of the medium or its chemical composition. In conclusion, the redox potential and the oxygen pressure influence the growth of microorganism. To produce high levels of biomass, dissolved oxygen should be above the critical concentration, which varies depending on the bacterium and the growth medium (Ward, 1991).

iv. Nutrients

The nutrients of the medium should meet the basic needs for growth and cell metabolism, providing a suitable supply of energy for synthesis and cell maintenance (Stanbury *et al*., 1995). Nutrients can be divided into macronutrients (in quantity) and micronutrients (needed in small quantities).

In a typical cell, carbon and nitrogen represent about 50 and 12% of dry weight respectively, and are the two most abundant elements and those that are needed in larger quantities. Carbon is essential in the composition of carbohydrates, lipids and proteins that provide the energy necessary for the growth and act as bricks of cellular material. Nitrogen is the major constituent of proteins, nucleic acids and other cellular constituents. Can be found and assimilated into organic form (amino acids, peptides) or inorganic (nitrates, nitrites, ammonium salts). There are some bacteria that are capable of assimilating this element in gaseous form, through the fixing process.

As macronutrients there are also phosphorus, sulfur, potassium, magnesium, sodium, calcium and iron, but are generally less relevance.

Micronutrients, such as chromium, cobalt, copper, manganese, molybdenum, nickel, selenium, zinc are metals having an important role in enzymatic processes. Other organic compounds required by some organisms, in small quantities, are growth factors, such as vitamins, amino acids, purines and pyrimidines.

As mentioned, microbial growth is greatly affected by chemical and physical nature of their surroundings instead of variations in nutrient levels and particularly the nutrient limitation. For successful cultivation of microorganisms it is not only necessary to maintain proper environmental conditions but also it is essential to supply proper and balanced nutrients, therefore the first step in optimization strategy is the development of a defined or semi-defined medium which supports good culture growth of the biocontrol agent.

Depending on the nature or the material used, the fermentation medium can be chemically defined (synthetic, defined) or undefined (natural, complex). A defined medium is composed of pure chemicals in precisely known proportions, while a complex medium is formulated by including ingredients of natural origin, the compositions of which are not completely known.

Chemically defined media have as their main advantage standardization and stability over time, thus, they are usually preferred in laboratory research since they permit one to determine the specific requirements for growth and product formation by systematically adding or eliminating chemical species from the formulation, with minimal complicated medium interactions and reproducible culture conditions (Zhang & Greasham, 1999). The culture media that promote high productivity usually contain organic nitrogen sources such as yeast extracts, peptones and meat extracts. These substrates contain amino acids and vitamins that promote bacterial growth and cause high growth rates and high yields of biomass (Crueger & Crueger, 1993).

The nutrients comprise 30 to 40% of the total cost of fermentation process (Zhang et *al*., 2007) being the carbon source usually the most costly component (Stanbury *et al*., 1994). At an industrial scale the use of standard chemical means is unusual. The use of low cost raw materials and available in abundance, such as byproducts of industry, is one way to reduce costs substantially. Therefore once defined the most appropriate culture medium, there is a reformulation of it through the substitution of some nutritional components of the standard medium, for alternative and low cost substrates (Wraight *et al*., 2001).

a. Agro-industrial byproducts

The agricultural activity and food industry annually produce worldwide large volumes of waste (Couto, 2008; Giese *et al*., 2008; Bacha *et al*., 2011). The waste solid, liquid or materials from air emissions may constitute a problem for the industry that generates them, since they cannot be stored indefinitely (Pelizer *et al*., 2007). In order to avoid environmental problems, waste must be subject to treatment and legal framework according to environmental regulations, often requiring significant investments.

The waste can contain many reusable substances. Depending upon the existence of appropriate technology, they can be converted to commercial products or raw material in new processes. Several substances in food production are suitable for

separation and recycling at the end of its life cycle, even if the separation process and recycling is not fully efficient (Laufenberg *et al*., 2003; 2004).

The high sugar composition of agro-industrial by-products makes them a sustainable alternative from an environmentally and economically point of view, so they have been used as substrates to produce industrially relevant products such as antibiotics (Asagbra *et al*., 2005), bioethanol (Ruiz *et al.*, 2007; Raposo *et al.*, 2008, Velásquez Arredondo *et al.*, 2010; Lima-Costa *et al*., 2012), organic acids (Bustos *et al*., 2004; Nigan, 2009) xylitol (Tada et al., 2004; Carvalho *et al.*, 2005), polysaccharides and polymers (Castillo *et al*., 2009; Poli *et al.*, 2011; Song *et al.*, 2011).

However there are some limitations to the use of by-products, which are evident by their lack of homogeneity, the presence of impurities, the variability due to the origin and season (Stanbury *et al*., 1995, Costa *et al*., 2001; 2002; Teixidó *et al.*, 2011) and seasonality. Commercial products or by-products of the food industry such as, malt extract, beer extract, molasses, fruit concentrates, peanut hulls, soybean hulls, corn cobs, fish flour, among others, are described as a raw material in the production of biocontrol agents (Fravel *et al*., 1999; Choi & Park, 2003; Abadias *et al*., 2003; Patiño-Vera *et al*., 2005; Verma *et al*., 2007; Bai *et al*., 2008; Peighami-Ashnaei *et al*., 2009; Shi *et al.*, 2009; Sartori *et al*., 2012) and in the production of metabolites synthesized by the biocontrol agents (Mizumoto *et al*., 2006).

In an attempt to find an inexpensive production medium, Yánez-Mendizábal *et al*. (2011) optimized the production conditions of the antagonist *Bacillus subtilis* CPA-8, and concluded that the best results were obtained with defatted soy flour combined with molasses. With the same goal, Costa *et al*. (2001) demonstrated that *P. agglomerans* CPA-2 can be produced in different media, using various organic nitrogen sources such as yeast extract, dry yeast or soya powder and accessible carbon source such as sucrose and molasses.

Although the use of by-products could lead to a reasonable decrease in cost production, their use in the production of biocontrol agents is only feasible if the effectiveness of the biomass produced is not compromised. Industrial by-products used in the production of *Penicillium frequentans* (De Cal *et al*., 2002) or *Epicoccum nigrum* (Larena *et al*., 2004) and *Rhodosporidium paludigenum* (Wang *et al*., 2011) were used with good yields, without affect the ability antagonist.

i. Carob industry byproducts

The carob tree (*Ceratonia siliqua* L.) is an evergreen tree, native of the Mediterranean region, belonging to the family *Leguminoseae*, and the subfamily *Caesalpinaceae*. It is a typically culture of semi-arid areas of southern Europe and parts of North America and is presented as a cash crop and a valuable resource for reforestation and erosion control on not cultivated land (Roseiro *et al*., 1991 a,b; Rizzo *et al*., 2004; Biner *et al*., 2007).

Much of the economic value of the crop is associated with the seed of the fruit, from where the gum is extracted, consisting of complex carbohydrates (galactomannans) (Batlle & Tous, 1997). These polysaccharides, commonly dubbed LBG (locust bean gum) have a high quality as thickener, stabilizer, emulsifier, gelling agents (food additive E410) and many uses in the food, pharmaceutical, textile and cosmetics (Barracosa *et al*., 2007). However, the seed is only 10% of the weight of the fruit and the pulp is the rest (Fig. 5a,b). Pulp constitutes a by-product which has primarily been used in animal feed. Several authors have reported that the composition of the carob pulp has a high sugar content, primarily sucrose (over 30%), fructose and glucose (Petit & Pinilla, 1995; Batlle & Tous, 1997; Yousif & Alghazawi, 1999; Bulut *et al*., 2004; Biner *et al*., 2007; Santos *et al*., 2005; Ayaz *et al*., 2007; Tetik *et al.*, 2011). The carob pulp also contains appreciable amounts of fiber, protein (3-4%), a low level of fat (0.4-0.8%) (Avallone *et al*., 1997), calcium, sodium, potassium (Ozcan *et al*., 2007), a high level of condensed tannins and a low content of water-soluble tannins (Avallone *et al*., 1997; Kumazawa *et al*., 2002; Makris & Kelafas, 2004). As previously mentioned, the carob pulp has been used as animal feed, but also on human consumption, in the preparation of antiemetic and antidiarrheal products, pastry and syrups. It is also used as a substitute for cocoa, with the advantage of being free of caffeine and theobromine, since chocolate and cocoa contain relatively high amounts of these two antinutrients (Craig & Nguyen, 2006). However, most of the carob pulp remains as a residue, representing a cheap and available raw material to produce a value-added product. The use of by-products from carob industry has been studied in the production of: dextran and fructose (Santos *et al*., 2005), citric acid (Roukas, 1999), bioethanol (Roukas, 1996; Raposo *et al*., 2009; Lima-Costa *et al*., 2012), fertilizer/bio-stimulant (Parrado *et al*., 2008), DHA (docosahexaenoic acid) (Mendes *et al*., 2007).

ii. ***Citrus juice industry byproducts***

In recent decades citrus production has been increased. Brazil, the Mediterranean countries (particularly Spain and Italy), USA and China are the major producing countries, representing more than two thirds of global production (Rivas *et al*., 2008; Ylitervo, 2008). Much of the outputs of these fruit are for the juice production. Fruit are washed, perforated for the removal of oils and squeezed to extract the juice. The resulting material processing is the bagasse (Fig. 5c,d), representing 50% of the fruit weight (Giese *et al*., 2008), and consists in seeds, peel and pulp and is usually used to animal feed. In a first step, the residue is triturated and the pH adjusted by adding NaOH or CaO, to facilitate the degradation of pectin, in order to increase the efficiency of dewatering presses in the bagasse (Yoo *et al*., 2011). From the pressing operation results a liquid product, the liquor, with a soluble solids of about 10-12.5 °Brix, depending on the amount of water added in the process and on fruit variety. Much of soluble solids present in the liquor (60-75%) consist in sugars, primarily glucose, fructose, sucrose and small amounts of pentoses (Roçafa Junior *et al*., 2005).

The use of by-products from citrus juice industry has been investigated in producing bioenergy (Thomsen, 2005), ethanol (Ylitervo, 2008), β-carotene (Ciegler *et al*., 1962), enzymes (Giese *et al*., 2008; Mamma *et al*., 2008) food supplements (Adoki, 2002; Bacha *et al*., 2011), biosurfactant produced by *B. subtilis* SPB1 (Ghribi *et al*., 2011) and antimicrobial compounds produced by *B. subtilis* LS 1-2 (Yoo *et al*., 2011).

Considering the characteristics of by-products from carob and citrus juices industry, as well as the availability of these raw materials in the region where the major part of this work was developed, their use in the production of biocontrol agents emerged as a strong evidence. Thus, besides a reduction of production costs, it was also intended the valorization of a by-product (in the case of carob and citrus bagasse) and the search for an alternative to a product (liquor) with high pollution load, difficult to treat, which is generating serious environmental problems, and that lastly can cause the closure of this industry, with devastating effects for the local economy.

Figure 1.5. By-products of industry: a) carob pulp; b) carob pulp dried and grounded; c) production unit citrus pulp; d) Citrus bagasse.

b. Geometries designs

In the optimization of the production process the experiments of different conditions should ensure that the obtained results from small scale trials are capable to be transferred to the commercial production process (de Carvalho *et al*., 2007). From experiments in flasks follows the scale-up in laboratory reactors (Fig. 7a,b) and finally to a pilot scale and industrial scale.

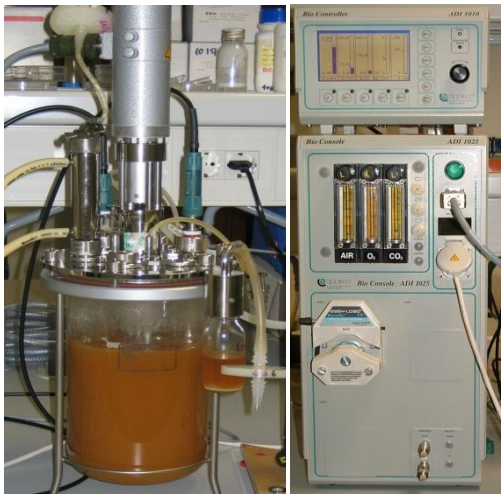


Figure 1.6. Biological reactors a) Biocontrol unit, Applikon Adi 1010 and console Applikon 1025; b) tank mechanically agitated (kindly loaned by "Laboratório de Engenharia e Biotecnologia Ambiental (LEBA)" from "Centro de Investigação Marinha e Ambiental (CIMA)" to realize the experiments.

The reactors, process units where biological reaction is performed, cover a broad spectrum and scale applications (Vogel, 1996; Teixeira *et al*., 2007), such as biomass production (Costa *et al*., 2001; Mounir *et al*., 2007), beverages (Pilkington *et al*., 1998; Kourkoutas *et al*., 2002), plant cells (Lima-Costa *et al*., 1997, 2002, Lima-Costa & Raposo, 2007), food (Caplice & Fitzgerald, 1999), products with high added value for health (Bilodeau & Mantovani, 2006; Hu *et al*., 2006) and pharmaceutical industry (Hambor, 2012) and also biological remediation of effluents (Wang & Menon, 2008).

In general and depending on the nature of the biocontrol agent, the methods used for production in a large scale are liquid or solid phase fermentation, taking advantage in leading edge technology used in the food and pharmaceutical industry (Montesinos, 2003). Bacteria and yeast are typically produced in a liquid phase, while the fungi are produced in solid phase (Fravel *et al*., 1999).

There are different reactor geometries, the choice should be appropriate depending on the microorganism and the process. The most commonly used are the mechanically stirred tank (STR, Stirred tank reactor), fluidized bed reactor, immobilized Cell bioreactor, bubble column bioreactors and air-lift (Teixeira *et al.*, 2007).

The STR, the most used in industrial processes, performs several functions in simultaneously, assists in mass transfer and heat and stirs and homogenizes the suspensions (Ungerman & Heindel, 2007). This type of configuration allows an easy control of the agitation and gas dispersion by simply changing the stirrer speed. However, this system has low oxygen transfer efficiency, requires large agitation power, which generates heat and shear stresses, making it inappropriate for cell cultures sensitive to this type of forces (Teixeira *et al.*, 2007).

Generally cylindrical, the laboratory-scale reactors are mostly in glass, at a pilot and industrial scale, stainless steel is the elected construction material, given its characteristics of easy cleaning, sanitizing and chemical inertness. The dimensions are standard published by the International Standards Organization and the British Standards Institution. The relation height/diameter (H/D) of the vessel has a large impact on the most important process parameters for fermentation, particularly agitation and aeration (Aiba *et al*., 1979; Fernandes, 2007). The most economic relation is close to 1, that is has a lower surface and therefore requires less material in construction, however is also the least recommended for fermentations with more demanding aeration. In the latter cases the units are further recommended that have a H/D between 2 and 3, which implies greater efficiency in the transfer of gas-liquid mass, giving the greater residence time of the gas phase.

The oxygen mass transfer as well the elimination of gradients, nutrients concentration, temperature, pH, is dependent upon a good mixing. The mixture, a physical process that ensures homogeneity of the system, is one of the important operations in bioprocesses, and is achieved in agitated tanks by the existence of stirrers, dispersers and baffles.

Based on the flow produced, impellers can be classified as axial and radial. In axial flow, the liquid is directed towards the base of the reactor, parallel to the axis of the impeller, are examples the marine-propeller (Fig. 7a), 45º pitched blade turbine and Lightnin, are indicated for media with high viscosity. In radial-flow the fluid is directed to the wall of the reactor, are examples, Rushton turbine (Fig. 7b), Pitched bladed turbine, Smith turbine. These impellers are recommended for applications where it is important to mix the gas dispersion with an intensive stirring (Collignona *et al.*, 2007; Lima-Costa & Raposo, 2007; Ungerman & Heindel, 2007).

The baffles are bars placed upright parallel to the walls of the reactor and depending on the viscosity of the liquid, may be more or less apart from the walls, their goal is to increase turbulence, avoiding areas of stagnation and thus increase the oxygenation of the medium.

In biological reactors, it is common the use of three types of spargers: holes, "nozzle or L" (Fig. 7c) and porous (Fig. 7d). The porous spargers of metal, ceramic or glass, for low gas flow rates above 0.1 vvm (Matthews, 2008), exhibit great resistance to the flow of gas and so its use is limited to some applications. The holes spargers as the name indicates there are several holes in a cylindrical tube with a ring-shaped or cross. "L" dispersers are more conventional for high gas flow rates, above 2 vvm (Matthews, 2008), consisting of a simple opening or partially closed pipe, providing a stream of air bubbles, offering low resistance to flow and air and low risk of blockage.

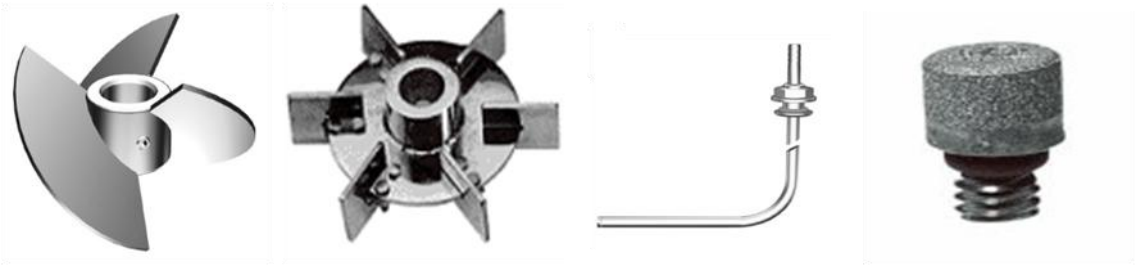


Figure 1.7. a) marine propeller; b) Rushton impeller; c) “L-shape”; d); impeller porous.

As mentioned earlier in this work, for some microorganisms, the presence of oxygen is essential to their development. Thus, in aerobic biological process, in which the presence of oxygen is indispensable, the availability oxygen may be predominant in the process of scale-up (Lima-Costa *et al*., 1997; Garcia-Ochoa *et al*., 2000; Garcia-Ochoa & Gomez, 2009).

As a result of its low water solubility (7-9 mg/L), oxygen is often the limiting factor in aerobic fermentation. In a typical culture the concentration of the dissolved oxygen in the medium decreases with the growth time until reaches the stationary phase, followed by an increase of this parameter (Gomez *et al*., 2006). When the oxygen uptake by the cells exceeds its supply, critical concentrations are reached with adverse effects, reflected in specific growth rate, biomass concentration and productivity (Rocha-Valadez *et al*., 2007). The concentration of the dissolved oxygen in the suspension depends on oxygen transfer rate from the gas phase into the liquid, the rate at which oxygen is transported into the cells and oxygen uptake rate (OUR) by the microorganism. The oxygen transfer rate (OTR) described and analyzed by the mass transfer coefficient or $K_L a$ is influenced by various parameters such as the physicochemical properties of the culture, operating conditions, bioreactor parameters geometric, the concentration and morphology of the microorganism (Cascaval *et al*. 2,006; Garcia-Ochoa & Gomez, 2009).

The STRs provide high transfer rates of mass and heat, as well as a great mix. In these systems, the stirring speed, the type and number of stirrers and the gas flow used are the factors that most affect the mixing and mass transfer (Garcia-Ochoa & Gomez 2009). Aiming to improve the oxygen mass rate transfer in the cultures, it is common to increase the speed of agitation. However, this increase causes a change of hydrodynamic conditions, increasing the forces of division and can damage the cells (Rocha-Valadez *et al*., 2007).

c. Operation Mode

Depending on the nutrient supply, the reactors can operate in batch mode, fed-batch and continuous.

In batch mode all nutrients from the culture medium are present in the startup process, there are no substrate addition or removal of culture medium throughout the process, so that the volume remains approximately constant. In fed-batch mode, the substrates are added in different stages of the process, and the removal of the culture medium is performed only at end of fermentation, which cause a gradual increase of the volume. In the continuous mode of operation, and like in the discontinuous mode, the volume remains approximately constant, since while substrate is added to reactor, the effluent is withdrawn at similar fluxes Compared to batch mode, continuous mode allows the maintenance and the control of the specific growth rate, a constant biomass

concentration by varying the dilution rate, a high biomass productivity as well as a shorter time of cleaning and sterilization. However the problems of contamination and mechanical maintenance increased and the increased risk of aggregation can cause washout. , It is not possible to obtain products that are not related to the growth phase.,

Depending on the intended product, the technology and operating mode should be adapted. The batch mode is the most common in the producing of biocontrol agents, however fed-batch mode, which prevents the repressive effects of a high concentration of a limiting substrate, was studied in producing of *Bacillus thuringiensis* (Chen *et al*., 2003) and *Aureobasidium pullulans* (Mounir *et al*., 2007). Abadias *et al*. (2003) tested the fed-batch mode to produce the biocontrol agent *Candida sake* and concluded that growth obtained under these conditions was not improved when compared with the growth in batch mode.

iii. Formulation

After the optimization of the production conditions of the microorganism, follows the separation of the produced biomass from the culture medium, to this, it is common to use centrifugation, filtration and flocculation (Teixidó *et al*., 2011). The biomass produced must then be applied or stored for a few days in conditions to maintain its viability. However, with time, fresh cells lose viability (De Cal *et al*., 2001; Larena *et al*., 2004) and effectiveness becoming useless. The formulation appears as an answer to this problem and aims to obtain a commercial presentation which maintains or improves the efficacy, extending the shelf life of the product, ensuring the stability, allowing easy handling and application. Furthermore, if it is formulated into a dry form, the product should be easily rehydrated (Melin *et al*., 2011).

The formulations of biological control agents may be liquid or solid (Nunes, 2012), can range from fresh cells of the microorganism simply compressed and stored at 4 ºC until complex systems of lyophilization and conservation in modified atmosphere (Powell, 1992). The liquid formulation consists in a suspension of the biomass in an oily base (oils and emulsifying agents to facilitate their dispersion in water) or an aqueous base (liquid which could be added stabilizers, surfactants, or other additional nutrients) (Fages, 1992), which is suitable for microorganisms whose viability is affected by the drying process. More economic than drying technology, the liquid formulation presents as main disadvantages the need for refrigeration during storage and distribution and a short shelf life.

The dehydration of the product and maintenance of a dry environment is one of the best ways to formulate microorganisms, so that the product can integrate the storage and distribution channels (Rhodes, 1993). The solid formulation is the only one that guarantees a lower risk of contamination, higher self life, and greater tolerance to higher temperatures during storage and easier application (Li & Tian, 2006). Unfortunately, not all microorganisms are liable to be dried many tend to lose viability during drying and storage processes. The most usual processes of dehydration are: freeze-drying, spray drying and fluidized bed drying. These processes alter the physical state of the water, by varying the temperature or pressure, or by the combined action of both parameters (Usall *et al*., 2009). To avoid or reduce the loss of viability during the formulation process, stabilizers or protective substances can be added or induce the accumulation of intracellular solutes, such as poly hydroxy alcohols, carbohydrates or even amino acids. The accumulation of compatible solutes is achieved by altering the conditions and the culture medium, for example by changing the water activity in the growth medium of the microorganism, promoting an osmotic equilibrium between the inside and outside of the cell while preserving the function of proteins (van Eck *et al*., 1993).

Before the registration step and entry into the market, the biocontrol agent still passes through several steps and is subjected to careful testing, including toxicology tests, efficacy and improvement tests. The fresh or formulated biomass is tested in a semi-commercial (Fig. 8a) and commercial scale, involving a large amount of fruits in different locations and with different fruit varieties, with different application and storage conditions (Fig. 8b) (Torres *et al*., 2007). In parallel to the tests of effectiveness the population dynamics of biocontrol agent are studied (Fig. 8c), with aim to assess the ability of the microorganism to colonize the fruit and survive in different storage conditions (Nunes *et al*., 2008).

Figure 1.8. Semi-commercial trials: a) comparing efficacy, left: apples treated with the biocontrol agent, right: control treatment; b) cold storage of different varieties after treatment; c) population dynamics assay in apples.

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Objectives

Objetivos

OBJETIVOS

Este trabalho teve como principal objetivo identificar e produzir agentes de biocontrolo em dois modelos distintos, uma bactéria e uma levedura, das principais doenças de pós-colheita de citrinos e pomóideas.

Deste modo estabeleceram-se objetivos particulares:

1. Procura de uma bactéria e uma levedura como agentes de biocontrol das principais doenças de pós-colheita de citrinos e pomóideas,
   1.1. Isolar e avaliar a capacidade antagonista de microrganismos a nível primário;
   1.2. Determinar a eficácia de *Pantoea agglomerans* PBC-1 e *Metschinikowia andauensis* PBC-2 em condições laboratoriais e semi-comerciais;
   1.3. Avaliar a capacidade de *P. agglomerans* PBC-1 e de *M. andauensis* PBC-2 colonizar fruta.

2. Desenvolver um meio de cultura e as condições de produção de biomassa eficaz dos agentes de biocontrol:
   2.1. Otimizar o meio de cultura, (fontes de carbono e azoto e suas concentrações) e as condições de produção de forma a garantir elevada produtividade e rendimento;
   2.2. Otimizar a produção dos agentes de biocontrolo, em reator mecanicamente agitado;
   2.3. Avaliar o efeito do meio de cultura na atividade antagonista dos agentes de biocontrol.

3. Desenvolver um meio de cultura de baixo custo usando subprodutos da indústria alimentar como fonte de carbono
   3.1. Avaliar o uso de extratos de subprodutos da indústria de alfarroba na produção de *P. agglomerans* PBC-1 e de desperdícios da indústria de sumos de citrinos na produção de *M. andauensis* PBC-2;
   3.2. Otimizar a produção dos agentes de biocontrolo, em reator mecanicamente agitado;
   Avaliar o efeito dos meios de cultura na eficácia dos agentes de biocontrolo;

4. Estudar os possíveis modos de ação de *M. andauensis* PBC-2;

OBJECTIVES

This work has as main objectives to identify and produce a biocontrol agent, two distinct models, one bacterium and one yeast, of the major postharvest diseases of citrus and pome fruit.

Therefore, particular objectives were established, it was intended:

1. Search a bacterium and a yeast as biocontrol agents of the major postharvest diseases of citrus and pome,
   1.1. Isolate and assess the antagonistic ability of microorganisms in primary screenings;
   1.2. Study the efficacy of *Pantoea agglomerans* PBC-1 and *Metschinikowia andauensis* PBC-2 in laboratory and semi-commercial trials;
   1.3. Evaluate the fruit colonization capacity of *P. agglomerans* PBC-1 and *M. andauensis* PBC-2

2. Develop a culture medium and effective biomass production conditions of the biocontrol agents,
   2.1. Optimize the culture medium (carbon and nitrogen sources) and operational conditions to ensure high productivity and yield;
   2.2. Optimize the production of the biocontrol agents in a biorector;
   2.3. Evaluate the effect of culture media on the efficacy of the biocontrol agents;

3. Develop a low-cost culture medium using as carbon source by-products of food industry
   3.1 Evaluate the use of extracts of carob industry byproducts in the production of *P. agglomerans* PBC-1 and wastes of citrus juice industry in the production of *M. andauensis* PBC-2
   3.2 Optimize the production of the biocontrol agents in a bioreactor
   3.3 Evaluate the effect of culture media on the efficacy of the biocontrol agents;

4. Study the possible modes of action of *M. andauensis* PBC-2.

Chapter 2

Metschnikowia andauensis as new biocontrol agent of fruit postharvest diseases

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Metschnikowia andauensis as a new biocontrol agent of fruit postharvest diseases

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ABSTRACT

Potential antagonists were isolated from the epiphytic flora associated with oranges and pome fruit. A total of 1465 microorganisms were tested in a preliminary screening against blue and green moulds on pome and citrus fruit, respectively. Among them, approximately 3% reduced incidence and severity by more than 50% and 4 microorganisms fulfilled the selection criteria of reduction in severity and incidence by 75%. The most effective was a yeast identified as *Metschnikowia andauensis*, strain NCYC 3728 (PBC-2), isolated from the surface of 'Bravo de Esmolfe' apple fruit cultivated in North Portugal. The biocontrol activity of *M. andauensis* PBC-2 was dependent on its applied concentration. At 5×10^6 cfu/mL incidence (% of infected wounds) and severity (lesion diameter) were reduced by 62 and 70%, respectively and at 1×10^7 cfu/mL, the greatest reduction was achieved, 90% of incidence and 95% of severity. The broad spectrum of action of *M. andauensis* PBC-2 was evaluated with effective control being achieved against *Rhizopus stolonifer*, *Penicillium expansum* and *Botritys cinerea*, on 'Rocha' pears and on different apple cultivars and against *Penicillium digitatum* and *Penicillium italicum* on mandarins and oranges. In semi-commercial trials in cold storage, the reduction of blue mould was 90%. Rapid colonization of fresh apple fruit wounds was observed during the first 24 h of cold storage, followed by a significant population increase during the first 15 days of storage and then the population remained stable until the end of storage.

1. Introduction

Chemical pesticides for postharvest protection of fruit and vegetables have been extensively used over the past 70 years due to their effectiveness and ease-of-use. The widespread use of chemicals will continue, although the declining effectiveness of registered fungicides, public pressure to reduce fungicide use, and public demand for produce free of synthetic pesticides, has heightened interest in the development of safer alternatives that are effective and pose less risk to human health and the environment (Droby, 2006; Sipiczki, 2006).

During the last 25 years, several research programs have been developed around the world and numerous biological control agents have been investigated against different postharvest diseases of fruit (Nunes et al., 2009). As a result, some microorganisms have been reported and patented as postharvest biocontrol agents (Janisiewicz and Roitman, 1988; Chalutz and Wilson, 1990; McLaughlin et al., 1992; Janisiewicz and Jeffers, 1997; Droby et al., 1998; Viñas et al., 1998; Fan et al., 2000; Janisiewicz et al., 2001; Kurtzman and Droby, 2001; Nunes et al., 2001; Adikaram et al., 2002; Zheng et al., 2005; Govender and Korsten, 2006). Despite this, only a few commercial products are available, such as Biosave™ (*Pseudomonas syringae*, Jet harvest solutions, USA), Shemer™ (*Metschnikowia fructicola*, Bayer Crop Science, AG), Candifruit™ (*Candida sake* CPA-1, Spicam-Inagra, Spain), Pantovital™ (*Pantoea agglomerans*, Biodurcal S.L., Spain), Serenade™ (*Bacillus subtilis*, AgraQuest, USA) and Boniprotect™ (*Aureobasidium Pullulans*, Bioprotect, Germany).

The identification, development and commercialization of a biocontrol product is an extensive and costly process, so at the initial stages of a project it is important to spend considerable time developing a product concept and trying to anticipate any possible obstacles to commercialization (Droby et al., 2009).

The development of a biological control agent requires several steps. The first is the isolation and screening process which will largely influence the efficacy and ultimately its success under commercial conditions (Droby et al., 2009). Different strategies have been used; however, most of the research has been focused on isolating naturally occurring microorganisms from fruit just before harvest or during storage (Nunes et al., 2009). The fructoplane has provided the most abundant and desirable source for isolating antagonists against postharvest fruit pathogens (Janisiewicz, 1988, 1997; Viñas et al., 1998; Jijakli et al., 1999; Nunes et al., 2001, 2007; Manso et al., 2010). However, the antagonists may also come from other closely related or unrelated sources. The phylloplane has also been a good source of antagonists (Janisiewicz and Korsten, 2002).

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A poorly conceived screening strategy can be a costly mistake since the selection of the candidate microorganisms comes at the beginning of the long development process (Roberts and Lohrke, 2003). Rapid methods of evaluation are required for preliminary screening of large numbers of candidates, as an example, the use of *in vitro* tests, but results obtained from these methods must be correlated with *in vivo* efficacy (Boland, 1990). However, a shortcoming of this screening strategy is that *in vitro* primary screening identifies antagonists with the ability to produce antibiotics and discard potential non-antibiotic-producing antagonists (Cheah et al., 1996). Thus although direct screening on fruit is very laborious, it is the most efficient methodology to obtain antagonistic microorganisms of postharvest wound pathogens (Janisiewicz, 1997).

Postharvest biocontrol agents are applied to consumable products, therefore they need to have strict requirements including non-production of toxic metabolites, thus although there are numerous bacteria known as biocontrol agents, much interest has been given to yeasts. Yeasts would be suitable biocontrol agents because of their characteristics: the mode of action is normally not based on the production of toxic compounds, they have the ability to colonize a surface for long periods, they can produce extracellular polysaccharides that enhance their survival and restrict wound colonization and slow germination of fungi, and they can rapidly use available nutrients and be minimally affected by pesticides (Janisiewicz, 1988).

The aim of the present work was to isolate yeasts antagonists that would efficiently protect pome and citrus fruit against major postharvest diseases.

2. Material and methods

2.1. Isolation of microorganisms

Epiphytic microorganisms were isolated from the surface of leaves, pome and citrus fruit, picked during the growing season from different orchards. The microbiota of pome fruit stored for 3 months at $1 \pm 0.5\,^{\circ}C$, was also periodically collected.

Fruit and leaves were washed in 350 mL of sterile water on a rotating shaker (Selecta, Rotabit, Spain) for 10 min at 150 rpm. The water was discarded and fruit and leaves were washed once again in 200 mL of phosphate buffer 0.05 M, pH 6.5 in an ultrasonic bath (Selecta, Ultrasons, Spain) for 10 min. Samples of 0.1 mL were taken and plated in NYDA [8 g/L nutrient broth (Biokar Diagnostics); 5 g/L yeast extract (Biokar Diagnostics); 10 g/L glucose (Riedel-de-Haën); 15 g/L agar (Vaz Pereira)] and incubated at $25 \pm 1\,^{\circ}C$. After 48 h incubation, single colonies of yeasts were isolated according to their different characteristics. Isolates were purified on NYDA and maintained at $4 \pm 1\,^{\circ}C$.

2.2. Fruit

'Valencia Late' and 'Lanelate' oranges (*Citrus sinensis*) and 'Clementina Fina' mandarins (*Citrus clementina*) from commercial orchards in Algarve, Portugal, were used in the experiments, 1 or 7 days after harvest. 'Rocha' pears (*Pyrus communis* L.), a Protected Designation of Origin (PDO) cultivar, and 'Golden Delicious', 'Royal Gala', 'Bravo de Esmolfe' (PDO), 'Fuji', 'Granny Smith' and 'Starking' apple fruit (*Malus* × *domestica* Borkh.) from commercial orchards in Região Oeste, Portugal, were used after harvest or with less than 3 months in cold storage.

2.3. Pathogens

Penicillium digitatum and *Penicillium italicum* isolated from decayed citrus fruit and *Penicillium expansum*, *Botritys cinerea* and *Rhizopus stolonifer* isolated from decayed pome fruit, were selected based on their high virulence and were periodically transferred through fruit.

Petri dishes of PDA (Biokar Diagnostics) were inoculated with the fungal strains and incubated at $25 \pm 1\,^{\circ}C$ for 7–10 days. Conidial suspensions of the pathogens were prepared in Tween 80 in sterile water and the concentrations were determined with a haemacytometer and adjusted as appropriate.

2.4. Screening for inhibitory activity on wounded fruit

The antagonistic ability of the isolates was evaluated *in vivo* screenings in 'Golden Delicious' apples, 'Rocha' pears and in 'Valencia Late' or 'Lanelate' oranges. Microorganism suspensions in phosphate buffer (0.05 M, pH 6.5) were prepared from 48 h old-NYDA-grown cultures.

Citrus fruit were inoculated by wounding the flavedo at two locations (midway between the calyx and at the stem end) with a stainless steel needle (2 mm deep by 1 mm wide) previously immersed into the suspension of *P. digitatum* at 10^5 spores/mL, followed after 2 h by the inoculation of 15 μL of each isolate. Each fruit with two wounds constituted a single replicate and each treatment was repeated five times. Incidence (percentage of decayed fruit) was determined after 7 days at $20 \pm 1\,^{\circ}C$ and $80 \pm 5\%$ relative humidity (RH).

Pome fruit were wounded at two locations (midway between the stem and calyx end) with a stainless steel needle (2 mm deep by 1 mm wide). Then 20 μL of the suspension of each isolate was inoculated into the wound, followed after 2 h by the inoculation of 15 μL of a suspension of *P. expansum* at 10^4 spores/mL. Each fruit with two wounds constituted a single replicate and each treatment was repeated three times. Fruit were stored at $20 \pm 1\,^{\circ}C$ and $80 \pm 5\%$ HR for 7 days at the end of which, incidence and severity (lesion diameter) was determined. For all fruit the control treatment consisted of replacing the tested microorganisms by the same volume of phosphate buffer. The yeast isolates capable of reducing incidence and severity of disease by 75% were selected and revaluated in screening assays with a higher number of fruit per replication.

2.5. Screening for minimum effective concentration

Metschnikowia andauensis (PBC-2) isolated from the surface of 'Bravo de Esmolfe' apple and identified by the National Collection of Yeast Cultures (NCYC) (UK) and deposited with the number NCYC3728^T, was the yeast isolate selected since it provided greater antagonistic capacity in both pome and citrus fruit.

The minimum effective concentration of the potential biocontrol agent *M. andauensis* against *P. expansum* was determined in 'Golden Delicious' apples. The yeast was growth in NYDB (NYDA without agar) and incubated under orbital agitation, 150 rpm, at $25 \pm 1\,^{\circ}C$ for 36–40 h. The medium was centrifuged at $1920 \times g$ (Hettich Zentrifugen Universal 320, Germany) for 20 min and harvested cells were resuspended in phosphate buffer. Desired concentrations were adjusted by measuring the optical density at 420 nm in a spectrophotometer (Spectrophotometer UV-Vis, Shimadzu-UV160, USA) according to a standard curve. The viable population of each suspension tested was checked in NYDA using the surface method, serial dilutions were made in sterile phosphate buffer and the plates incubated at $25 \pm 1\,^{\circ}C$. The screening tests were carried out as described above. Five fruit, each one with two wounds, constituted a replicate and each concentration was tested three times.

2.6. Broad spectrum activity and biocontrol activity under cold storage conditions

To select and develop a successful biocontrol agent it is necessary to evaluate its performance under different conditions, similar to real application, and in a wide range of hosts and pathogens. Therefore to broaden the biocontrol activity spectrum, the effectiveness of *M. andauensis* NCYC3728 (PBC-2) at 10^7 cfu/mL was evaluated against *P. expansum*, *B. cinerea* and *R. stolonifer* at 10^4 spores/mL, in different apple cultivars, ('Royal Gala', 'Fuji', 'Starking', 'Granny Smith' and 'Bravo de Esmolfe') and in 'Rocha' pears, and against *P. digitatum* and *P. italicum* at 10^6 spores/mL in 'Lanelate' oranges and at 10^5 spores/mL in 'Clementina Fina' mandarins. Pome and citrus fruit were wounded and treated as described above. Ten fruit with two wounds constituted a single replicate and each treatment was repeated four times. The test was repeated twice.

To evaluate the effectiveness of *M. andauensis* NCYC3728 (PBC-2) at 10^7 cfu/mL against *P. expansum* under cold storage conditions 'Golden Delicious' apples and 'Rocha' pears were wounded and treated as described above. Twenty fruit with two wounds constituted a single replicate and each treatment per type of fruit was repeated four times. Fruit were stored at $1 \pm 0.5\,^{\circ}C$ and $80 \pm 5\%$ RH for 90 days. Every 30 days, incidence (% of infected wounds) and severity (lesion diameter, including all wounds) were determined. The test was repeated twice over two storage seasons.

2.7. Semi-commercial trials on 'Golden Delicious' apples under cold storage

In order to evaluate antagonistic activity of *M. andauensis* NCYC3728 (PBC-2) in 'Golden Delicious' apples against *P. expansum* on a semi-commercial scale, each fruit was wounded as described above. Fruit were submerged for 30 s in a *M. andauensis* aqueous suspension at 10^7 cfu/mL. After 1 h, fruit were submerged again for 30 s in a suspension of *P. expansum* at 10^4 spores/mL. The antagonist efficacy, in 2010–11 season, was compared with the efficacy of fungicide imazalil at a recommended concentration for standard postharvest treatment (0.5%). Forty fruit constituted a single replicate and each treatment was replicated four times. The incidence and severity were determined 90 days after cold storage at $1 \pm 0.5\,^{\circ}C$ and $80 \pm 5\%$ HR. The assays were carried out during 2007–08, 2008–09, 2009–10 and 2010–11 seasons.

2.8. Population dynamics on 'Golden Delicious' apple surface

Population dynamics of *M. andauensis* NCYC3728 (PBC-2) were determined on the surface of wounded 'Golden Delicious' apples. In this test the fruit were wounded as described above and submerged for 30 s in an aqueous suspension of *M. andauensis* at 10^7 cfu/mL, then placed on tray packs and incubated at $1 \pm 0.5\,^{\circ}C$ and $80 \pm 5\%$ RH for 90 days, followed by 7 days at $20\,^{\circ}C$ and $80 \pm 5\%$ RH. Fruit samples were taken to determine the number of viable cells of *M. andauensis* at 0, 1, 7, 30, 60, 90 and 97 days after inoculation. Twenty-five pieces of peel surface of 2.5 cm^2 were removed with a cork borer from each apple, shaken in 100 mL sterile phosphate buffer (pH 6.8) on a rotary shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. Serial dilutions of the washings were made and plated on NYDA. Colonies were counted after incubation at $25\,^{\circ}C$ in the dark for 48 h. Population sizes were expressed as cfu/cm^2 of apple surface. Four fruit constituted a single replicate with four replicates per sample time. The experiment was carried out twice.

Number of isolates with inhibitory activity on wounded fruit.

Incidence reduction (%)	Severity reduction (%)	No. of microorganisms
0	≤25	975
≤50	≤50	540
≤75	≤75	46
≥75	≥75	4

2.9. Studies of acid tolerance in a simulated gastric fluid

The survival of *M. andauensis* NCYC3728 (PBC-2) in a simulated gastric fluid was evaluated following a modified method adapted from Chan et al. (2010). Simulated gastric fluid was prepared by dissolving 3.2 g/L pepsin (Sigma) and 2.0 g/L sodium chloride (Panreac) into distilled water, and the pH was adjusted to 2 by addition of 0.5 M HCl (Applichem). Twenty microlitre of *M. andauensis* at 10^7 cfu/mL were added to Erlenmeyer flasks containing 20 mL of simulated gastric fluid. The flasks were incubated at $37\,^{\circ}C$ on a rotary shaker at 20 rpm for 72 h. Samples were taken at 0, 4, 6, 24, 30, 48 and 54 h, and serial dilutions were made and plated on NYDA to determine the number of viable cells. Colonies were counted after incubation at $25\,^{\circ}C$ in the dark for 48 h and expressed as cfu/mL. The experiment was repeated twice with three replicates per test.

2.10. Statistical analysis

Effects of treatments on the incidence and severity of infected wounds were analysed by an analysis of variance with the program SPSS (SPSS Inc., version 16 for Windows, Chicago, IL). Statistical significance was applied at the level $P < 0.05$. When the analysis of variance was statistically significant, Duncan's multiple range test was employed to separate means. Data of antagonist populations (cfu/cm^2 and cfu/mL) were transformed to logarithms to improve the homogeneity of variances. Data from the separate experiments were combined when statistical analysis determined that variances were homogeneous.

3. Results

3.1. Isolation of microorganisms and screening for inhibitory activity on wounded fruit

Among more than 1800 microorganisms, isolated from the fructoplane and phylloplane of different pome and citrus orchards, 80% were screened for inhibitory activity against *P. expansum* or *P. digitatum*. From the screened microorganisms, 67% showed no ability to reduce the number of infected wounds, 22% reduced pathogen incidence by less than 25% and severity by more than 25%, and 7.6% reduced incidence and severity by more than 25%. However no more than 3% reduced incidence and severity by more than 50% and only 4 isolates (3 yeasts and 1 bacterium strain) fulfilled the minimum selection criteria: reduction of disease incidence and severity by 75% or more (Table 1). The three yeast strains that met the minimum criteria were tested again in 'Golden Delicious' apples and 'Rocha' pears against *P. expansum* and in 'Valencia Late' oranges against *P. digitatum*. The most effective isolate was *M. andauensis* NCYC3728 (PBC-2), which was selected for further assays.

3.2. Screening for minimum effective concentration

The application of the antagonist *M. andauensis* PBC-2 significantly inhibited pathogen development at all tested concentrations. The increase in antagonist concentration increased the reduction of pathogen incidence and severity (Fig. 1). For the lower

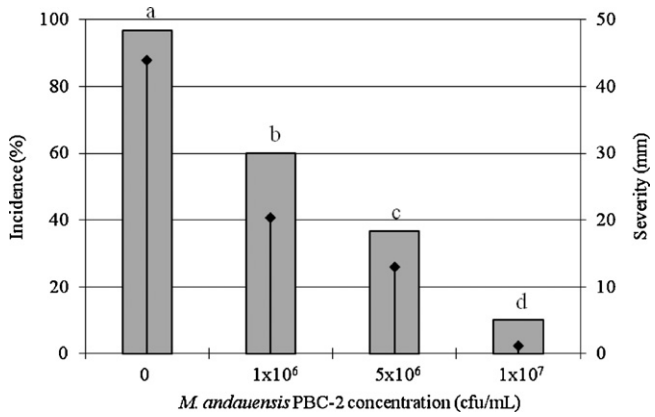

Fig. 1. Incidence (■) and severity (♦) of blue mould on wounded 'Golden Delicious' apples treated with *M. andauensis* PBC-2 at different concentrations, followed by the pathogen, *P. expansum*, at 10^4 spores/mL, after 7 days at 20 ± 1 °C and 80 ± 5% RH. Each fruit were wounded twice; five fruit, with two wounds, constituted a replicate and each treatment were repeated three times. Within incidence, columns and lines with different letters are significantly different, using Duncan's test ($P < 0.05$).

concentration tested (10^6 cfu/mL), incidence and severity suppression was less than 38 and 54%, respectively. Higher reductions of incidence and severity were observed with the intermediate concentration 62 and 70%, respectively; the higher dose, 10^7 cfu/mL, promoted the greatest reduction, 90 and 95% of incidence and severity respectively.

3.3. Broad spectrum activity and biocontrol activity under cold storage conditions

The effectiveness of *M. andauensis* NCYC3728 (PBC-2) was evaluated under different conditions and in a wide range of hosts and pathogens. Green and blue moulds were significantly higher on untreated mandarins or oranges when compared with fruit treated with the antagonist *M. andauensis* PBC-2 (Fig. 2). *M. andauensis* PBC-2, at 10^7 cfu/mL, reduced incidence of both pathogens by 73 and 77% on mandarins infected at 10^5 spores/mL (Fig. 2A). On oranges infected with *P. digitatum* and *P. italicum* at 10^6 spores/mL (Fig. 2B), reductions achieved were 74 and 83%, respectively.

The results of the effectiveness of *M. andauensis* PBC-2 in different apple cultivars against different postharvest pathogens are shown in Fig. 3. The development of *P. expansum*, *R. stolonifer* and *B. cinerea* was completely suppressed on 'Bravo de Esmolfe' apples treated with *M. andauensis* PBC-2. This was the cultivar that showed less susceptibility to decay. The incidence of blue mould (Fig. 3A) was reduced by 100, 96, 91 and 85% on 'Starking', 'Granny Smith', 'Fuji' and 'Royal Gala' apples, respectively. Grey mould was completely controlled on 'Granny Smith' apples (Fig. 3B). On 'Fuji' apples the reduction was 75% and on 'Royal Gala' and 'Starking' apples the reduction was 71%. The development of *R. stolonifer* was reduced by 82% on 'Royal Gala' and on 'Granny Smith' apples and lower reductions were observed with the other cultivars (Fig. 3C).

The results of the effectiveness of *M. andauensis* PBC-2 to control blue mould in cold storage of 'Rocha' pears and 'Golden Delicious' apples are shown in Fig. 4. During all storage periods, *M. andauensis* PBC-2 significantly inhibited development of blue mould. After 2 months of cold storage the incidence on control fruit (not treated) was 78 and 68% on pears and apples respectively, and with treated fruit the percentage of decayed fruit was 21 and 14% for pears and apples respectively. The application of the biocontrol agent *M. andauensis* PBC-2 reduced incidence and severity of blue mould on pears stored at 1 °C for 60 days by 73 and 79%, respectively (Fig. 4A). Similar reductions were observed

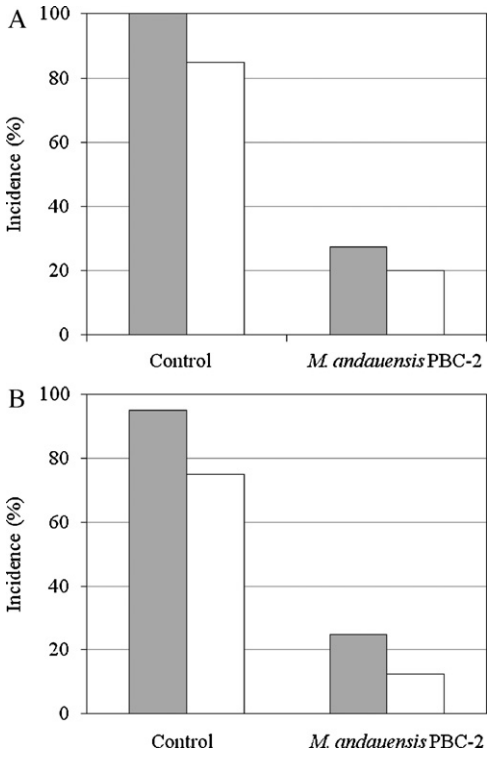

Fig. 2. Incidence of *P. digitatum* (■) and *P. italicum* (□), A: at 10^5 spores/mL, on wound-inoculated 'Clementina Fina' mandarins, B: at 10^6 spores/mL, on wound-inoculated 'Lanelate' oranges, treated with *M. andauensis* PBC-2 at 10^7 cfu/mL. Incidence was assessed after 7 days at 20 ± 1 °C and 80 ± 5% RH. Ten fruit constituted a replicate and each treatment was repeated four times.

with apples (Fig. 4B). After 90 days of cold storage the incidence on untreated pears and apples was near 100%, and higher on pears than apples. The treatments with the antagonist *M. andauensis* PBC-2 significantly inhibited *P. expansum* development on both fruit by 78%.

3.4. Semi-commercial trials on 'Golden Delicious' apples under cold storage

The results of semi-commercial trials on 'Golden Delicious' apples over 4 seasons are shown in Fig. 5. Significant reduction in blue mould occurred after the application of fresh cells of *M. andauensis* PBC-2 simulating commercial conditions. Blue mould incidence and severity on untreated fruit, after 3 months at 1 ± 0.5 °C and 7 days at room temperature, were 92% and 26 mm, respectively. On treated fruit, incidence and severity were 9% and 6 mm, respectively. No significant differences were observed between the application of the antagonist and the commercial fungicide imazalil.

3.5. Population dynamics on 'Golden Delicious' apple surfaces

Population dynamics of *M. andauensis* PBC-2 within fruit wounds are shown in Fig. 6. At time 0, the initial population recovered from fruit was 1.8×10^4 cfu/cm^2 and after 24 h of cold storage the population increased. During the first 15 days the population increased 3.5-fold, achieving the maximum population level, and then remained relatively unchanged until day 30. After that time, the population started to decrease until the end of cold storage, achieving 2.2×10^4 cfu/cm^2 at day 90. Through the 7 days of shelf-life at 20 °C, the viable population increased 1.5-fold.

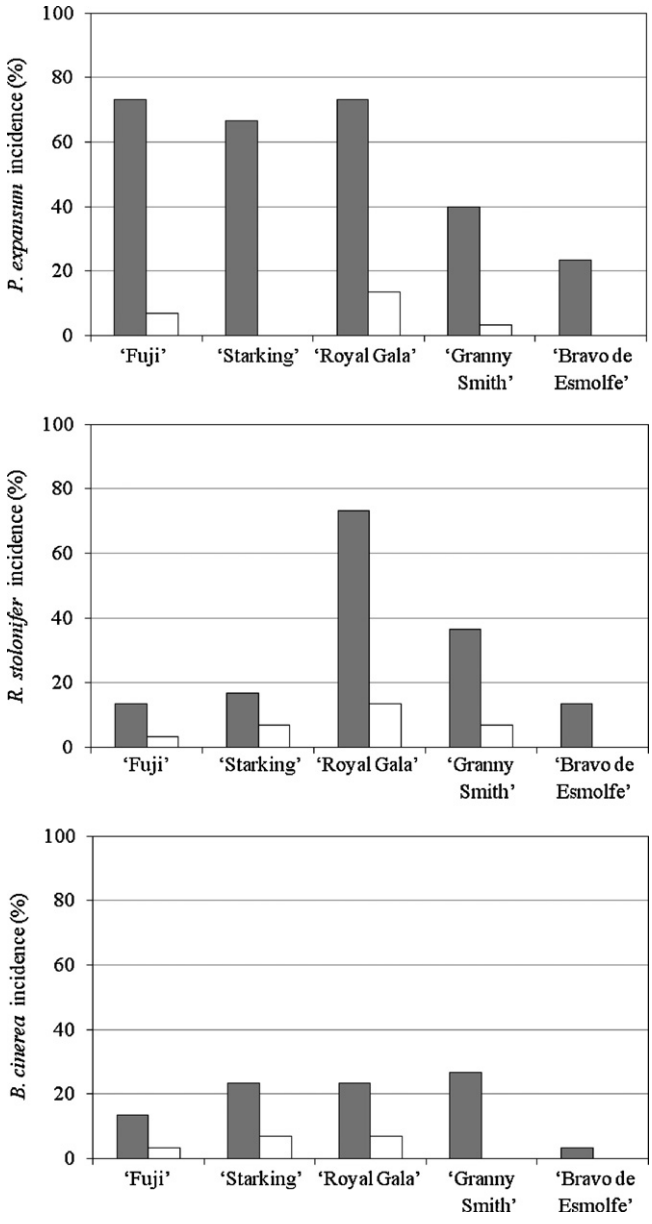

Fig. 3. Postharvest decay of wounded apple of different cultivars, untreated (□) or treated with *M. andauensis* PBC-2 at 10^7 cfu/mL (■) and infected with 10^4 spores/mL of A: *P. expansum*; B: *R. stolonifer*; and C: *B. cinerea*. Decay was assessed after 7 days at 20 ± 1 °C and 80 ± 5% RH.

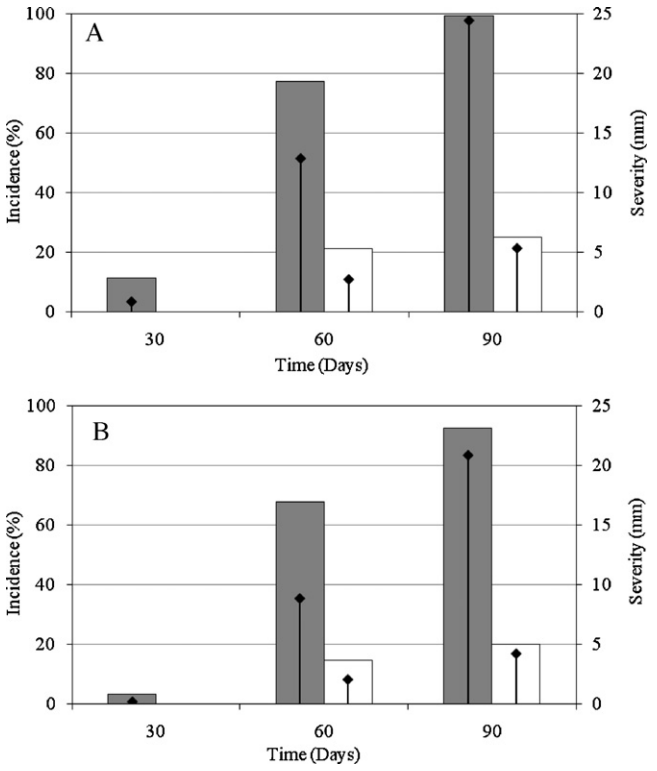

Fig. 4. Postharvest incidence of blue mould on wounded A: 'Rocha' pear and B: 'Golden Delicious' apples, untreated □ or treated with *M. andauensis* PBC-2 at 10^7 cfu/mL (■) and infected with 10^4 spores/mL of *P. expansum* during 90 days of storage at 1 ± 0.5 °C and 80 ± 5% RH. (♦) Represents blue mould severity.

3.6. Studies of acid tolerance in a simulated gastric fluid

The survival of *M. andauensis* PBC-2 in the simulated gastric fluid is shown in Fig. 7. No growth was observed, and the population started to decrease immediately after exposure to simulated gastric fluid, and decreased exponentially over time. No surviving cells were observed at 48 h.

4. Discussion

The development of a biological control agent requires several steps. The first is isolation, disease control efficacy, identification and selection. Isolating potential biocontrol agents from appropriate tissues and under appropriate environmental conditions helps ensure that the microbial antagonists isolated will be well adapted to survival and activity on the specific tissues requiring protection (Slininger et al., 2003). For that reason, we started to recover isolates from the surface of pome and citrus leaves and fruit picked from different orchards throughout the growing season and during fruit storage in different packinghouses. The recovered isolates were evaluated for their antagonistic activity *in vivo* screenings, against blue mould on pome fruit and green mould on citrus fruit. From the total of 1465 microorganisms tested, approximately 7.6% diminished severity and incidence by more than 25% and less than 3% reduced incidence and severity by more than 50%. Similar results were reported by Viñas et al. (1998), from 933 isolates tested, 9.8% reduced lesion diameter by more than 50%, but only 3.3% reduced incidence by more than 50% and severity by more than 75%. After the initial screening of 210 potential antagonists in peaches, Zhang et al. (2009) showed that only 3 isolates (1.4%) were the efficient

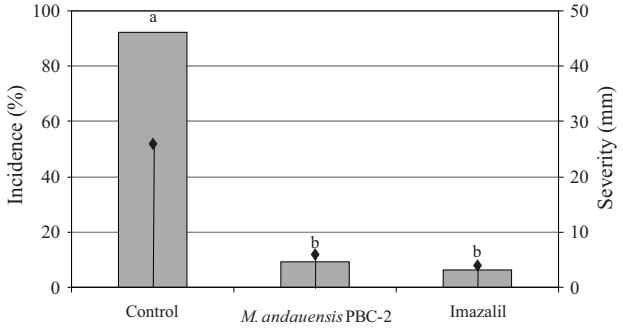

Fig. 5. Postharvest incidence (■) and severity (♦) of blue mould on wounded 'Golden Delicious' apples untreated, treated with *M. andauensis* PBC-2 at 10^7 cfu/mL or with fungicide imazalil at 0.5% and then inoculated with 10^4 spores/mL of *P. expansum* followed by 3 months of storage at 1 ± 0.5 °C and 80 ± 5% RH and 7 days at 20 ± 1 °C. Columns and rows with the same letter are not significantly different according to Duncan Test ($P < 0.05$). Columns and rows represent the means experiments during four seasons.

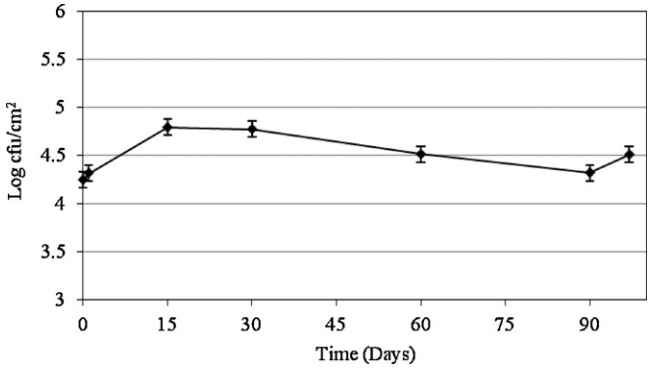

Fig. 6. Population dynamics of *M. andauensis* PBC-2 within wounds on 'Golden Delicious' apples. Fruit were wounded, treated by dipping for 30 s in a suspension of *M. andauensis* PBC-2 at 10^7 cfu/mL and stored at 1 ± 0.5 °C and 80 ± 5% RH for 90 days, followed by 7 days at 20 ± 1 °C. Symbols represent the means of four replicates of two experiments and the vertical bars indicate mean standard error.

antagonists to reduce decay caused by *Monilia laxa*. Studies performed by Nunes et al. (2001) on pears against *P. expansum*, by Karabulut and Baykal (2003) on peaches against *P. expansum* and *B. cinerea*, and by Taqarort et al. (2008) on citrus against *P. digitatum*, showed that microorganisms with potential as biocontrol agents account for only 2.0–6.8% of the total of epiphytic isolates. Although the results mentioned above have similarities with each other, and are identical to our study, the number of isolates with antagonistic activity is quite dependent on the screening method and the minimum selection criteria applied. Due to the high number of isolates recovered in the present work, the minimum selection criteria was narrowed and only one bacterium and three yeasts fulfilled the minimum criteria: reduction of incidence and severity of disease by 75% or more. Although there are several bacteria known as biocontrol agents, interest has been given to yeasts, since their mode of action it is not based in the production of toxic compounds, which is important once the antagonists are applied to consumable products. Moreover, yeasts are easily cultivated with simple nutritional requirements, easily produced on a large scale (Droby and Chalutz, 1994), can colonize a surface for long periods under dry conditions, produce extracellular polysaccharides that enhance their survival and restrict wound colonization and flow germination of fungi, can rapidly use available nutrients and be minimally impacted by pesticides (Janisiewicz, 1988).

In this work the attention was focused only on yeasts and the most effective one, *M. andauensis* NCYC3728 (PBC-2), was isolated from 'Bravo de Esmolfe' apples, a typical Portuguese apple cultivar classified as a PDO, which means that it corresponds to a traditional commodity, produced under strict controlled conditions and labelled with a specific certification. This cultivar has good sensory properties, namely sweetness and flavour. *M. andauensis* NCYC3728 (PBC-2) was selected for further studies and showed excellent protection on pome and citrus fruit, and a patent of application as a biocontrol agent has been applied (Nunes and Manso, 2010). Two more species of this genus, *Metschnikowia fruticola* and *Metschnikowia pulcherrima*, have been described as effective biocontrol agents against *P. digitatum* (Kinay and Yildz, 2008) and *P. italicum* (Droby, 2006) on citrus, *P. expansum* on apples (Janisiewicz et al., 2001), *B. cinerea* on grapes (Kurtzman and Droby, 2001), apples (Piano et al., 1997; Janisiewicz et al., 2001) and on strawberries (Karabulut et al., 2004). The species *M. andauensis* was recently identified by Molnár and Prillinger (2005) and our work is the first reporting biocontrol activity of this species. In fact, to our knowledge, there are only three prior reports mentioning this species (Molnár and Prillinger, 2005; Xue et al., 2006; Molnár et al., 2008).

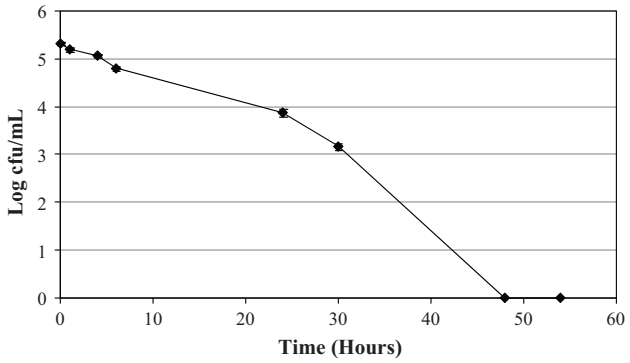

Fig. 7. Survival of *M. andauensis* PBC-2 in simulated gastric fluid (pH 2) at 37 °C. *M. andauensis* PBC-2 was inoculated in simulated gastric fluid and incubated at 37 °C at 20 rpm for 54 h. Symbols represent the means of three replicates of two experiments and the vertical bars indicate mean standard error.

Some reports have demonstrated a direct relationship between the population density of an antagonist and its effectiveness as a postharvest biological control treatment (Karabulut et al., 2004; Zhang et al., 2009). Our results also showed that the antagonistic activity of *M. andauensis* NCYC3728 (PBC-2) was dependent on the concentration of the antagonist. Concentrations of 1×10^6 and 5×10^6 cfu/mL reduced incidence by 38% and 62%, respectively, while 1×10^7 cfu/mL caused a reduction of 90 and 95% of incidence and severity, respectively, on wounded apples infected with *P. expansum*. The potential for using lower inoculum doses for achieving control is very important from a cost perspective. From a practical point of view, the limit for yeasts is about 5×10^7 cfu/mL (Janisiewicz, 1988). The concentration of *M. andauensis* NCYC3728 (PBC-2) that provides a satisfactory level of control is low enough to be considered feasible for commercial use and is lower than that recommended for yeasts.

As reported by Wilson and Wisniewski (1994), a quantitative balance exists at the wound site between the number of antagonist and pathogen propagules, which affects the outcome of the interaction and determines whether the wound becomes the site of infection. The dose response relationship demonstrated in our study was also found by Piano et al. (1997) and Schena et al. (2000), with different isolates of *Metschnikowia* spp. on apples, table grapes and cherry tomatoes against different postharvest pathogens. These data indirectly suggest that competition for space and nutrients has an important role in the mode of action of these yeasts. Studies of the mode of action of *M. andauensis* NCYC3728 (PBC-2), performed by Manso et al. (2011), indicated that the production of lytic enzymes or inhibitory compounds, such as antibiotics, as well as the competition for iron, were not responsible for the antagonistic properties of this yeast.

To select an antagonist suitable for postharvest application, it is necessary to look for those that are well adapted to survival and growth in wounds under storage conditions, and that have adaptive advantage over a wide range of crops, pathogens and commodities (Wilson and Wisniewski, 1994). The results obtained with the application of *M. andauensis* NCYC3728 (PBC-2) on different pome cultivars, against *R. stolonifer*, *P. expansum* and *B. cinerea*, on citrus fruit against *P. digitatum* and *P. italicum*, as well as the efficacy obtained in the experiments on pears and apples stored under cold conditions, has shown fulfillment of these requisites. The efficacy of *M. andauensis* NCYC3728 (PBC-2) was proved in semi-commercial trials, on apples stored at 1 °C, simulating the temperature normally used during cold storage. The effectiveness of the biocontrol agent could be attributed to the ability of maintaining a high population of viable cells on the fruit surface under storage conditions. Rapid colonization of apple wounds was observed during the first 24 h, increasing until 15 days and then the population stabilized. This indicates excellent adaptation to cold storage, a necessary feature

for a postharvest agent and a good indication of their commercial potential. The effect of cold storage conditions of some strains of *M. pulcherrima* and *M. fruticola* has been also reported. Karabulut et al. (2004) showed that the population of *M. fruticola* on harvested strawberries slightly decreased during storage (3 days at 0 °C) and shelf-life (2 days at 20 °C). Piano et al. (1997) observed that on apples stored at 4 °C, the population density of *M. pulcherrima* on wounds also increased during the first hours and continued over 96–128 h after application. Janisiewicz et al. (2001) demonstrated that strains of *M. pulcherrima*, increased populations by approximately two log units in apple wounds during 1 month of storage at 1 °C followed by 5 days at 24 °C, concluding that this species is an excellent colonizer of apple wounds and can thrive on apple as a substrate. In the present study, the population of *M. andauensis* NCYC3728 (PBC-2) on apple surfaces increased again during the shelf-life period. This is particularly important due to the vulnerability of the fruit during the shelf-life period. Satisfactory control of pathogen development depends on the establishment of the biocontrol agent, especially when the main mode of action is competition for nutrients and/or space.

Safety tests, such as the acute oral toxicity, are important to characterize and register a biocontrol agent. Though additional safety tests are required, in our experiments we showed that *M. andauensis* NCYC3728 (PBC-2) at 37 °C, the human body temperature (data not shown), did not grow on simulated gastric fluid, indicating that it might not grow in the human stomach.

In conclusion, *M. andauensis* NCYC3728 (PBC-2) is a new biocontrol agent that could be used effectively, at a concentration feasible for commercial use, against different fungal pathogens on citrus fruit and on a broad range of pome fruit, under cold storage and shelf-life conditions. Future research will focus on the optimization of the growth parameters, the establishment of *M. andauensis* NCYC 3728 (PCB-2) optimal conditions for high-cell density production and elucidation of mode of action.

Acknowledgments

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Chapter 3

Growth kinetics of the biocontrol agent *Metschnikowia andauensis* PBC-2 in submerged batch cultures

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Submitted

Growth kinetics of the biocontrol agent *Metschnikowia andauensis* PBC-2 in submerged batch cultures

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 others like 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*.

Key words: 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 e*t al.*, 1999). For yeasts and bacterial strains, commercial production of biomass for preparations generally requires liquid culture production in large, industrial scale fermenters (Roberts & 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 semi-defined 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 gladiodi* 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 & 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 & 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 & 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 & Yildz, 2008) and *Penicillium italicum* (Droby, 2006) on citrus, against *B. cinerea* on grapes (Schena *et al.*, 2000; Kurtzman & 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 & 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 anduensis PBC-2, used in this study, was originally isolated from the carposphere of 'Bravo de Esmolfe' apples. This strain was stored at -80 ºC in liquid medium with 20 % (v/v) glycerol. 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 ºC.

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 ºC 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 solution. After incubation at 25±0.5 ºC 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 flashes 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 the biocontrol agent *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 10^5 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 ºC 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 ºC and 30±0.5 ºC.

2.3.2. Effect of nitrogen sources concentration

The effect of nitrogen sources concentration was tested in YPD medium, at 25±0.5 ºC, 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.

2.3.2. Effect of pH and temperature

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

2.3.3. 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 ºC, during 60 h. At the beginning of the experiment and at regular intervals after 12 h of incubation, the pH was measured. Different samples were taken from the flasks to measure the Optical Density (OD_{540nm}) 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+ Z001023551) 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 1 vvm (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, the 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 (OD_{590nm}), 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 10^7 cfu/ml using a spectrophotometer.

Conidial suspensions were prepared from 7-10 d old cultures, in sterile distilled water containing Tween-80. Pathogen concentration was adjusted to 10^4 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. Fruits were artificially wounded at the equatorial region (2 mm deep by 1 mm wide; 2 wounds per fruit).

Twenty microlitres of the *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. 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, no statistical differences were observed between 25 and 30 ºC. YPD medium was selected for the following experiments.

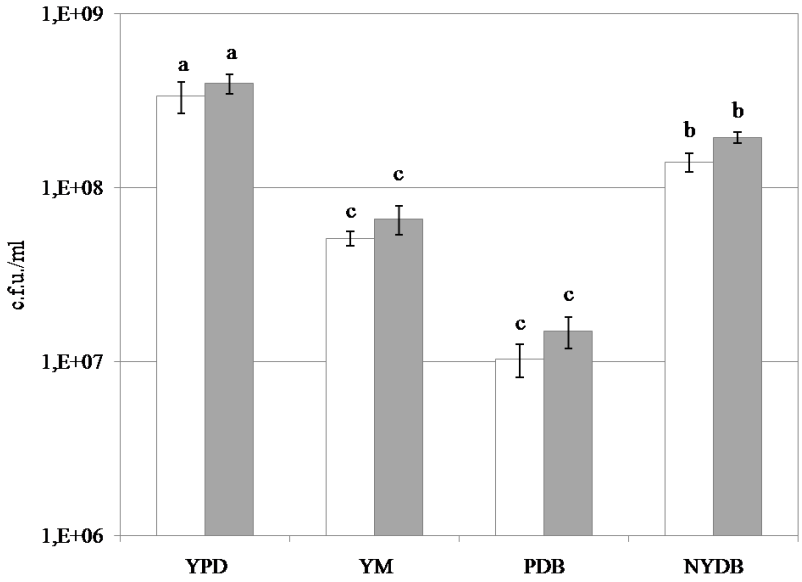

Figure 1. Production of *M. andauensis* strain PBC-2 in different standard media, at 25 ºC □ and 30 ºC ■. Growth was carried out in 250 ml conical flasks, shaken at 150 rev/min for 40 h. Columns with different letters are significantly different for each medium using Duncan test ($P< 0.05$) and the vertical bars indicate the standard deviation.

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 ºC. 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.

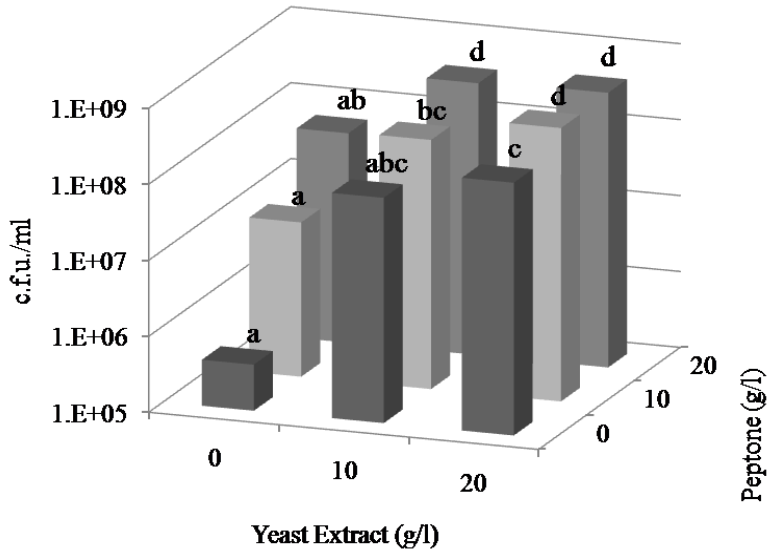

Figure 2. Effect of yeast extract and peptone concentrations on the growth of *M. andauensis* PBC-2, in YPD medium, after 40 h of incubation, at 150 rev/min and 25 ºC. The experiments were performed twice, in triplicates under identical conditions.

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. The viable population reached with yeast extract at 10 and 20 g/l, without peptone, was 9.2×10^7 and 2.1×10^8 cfu/ml, respectively. In this experiment, the maximum population achieved was 4.2×10^8 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 ºC, 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 ºC, respectively. Maximum population was achieved at pH 6.5 at 25 and 30 ºC, nonetheless no significant differences were noted between both temperatures. The selected pH and temperature for the subsequent tests were 25 ºC and an initial pH at 6.5.

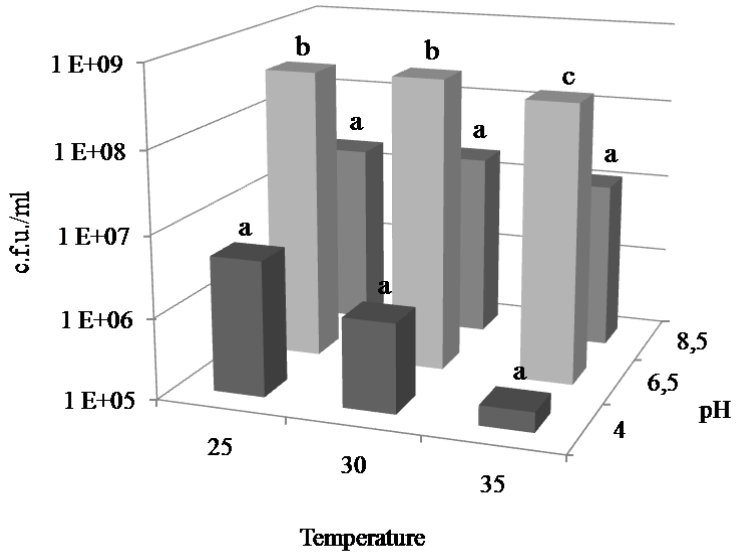

Figure 3. Effect of temperature and pH on the growth of *M. andauensis* PBC-2, in YPD medium, after 40 h of incubation at 150 rev/min. The experiments were performed twice, in triplicates under identical conditions.

3.4. Variation in carbon source and concentration

The effect of the nature of the carbon source was evaluated in YPD modified medium, replacing the usual carbon source, glucose, by others like sucrose or 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.

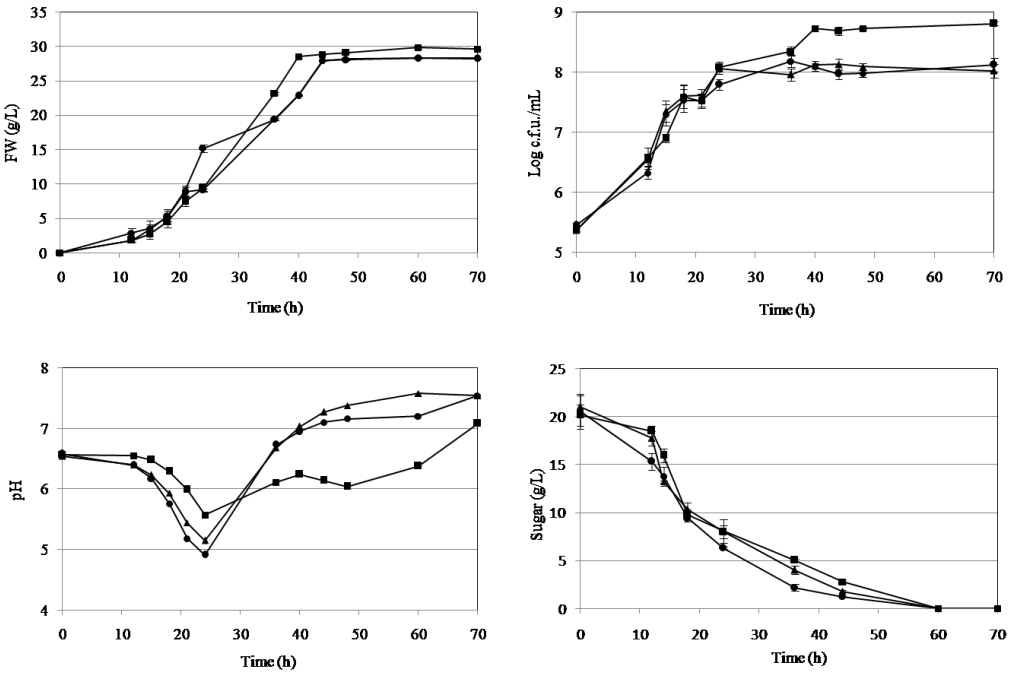

Figure 4. Growth of *M. andauensis* PBC-2 in YPD modified medium with different carbon sources, sucrose (squares), glucose (circles), fructose (triangles). a: fresh cell weight (FCW), g/l; b: viable cells, expressed in log cfu/ml; c: pH; d: sugar depletion, g/l. Cultures were grown in 50 ml of media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

Table 1. Growth parameters of *M. andauensis* PBC-2, in shake flasks experiments in YPD modified medium with different carbon sources, at 25 ºC, stirring at 150 rpm.

Carbon source	Fructose	Glucose	Sucrose
μg (1/h)	0.083±0.014a	0.098±0.011ab	0.107±0.008b
P max (g/lh)	0.603±0.013a	0.599±0.012a	0.669±.007b
Rs (g/lh)	0.368±0.013a	0.360±0.030a	0.364±0.031a
Yx/s (g/g)	1.350±0.129a	1.401±0.234a	1.487±0.153a

Values are means ± SD 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, *P*max biomass productivity, *Yx/s* biomass yield, *Rs* substrate uptake rate.

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 1.2×10^8, 5.3×10^8 and 1.3×10^8 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.

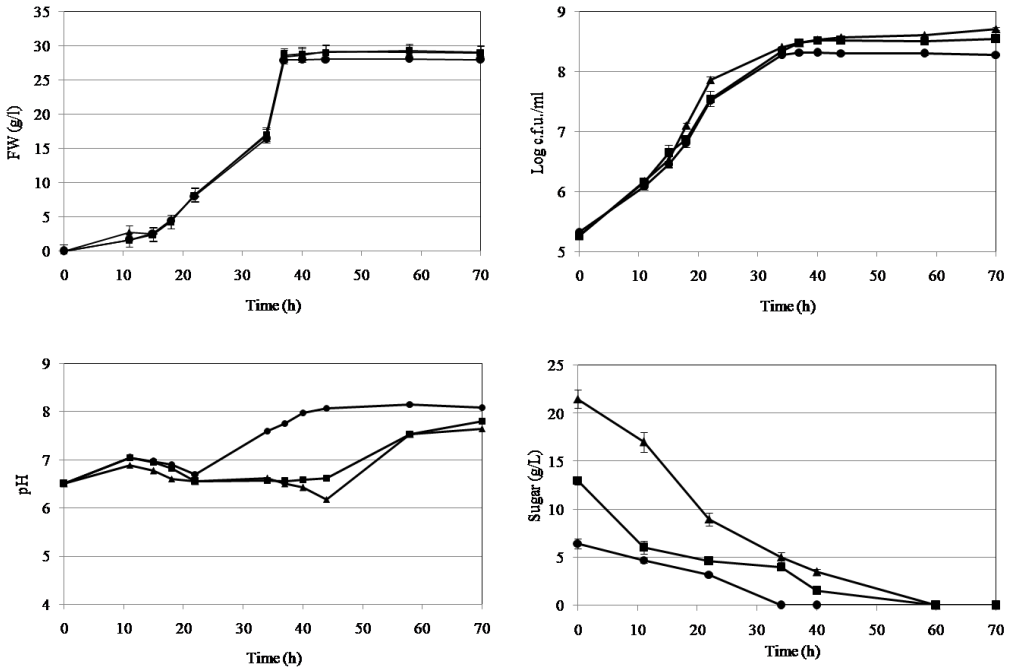

Figure 5. Growth of *M. andauensis* PBC-2 in YPS medium, with different sucrose concentrations, 5 g/l (circles), 12.5 g/l (squares), 20 g/l (triangles). a: fresh cell weight (FCW), g/l; b: pH; c viable cells, expressed in log cfu./ml; d: sugar consumption, g/l. Cultures were grown in 50 ml of media shaken 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.

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 2.1×10^8, 3.1×10^8 and 3.0×10^8 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.

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

Sugar Concentration (g/l)	5	12.5	20
μg (1/h)	0.102±0.002a	0.106±0.003a	0.095±0.014a
P max (g/lh)	0.638±0.002a	0.665±0.004b	0.653±0.011ab
Rs (g/lh)	0.246±0.070a	0.194±0.060a	0.373±0.011b
Yx/s (g/g)	4.515±0.588a	2.253±0.178b	1.357±0.104c

Values are means ± SD 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, *P*max biomass productivity, *Yx/s* biomass yield, *Rs* substrate uptake rate.

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 ºC.

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 3.1×10^8 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 O_2 /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 70 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.

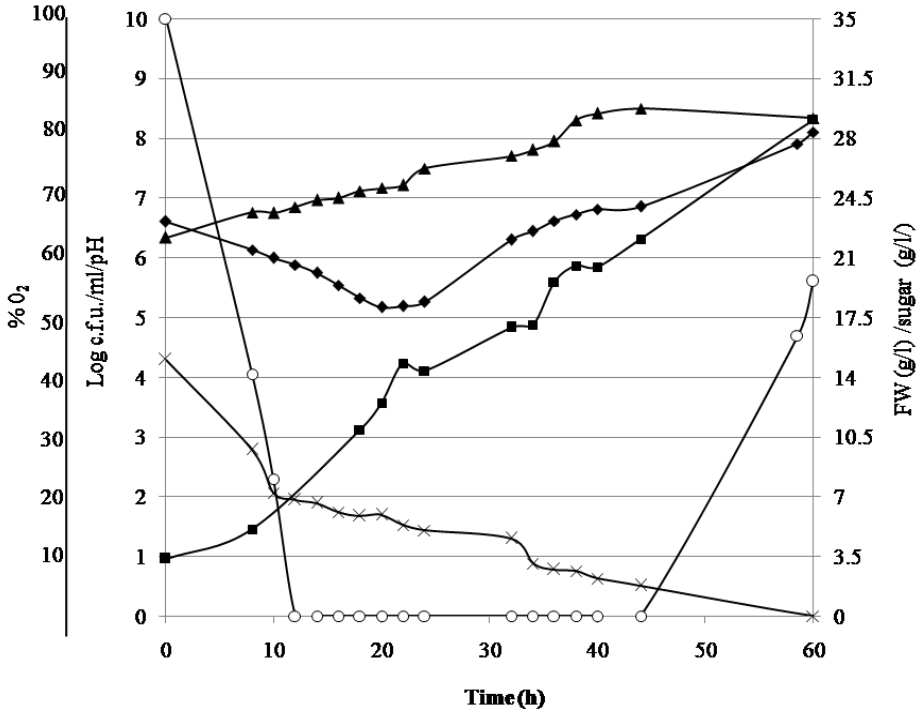

Figure 6. Time course of viable cells cfu/ml (triangles), fresh cell weight (squares), pH value (diamonds), oxygen concentration (circles), consumption of sugar (times) in batch cultivation of *M. andauensis* PBC-2, performed in STR with airflow rate of 1 vvm, L-shaped sparger, 300 rev/min., Rushton-type turbine, at 25 ºC, in YPS medium (sucrose 12.5 g/l, yeast extract 10 g/1, peptone 20 g/l), inoculated at initial concentration at 10^6 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.

3.6. Efficacy assay

Figure 7 shows the results of the effectiveness of *M. andauensis* PBC-2 in different apple cultivars against blue mould. *P. expansum* incidence was reduced by 76, 74 and 63 % on 'Bravo de Esmolfe', 'Jonagolg' 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.

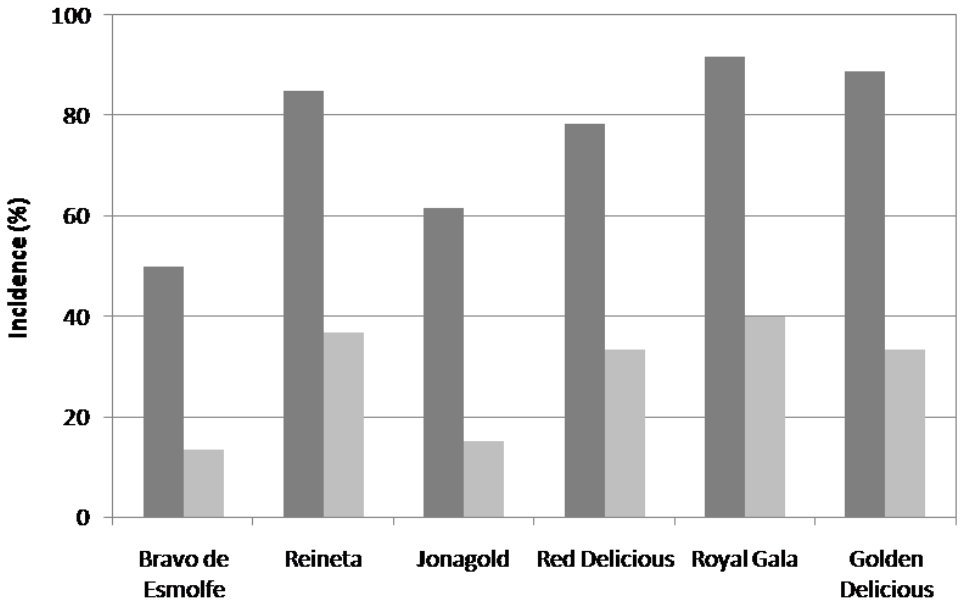

Figure 7. Postharvest incidence of blue mould on wounded apples of different cultivars, untreated (■) or treated with *M. andauensis* PBC-2 at 10^7 cfu/ml (■) and infected with 10^4 conidia/ml of *P. expansum.* Incidence was assessed after 7 days at 20 ± 1 ◦C. Ten fruit constituted a replicate and each 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; Zhang *et al.*, 2009), were tested. The best performance was achieved in YPD medium at both studied temperatures (25 and 30 ºC). 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 & 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 & Droby, 2001), *M. reukafii* (Nozaki *et al.*, 2003), *M. bicuspidate* (Fell & Pitt, 1969), *M. shivogae* (Lachance *et al.*, 2008), *M. vanudenii* (Giménez-Jurado *et al.*, 2003), *M. sinensis*, *M. zizyphicola*, *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, 4×10^5 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 4.2×10^8 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. 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 & 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 ºC. 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 3.1×10^8 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 PBC-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 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* is only present 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 amyloliquefaciens.*

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.

Acknowledgments

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Chapter 4

Production of the biocontrol agent *Pantoea agglomerans* PBC-1 in a stirred tank reactor by batch and fed-batch cultures

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Production of the biocontrol agent *Pantoea agglomerans* PBC-1 in a stirred tank reactor by batch and fed-batch cultures

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Abstract Concerns about food safety as well as the development of resistance to many fungicides by major postharvest pathogens have increased recently. Biological control, using microorganisms antagonistic to the fungal plant pathogens, appears to be promising as an alternative to fungicides. The microbial biocontrol agent has to be produced on an industrial scale, maintaining its biocontrol efficacy. The purpose of the current study was to optimize the conditions for microbial biomass production of the biocontrol agent *Pantoea agglomerans* PBC-1 in a 2-l mechanically stirred reactor (STR), defining mixing and mass transfer technological parameters and the growth kinetics for different saccharides. In the batch mode, different impellers and spargers were tested. Despite the oxygen mass transfer improvement achieved with marine propeller combined with porous sparger, the biomass did not increase, if compared with the use of a Rushton turbine and L-sparger, pointing out the relevance of a radial flux for better broth homogenization. Different carbon sources were used: sucrose, glucose and fructose; each of which led to viable populations 3.9×10^9, 1.4×10^9, 3.9×10^9 c.f.u/ml, respectively, after 20 h of incubation. Fed-batch technology allows the maintenance of high cell viability for longer periods of time in the stationary growth phase, which can be crucial for the scale-up of biocontrol agent production process that is achieved together with a reduction of 85% on the incidence caused by the pathogens, brought about by fresh microbial biomass preparation on artificially wounded apples or oranges, stored for 7 days at 25°C against *Penicillium expansum* and *Penicillium digitatum*.

Keywords Batch culture · Fed-batch culture · Mixing · Mass transfer · Cell growth · Stirred bioreactor · Biocontrol agent · *Pantoea agglomerans*

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Introduction

Postharvest fungal decays are predominantly controlled by fungicides. Concerns about the environment and human health as well as the development of resistance to many fungicides by major postharvest pathogens have generated an urgent need for the development of safe alternative methods. Biological control, using microorganisms antagonistic to the fungal plant pathogens, has gained considerable attention and appears to be promising as a viable supplement or alternative to chemical control (Spadaro and Gullino 2004; Nunes et al. 2009). A whole range of living organisms has been proposed as potential biocontrol agents, on pome fruits (Viñas et al. 1998; Nunes et al. 2001; Torres et al. 2005; Mounir et al. 2007; Nunes et al. 2007), stone fruit (Pusey and Wilson 1984; Bonaterra et al. 2003), grapes, cherries (Kurtzman and Droby 2001; Schena et al. 2003) and citrus fruits (Chalutz and Wilson 1990; Smilanick and Denis-Arrue 1992). However, the success and widespread use of biofungicides, remains limited. This is due to several reasons, among which is the inconsistency, variability of the efficacy under commercial conditions and production of shelf-stable formulations. In order to obtain semi-commercial quantities of the microbial agent, it is necessary to scale up the production process, at least to the level of pilot plant, using a low cost culture medium (Patiño-Vera et al. 2005). There are many factors

involved in optimization of biomass production, including the temperature, pH and nutrients (Lee et al. 1991). The nutrients for a medium must satisfy the basic requirements for cell biomass and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance (Stanbury et al. 1995). The inoculum is another important factor to be studied; it must be healthy, active and with an adequate cell density to minimize the lag phase.

Besides this, operational bioprocess optimization also determines the success of biocontrol agent production. Stirred tank reactors (STRs) are commonly used in industrial-scale fermentation processes because of their good fluid-mixing and gas transfer conditions, with a high mass and heat transfer and scale-up characteristics (Ungerman and Heindel 2007).

The performance of the microbial culture is dependent on a good mixing and mass transfer, conditioned by the choice of the sparger geometry and the impeller design. These parameters influence the volumetric mass transfer coefficient, K_La, an experimental factor extremely important in aerobic cultures with severe oxygen demands. With the intention of understanding the impact of mass transfer and mixing in the performance of stirred bioreactors, different reports have appeared in the literature (Puthli et al. 2005; Cascaval et al. 2006; Hadjiev et al. 2006; Puthli et al. 2006; Raposo and Lima-Costa 2006; Fujasová et al. 2007; Kao et al. 2007; Littlejohns and Daugulis 2007; Pollard et al. 2007; Potumarthi et al. 2007).

It has been observed that an L-sparger forms large gas bubbles with a small gas–liquid interfacial area and low residence time in the liquid phase (Doran 1995). On the other hand, the porous sparger which forms small bubbles, hence is more adequate for a good mass transfer for aerobic cultures. K_La determined with the L-shaped sparger presents lower values when compared with the porous sparger, for the same impeller (Raposo and Lima-Costa 2008). These observations were reinforced by other authors (Leckie et al. 1991a, 1991b).

The Rushton blade disc turbine has traditionally been the most popular impeller; it is simple in design and robust in construction (Irvine 1990; Gogate et al. 2000) and promotes radial flux against the axial flux defined by a marine propeller.

The hydrodynamics of stirred bioreactors is also crucial for the cell growth and viability of the biocontrol agent. Excessive shear forces may cause damage to microbial cells (Belo et al. 2003; Ungerman and Heindel 2007). Although using similar mass transfer conditions (K_La), Raposo and Lima-Costa (2008) showed different hydrodynamic behaviour of growth cultures. These differences, apparently due to the sensitivity of the cell line to mechanical stirring led the authors to support that the operational parameters, impeller design and stirred velocity have a preponderant effect on growth cultures.

Compared with conventional batch culture, fed-batch culture may have several advantages, including increased fermentation time, eventual higher productivity, extended exponential and stationary growth phases and reduced toxic effects of medium components which are present at high concentration (Stanbury et al. 1995).

The selected microorganism used in the present study was strain PBC-1, a bacterium identified as *Pantoea agglomerans* by the Centraalbureau Voor Schimmelcultures (CBS), (Delft, The Netherlands). *Pantoea agglomerans* is a facultatively anaerobic Gram-negative bacterium that belongs to the family Enterobacteriaceae. The strain PBC-1 was isolated from the surface of 'Valencia late' oranges and exhibited activity against *Penicillium expansum*, *Botrytis cinerea*, *Rhizopus stolonifer* on pome fruits and *P. digitatum* and *P. italicum* in citrus fruits.

This work focused on establishing *P. agglomerans* PCB-1 operational optimal conditions for high-cell density biocontrol agent production in batch and fed-batch culture modes in an STR bioreactor. Mixing and oxygen mass transfer were studied as crucial technological parameters and the growth kinetics of PCB-1 was described for different sugars, as well for different impeller and sparger designs. To the best of our knowledge, this is the first work that describes geometry of a stirred bioreactor (STR) for the aerobic production of a biocontrol agent, reporting volumetric oxygen mass transfer correlated with kinetic growth parameters.

Materials and methods

Microorganism

Pantoea agglomerans PBC-1, used in this study, isolated and identified as described above was stored at −80°C in liquid medium with 20% (v/v) glycerol. When required for fermentation studies, *P. agglomerans* PBC-1 was streaked on NYDA medium (per l: 8 g nutrient broth, Biokar Diagnostics BK003HA; 6 g yeast extract, Biokar Diagnostics A1202HA; 10 g glucose, Riedel-de-Haën 16325; 15 g agar) and incubated at 30°C. For inoculum preparation, individual colonies grown for 24 h at 30°C were transferred with a sterile loop into a 250 ml shake flask containing 50 ml of NYDB (the same as NYDA without agar) for flask assays and for bioreactor assays. The pre-culture was incubated at 30°C, in a rotary shaker-flask incubator (Pentlab, Neifo refrigerada, Portugal) at 150 rev/min, until it reached the exponential phase. These fresh cells were used to inoculate the flasks or the bioreactor.

Batch experiments in shake flasks

Variation in carbon composition and concentration of liquid medium were tested in shake flasks assays. Sucrose (Panreac 141621.1211), fructose (Fluka Biochemistry 47740) and glucose (Riedel-de-Haën 16325) at 10 g/l were screened as carbon sources. The effect of carbon source concentration was studied without any carbon source addition or with sucrose at 5, 10, 15, 20 g/l. Yeast extract at 5 g/l was used as nitrogen source in all studies. Each flask assay was conducted in three replicates and each experiment was repeated twice.

Fresh cells of *P. agglomerans* PBC-1, at an initial concentration of 1×10^5 c.f.u/ml were inoculated into 250 ml conical flasks, with 50 ml of culture medium. Flasks were incubated, at 30°C under orbital agitation at 150 rev/min. At the beginning of the experiment and every 2 h after 12 h of inoculation, samples were taken for analysis over 45 h. Optical Density (OD_{640nm}), measured in a spectrophotometer (Amersham-Pharmacia Biotech, Ultrospec 1100pro, Sweden), pH, colony-forming units (c.f.u/ml) and the sugar content were determined.

Viable cells were estimated in duplicate on NYDA, using the surface method. The sample dilutions were made in sterile phosphate buffer and the plates incubated at 30°C for 24 h.

Carbon source was analysed by HPLC (Xie et al. 2009), in samples previously centrifuged at 13,400×*g* for 10 min (Eppendorf, Centrifuge 5415D, Germany) and filtered through a 0.22 μm membrane filter. Analyses were performed on a Beckman System Gold HPLC (Beckman, USA) equipped with a Jasco Refractive Index model 1530 (Jasco, Japan). Frutose, glucose and sucrose were analysed using a Purospher STAR NH2 column (Merck KGaA, Germany) in an isocratic system, acetonitrile: water (75:25) at 1 ml/min and 35°C.

Fresh weight was determined by a fresh weight vs. optical density correlation curve.

Batch experiments in STR

The batch experiments were conducted in a 3-l (2.4-l working volume) stirred tank reactor, STR, (BioBundle System, Apllikon, Holland) operated at 30°C and 250 rev/min. The growth medium was composed by yeast extract at 5 g/l, sucrose at 5 g/l and antifoam B (Sigma A-5757) at 0.1 ml/l, at a pH 6.5, sterilised at 121°C for 20 min.

With intent to understand the impact of mass transfer and mixing in the biomass viability and production of *P. agglomerans* PBC-1, different aeration sparger geometry, L-sparger or porous and impeller designs, Rushton-type turbine (radial flow) or marine propeller (axial flow), were studied.

In the experiments with Rushton turbine and L-sparger the aeration was set at 100 l/h (0.69 v/v/m, air volume/liquid volume per minute), and in the ones with marine propeller a porous sparger was used and the aeration was set at 30 l/h (0.20 v/v/m), as described by Raposo and Lima-Costa (2006). The initial concentration used in these experiments was 10^6 c.f.u/ml.

To study the effect of the initial inoculum concentration, the Rushton turbine and L-shaped sparger were used. Bioreactor was set to 100 l/h (0.69 v/v/m) and inoculated in order to start the experiment with the concentration of 1×10^6 or 1×10^7 c.f.u/ml.

In all experiments and during cultivation, pH, temperature and dissolved oxygen were constantly monitored on-line using specific probes and registered with the BioeXpert program, version 2. Samples were taken at the beginning of the experiment and every 2 h, the OD_{640}, viability counts were determined as describe above. Cells were harvest by centrifugation at 1,920×*g* in 15 ml centrifuge tubes, the fresh weight was determined and the sugar concentration was measured in the supernatant fluid grown. Each independent assay was repeated twice.

Fed-batch experiments

The fed-batch growth medium was inoculated with preculture as described above, at an initial concentration of 1×10^7 c.f.u/ml. Rushton turbine and L-sparger were used and aeration was set at 100 l/h 250 rev/min and 30°C. Samples were taken at the beginning of the experiment and every 2 h after inoculation and optical density, viable population and fresh weight were determined. The sugar concentration of each sample was determined by HPLC and when the sugar concentration was <1 g/l in the supernatant fluid, the fermenter was fed with batches of 300 ml of a sucrose solution (40 g/l) by a peristaltic pump at a flow rate of 62.5 ml/min. Experiments were repeated twice.

Biocontrol activity

The efficacy of the biomass produced, was evaluated in apple and orange fruits. Cells were harvest by centrifugation at 5,418×*g* (Beckman, Avanti J-14, Coulter, USA) and resuspended in phosphate buffer. The concentration of suspension was adjusted to 1×10^8 c.f.u/ml using a spectrophotometer. The *P. expansum* and *P. digitatum* isolated from decayed apples and oranges, respectively, are the most virulent isolates of our collection and were periodically transferred through fruits.

The concentration of the conidial suspensions of the pathogens was prepared from 7-day-old cultures grown on PDA (Biokar Diagnostics BK095HA) medium and

determined with a haemacytometer. Apples, cv. 'Golden Delicious' were obtained from commercial orchards in Alcobaça, Portugal, stored at 1°C, for no more than 3 months. Before experiment, fruits were dipped in a NaOCl 0.5% solution for 1 min and rinsed with water. The apples were wounded with a steel needle, making an injury 2 mm deep by 1 mm wide at the stem and calyx end. Then 20 µl of the suspension of *P. agglomerans* PBC-1, prepared as above were inoculated into the wound. In the control treatment the same volume of phosphate buffer was pipette into the wound. After drying 15 µl of an aqueous suspension of *P. expansum* at 1×10^4 spores/ml were inoculated into the wound. Fruits were stored at 20 ± 1°C and 80 ± 5% HR for 7 days, at the end of which the incidence (percentage of infected wounds) were determined. Ten fruits constituted a single replicate, and each treatment was repeated four times.

Oranges cv. 'Valencia late' from Algarve, Portugal orchards were inoculated by wounding the flavedo with the steel rod, previously immersed into a 1×10^6 spores/ml conidial suspension of *P. digitatum*, on the equator of each fruit. After 2 h 15 µl of the suspension of *P. agglomerans* PBC-1 at 1×10^8 c.f.u/ml was inoculated into the wound. Incidence was determined after 7 days of storage at 20 ± 1°C and 80 ± 5% HR. Twenty fruits constituted a single replicate and each treatment were repeated four times.

Statistical analysis

Each experiment was repeated twice to confirm the reproducibility of the results. Data for growth parameters of *P. agglomerans* PBC-1, produced in media with different carbon source and sucrose concentrations, were subjected to analysis of variance and mean separation using Duncan′s Multiple Range Test ($P < 0.05$) with SPSS software (SPSS Inc., version 16 for Windows, Chicago, IL).

Incidence of mould contamination in fruits were analysed by a test of variance applied to the arcsine of the square root of the proportion of infected fruit, followed by Duncan′s Test for separation of means when the variable was statistically significant ($P < 0.05$).

Results

Batch experiments in shake flasks

Cultures of *P. agglomerans* PBC-1 were grown in shake flasks, using different carbon sources. The biomass growth profiles were very similar, but in the run using glucose as carbon source, the lag phase was shorter (Fig. 1). However,

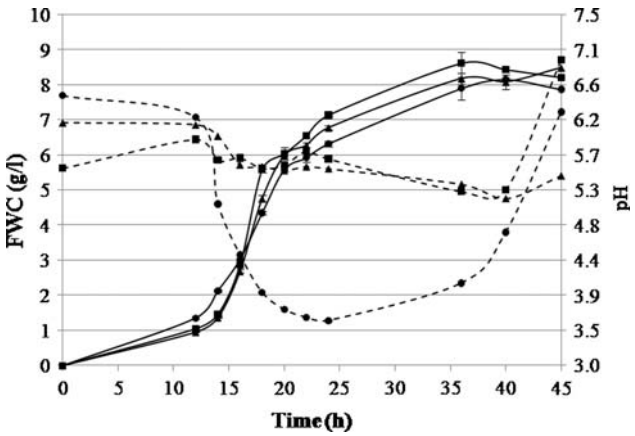

Fig. 1 Growth profiles (*solid lines*) and pH (*dashed lines*) for different carbon sources, glucose (*filled circle*) fructose (*filled square*) and sucrose (*filled triangle*) of *Pantoea agglomerans* PBC-1. Cultures were grown in 50 ml of media with 10 g/l of carbon source, shaken at 150 rev/min at 30°C

after 16 h of incubation, no differences were observed. At 20 h of incubation the viable population was 3.9×10^9, 3.9×10^9 and 1.4×10^9 c.f.u/ml in sucrose, fructose and glucose, respectively (data not shown). During the exponential phase the pH decreased in all carbon sources tested, particularly for glucose, getting a reduction from 6.5 to 3.6 at 24 h (Fig. 1).

The higher specific growth rate was reached with sucrose and fructose (Table 1).

These two carbon sources were also the ones that led to the highest level of biomass productivity and viable populations.

Nevertheless with respect to biomass yields the variations between sources become less evident. Glucose was the source that presents the lower consumption rate (Table 1). On the other hand, fructose was the first substrate to be consumed, which is in agreement with the comparative values of carbon source consumption rates (Table 1). After 14 h of incubation 70% of this sugar was

Table 1 Specific growth rate, substrate consumption rate, biomass yield and biomass productivity of *Pantoea agglomerans* PBC-1, grown in media with different carbon sources (10 g/l), at 30°C, stirring at 150 rev/min

Carbon source (10 g/l)	µg (1/h)	R_s (g/l h)	$Y_{x/s}$ (g/g)	P_{max} (g/l h)
Sucrose	0.28a	0.43b	0.82a	0.27a
Glucose	0.19b	0.40c	0.80a	0.25b
Fructose	0.28a	0.46a	0.81a	0.28a

Means followed by same letters are significantly equal, according to Duncan′s multiple range test ($P < 0.05$)

µg, specific growth rate; R_s, substrate uptake rate; $Y_{x/s,}$ biomass yield; P_{max}, biomass productivity

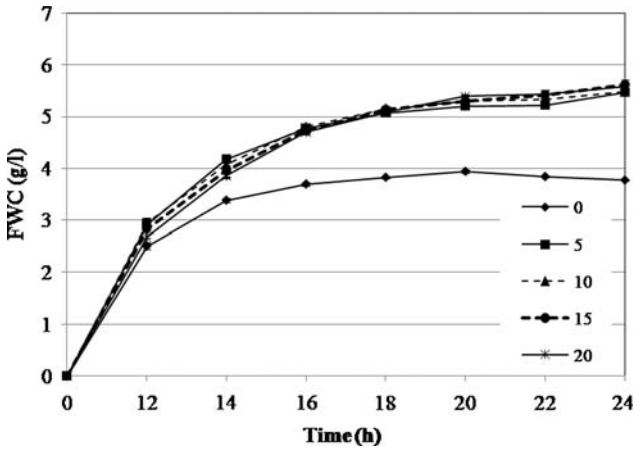

Fig. 2 Effect of different sucrose concentrations (g/l) on growth of *Pantoea agglomerans* PBC-1. Cultures were grown in 50 ml of media shaken at 150 rev/min at 30°C

Table 2 Specific growth rate, substrate consumption rate, biomass yield and biomass productivity of *Pantoea agglomerans* PBC-1, grown in media with different sucrose concentrations, at 30°C, stirring at 150 rev/min

Sucrose concentration (g/l)	μg (1/h)	R_s (g/l h)	$Y_{x/s}$ (g/g)	P_{max} (g/l h)
0	0.10a			0.21a
5	0.12a	0.19d	1.10d	0.24b
10	0.12a	0.33c	0.69c	0.24b
15	0.13a	0.45b	0.52b	0.25b
20	0.14a	0.61a	0.40a	0.25b

Means followed by same letters are significantly equal, according to Duncan's multiple range test ($P < 0.05$)

μg, specific growth rate; R_s, substrate uptake rate; $Y_{x/s}$, biomass yield; P_{max}, biomass productivity

consumed. Since the maximum specific growth rate and biomass productivities were achieved with sucrose and fructose (Table 1), and no difference was observed in the other studied parameters, sucrose was selected as carbon source for the following experiments, given it is a cheap and available carbon source.

Figure 2 shows that *P. agglomerans* PCB-1 can grow on yeast extract, without addition of any carbon source. However, when sucrose was added to the medium, the growth rate increased for all sucrose concentrations tested. At the beginning of the stationary phase 18 h, the viable populations were similar in all sucrose media, 3-4 × 10^9 c.f.u/ml, and at this point the viable population has been multiplied by 4 log (data not shown).

The carbon source was completely depleted after 24 h in shake flasks that had an initial sucrose concentration of 5 g/l, whereas for 10, 15 and 20 g/l, the sucrose concentration in the broth supernatants was close to 2, 4, 6 g/l, respectively.

Results in Table 2 show that biomass yield decreased with the increase of sucrose concentrations. For the different sugar concentration, the values of biomass productivities (Table 2) were very similar. However, substrate uptake rate was higher for 10, 15 and 20 g/l, compared with the 5 g/l. Nevertheless this increase in sucrose uptake was not accompanied by increasing biomass, suggesting that sugar is not a limiting nutrient in the assayed conditions.

Batch experiments in STR

In order to understand the impact of mass transfer and mixing in the production of the biological control agent *P. agglomerans* PBC-1 and to optimize the oxygen availability in the broth, different aeration sparger geometries and impeller designs were studied, at the same inoculum concentrations 1 × 10^6 c.f.u/ml.

The fermentation using a marine impeller and porous sparger which presents higher K_La is shown in Fig. 3. It is possible to observe a lag phase of 6–8 h and after 20–24 h the viable population achieved 4–5 × 10^9 c.f.u/ml.

During the exponential phase it is possible to register a decrease in the pH value and its increase at the beginning of the stationary phase. The oxygen decreases as result of cell respiration, but it was never exhausted, with the minimum values reached during the exponential phase, at the end of which the oxygen rose again to its initial phase.

Figure 4 illustrates the batch fermentation with L-sparger and Rushton turbine, and it was possible to observe a lag phase of 8 h, followed by an exponential phase of approximately 10–12 h. At this time the viable population was in the order of 3 × 10^9 c.f.u/ml, after this moment a long stationary phase started with a sugar depletion similar to that observed when porous sparger and marine propeller were used, at the same inoculum conditions.

Although the coefficient of volumetric oxygen mass transfer, K_La presents a lower value for Rushton turbine/L-sparger, the biomass accumulation did improved in those assayed conditions (Figs. 3, 4). Table 3 also depicts that specific growth rate, biomass yield and productivity were significantly higher for Rushton turbine/L-shaped sparger than for marine propeller/porous sparger, suggesting that radial flow is more adequate for broth homogenization in the reported conditions.

The effect of initial concentration of inoculum was studied at 1 × 10^6 and 1 × 10^7 c.f.u/ml using the Rushton turbine and L-sparger and is depicted in Figs. 4, 5, respectively. In the fermenter inoculated at initial concentration 1 × 10^7 c.f.u/ml the lag phase was shortened to 5 h, comparatively with inoculation of 1 × 10^6 c.f.u/ml and the culture began the exponential phase 3 h after inoculation,

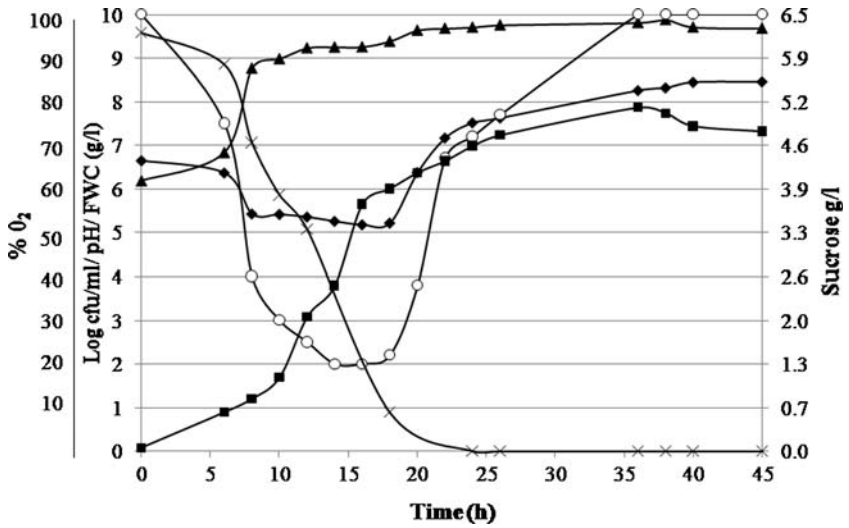

Fig. 3 Time course of viable cells c.f.u/ml (*filled triangle*), fresh weight cell (*filled square*), pH value (*filled diamond*), oxygen concentration (*open circle*), depletion of sucrose (*times*) in batch cultivation of *Pantoea agglomerans* PBC-1, performed with aeration flux of 30 l/h, porous sparger 250 rev/min, marine impeller, at 30°C, in medium with sucrose 5 g/l and yeast extract 5 g/l, inoculated at initial concentration at 10^6 c.f.u/ml

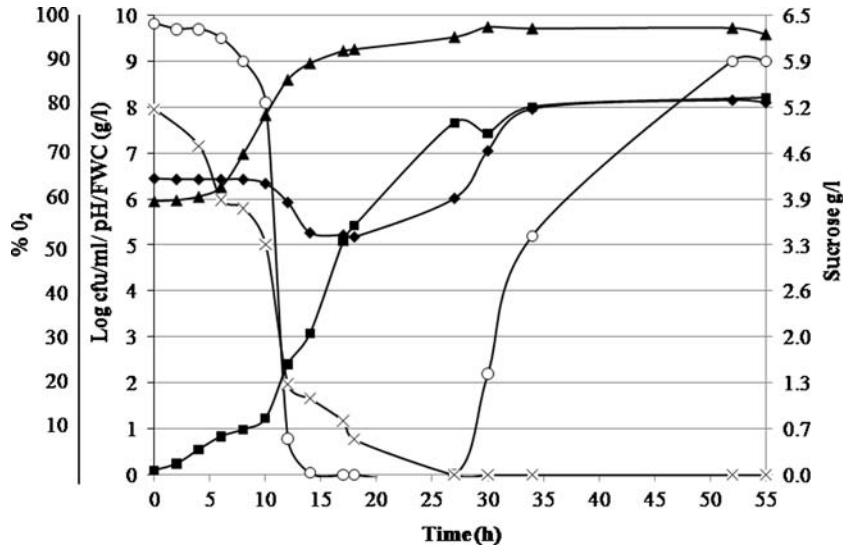

Fig. 4 Time course of viable cells c.f.u/ml (*filled triangle*), fresh weight cell (*filled square*), pH value (*filled diamond*), oxygen concentration (*open circle*), depletion of sucrose (*times*) in batch cultivation of *Pantoea agglomerans* PBC-1, performed with aeration flux of 100 l/h, L-shaped sparger 250 rev/min, Rushton turbine, at 30°C, in medium with sucrose 5 g/l and yeast extract 5 g/l, inoculated at initial concentration at 10^6 c.f.u/ml

Table 3 Growth parameters of *Pantoea agglomerans* PBC-1 produced in STR at different conditions

	Initial K_La (1/h)	μg (1/h)	R_s (g/l h)	$Y_{x/s}$ (g/g)	P_{max} (g/l h)	X_{max} (g)
Marine/porous, 10^6 c.f.u/ml	14.76	0.13	0.30	1.25	0.27	7.86
Rushton/L-sparger, 10^6 c.f.u/ml	9.74	0.20	0.29	1.53	0.27	8.20
Rushton/L-sparger, 10^7 c.f.u./ml	10.06	0.21	0.42	1.27	0.26	7.15
Fed batch, 10^7 c.f.u/ml	9.56	0.19	0.26	0.72	0.26	7.93

K_La, volumetric oxygen mass transfer coefficient; μg, specific growth rate; R_s, substrate uptake rate; $Y_{X/S}$, biomass yield; P_{max}, biomass productivity; X_{max}, maximum biomass

reaching similar values of biomass after the exponential phase, than culture inoculated at 1×10^6 c.f.u/ml.

Depending on the inocula, differences in pH and oxygen and sugar concentration were observed. Although the values of pH were similar in both experiments, the time to achieve these values was dependent on the initial inoculum. During the course of the fermentation run of 1×10^7 c.f.u/ml initial inoculum, the pH decreased gradually from 6.5 to 5.2 during the first 9 h and remained low until 23 h. At this point an increase to pH 8 was observed, while in the fermenter at 1×10^6 c.f.u/ml 15 h were necessary to reach the value of 5.2. A similar delay in the fermenter at 1×10^6 c.f.u/ml was observed in the oxygen and sugar concentration. In both runs a rapid decrease in concentration of sugar and oxygen was observed, however, this decrease was much more

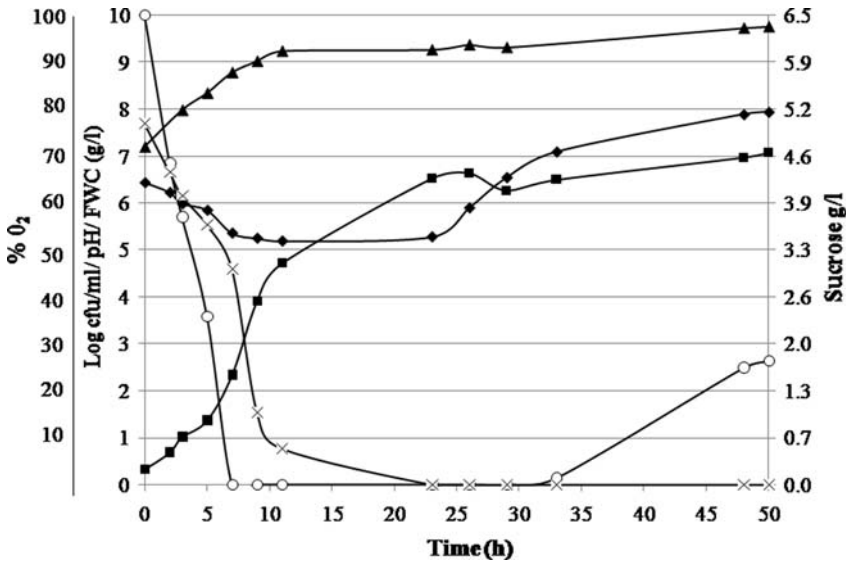

Fig. 5 Time course of viable cells c.f.u/ml (*filled triangle*), fresh weight cell (*filled square*), pH value (*filled diamond*), oxygen concentration (*open circle*), depletion of sucrose (*times*) in batch cultivation of *Pantoea agglomerans* PBC-1, performed with aeration flux of 100 l/h, L-shaped sparger 250 rev/min, Rushton turbine, at 30°C, in medium with sucrose 5 g/l and yeast extract 5 g/l, inoculated at initial concentration at 10^7 c.f.u/ml

pronounced, and detected right from the first hours in the fermenter with higher initial inoculum. These decreases are related to the high respiration rate (data not shown) and substrate uptake rate (Table 3), caused by a high density population in the fermenter at 1×10^7 c.f.u/ml and after 11 h of fermentation, the viable cells were multiplied more than 2 log-fold, reached 2–3 $\times$ 10^9 c.f.u/ml.

In Table 3 are summarized the parameters of growth determined in the different trial conditions. Specific growth rates were not different for both assayed inocula 1×10^6 and 1×10^7 c.f.u/ml, using a L-sparger and Rushton turbine and were higher when compared with the fermentation at 1×10^6 c.f.u/ml in which porous sparger and marine impeller were used. In these conditions a lower substrate uptake rate (0.30 and 0.29 g/l/h) was observed compared with the run at 1×10^7 c.f.u/ml (0.42 g/l/h). No significant differences were observed in biomass productivities for both inocula.

Fed-batch experiments

With the objective of obtaining a high cell density of *P. agglomerans* PBC-1, fed-batch fermentation was performed (Fig. 6). The sugar content in the supernatant was determined at each sampling time and when the sugar concentration was inferior to 1 g/l, batches of sucrose solution (40 g/l) were added periodically to the fermenter, at 16, 32 and 40 h, when sucrose attained at about 13% of the initial sugar content.

Similar growth profiles and kinetic parameters (Table 3) were obtained in batch cultures, when comparison was done between the same inoculum concentration (1×10^7 c.f.u/ml)

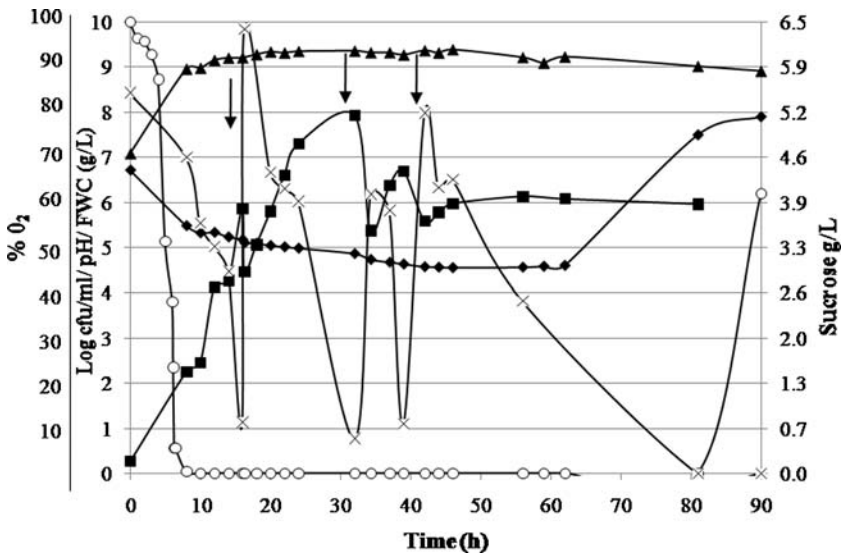

Fig. 6 Time course of viable cells c.f.u/ml (*filled triangle*), fresh weight cell (*filled square*), pH value (*filled diamond*), oxygen concentration (*open circle*), depletion of sucrose (*times*) in Fed-batch cultivation of *Pantoea agglomerans* PBC-1, performed with aeration flux of 100 l/h, L-shaped sparger 250 rev/min, Rushton turbine, at 30°C, in medium with sucrose 5 g/l and yeast extract 5 g/l, inoculated at initial concentration at 10^7 c.f.u/ml. *Arrows* represent sugar addition

fermentation runs. In fed-batch culture a longer exponential phase (30 h) was observed and higher cell viability than in batch cultures. This high value remained unchanged during the stationary growth phase (90 h).

Antagonistic activity of *P. agglomerans* PBC-1

The fresh biomass of *P. agglomerans* PBC-1 produced was evaluated for its antagonistic activity against *P. expansum* on 'Golden Delicious' apples and 'Valencia Late' oranges. Apples were wounded and treated with a suspension of fresh cells of *P. agglomerans* PBC-1, at 1×10^8 c.f.u/ml, followed by inoculation of the pathogen, *P. expansum* at 1×10^4 spores/ml. The incidence after 7 days at 20°C and 80 ± 5% HR, on treated fruits was reduced to about 86% relative to the untreated control (data not shown).

In oranges, fruits were wounded and inoculated with the pathogen simultaneously, followed by inoculation with the biocontrol agent. Once again the application of the biocontrol agent permitted a severe reduction of about 85% in the incidence of *P. digitatum* when compared to untreated fruits (data not shown).

Discussion

In this paper the mixing behaviour and growth kinetics of *P. agglomerans* PBC-1 were studied. Three different carbon sources, fructose, glucose and fructose were screened for their capacity to support growth of *P. agglomerans* PBC-1 cultures. The results showed that this biocontrol agent can utilize these carbon sources to biomass production, which represent an advantage and a proposal to future investigations, since food industry by-products like cane molasses, juices and fruit pellets that can be used as raw-material for biomass production at low cost, consist primarily of those sugars.

The viable populations achieved with yeast extract at 5 g/l, as nitrogen source and glucose, fructose and sucrose at 10 g/l, as carbon sources were 1.4×10^9, 3.9×10^9 and 3.9×10^9 c.f.u/ml, after 20 h of incubation. The high biomass achieved with sucrose, as well as the feasibility and accessibility of this carbon source, led to the selection of this carbon source for further experiments. The preference for sucrose as carbon source was also made by Visnovsky et al. (2008) in the production of *Serratia entomophila*, an Enterobacterium that has been developed as a commercial biopesticide. In this study sucrose, fructose, glucose, molasses, and a mixture of glucose and fructose were used to study the influence of culture media composition, and sucrose yielded the higher viable cell densities. Otherwise in a study of medium optimization to produce the biofungicide phenazine-1-carboxlic (PCA) by *Pseudomonas* sp. M18G, glucose was chosen as the optimal carbon source (He et al. 2008). Some authors considered glucose as the main carbon source by all microorganisms because of its size, rapid uptake, utilization and cellular energy conversion (Abadias et al. 2003).

In the present work a viable population of 2.8×10^9 c.f.u/ml was reached after 20 h of incubation, in a medium without carbon source and with 5 g of yeast extract/l as nitrogen source. The doubling of nitrogen source was necessary to obtain 2×10^9 c.f.u/ml (Costa et al. 2002) after 20 h of incubation of *P. agglomerans* CPA-2. Once again the capacity of yeast extract to support microbial growth by itself has been demonstrated and the high content of amino acids, minerals, vitamins and carbohydrates (Zhang et al. 2007) make it a common and an excellent medium component for industrial cultivation of many microorganisms.

The effect of the initial inoculum was studied at 1×10^6 and 1×10^7 c.f.u/ml, and the main difference between experiments was the duration of the lag phase, which was shortened to 5 h in the fermenter inoculated at 1×10^7 c.f.u/ml compared with inoculation at 1×10^6 c.f.u/ml. A shorter lag phase can reduce the cultivation period which represents a reduction in the biocontrol production costs.

Usually, in a typical aerobic batch culture, the dissolved oxygen concentration decreases during growth culture, especially in the exponential phase when the culture has active growth. When the microorganism reaches the stationary phase, the oxygen demand is minor and then the dissolved oxygen concentration increases. This evolution can be observed in many cultures of bacteria, yeast, and fungi. In the culture of *P. agglomerans* PCB-1, the dissolved oxygen concentration decreases severely in a few hours, until low levels, and only increases when the microorganism reaches an advanced stationary growth phase. In order to avoid oxygen limitation during growth, an increase in the gas flow rate or in the stirrer speed can be applied; however, this can promote great turbulence in the fluid growth and cause damage to the cells. Another possibility to improve the volumetric oxygen mass transfer coefficient, K_La and subsequently, increase dissolved oxygen in the broth is to optimize the aeration sparger geometry and impeller designs. In the current research, mixing trials were performed using marine propeller and Rushton turbine combined with porous and L-shape spargers for the production of *P. agglomerans* PBC-1. The comparison between different geometry evidences that biomass accumulation was improved when Rushton turbine and L-sparger were used. The specific growth rate and biomass yield were significantly higher than what was obtained with marine propeller and porous sparger (Table 3), suggesting that radial flow is more adequate for broth homogenization in the reported conditions. It seems

that the oxygen depletion was not a constrain for the biomass production of *P. agglomerans* PBC-1 and it was also observed that higher K_La (Table 3) does not favor biomass production, suggesting that broth homogenization is optimized and conditioned by agitator geometry which defines the fluid regime in the vessel.

The balance between the time of culture to get high concentration of biomass, viable population and the growth parameters, are indicators that the batch cultivation of *P. agglomerans* PBC-1, performed with Rushton turbine and L-sparger is the best option as it promotes a good mixing conditions leading to high cell-density population.

However, there are studies indicating that dissolved oxygen can favor biomass production (Fredlund et al. 2004; Verma et al. 2006). Visnovsky et al. (2008) studied the bacterium *Serratia entomophila* (Enterobacteriaceae) and proved that dissolved oxygen concentration influences biomass production, increasing the cell density 5-fold.

Large-scale production of a biocontrol agent is an essential step for the commercialization. Some reports described the use of fed-batch technology to achieve high cell biomass (de Mare et al. 2005; El-Enshasy et al. 2007; Mounir et al. 2007; Visnovsky et al. 2008). In this particular case the reactor was fed with sugar batches after 16, 32 and 40 h of incubation, the growth profile and parameters analysed showed that was possible to extend the growth exponential and stationary phases and maintain high stability on cell viability for longer periods of time which can be crucial to scaling up the process. Allowing the production of biocontrol agent with high viability and adequate fresh biomass during the process production until 90 h after inoculation or even more, is therefore a plausible advantage on industrial biocontrol agent production. However, the final biomass obtained in fed-batch did not improve compared to batch cultures, as also reported by Abadias et al. (2003) with *Candida sake* grown in a fed-batch fermenter, with glucose as carbon source, which suggests that carbon source was not the limiting nutrient in production in these strains. Further studies should be carried out in order to clarify kinetic and model growth of *P. agglomerans* bacteria.

Once again the oxygen depletion seems not to influence the growth. This aspect can be attributed to the fact that *Pantoea* is an Enterobacterium, with facultative anaerobic characteristics, which would permit a switch to aerobic respiration when oxygen is available or instead, to fermentation pathway, in the absence or scarcity of oxygen.

The fresh biomass of *P. agglomerans* PBC-1 was evaluated for its antagonistic activity against the *P. expansum* in apples and against *P. digitatum* in oranges. The incidence caused by each pathogen in apples and oranges was significantly reduced to about 86%, after 7 days of storage at room temperature. Our results are in accordance with those of Nunes et al. (2001), (2002) who observed a decrease of 72% of incidence of *P. expansum* rots, at 1×10^4 spores/ml in apples, with the application of fresh cells of *P. agglomerans* CPA-2, at 1×10^8 c.f.u/ml, produced in shake flasks in NYDB medium. Likewise, in another study, the application of fresh cells of *P. agglomerans* CPA-2, at 2×10^8 c.f.u/ml, in NYDB medium, promoted a reduction of incidence of *P. digitatum* in wounded oranges, of 71% (Teixidó et al. 2001).

The bioassays proved the dual efficacy of fresh cells of the biocontrol agent *P. agglomerans* PBC-1, against *P. expansum* or *P. digitatum* on wounded apples and oranges, respectively.

Sucrose was revealed to be an adequate carbon source for *P. agglomerans* PBC-1 growth, in batch fermentations and due the availability of this sugar, as well. The relevance of a radial flux for better broth homogenization in a biological reactor, achieved with the use of Rushton turbine and L-sparger, was demonstrated by the comparison of the performance of different impellers and air spargers.

Fed-batch technology allows the maintenance of high cell viability for longer periods of time in the stationary growth phase, which can be crucial for up-scale the bioprocess. Production is a critical step on the development of biocontrol agents; the mixing behaviour and culture growth kinetics studies are relevant contributions for further designing a pilot-scale STR, using in preference a low cost nutrient medium.

The next steps in the improvement of the industrial production of *P. agglomerans* PBC-1 should include studies of microbial biomass production, using food industry by-products that have in their composition the carbon sources tested in this study, as a way to reduce the costs of the biocontrol agent production.

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Chapter 5

Carob pulp as raw material for production of the biocontrol agent *P. agglomerans* PBC-1

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Carob pulp as raw material for production of the biocontrol agent *P. agglomerans* PBC-1

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Abstract Large-scale production has been the major obstacle to the success of many biopesticides. The spreading of microbial biocontrol agents against postharvest disease, as a safe and environmentally friendly alternative to synthetic fungicides, is quite dependent on their industrial mass production from low-cost raw materials. Considerable interest has been shown in using agricultural waste products and by-products from food industry as nitrogen and carbon sources. In this work, carob pulp aqueous extracts were used as carbon source in the production of the biocontrol agent *Pantoea agglomerans* PBC-1. Optimal sugar extraction was achieved at a solid/liquid ratio of 1:10 (w/v), at 25°C, for 1 h. Batch experiments were performed in shake flasks, at different concentrations and in stirred reactors at two initial inoculums concentrations, 10^6 and 10^7 cfu ml^{-1}. The initial sugar concentration of 5 g l^{-1} allowed rapid growth (0.16 h^{-1}) and high biomass productivity (0.28 g l^{-1} h^{-1}) and was chosen as the value for use in stirred reactor experiments. After 22 and 32 h of fermentation the viable population reached was 3.2×10^9 and 6.2×10^9 cfu ml^{-1} in the fermenter inoculated at 10^6 cfu ml^{-1} and 2.7×10^9 and 6.7×10^9 cfu ml^{-1} in the bioreactor inoculated at 10^7 cfu ml^{-1}. A 78% reduction of the pathogen incidence was achieved with PBC-1 at 1×10^8 cfu ml^{-1}, grown in medium with carob extracts, on artificially wounded apples stored after 7 days at 25°C against *P. expansum*.

Keywords Biological control · Carob by-products · Stirred reactor · Batch cultures · Postharvest

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Introduction

Fruits are perishable products, especially during the postharvest phase, when considerable losses can occur due to fungal diseases. Fungal propagules are present in the air, water and equipment in packinghouses [41] where they can easily enter the fruit via wounds, a susceptible entry point for pathogens [15]. Postharvest treatments are therefore often necessary during storage. The control of postharvest pathogens relies, mainly, on the use of synthetic fungicides, but the repeated use of certain fungicides in packinghouses has led to the appearance of fungal resistant populations, as well as the appearance of diseases which have increased their severity by the use of specific chemical products.

In addition to the growing concern for human safety and protection of the environment, there has been an increase in interest in safe food, free of chemical residues, driving the development of sustainable agriculture, integrated crop management and organic production and renewing interest in the search for alternative and safer control measures [30].

During recent years microorganisms used against postharvest disease have gained considerable attention and achieved a practical application [23, 47]. Antagonistic bacteria or yeasts are used as biofungicides primarily to colonize and protect wounds on the fruit surface resulting in the exclusion or inhibition of the pathogens and less diseases [24]. However, the commercial success of these

products remains limited and there are currently just a few available products such as Biosave (*Pseudomonas syringae*, Jet Harvest Solutions) registered in the USA, and Shemer (*Metschnikowia fructicola,* Agrogreen) registered in Israel. Recently Candifruit (*Candida sake* CPA-1, Spicam Inagra) and Boniprotect (*Aureobasidium Pullulans*, Bio-protect) were registered in Spain and Germany, respectively, for use on pome fruit. Aspire (*Candida oleophila*) and YieldPlus (*Cryptococcus albidus*) are no longer available; however, BioNext (Belgium) and Leasaffre International (France) are developing a commercial product, based on the same yeast used in Aspire and a product based on the yeast *Candida saitoana* is being developed by Neova Technologies (Abbotsford, British Columbia, Canada) [14, 45].

Many studies have described the bacterium *Pantoea agglomerans* as a biocontrol agent [7, 8, 21, 28, 43]. The strain CPA-2 of *P. agglomerans*, isolated from the surface of apples and an effective antagonist against the major fungal pathogens of apples and pears [28, 29], is being commercialized in Spain as a solid formulation named Pantovital by Biodurcal S. L. [42, 45].

The bacterium *P. agglomerans* PBC-1 used in the present study was originally isolated from the surface of oranges and its antagonistic activity was evaluated with success against *Penicillium expansum, Botrytis cinerea* and *Rhizopus stolonifer*, in pome and *Penicillium digitatum* and *Penicillium italicum* citrus fruits. This strain is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae,* and it is an anaerobic facultative microorganism. Our recent studies [25] on *P. agglomerans* PBC-1 production demonstrated that this biocontrol agent can use sucrose, glucose and fructose as carbon sources, reaching good viable populations, 3.9×10^9, 1.4×10^9 and 3.9×10^9 cfu ml^{-1}, respectively, after 20 h of incubation. This fact represents an advantage since food industry by-products that can be used as raw material at low cost, consist primarily of those sugars.

Industrial scale-up of the biomass production has been the major obstacle to the success of many biopesticides [13, 46]. The spreading of biocontrol agents, as an alternative to synthetic fungicides, is quite dependent on their mass production and of the industrial development of a culture medium, which can produce effective and high amounts of biomass at low cost [22]. In industrial fermentations, it is very rare that a conventional chemical defined medium is used. The medium cost is one of the major operational costs, representing 30–40% of the total [49], so the use of cheap and widely available raw materials is an economical requisite and has a substantial impact on overall cost reduction efforts. Considerable interest has been shown in using agricultural product wastes [9] and by-products from food industry as nitrogen or carbon sources [1], as an option to reduce costs. The use of commercial products or by-products from food industry, such as malt extract, dry beer extract, soy powder, fruit concentrate and molasses were studied for the biomass production of biocontrol agents [1, 11, 32, 46].

Interest in carob pod as a cheap raw material for various products has been increasing in recent years. Some investigations explored carob pulp as a readily available and inexpensive material for the production of dextran and fructose [40], citric acid [39] and bioethanol [35, 38]. Carob (*Ceratonia siliqua* L.), a perennial leguminous tree, is an important component of the Mediterranean vegetation and its cultivation in marginal and prevailing calcareous soils of the Mediterranean region is important environmentally and economically [5]. According to recent data, carob pod production worldwide amounts to nearly 400,000 tons per year from about 200,000 ha [25, 39]. Carob tree plays an important role in the economy of the south of Portugal (Algarve) where 50,000 tons of carob fruit is produced each year, making the region the third largest producer in the world [5, 6, 40]. The price of carob pod has fluctuated and at the time of writing was about 205 €/ton (personal communication).

The carob seeds represent 10% of the weight of the fruit. The seeds comprise coat (30–33%), endosperm (42–46%) and embryo or germ (23–25%) [5]. The seed endosperm is a molecule composed of mannose and galactose sugar units (ratio 4:1) and this polysaccharide (CBG or locust bean gum, LBG) presents high viscosity in water, over a wide range of temperatures and pH. This gum is used as a thickener, stabilizer, binder and gelling agent in the food industry and in several technical applications [10], including the manufacture of chemicals, paper and cosmetics and the pharmaceutical industry [3, 25, 40]. The seed embryo has 50% protein content and is used for human and pet food. World demand for gum equates to about 30,000 tons of carob seed. The seed is processed in Portugal and the products obtained are exported to Japan, Holland, Denmark and USA [10]. The carob seed price, in 2009 reached 2,000 €/ton (personal communication). The pulp represents the other 90% of the fruit, and its composition depends on the variety, climate and growing technique [5, 10]. Several authors reported that the carob pulp composition has a high content of sugars, mainly sucrose (more than 30%), fructose and glucose: a range of values are reported e.g. 20–50% [34], 45% [3], 48–56% [48] and 28–82% [6]. Carob pod also contains appreciable amounts of protein 3–4%, a low level of fat 0.4–0.8% [3, 48], a high level of condensed tannins and a low content of hydrosoluble tannins [3]. Significant differences in the tannins content were reported by several authors and can be attributed to the extraction process used [25, 36, 37].

Carob pulp has been used as animal fodder and also for human consumption. It is also used in the preparation of antidiarrhoeal and antiemetic products, pastry baking, syrups and as a cocoa substitute, with the advantage of being caffeine- and theobromine-free, whereas chocolate and cocoa contain relatively high amounts of these two antinutrients [12]. However, most carob pods are discarded and not effectively utilized at present. For that reason the by-product of this industry represents a cheap and available raw material to produce a value-added product.

The feasible use of food industry by-products as raw material in a potential industrial medium is quite dependent on the composition and raw material processing and, in this specific case, the raw material has to be free of or has to have residual values of bacterial growth inhibitors to permit high amounts of biomass production [1]. The antimicrobial effect of carob extracts in several organisms was reported by Henis et al. [19]. The polyphenols, condensed tannins and hydrolysable tannins in carob pulp can inhibit bacterial growth, eventually preventing the use of this raw material as carbon source for fermentations.

The objective of this investigation was the production of the biocontrol agent *P. agglomerans* PBC-1 with high antagonistic activity against pathogens of fruit, by using carob kibbles, an agro-industrial by-product, as a low-cost carbon source, optimizing the solid/liquid sugar extraction from the carob pulp.

Materials and methods

Sugar extraction from carob pulp

A carob industry by-product from a Portuguese carob processing factory was used. The kibbles of carob pulp were milled to particles on an electric mill. Sugar extractions were conducted in one step; to maximize sugar aqueous extraction, different solid/liquid ratios were tested. Extracts were prepared in conical flasks by homogenizing the mixture with distilled water at different ratios viz. 0.5:10, 1:10, 2:10 and 3:10 (w/v). Flasks were placed in a water bath and agitated at 100 rpm and 25°C for 1 h. The suspensions were vacuum-filtered and the volume of liquid was recovered and measured. The extracts were then centrifuged at 7,500*g* for 20 min at 4°C using an Avanti J-14 centrifuge (Beckman Coulter, USA) and then filtered through a 0.22-μm membrane filter before analysis by HPLC. In order to optimize the extraction process the most favourable ratio was studied at different temperatures (25, 40, 60 and 75°C) and extraction times (1, 2 and 3 h) by using the methodology previously described. Each extraction tested was repeated twice with three replicates per experiment.

The yield of the extraction process was determined by considering that 50% of the carob pulp is composed by sugars [3, 6, 34, 48].

HPLC analysis

The composition of sugars, in carob extracts and in supernatant culture broth, 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 KGaA, Germany, was used at room temperature. The mobile phase was acetonitrile/water (75:25 v/v) applied at a flow rate of 1 ml min^{-1} [33]. 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.

Biocontrol agent

Pantoea agglomerans PBC-1 was isolated from the surface of apples and has been tested for many years as a control agent against the major postharvest pathogens of pome and citrus fruits. The bacterium was stored at −80°C in liquid medium with 20% (v/v) glycerol. From these stock cultures, agar plates containing NYDA (8 g l^{-1} nutrient broth, Biokar Diagnostics BK003HA; 5 g l^{-1} yeast extract, Biokar Diagnostics A1202HA; 10 g/1 glucose, Riedel-de-Haën 16325; 15 g l^{-1} agar, Vaz Pereira) were incubated and cells were activated by incubation at 30°C.

P. agglomerans PBC-1 production in shake flasks with carob extract

The possibility of using the aqueous sugar extract from carob kibbles, as carbon source, in the production of the biological control agent was tested at different concentrations, 5, 10, 15 and 20 g l^{-1}. Yeast extract at 5 g l^{-1} was used as nitrogen source. Nitrogen and carbon sources were sterilized separately by autoclaving for 15 min at 121°C. Fresh cells of *P. agglomerans* PBC-1, at an initial concentration of 1×10^5 cfu ml^{-1}, were inoculated in 250-ml conical flasks with 50 ml of culture medium. Flasks were incubated, at 30°C under orbital agitation, at 150 rpm in an orbital incubator (Pentlab, Portugal) and determination of viable cells (cfu ml^{-1}), optical density, pH and sugar contents in the broth was carried out following the methodology described by Manso et al. [26]. To determine the possible inhibitory effect arising from phenol, its concentration was measured spectrophotometrically at 270 nm (Genisys 10 uv, Thermo Electron Corporation, USA), using the method described by Anselmo

et al. [2]. Each bioassay was conducted in three replicates, and was repeated twice.

P. agglomerans PBC-1 production in stirred reactor with carob extract

Biomass production was evaluated at 1×10^6 and 1×10^7 cfu ml^{-1}, in a 3-l (2.4-l working volume) stirred reactor, STR (ADI 1010/1025, Apllikon, Holland) operated at 30°C, 250 rpm, aeration 100 l h^{-1} (0.69 vvm). A Rusthon-type turbine and an L-shaped sparger were employed. The medium comprised yeast extract at 5 g l^{-1}, antifoam B (Sigma A-5757) at 0.1 ml l^{-1} and a dilution of the initial carob extract, previously prepared, in order to reach an initial sugar concentration of 5 g l^{-1}.

The fermentation was monitored online by using the BioXpert program, version 2.1. The temperature, pH and dissolved oxygen were measures by specific probes and the values were constantly registered. Samples were taken, immediately after inoculation and at regular intervals, to determine the OD_{640}, viability population counts and sugar concentrations, as previously described [26]. Fresh weight biomass was determinated after centrifugation at 1,920*g* (Universal 320, Hettich Zentrifugen, Germany), for 15 min.

Efficacy of *P. agglomerans* PBC-1 produced with carob extract

The antagonistic effect of fresh cells of *P. agglomerans* PBC-1, produced with carob aqueous extracts, in shake flasks, agitated at 150 rpm was evaluated. Bacterial suspensions at 1×10^8 cfu ml^{-1} were prepared in phosphate buffer, after harvest by centrifugation at the stationary phase.

The 'Golden Delicious' apples used in this experiment were obtained from commercial orchards in Região Oeste, Portugal. Fruits were stored at 1°C, and 1 day before the assays apples were washed in NaOCl 0.5% solution, rinsed with water and maintained at room temperature. Apples were wounded with a stainless steel rod (1-mm-wide and 2-mm-long tip) at the stem and calyx end. Twenty microlitres of phosphate buffer (control) or antagonist suspensions was inoculated in the wound. Two hours later wounds were inoculated with 15 µl of an aqueous suspension of *Penicillium expansum*, at 10^4 spores ml^{-1}, prepared from 10-day-old culture incubated on Potato Dextrose Agar (Biokar Diagnostics) at 25°C. The concentration of the conidial suspension was determined with a haemocytometer and diluted to a suitable concentration.

The treated fruits were then stored at 20°C and 85% RH (relative humidity) for 7 days, after which the percentage of infected wounds (incidence) was evaluated. Each treatment was repeated four times with ten fruits per replicate.

Statistical analysis

The program SPSS (version 17 for Windows, SPSS Inc., Chicago, IL), was used for statistical analysis to process the data and Student–Newman–Keuls test was employed at $P < 0.05$ to separate the values in the carob extraction experiments and in the parameters of *P. agglomerans* PBC-1.

Incidence of mould contamination in fruits was analysed by a test of variance applied to the arcsine of the square root of the proportion of infected fruit.

Results and discussion

Sugar extraction from carob pulp

Carob pulp was milled and, in order to maximize solid/liquid sugar extraction, several aqueous mixtures using different solid/liquid ratios were prepared. Table 1 lists the sugars and respective concentrations obtained with different milled pulp/water ratios, as well as the percentage of liquid recovered. There are differences in the sugars extracted from the different ratios studied. With a higher solid/liquid ratio, a solution with higher sugar concentration was obtained. This could lead to the idea of choosing larger ratios for higher sugar concentrations. However, the percentage of liquid recovered and the yield of extraction must be taken into account. Considering that the concentration of sugars in the carob pulp is around 50% [3, 6, 34, 48], for a theoretical 100% yield extraction, 50 g l^{-1} of sugars will be extracted for each 100 g of carob pulp.

The 3:10 ratio afforded the lowest yield of sugar extracted (78.56%), since the extraction process produced a low volume of a high concentrated sugar solution and a residue still rich in sugar. The sugar solution obtained reaches the solubility coefficient for the working temperature. The highest solid/liquid ratios are also those where the volume of liquid recovered is minor and the result is a mixture with a low amount of water, difficult to extract, even by using vacuum filtration.

Our results are in agreement with those of Petit and Pinilla [34] who studied the production and purification of syrup from carob pulps and saw that the efficiency of sugar extraction increased when the pulp/water ratio decreased. At the same time, the sugar concentration measured decreased due to dilution. With a 1:10 ratio (w/v) of pulp water, at room temperature, neutral pH, for 1 h, it was possible to achieve a sugar extraction yield of 94.4%. Similar efficiency was achieved in the present work, under the same conditions.

In an endothermic process, the solubility of a substance strongly depends on the used solvent, as well as on pressure

Table 1 Sugar extraction with different solid/liquid ratios (w/v) of carob pod, at 25°C, for 1 h

Ratio (w/v)	Fructose (g l^{-1})	Glucose (g l^{-1})	Sucrose (g l^{-1})	Total sugar (g l^{-1})	Liquid recovered[1] (%)	Yield sugar extraction[2] (%)
0.5:10	6.44 ± 0.08a	4.83 ± 0.48a	11.84 ± 1.55a	23.11 ± 2.52a	91.31 ± 1.08a	92.43 ± 2.82a
1:10	12.21 ± 0.35b	7.27 ± 0.59b	27.61 ± 0.63b	47.09 ± 1.06b	90.86 ± 1.86a	94.17 ± 2.12a
2:10	21.31 ± 0.15c	12.09 ± 0.64c	48.82 ± 1.44c	82.22 ± 2.09c	70.40 ± 1.60b	82.22 ± 2.09a
3:10	33.79 ± 0.08d	20.29 ± 0.20d	63.77 ± 2.44d	117.84 ± 2.97d	37.68 ± 1.33c	78.56 ± 3.01a

Values are means ± SD of two experiments with three replicates. Within a column, values followed by the same letter are not statistically different according to Student-Newman–Keuls ($P < 0.05$)

[1] % Liquid recovered = volume liquid recovered (ml)/total volume of liquid (ml) × 100%

[2] % Yield sugar extraction = total sugar concentration (g l^{-1})/theoretical sugar concentration, i.e. half of the carob pulp mass (g l^{-1}), × 100%

Table 2 Sugar extraction at 1:10 (w/v) ratio of carob pod, tested at different times and temperatures

Temperature (°C)	Time (h)	Fructose (g l^{-1})	Glucose (g l^{-1})	Sucrose (g l^{-1})	Total sugar (g l^{-1})	Yield sugar extraction[1] (%)
25	1	12.03 ± 0.60a	6.95 ± 0.05a	26.14 ± 0.52a	45.12 ± 1.09a	90.24 ± 2.19a
	2	12.39 ± 0.39a	7.69 ± 0.03a	25.25 ± 0.47a	45.33 ± 0.33a	90.66 ± 0.65a
	3	12.14 ± 0.08a	7.36 ± 0.10a	26.98 ± 0.45a	46.48 ± 0.61a	92.96 ± 1.22a
40	1	12.02 ± 0.12a	7.25 ± 0.03a	24.35 ± 0.37a	43.62 ± 0.38a	87.24 ± 0.75a
	2	12.17 ± 0.15a	7.30 ± 0.06a	24.71 ± 0.22a	44.18 ± 0.36a	88.36 ± 0.72a
	3	13.91 ± 0.57a	10.48 ± 0.82b	18.67 ± 3.40b	43.06 ± 3.31a	86.12 ± 6.62a
60	1	13.20 ± 0.67a	9.81 ± 0.17b	18.50 ± 0.98b	41.51 ± 1.80a	83.02 ± 3.60a
	2	12.12 ± 0.11a	7.88 ± 0.60a	24.57 ± 1.34a	44.57 ± 1.95a	89.14 ± 3.90a
	3	13.53 ± 0.49a	10.74 ± 0.76b	16.92 ± 0.98b	41.19 ± 2.22a	82.38 ± 4.44a
75	1	13.28 ± 1.24a	9.60 ± 1.17b	18.26 ± 2.18b	41.14 ± 4.58a	82.28 ± 9.15a
	2	11.13 ± 0.96a	7.14 ± 1.13a	23.90 ± 1.60a	42.17 ± 3.36a	84.34 ± 6.72a
	3	12.65 ± 2.23a	7.85 ± 0.80a	25.07 ± 3.24a	45.57 ± 6.25a	91.14 ± 12.50a

Values are means ± SD of two experiments with three replicates. Within a column, values followed by the same letter are not statistically different according to Student–Newman–Keuls ($P < 0.05$)

[1] % Yield sugar extraction = total sugar concentration (g l^{-1})/theoretical sugar concentration, i.e. half of the carob pulp mass (g l^{-1}), × 100%

and temperature. In these processes the solubility increases with rising temperature. In order to obtain the maximum efficiency in the sugar extraction process, time and temperature were studied to find the optimal conditions. The results are presented in Table 2. Contrasting the results obtained by Turhan et al. [44] in which the largest % of sugar was extracted at the highest of the three temperatures studied, 20, 50 and 85°C, in our study, the increase of the temperature does not improve sugar extraction.

The effect of temperature extraction was also studied by Petit and Pinilla [34] in the range between 15 and 45°C. The efficiency of extraction remained constant at around 40% between 15 and 30°C, and decreased to 30–34% at higher temperatures. Those authors ascribed this fact to an increase of the soluble tannins extracted. As in our study, the optimum working temperature chosen was room temperature. The application of higher temperature, particularly over 20°C, also results in solubilization of phenolic compounds, which is an undesirable effect [36, 44].

It was also reported by Roseiro et al. [37] that the final sugar concentration of carob pulp extracts is dependent on the extraction time. In that work, after 6 h of extraction, the sugar concentration reached a constant value for different solid/liquid ratios when performed at 20°C. However, our results showed that under the tested conditions, the extraction time does not cause significant differences in extraction efficiency (Table 2).

P. agglomerans PBC-1 production in shake flasks with carob extract

The ability of the biocontrol agent *P. agglomerans* PBC-1 to use the sugars contained in the extracts of carob pulp previously prepared, as a carbon source, was evaluated at four different concentrations viz. 5, 10, 15 and 20 g l^{-1} (Fig. 1).

Figure 1a depicts that the difference between growth profiles was more evident when expressed as fresh cell

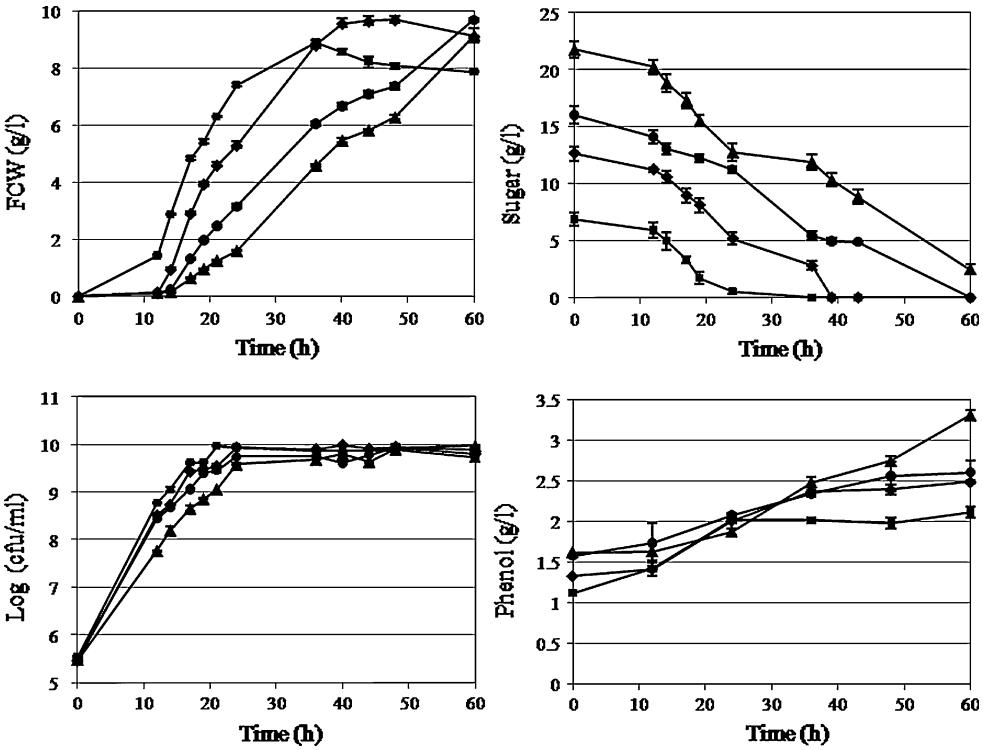

Fig. 1 Growth of *P. agglomerans* PBC-1 with different sugar concentration 5 g l^{-1} (*squares*), 10 g l^{-1} (*diamonds*), 15 g l^{-1} (*circles*), 20 g l^{-1} (*triangles*) from carob extracts. **a** Fresh cell weight (*FCW*), g l^{-1}; **b** sugar depletion, g l^{-1}; **c** viable cells, expressed in log cfu ml^{-1}; **d** phenol concentration in the media, g l^{-1}. Cultures were grown in 50 ml of media shaken at 150 rpm at 30°C. The experiments were performed twice, in triplicates under identical conditions and the *vertical bars* indicate the standard deviation

Table 3 Growth parameters of *P. agglomerans* PBC-1, in shake flasks experiments in medium with different sugar concentrations (5, 10, 15, 20 g l^{-1}) from carob extracts

Carbon concentration (g l^{-1})	5	10	15	20
μ_g (h^{-1})	0.158 ± 0.004a	0.145 ± 0.021a	0.075 ± 0.002b	0.084 ± 0.001b
P_{max} (g l^{-1} h^{-1})	0.280 ± 0.002a	0.249 ± 0.003b	0.188 ± 0.005c	0.171 ± 0.004d
$Y_{X/S}$ (g g^{-1})	1.303 ± 0.015a	0.766 ± 0.023b	0.585 ± 0.028c	0.474 ± 0.026d
R_S (g l^{-1} h^{-1})	0.220 ± 0.011a	0.330 ± 0.011b	0.290 ± 0.012b	0.310 ± 0.005b
X_{24h} (g l^{-1})	7.406 ± 0.102a	5.29 ± 0.314b	3.160 ± 0.183c	1.599 ± 0.051d
X_{36h} (g l^{-1})	8.916 ± 0.112a	8.808 ± 0.346a	6.050 ± 0.185b	4.600 ± 0.101c

Values are means ± SD of three replicates. Within a row, values followed by the same letter are not statistically different according to Student–Newman–Keuls ($P < 0.05$)

μ_g specific growth rate, P_{max} biomass productivity, $Y_{X/S}$ biomass yield, R_S substrate uptake rate, X_{24h} biomass obtained at 24 h, X_{36h} biomass obtained at 36 h

weight. The lag phase was shorter at the lower sugar concentration (5 g l^{-1}); a rapid growth until 36 h of incubation was observed, followed by a decrease, coincident with the sugar depletion in the broth (Fig. 1b). The most significant initial cell growth was promoted at 5 g l^{-1} and 10 g l^{-1} of sugar concentration, which showed the highest specific growth rate (0.16 and 0.15 h^{-1}). Significant differences were observed in biomass productivity and in amount of biomass accumulated after 24 h of fermentation, among the four sugar concentrations tested. The highest biomass productivities were obtained at the lowest sugar concentrations (Table 3).

The initial sugar concentration of 5 g l^{-1} allowed the highest amount of biomass after 24 h of fermentation. However, 36 h after inoculation a smaller biomass increment occurred precisely in the lower sugar concentration, an increase of 1.5 g, contrasting with approximately 3.5 g of increment at 10 g l^{-1}, suggesting carbon source limitation at 5 g l^{-1}, after 24 h of fermentation, as verified in Fig. 1b. Nevertheless, the highest amount of biomass accumulated was registered at the lower sugar concentration, with no significant differences between 5 and 10 g l^{-1}. The depletion of the carbon source observed for all concentrations, concomitant with the beginning of the stationary phase (10, 15 or 20 g l^{-1}) or the induction of the decline phase (5 g l^{-1}), confirmed that, under these conditions, carob sugar extract may be the limiting growth parameter of *P. agglomerans* PBC-1, underlining the

relevance of the choice of an adequate sugar concentration for higher biomass production during the exponential growth phase. The lower sugar consumption rate was observed at 5 g l^{-1}, with significant differences to the others sugar concentrations (Table 3).

The analysis of Fig. 1c evidences no differences between viable populations for the assayed sugar concentrations, during exponential and stationary growth phases. After 21 h of inoculation, the maximum viable population achieved for 5 g l^{-1} was 9×10^9 cfu ml^{-1}; similar viable population was obtained after 24 h at 10 g l^{-1} and after 36 h at 15 and 20 g l^{-1}.

Similar growth profiles were obtained in previous studies on the production of this biocontrol agent at different sucrose concentrations 5, 10, 15 and 20 g l^{-1} [26]. In those studies, at the beginning of the stationary phase, 18 h, the viable populations were similar in all sucrose concentrations, 3–4 $\times 10^9$ cfu ml^{-1}. Sucrose at 5 g l^{-1} was chosen for the following scale-up experiments, since it was the concentration that led to achieve the higher biomass yield [26]. In our study the viable populations achieved after 19 h of inoculation was 4.2×10^9, 3×10^9, 2.5×10^9 and 7.2×10^8 cfu ml^{-1}, at 5, 10, 15 and 20 g l^{-1}, respectively. These results suggest that carob pulp is not only a raw material with low cost, but also allows greater viable populations and amount of biomass.

The use of industry by-products in the growth of another strain of *P. agglomerans* (CPA-2) was tested by Costa et al. [11]; molasses, as carbon source, combined with yeast extract at 10 g l^{-1} promoted the higher viable populations, around 3.5×10^9 cfu ml^{-1}, after 20 h of fermentation. In our study, carob industry by-products at 5 g l^{-1}, combined with half of the yeast extract concentration used by Costa et al. [11] provided higher viable populations of the biocontrol agent PBC-1.

The feasible use of food industry by-products as raw material in a potential industrial medium is quite dependent on the composition and raw material processing and, in this specific case, the raw material has to be free of or has to have residual values of bacterial growth inhibitors to permit high amounts of biomass production [1]. Carob pulp is largely defined in terms of its contents of polyphenols, condensed tannins and hydrolysable tannins [3, 4]; hence the quantification of the phenol content in the growth medium and its effect on biocontrol agent growth is particularly important.

In Fig. 1d, it is possible to observe an increase of phenol content during the cultivation period of the biocontrol agent for all sugar concentrations tested. As expected in the bioassays in which the carob sugar solution was less diluted, the phenol content was higher. The possible susceptibility of *P. agglomerans* PBC-1 to the compounds present in carob extracts, especially phenol, is more apparent with the lower specific growth rate observed in higher sugar concentrations and related to the higher phenol content (Fig. 1a, d). In contrast, the phenol content present in the shaker with 5 g l^{-1} of initial sugar concentration does not appear to adversely affect the growth of the biocontrol agent.

The antimicrobial spectrum of some natural substances containing phenol groups, including carob extracts, was evaluated by Henis et al. [19], in several organisms. In that study, carob pulp extracts inhibited *Cellvibrio fulvus* and *Clostridium cellulosolvens* at 15 μg ml^{-1}, *Sporocytophaga myxococcoides* at 45 μg ml^{-1} and *Bacillus subtilis* at 75 μg ml^{-1}.

The selection of the carob extract concentration (5 g l^{-1}) for further reactor assays is a compromise between the concentration of sugars that promotes the greatest amount of biomass in the shortest period of time and the phenol content present in the extract, which should not inhibit the microbial growth.

P. agglomerans PBC-1 production in stirred bioreactor with carob extract

The lower sugar concentration studied in shake flasks, 5 g l^{-1}, was chosen to proceed to scale-up experiments in a 3-l mechanically agitated reactor, equipped with a Rushton turbine and an L-shaped sparger. The effect of initial inoculum at 10^6 and 10^7 cfu ml^{-1} is shown in Fig. 2. For all parameters analysed the profiles were similar in both experiments; however, there was a consistent difference in the duration of the lag phase. In the fermenter inoculated at the initial concentration of 10^7 cfu ml^{-1}, the lag phase was shortened. The beginning of the exponential phase occurs approximately 4 h after inoculation for 10^7 cfu ml^{-1}. The same was observed in growth curves of this strain *P. agglomerans* PBC-1 supplied with sucrose as carbon source [26], which represents a reduction in the bioprocess costs. Table 4 displays the growth kinetic parameters of *P. agglomerans* PBC-1 culture tested at two different inocula concentrations. The specific growth rates, the biomass productivities and biomass accumulated after 22 and 32 h of inoculation were statistically similar.

In both experiments, the oxygen was completely consumed at early stages of the growth, after 5 and 10 h of inoculation, at the higher and lower inoculum concentrations, respectively (Fig. 2). The high oxygen consumption rates, 56 and 34.8 μmol O_2 l^{-1} h^{-1}, respectively at 10^7 and 10^6 cfu ml^{-1}, shows a severe oxygen need of this microbial culture in the stirred bioreactor, which is a clear indication of the aerobic metabolism at that growth phase. This fact is apparently a limitation on the biomass production in the bioreactor, in comparison with shake flasks, where the biomass productivities, specific growth rates and maximum

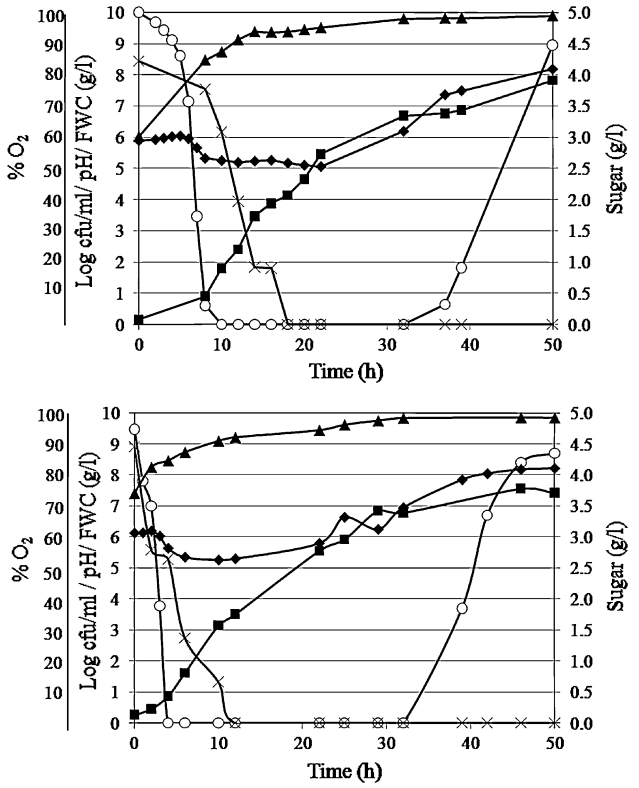

Fig. 2 Time course of viable cells cfu ml^{-1} (*triangles*), fresh cell weight (*squares*), pH value (*diamonds*), oxygen concentration (*circles*), consumption of sugar (*times*) in batch cultivation of *P. agglomerans* PBC-1, performed with aeration flux of 100 l h^{-1}, L-shaped sparger, 250 rpm, Rushton turbine, at 30°C, in medium with carob extracts with a sugar content at 5 g l^{-1} and yeast extract 5 g l^{-1}, **a** inoculated at initial concentration at 10^6 cfu ml^{-1}, **b** inoculated at initial concentration at 10^7 cfu ml^{-1}. The experiments were performed three times. pH, oxygen concentration were measured online; fresh cell weight, viable cells cfu ml^{-1} and sugars data are averages of three replicates

Table 4 Effect of inoculum size on the growth *P. agglomerans* PBC-1, using carob aqueous extracts at an initial sugar concentration of 5 g l^{-1} and yeast extract at 10 g l^{-1}, in STR operated at 30°C, 250 rpm, 0.69 initial vvm

	10^6 cfu ml^{-1}	10^7 cfu ml^{-1}
$K_L a$ initial (h^{-1})	9.541 ± 0.494a	8.283 ± 0.679a
μ_g (h^{-1})	0.110 ± 0.005a	0.119 ± 0.009a
P_{max} (g l^{-1} h^{-1})	0.163 ± 0.012a	0.181 ± 0.007a
$Y_{X/S}$ (g g^{-1})	1.527 ± 0.149a	1.468 ± 0.032a
R_S (g l^{-1} h^{-1})	0.223 ± 0.021a	0.343 ± 0.003b
X_{22h} (g l^{-1})	5.460 ± 0.750a	5.554 ± 0.701a
X_{32h} (g l^{-1})	6.690 ± 0.873a	6.874 ± 0.900a

Values are means ± SD of three replicates. Within a row, values followed by the same letter are not statistically different ($P < 0.05$)

$K_L a$ volumetric oxygen mass transfer coefficient, μ_g specific growth rate, P_{max} biomass productivity, $Y_{X/S}$ biomass yield, R_S substrate uptake rate, X_{22h} biomass obtained at 22 h, X_{32h} biomass obtained at 32 h

biomass were favored (Table 3). Shake flasks allow oxygen equilibrium, between liquid and gaseous phase and subsequent continuous gaseous changes inside the flasks, avoiding the complete depletion of the oxygen.

The effect of abrupt oxygen depletion on the growth of aerobic organisms is a technological bottleneck for industrial scale-up in stirred tank reactors [17, 50] and several research developments are ongoing in an effort to solve that. The addition of a water-immiscible phase, in which oxygen has a higher solubility, e.g. oxygen vectors (hydrocarbons or fluorocarbons) [16, 18] or magnetic nanoparticles [27, 31], has been proposed by several authors as an alternative means of improving oxygen transfer rate, without increasing the energy consumption for mixing or aeration.

The sugar depletion in the bioreactor also occurred earlier in the growth curve, 18 and 12 h after the inoculation with 10^6 and 10^7 cfu ml^{-1}, respectively, when compared with the shake flask bioassay, performed with 5 g l^{-1} carob extract (Fig. 1b). The carbon consumption rate displayed no difference between the bioreactor inoculated at 10^6 cfu ml^{-1} and shake flask, both performed at 5 g l^{-1} of initial sugar concentration. However, the bioreactor inoculated at higher inoculum concentration registered a higher carbon source consumption rate (0.34 g l^{-1} h^{-1}).

The purpose of production is to obtain the greatest amount of efficacious biomass in the shortest period of time [30], which means high cell density and viable and effective antagonist activity. Industrial mass production of biocontrol agent must be a cost-effective process and fermentation should be implemented within 24–30 h [20]. In the fermenter inoculated at 10^7 cfu ml^{-1}, after 10 h, the viable population reached 10^9 cfu ml^{-1}. The same level of viable populations was achieved in the fermenter inoculated at 10^6 cfu ml^{-1}, 14 h after inoculation. After 22 h of fermentation the biomass accumulated in the reactors inoculated at 10^6 and 10^7 cfu ml^{-1} was 5.46 and 5.55 g l^{-1}, respectively, and 10 h later the biomass was 6.69 and 6.87 g l^{-1}, an increment of 1.23 and 1.32 g l^{-1}, respectively. The viable population achieved after 22 and 32 h of inoculation was 3.2×10^9 and 6.2×10^9 cfu ml^{-1} in the fermenter inoculated at the lower concentration and 2.7×10^9 and 6.7×10^9 cfu ml^{-1} in the fermenter inoculated at the higher concentration. Manso et al. [26] reported similar viable populations achieved with *P. agglomerans* PBC-1 cultivated with sucrose 5 g l^{-1}, in stirred tank reactors, inoculated at 10^6 and 10^7 cfu ml^{-1}, which highlights the feasible use of carob pulp as raw material in the production of this biocontrol agent.

The maximum biomass values were obtained after 40 h of inoculation for the higher inoculum and 50 h for the lower inoculum and were similar to the obtained fresh

biomass in shake flasks after 36–40 h, using 5 g l^{-1} carob extract.

The time-prolongation cost of the production bioprocess, a relevant economical parameter, will be analysed in the project design, taking into account the need for high cell density and its bioactivity, balanced with the fermentation time, with the selection of low-cost raw material in order to get an economically viable biological industrial production of the *P. agglomerans* PBC-1.

Efficacy of *P. agglomerans* PBC-1 produced with carob extract

Once we had evaluated the ability of the biocontrol agent *P. agglomerans* PBC-1 to use carob sugar extracts as carbon source and found the best concentration, the effectiveness of the fresh biomass produced with these extracts was assayed. Apples were wounded, treated in a suspension of fresh cells of *P. agglomerans* at 10^8 cfu ml^{-1} and infected with *P. expansum* at 10^4 spores ml^{-1}. The incidence of the pathogen on treated fruits after 7 days of storage was reduced by approximately 78% compared with the control treatment (Fig. 3). In the assay performed under semi-commercial conditions, at 1°C, we observed 74.7% reduction in the incidence of the pathogen on treated apples, after 3 months of storage. We also verified the high PBC-1 viability under cold storage conditions, 4°C for 7 days, as only a slight reduction of about 18% of the biocontrol agent cell viability occurred.

The reduction in the pathogen development promoted by the biocontrol agent confirms the antagonistic ability of this bacterium, but also shows that this ability was not compromised by its growth media and the use of carob extracts as carbon source.

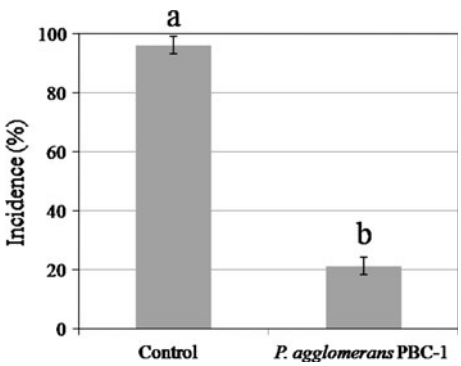

Fig. 3 Incidence of blue mould on wounded 'Golden Delicious' apples treated with *P. agglomerans* PBC-1 at 10^8 cfu ml^{-1}, followed by inoculation with the pathogen, *Penicillium expansum*, at 10^4 spores ml^{-1} after 7 days of incubation at 20 ± 1°C and 80 ± 5% RH. Each fruit was wounded twice. Ten fruits constituted a replicate and each treatment was repeated four times. *Columns* with *different letters* are significantly different ($P < 0.05$) and the *vertical bars* indicate the standard desviation

Another study where the composition of the culture medium seemed not to affect the antagonistic activity of a biological control agent was reported by Costa et al. [11]. In that study the application of fresh cells of *P. agglomerans* CPA-2, produced in media with different by-products (dry beer extract, molasses, soy powder) reduced the growth of the pathogen *P. digitatum* on oranges by approximately 66% and 77%. However, it is also possible to find reports in the literature where the composition of the culture medium affects the efficacy of the biocontrol agent: Peighami-Ashnaei et al. [32] obtained a higher level of control of *Botritys cinerea* on apples when treated with *Pseudomonas fluorescens* P-35 or *Bacillus subtilis* B-3, produced in a medium with molasses and yeast extract, than when treated with cells grown in medium with molasses and urea or nutrient broth.

The effectiveness of *P. agglomerans* PBC-1 could be attributed to maintenance of a high population of viable cells on the fruit surface during the storage period. The initial population recovered was 5×10^4 cfu/wound (data not show). A rapid colonization of apple wounds was observed during the first 24 h, increasing until 4 days and then remains stable until the end of the experiment.

Conclusion

In the present study, industrial carob by-products were demonstrated to be eligible as raw material. The aqueous extracts exhibit appreciable high soluble sugar concentration, which evidences the high potential of this by-product as a carbon source to produce high amounts of biomass of the biocontrol agent *P. agglomerans* PBC-1 at low cost.

Varying the extraction process conditions, time, temperature and solid/liquid ratio produces differences in sugar extraction efficiency. A solid/liquid ratio of 1:10 (w/v), at 25°C, afforded a good yield of sugar extraction (94.17%) is one of the less expensive and so was selected as the best combination for sugar extraction of carob by-products.

The biomass productivity, biomass yield and viable populations reached in a short period of time by using this raw material at low carob sugar concentrations (5 or 10 g l^{-1}), allied with high biocontrol activity in pome fruits, showed it to be a suitable process. For large-scale assays it will be necessary to reach a compromise between a higher concentration of sugars, which promotes the greatest amount of biomass in the shortest period of time, and a low phenol content present in the extract, which should not inhibit the growth. Studies are still needed to overcome the oxygen scarcity in cultures grown in bioreactors in order to improve the biomass productivity.

Further research also has to be done on the compounds present in carob aqueous extracts, highlighting the need to develop a clarifying method that reduces or eliminates them, thereby avoiding their detrimental growth effects.

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Chapter 6

Production of the biocontrol agent *Metschnikowia andauensis* PBC-2 using food industry byproducts

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Submitted

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Abstract

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. The choice of inexpensive raw materials is important to the overall economy of the process because they account for 50% of the final product cost. The use of food industry by-products and agricultural wastes as potential raw material for production of biocontrol agents represents not only a mean to reduce costs but an eco-friendly venture. The production of orange juice on an industrial level leads to a considerable quantity of solid and liquid wastes. The aim of the present work was to investigate the possibility of using citrus industry wastes in the production of *M. andauensis* PBC-2, preserving biocontrol efficacy. The results show that this biocontrol agent was able to use molasses, citrus bagasse and citrus liquor on its growth. PBC-2 production was scale-up in a STR, using the YL optimized medium, composed by 10 g/1 of yeast extract and citrus liquor performing a sugar concentration of 12.5 g/l. After 40 h of incubation a viable population of 3.4×10^8 cfu/ml was achieved and a biomass productivity of 0.435 g/l.h and yield of 1.502 g/g were observed. The antagonist activity of fresh cells of *M. andauensis* PBC-2 produced in the different media was assessed in fruits trials against *P. expansum*. The reduction of incidence was 100, 87, 90 and 73% in citrus liquor, citrus bagasse, YPS and molasses, respectively. In semi-commercial trial, on 'Golden Delicious' apples, in cold storage, PBC-2 produced in STR in YL medium, reduced the incidence mould from 90 to 20% and severity from 27.1 to 3.0 mm. *M. andauensis* PBC-2 populations survived in wounds and on the on the surface of apples, which may represent a competitive advantage to further reinfection or new damages.

Key words: culture medium, batch, fermentations, citrus waste, molasses

1. Introduction

Postharvest losses of fruits and vegetables are high, ranging from 10 and 40% depending on the species and technologies used in the packinghouses (Wilson &

Wisniewski, 1994; Arras & Arru, 1999). Fungal pathogens are the mainly responsible for such important economical losses, they usually infect the host through wounds made during harvest, handling and processing (Wilson & Wisniewski, 1989).

Control measures of postharvest fungal decay are still principally based on the protection of pre and postharvest infection with pre-and postharvest fungicide treatments (Jijakli, 2011). However the indiscriminate use of chemicals leads to the frequent decreased of fungicide efficacy by the development of resistant strains of pathogens. In addition, public concern and regulatory restrictions about the presence of fungicide residues on crops have emphasized the need to find alternative methods for disease control (Smilanick, 1994).

Microbial control agents can be effective alternatives to synthetic fungicides in controlling postharvest pathogens if high quality stable products can be mass-produced economically (Prabakaran & Balaraman, 2006).

In spite of the great body of information and progress in the field, the commercial use of biocontrol products is still very limited and accounts for only a very small fraction of the potential market (Droby, 2006). In fact, the biopesticides have captured a scant (about 1.4%) of overwall wold pesticides market (Dinham, 2005).

One factor limiting commercial interest in biocontrol is the high cost of production for most biocontrol agents (Fravel *et al.*, 1999). This may be due to high cost of substrate, low biomass productivity, or limited economies of scale.

In microbial fermentations, the cost of the fermentation medium can account for almost 30-40 % of the total cost (Miller & Churchill, 1986; Akerberg & Zacchi, 2000), therefore to ensure the economic success of the process, the cost of the medium must be kept as low as possible. This is especially important for biocontrol agents, which are usually applied in large quantities and must be cost comparable to competing chemical products.

Initial studies are directed at determining defined nutritional conditions which maximize growth. After that the optimized defined medium serves as a nutritional framework, from which a production medium can be formulated, replacing the nutritional components of the defined medium for cheap subtracts (Wraight *et al.*, 2001).

Agricultural activities and food industry generate considerable quantities of wastes which are rich in organic matter and could constitute new materials for value added products (Bacha *et al.*, 2011). The use of food industry by-products and

agricultural wastes as potential raw material for production of biocontrol agents represents not only a mean to reduce costs but an eco-friendly venture. Nonetheless, this idyllic raw material contains some restrictions, since they are not standardized and may contain impurities that will need to be removed; moreover their composition may vary with season and origin (Stanbury *et al.*, 1995; Teixidó *et al.*, 2011).

Cane molasses, fruit concentrate, carob extract, soy powder, wastewater sludges among others have been tied up on the production of biocontrol agents, in order to reduce costs (Costa *et al.*, 2001; Verma *et al.*, 2007; Manso *et al.*, 2010; Ghribi *et al.*, 2011; Heidari-Tajabadi *et al.*, 2011; Wang *et al.*, 2011; Sartori *et al.*, 2012; Yánez-Mendizábal *et al.*, 2012).

The citrus-processing industry generates huge amounts of citrus peel as a waste from the industrial extraction of citrus juices (Rivas *et al.*, 2008). This huge amount of waste is sent to waste-disposal locations, which involves substantial costs for handling and transport (Yang *et al.*, 2009) or in most cases, spread on soil in areas adjacent to the production locations (Martín *et al.*, 2010), producing odor and soil pollutions, representing a major problem for the industries involved (Liu *et al.*, 2006). Besides, this method of waste handling produces highly polluted wastewater in terms of chemical and biological oxygen values which can negatively affect the soil and the ground and superficial waters (Braddock, 1995).

For juice production, citrus fruits are washed, punched without peeling to remove citrus-oil, and directly pressed to extract citrus juice. The solid material arising by squeezing the fruit (citrus bagasse) consists of peel, seed and pulp, representing 50 % of the fruit weight. This waste material can be pelletized and incorporated into animal feeds. However, the presence of pectin and the water and sugar contents significantly increase the cost of drying (Yoo *et al.*, 2011). For this reason, in a first step the residue is crushed and the pH adjusted adding NaOH or CaO to facilitate pectin degradation and enhance the efficiency of water removal by the press. From the pressing operation, performed after the addition of CaO, results a liquid phase, called "liquor" which has a concentration of soluble solids roughly 10 to 12.5 °Brix, depending on the amount of water added in process and the variety of fruit processed. Much of soluble solids present in the liquor (60-75%) are composed of sugars, primarily glucose, fructose, sucrose and small amounts pentose (Roçafa Junior *et al.*, 2005). Due its high content of organic matter, this waste is an agent with high pollution potential (Tavares *et al.*, 1998).

In the search for alternative solutions to the problems associated with the discarding of this liquid effluent, many industries have been opting for the use of microorganisms as agents to reduce the organic matter and eliminate or reduce toxic compounds (Rezzadori *et al.*, 2012). However, d-limonene a major constituent of the effluent citric can inhibit the growth of microorganisms, because it acts as bacteriostatic agent, making the process ineffective, causing serious environmental problems. Therefore it is urgent to find solutions to these wastes otherwise the continuity of the industrial activity may be compromised, with devastating effects for the local economy.

Yeast posses many features which make them eligible as biocontrol agents in fruit and other foods. Yeast do not require complex nutritional media, have capacity to grow in fermenters on inexpensive media, ability to survive in a wide range of environmental conditions and do not produce anthropotoxic compounds (Bleve *et al.*, 2006).

The ascomycetous yeast species of the genus *Metschnikowia* frequently dominates the mycobiota of flowers, fruit (Phaff & Starmer, 1987; Lachance *et al.*, 2001), and leafs (Sláviková *et al.*, 2007). *Metschnikowia* species have been tested as biocontrol agents against postharvest diseases, mainly *Botrytis cinerea* and *Penicillium* spp. on apples, table grapes, grapefruit, cherry tomatoes, strawberries, nectarines, apricot (De Curtis *et al.*, 1996; Schena *et al.*, 2000; Janisiewicz *et al.*, 2001; Kurtzman & Droby, 2001; Spadaro *et al.*, 2002, 2004; Karabulut *et al.*, 2003, 2004; Ferrari *et al.*, 2007).

Recently, *Metschnikowia andauensis* PBC-2, a strain isolated from the surface of 'Bravo de Esmolfe' apple fruit cultivated in North Portugal, was report as a new biocontrol agent (Manso & Nunes, 2011). The results obtained on different pome cultivars against *R. stolonifer*, *P. expansum* and *B. cinerea* under cold conditions, on citrus fruit against *P. digitatum* and *P. italicum*, shows the high efficacy in controlling postharvest diseases in different fruit and the high ability of *M. andauensis* to survive and grow under different storage conditions, a desirable characteristics of a biocontrol agent as described by Wilson & Wisniewski (1994).

Previous experiments on the production of the *M. andauensis* PBC-2, have examined the influence of nutrients in basal media for the purpose of developing a culture medium that allow the greatest quantity of efficacious biomass in the shortest period of time. From four different media usually used on the production of yeast, the chosen was YPD. Nonetheless and with the intent to reduce costs without

compromising biomass productivity and yield, dextrose was replaced by commercial sucrose and the carbon concentration was reduced in 37.5 % (Manso *et al.*, 2013, submitted).

The aim of this study was to develop a cost-effective medium to maximize the biomass production of *M. andauensis* PBC-2, using citrus industry by-products and waste as an economical carbon source on account of its nutritional abundance and low price.

2. Material and Methods

2.1. Biocontrol agent

M. andauensis PBC-2 were isolated and has previously been demonstrated to have antagonistic activity against the main postharvest disease of pome and citrus fruit (Manso & Nunes, 2011). This strain was identified by Centraalbureau Voor Schimmelcultures, Institute of the Royal Netherlands Academy of Arts and Sciences and by National Collection of Yeast Cultures, and it is deposited in National Collection of Yeast Cultures as NCYC3728 (Institute of Food Research, Norwich Research Park, Norwich, UK).

For the preparation of experiments, a frozen stock culture, maintained in CRIOBILLES AEB 400100 (AES Laboratory, Combourg, France) at -80 ºC, was used to inoculate NYDA plates (8 g/l nutrient broth, Biokar BK003HA; 5 g/l yeast extract, Biokar A1202HA; 10 g/l glucose, Riedel-de-Haën 16325; 15 g/l agar, Vaz Pereira). After 48 h of incubation at 25±1 ºC, subcultures were prepared, stored at 4 ºC and used when needed.

Inoculum was prepared by transferring cells from solid cultures to 50-ml of YPD (10 g/l yeast extract; 20 g/l peptone Oxoid PL0037; 20 g/l glucose) held in 250 ml conical flasks. After 36-40 h of incubation at 25 ±1 ºC and orbital shaker (150 rev/min.), cells were harvest at 1920×g (Hettich Zentrifugen Universal 320, Germany) for 20 min and resuspended in phosphate buffer. Desired concentrations were adjusted by measuring the optical density at 590 nm in a spectrophotometer (Spectrophotometer UV-Vis, Shimadzu-UV160, USA) according to a standard curve.

2.1.2. Pathogen and fruit

P. expansum was isolated from infected apples fruit and maintained on potato dextrose agar (PDA, Biokar BK095HA) at 25 ºC. The pathogen was selected for is high virulence and was periodically inoculated into fruit and re-isolated onto PDA.

Spore suspension was obtained from 7-10-days-old cultures. The number of spores was calculated with a hemocytometer, and then the spore concentration was adjusted to 1×10^4 conidia/ml with sterile distilled water with Tween 80.

'Golden Delicious' apples and "Rocha" pears were harvested at commercial maturity. Fruits without wounds or rot were selected based on uniformity of size, disinfected with 2% v/v sodium hypochlorite for 2 min, rinsed with tap water and dried in air.

2.2. M. andauensis PBC-2 production

2.2.1. Shake flasks experiments with food industry by-products

Different food industry by-products for *M. andauensis* PBC-2 growth were used in shake flash experiments. The medium YPS (10 g/l yeast extract; 20 g/l; 12.5 g/l sucrose, Panreac 141621.1211) recently described to grow PBC-2 (Manso *et al.*, 2013, submitted), was used as standard. Cane molasses was used to compare biomass production, since it is a byproduct commonly used for the production of microorganisms, specifically biocontrol agents (Costa *et al.*, 2001; Abadias *et al.*, 2003; Yánez-Mendizábal *et al.*, 2012). All media were prepared in 250 ml conical flasks containing 50 ml of each tested medium, replacing the carbon source sucrose for cane molasses or citrus bagasse. Once inoculated at an initial concentration of approximately 1×10^5 cfu/ml, flasks were incubated at 25±1 ºC under orbital agitation 150 rev/min. Periodically pH was measured, samples were taken and viable cells were estimated. Viable cell concentrations were estimated as cfu/ml by plating ten-fold dilutions of the suspension in NYDA. Plates were incubated at 25±1 ºC for 48 h. Each flask assay was conducted in four replicates and the assay was repeated twice.

2.2.2. Citrus liquor preparation and M. andauensis PBC-2 production in shake flasks with citrus liquor

The pH of citrus liquor was adjusted from 10-11 to 6, adding citric acid 1 M. A rough filtration was made with gaze to eliminate larger particles like peels, seed fragments or

even insects. The liquid was centrifuged (Beckman, Avanti J-14, Coulter) at 6981×g for 15 min at 4 ºC, the intermediate phase was transferred and autoclaved at 121 ºC for 15 min. After sterilization the liquor was maintained at rest for sedimentation of small particles in suspension and sugar concentration was determined by HPLC. The clear liquor was pumped, and integrated in the medium composition, in order to obtain an initial sugar concentration of 12.5 g/l.

Three different media were tested: YPL (10 g/l yeast extract; 20 g/l peptone; 12.5 g/l of sugar concentration of liquor); YL (10 g/l yeast extract; 12.5 g/l of sugar concentration of liquor); CL (12.5 g/l of sugar concentration of liquor). *M. andauensis* PBC-2 cultures were prepared and incubated as above. The growth in each treatment was determined spectrometically to 590 nm and cell viability (cfu/ml) was estimated. Each flask assay was conducted in four replicates and the assay was repeated twice.

2.2.3. M. andauensis PBC-2 production in stirred reactor with citrus liquor

Batch fermentations were performed in a stirred reactor, STR ADI 1010/1025 (Apllikon, Holland) with 2.4 l working volume. The optimized medium YL, (10 g/l yeast extract; 12.5 g/l of sugar concentration of liquor) was prepared as described above and antifoam (1 ml/l) was added to culture medium followed by sterilization at 121 ºC for 15 min. Fermenter was inoculated with 10% of its volume of fresh inoculums suspension of *M. andauensis* PBC-2. Fermentation conditions were set at 25 ºC, 300 rev/min. and sparged with an initial airflow rate of 1 vvm (volume of air per volume of medium).

During cultivation, pH, temperature and dissolved oxygen, pO2, measured by specific probes were constantly monitored online by using the BioXpert program, version 2.1.

Samples were taken, immediately after inoculation and at regular intervals, to determine the OD590, viability population counts and sugar concentrations, as previously described. Fresh weight biomass was determinated after centrifugation at 1920g (Universal 320, Hettich Zentrifugen, Germany), for 15 min, and the supernatant was recuperated for the analysis of remaining carbon. Viable cells (cfu/ml) were determined as above. Fermentation experiment was performed twice.

2.3. HPLC analysis

The composition of sugars, in citrus liquor and in supernatant culture broth, 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 9 4.5 cm, 5-lm particle size) from Merck KGaA, Germany, was used at room temperature. The mobile phase was acetonitrile/water (75:25 v/v) applied at a flow rate of 1 ml/min. 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.

2.4. Evaluation of antagonist efficacy

2.4.1. Efficacy assay

To evaluate a possible adverse effect of the production medium, on the efficacy of the *M. andauensis* PBC-2, the biomass produced in the different optimized media was tested in pome fruits. Cultures were grown in molasses, citrus bagasse, citrus liquor and YPS media at 25 ºC and 150 rev/min. After 40 h, cells were separated from supernatants by centrifugation at 1920 g, (Hettich Zentrifugen Universal 320, Germany) for 20 min and then resuspended in sterile phosphate buffer (pH 6.8). Cell concentrations were adjusted to 10^7 cfu/ml using a spectrophotometer (Ultraspec 1100 pro, Amershampharmacia Biotech, Sweden).

Fruit were wounded at two locations (midway between the stem and calyx end) with a stainless steel needle (2 mm deep by 1 mm wide). A 20 µl suspension of cells of PBC-2, grown on each medium was applied to each wound. Sterile-distilled water served as a control. After the fruits were air-dried for 2 h, 15 µl of *P. expansum* suspension (1×10^4 conidia/ml) was inoculated into each wound. Fruit were stored at 20±1 ºC and 80±5% HR. Disease incidence and lesion diameter (severity) of apple fruits caused by *P. expansum* were determined after 7 days. Each treatment contained three replicates with 10 fruits per replicate and the experiment was repeated twice.

2.4.2. Semi-commercial trial using M. andauensis PBC-2

The antagonistic activity of *M. andauensis* PBC-2, produced in STR, in medium with citrus liquor, was assayed on a semi-commercial scale, in ‘Golden Delicious’ apples against *P. expansum*. Each fruit was wounded as described above and then submerged for 30 s in a *M. andauensis* aqueous suspension at 10^7 cfu/ml. After 1 h, fruit were

submerged again for 30 s in a suspension of *P. expansum* at 10^4 conidia/ml. Forty fruit constituted a single replicate and each treatment was replicated four times. The incidence and severity were determined 90 days after cold storage at 1±0.5 ºC and 80±5% HR.

2.4.3. Population dynamics on fruit surface

The populations of *M. andauensis* PBC-2 were monitored on wounded and unwounded 'Golden Delicious' apples and 'Rocha' pears. Wounded and unwounded fruits were submerged for 30 s in an aqueous suspension of *M. andauensis* at 10^7 cfu/ml, then placed on tray packs and incubated at 1±0.5 ◦C and 80±5% RH for 90 days, followed by 7 days at 20 ◦C and 80±5% RH. Fruit samples were taken to determine the number of viable cells of *M. andauensis* at 0, 1, 7, 30, 60, 90 and 97 days after inoculation. On apples, 4 fruit constituted each replicate and 25 pieces of peel surface of 2.5 cm^2 from each fruit were removed with a cork borer (100 pieces of peel per replicate). In the case of wounded fruits, wounds were included in one of the removed segments of peel. Peel surface segments were shaken in 100 ml sterile phosphate buffer (pH 6.8) on a rotary shaker for 20 min at 150 rev/min. and then sonicated for 10 min in an ultrasound bath.

Serial dilutions of the washings were made and plated on NYDA. Colonies were counted after incubation at 25 ◦C in the dark for 48 h. Population sizes were expressed as cfu/cm^2 of apple surface. There were four replicates per treatment and the experiment was carried out twice.

2.5. Statistical analysis

All statistical analyses were performed using Statistica version 8 (StatSoft Statistica). The incidence of decayed fruit was analyzed using analysis of variance conducted after square root transformation to improve homogeneity of variances. *M. andauensis* PBC-2 population was analyzed by two-way factorial analysis and population size (cfu/cm^2) was log transformed to improve homogeneity. Statistical significance was judged at the level of $P < 0.05$ and least significant difference (Duncan´s) procedure was used to separate means.

3. Results

3.1. Shake flasks experiments with food industry by-products

Figure 1 show that the biocontrol agent PBC-2 was able to metabolize the two alternative carbon sources, molasses and citrus bagasse. It is possible to observe a rapid growth in the first 16 h in all tested media, reaching a viable population of 8.6×10^7, 6.9×10^8, 7.1×10^8 cfu/ml, in the YPS medium, molasses and citrus bagasse, respectively. After 20 h of incubation, the differences between the media become more visible, the lower population was achieved with citrus bagasse. On the other hand, the medium with molasses shown a similar profile to the grown observed in the medium used as standard, the YPS. The biomass productivity observed was 0.301, 0.508 and 0.692 g/l.h, in the citrus media, molasses media and YPS.

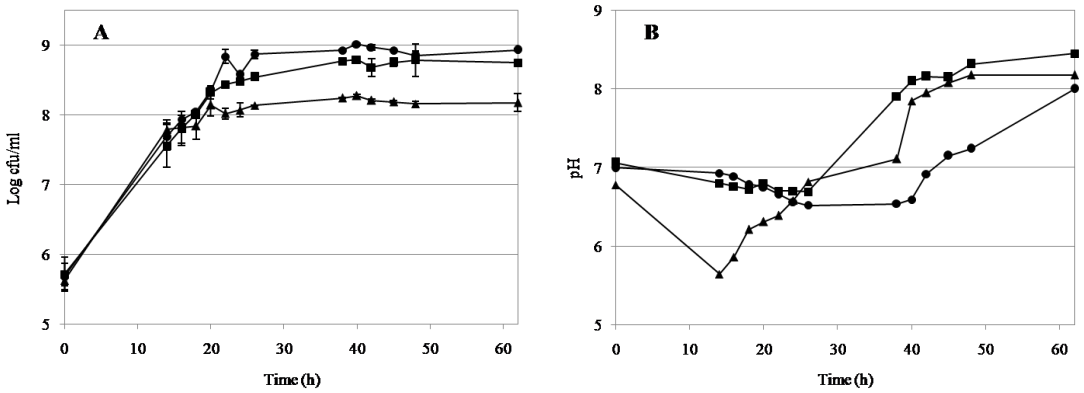

Figure 1. Growth profile of *M. andauensis* PBC-2 produced in YPS (circles) and in two low cost media, on which the usual carbon source was replaced by molasses (square) or citrus bagasse (triangles). Growth was carried out in 250 ml conical flasks at 25 ºC, shaken at 150 rev/min. A) viable cells, expressed in log cfu/ml; B) pH.

3.2. M. andauensis PBC-2 production in shake flasks with citrus liquor

In order to reduce the cost of production and reuse a product that constitutes a problem for the industry of citrus juice, a waste of this industry, the liquor was prepared to become able for medium incorporation.

The growth in the medium only with liquor shows that this waste satisfies the nutritional needs of PBC-2. The specific growth rate, biomass productivity and biomass yield reached only with citrus liquor were 0.092 1/h, 0.364 g/l.h and 1.184 g/g. However, when nitrogen was added to the medium, fresh weight (Fig. 2A) and viable population (Fig.2B) were higher, although there are no large differences between the

media with two or only one nitrogen source. The similarity between YPL and YL is perceptible by the identical biomass productivity (0.680 and 0.665 g/l.h) and specific growth rate (0.107 1/h).

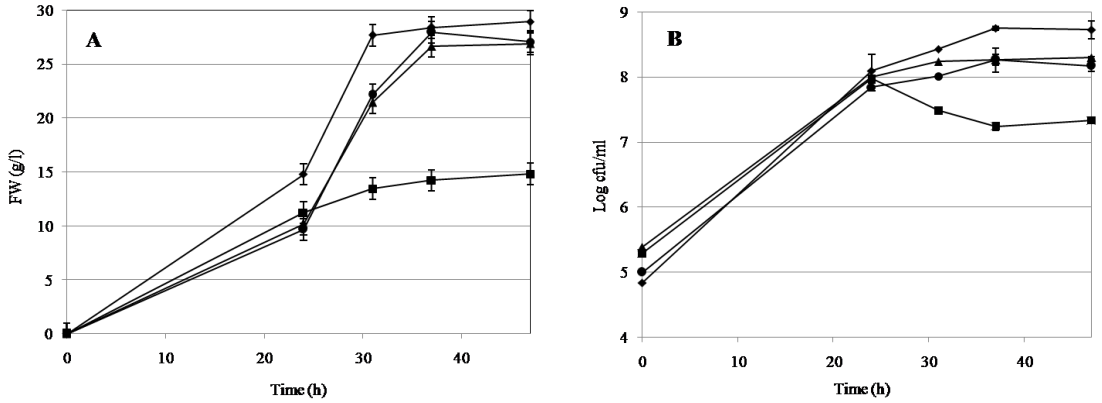

Figure 2. Growth profile of *M. andauensis* PBC-2 produced in 4 different media: YPS (diamonds); YPL (circles); YL (triangles); CL (squares). Growth was carried out in 250 ml conical flasks at 25 ºC, shaken at 150 rev/min. A) fresh cell weight FW, g/l; B) viable cells, expressed in log cfu/ml.

3.3. M. andauensis PBC-2 production in stirred reactor with citrus liquor

The production of *M. andauensis* PBC-2 was scaled-up from shake flasks to stirred tank reactor. The chosen media was YL (10 g/1 yeast extract and liquor performing sugar concentration at 12.5 g/l). Figure 3 shows the pH, pO2, sugar consumption, viable cells and biomass fresh weight, during the production process at 25 ºC.

There was an 8 h lag phase before growth, after which an exponential growth occurred. After 40 h, the viable population reached 3.4×10^8 cfu/ml, the biomass productivity and yield was 0.435 g/l.h and 1.502 g/g, respectively. The growth rate was 0.027 (1/h).

On the first 8 h of PBC-2 growth, oxygen was rapidly consumed; the oxygen consumption rate was 31 µmol O_2 /l.h, reaching 0% at that moment. 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 14 h the value decreased, after that period the pH slight increase with tendency to maintenance until the end of the experiment.

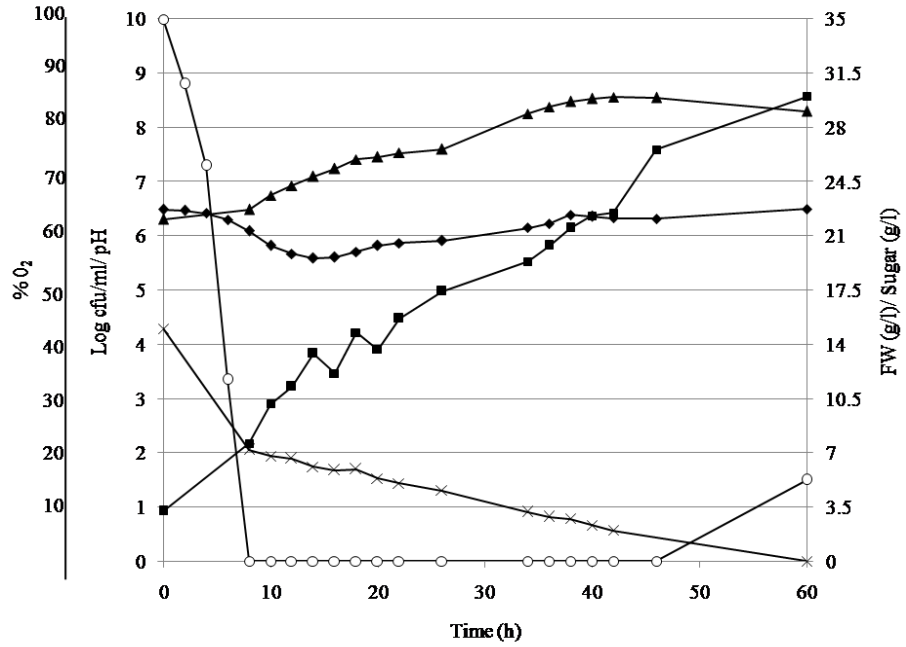

Figure 3. Time course of viable cells cfu/ml (triangles), fresh cell weight (squares), pH value (diamonds), oxygen concentration (circles), consumption of sugar (times) in batch cultivation of *M. andauensis* PBC-2, performed in STR with airflow rate of 1 vvm, L-shaped sparger, 300 rev/min., Rushton-type turbine, at 25 ºC, in YL medium (10 g/1 yeast extract and liquor performing sugar concentration at 12.5 g/l), inoculated at initial concentration at 10^6 cfu/ml. The experiments were performed three times. pH, oxygen concentration were measured online.

3.4. Efficacy assay

The effect of the production medium on biocontrol efficacy of *M. andauensis* PBC-2 was evaluated. After 7 days of storage, the biocontrol agent produced in citrus liquor medium, completely inhibited the pathogen development (Fig. 4), however no significant differences were observed between citrus liquor, citrus bagasse and YPS. The reduction of incidence was 87, 90 and 73 % in citrus bagasse, YPS and molasses, respectively.

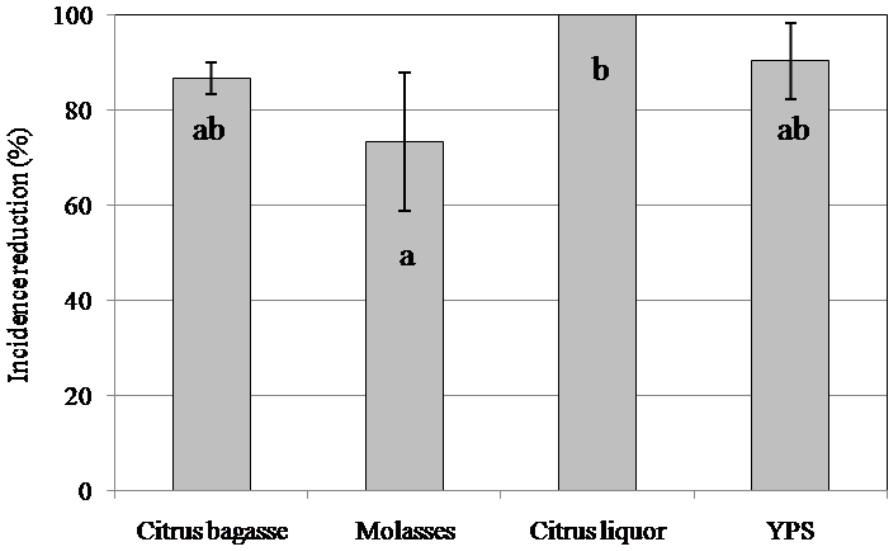

Figure 4. Efficacy of *M. andauensis* PBC-2 produced with food industry by-products and wastes. Fruit were wounded, untreated or treated with fresh cells of the biocontrol agent, at 10^7 cfu/ml, and infected with *P. expansum* at 10^4 conidia/ml. After air drying, fruits were stored at 20 ±1 ºC and 80±5% RH for 7 days and incidence and severity were determined. Columns represent the means of 4 repetitions.

3.5. Semi-commercial trial using M. andauensis PBC-2

The results of semi-commercial trials on 'Golden Delicious' apples are shown in Figure 5. After 3 months of cold storage, significant reduction in blue mould occurred after the application of fresh cells of *M. andauensis* PBC-2, produced in STR, in media with citrus liquor as carbon source. PBC-2 reduced the incidence mould on apples from 90 to 20% and severity from 27.1 to 3.0 mm.

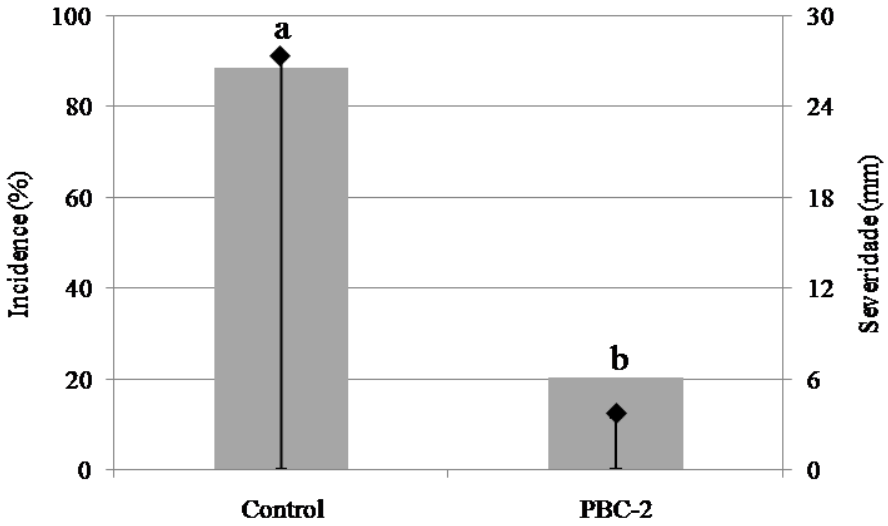

Figure 5. Postharvest incidence (■) and severity (♦) of blue mould on wounded 'Golden Delicious' apples untreated, treated with *M. andauensis* PBC-2 at 10^7 cfu/ml and then inoculated with 10^4 spores/ml of *P. expansum* followed by 3 months of storage at 1±0.5 ºC and 80±5% RH and 7 days at 20±1 ºC. Columns and rows with the same letter are not significantly different according to Duncan Test ($P < 0.05$). Columns and rows represent the means of 4 repetitions.

3.6. Population dynamics on fruit surface

Initial populations of *M. andauensis* PBC-2 on unwounded apples were slight higher than on wounded ones in 1 ºC storage condition (Figure 6). The patterns of growth of *M. andauensis* PBC-2 were similar on wounded and wounded fruits. In the first 24 h of cold storage a slight decrease on surface population was observed, this decrease was more evident on wounded fruits. After that moment surface population increase 12-fold on wounded and 1.5-fold on untreated fruits, returning to suffer a reduction until day 60, remaining stable until the end of cold storage. During the period of shelf-life population increased 1.2 and 2.1 fold on unwounded and wounded fruits.

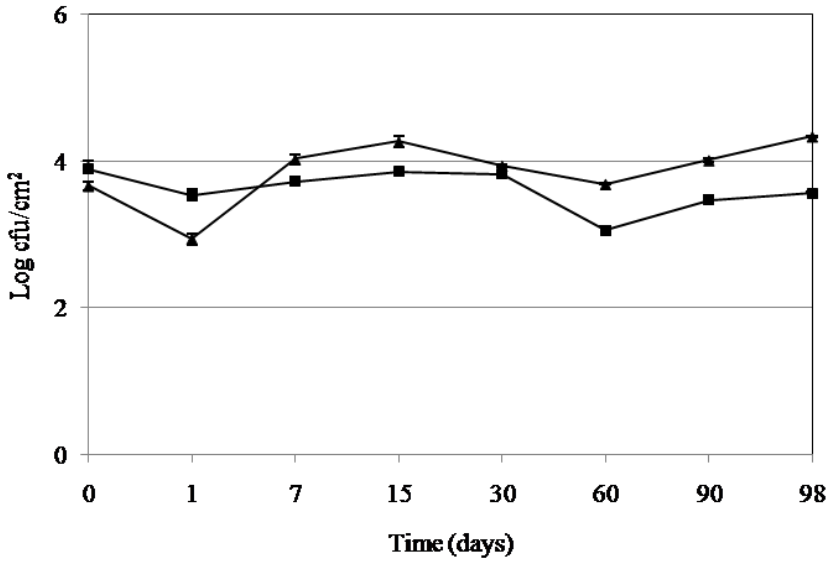

Figure 6. Population dynamics of *M. andauensis* PBC-2 on the surface of unwounded (squares) and wounded (triangles) 'Golden Delicious' apples, submerged for 30 s in a biocontrol agent suspension at 10^7 cfu/ml. Fruits were stored at 1±0.5 ºC and 80±5% RH for 90 days, followed by 7 days at 20±1 ºC.

4. Discussion

Companies are developing new products that are in the process of registration. Many of these companies are small, privately owned firms with a limited product line. Others are larger with more diverse product lines that include a variety of agrochemicals and biotechnological products, playing a significant role in the development and marketing products for the control of plant pathogens (Ardakani *et al.*, 2009). Whatever the size or facilities of the company high profit margin is a common goal, with this intent between other aspects, reduce the cost of production is essential. Since the cost of fermentation media can account for almost 30-40 % of the total cost (Miller & Churchill, 1986; Akerberg & Zacchi, 2000), commercial products and s are being studied to reduce this cost. In the present study the optimization of *M. andauensis* PBC-2 growth was based on the development of a low cost medium composed by inexpensive raw material that normally constitutes a waste product and a serious problem for the industries.

On a first approach, it was studied the ability of *M. andauensis* PBC-2 metabolize other carbon sources, in addition to those already studied in Manso *et al.* (2013, submitted). The results show that molasses and citrus bagasse supports rapid growth, nonetheless molasses provide higher viable population, similar to population

obtain on the standard medium (YPS, yeast extract, peptone and sucrose). Molasses are widely used in the production of microorganisms due to its high sugar content (approx. 50% w/w sucrose, glucose, and fructose), colloidal suspension, vitamins and nitrogen compounds (Costa *et al.*, 2001; Kotzamanidis *et al.*, 2002; Yánez-Mendizábal *et al.*, 2012). On the literature, it is possible to find studies where molasses replaces with success, the commercial carbon sources. Costa *et al.* (2001), Luna *et al.* (2002), Sartori *et al.* (2012) and Yánez-Mendizábal *et al.* (2012) showed that molasses based medium may be used for production of bacterial biocontrol agents. Likewise, molasses has been applied as carbon source in the biomass production of yeast species such as *Candida sake* (Abadias *et al.*, 2003), *Pichia anomala* (Kaur & Satyanarayana, 2005), *Rhodosporidium paludigenum* (Wang *et al.*, 2011).

Citrus fruits comprise an important group of fruit crops manufactured worldwide. In the fruit processing industry large amounts of waste materials are produced in the form of peel, pulp, seeds (Ylitervo, 2008). Citrus peels is the major solid produced, comprises around 50% of the fresh fruit weight (Giese *et al*., 2008) and contained 16.9% soluble sugars, 9.21% cellulose, 10.5% hemicellulose and 42.5% pectin as the most important components (Rivas et al., 2008), which offers potential applications in biotechnology. Our results show that one of these applications is the use of this by-product in the production of *M. andauensis* PBC-2. The use of citrus bagasse has been investigated for the production of bioenergy and/or biochemicals (Thomsen, 2005), ethanol (Ylitervo, 2008), β-carotene (Ciegler *et al.*, 1962), enzymes (Mamma *et al.*, 2008; Giese *et al.*, 2008) and protein-rich feed supplements (Adoki, 2002; Bacha *et al.*, 2011). Furthermore, citrus bagasse encompasses the production of biosurfactant from *B. subtilis* SPB1 (Ghribi *et al.*, 2011) and antimicrobial compounds produced by *B. subtilis* LS 1-2 against *E. coli*, *Staphylococcus aureus* and *Candida albicans*.

The liquor, a citrus waste resulting from pressing operation performed after addition of CaO, was assessed for *M. andauensis* PBC-2 biomass production. Previous to incorporation into the culture medium, the product was subjected to filtration and centrifugation, as well as an adequate adjustment to pH value.

Although, the by-products are a cheap alternative to produce biomass, they also have disadvantages, such as the presence of undesirable products or problems of non-homogeneity, which can limit their use in an industrial process (Zhang & Greasham, 1999). As mentioned before, in this case it was necessary to perform filtrations and

centrifugations, those operations are laborious, but they do not require expensive and unusual equipment, making more feasible the use if this.

Liquor was used in the growth assays, with and without addition of one or two nitrogen sources. Interestingly, the tests revealed that only liquor supports the growth of PBC-2, however, when nitrogen was added to the medium, fresh weight and viable population were higher, although there are no large differences between the media with two or only one nitrogen source.

The growth observed with liquor without additional nitrogen source, contrasted with the observations of Manso *et al.* 2013, submitted) and Spadaro *et al.* (2010). These authors observed that without nitrogen source, the population of *M andauensis* PBC-2 and *M. pulcherrima* remained practically equal to the initial population. The results led to the hypothesis that the liquor has nitrogen that the biocontrol agent PBC-2 can use. In fact, the experimental data presented by Rivas *et al.* (2008) showed that fermentation of orange peel autohydrolysate (liquor) by *A. niger* did not require any supplementation of additional nutrients, the citric acid was produced with promising yield, thereby showing the viability of citric acid production from this industrial waste.

In the growth assays with citrus bagasse or liquor, it was possible to observe a noted growth of the yeast, accompanied by a marked decrease of approximately 1 pH value, during the first hours of incubation. This decrease of pH in the culture broth may possibly adversely affect the growth of *M. andauensis* PBC-2. An example of the pH effects was expressed in the production of citric acid by *A. niger*, the strong pH decrease in the fermentation broth consequent to citric acid release limited its fermentability, making necessary the addition of calcium carbonate to neutralize the product. Thus, it is suggested in further studies the pH control.

In the scale-up of *M. andauensis* PBC-2 production in a STR a viable population of 3.4×10^8 cfu/ml was achieved after approximately 40 h of incubation, using the YL optimized medium, composed by yeast extract and citrus liquor. Similar results were obtained using the YPS optimized medium, with sucrose at 12.5 g/l. After approximately 40 h of incubation, the viable population of *M. andauensis* PCB-2 reached 3.1×10^8 cfu/ml. The biomass productivity and yield was 0.439 g/l.h and 1.416 g/g, respectively (Manso *et al.*, 2013, submitted), comparable to biomass productivity (0.435 g/l.h) and yield (1.502 g/g) observed in the present study. This result supported once again the possibility to produce *M. andauensis* at low cost and provided a reliable basis for the fermentation scaling-up process to an industrial level.

In this study the dissolved oxygen concentration decreases severely in the first hours of fermentation. Apparently, the oxygen starvation seems not influence the microorganism growth. Nonetheless in future studies this is an aspect to be considered, since yeasts belonging to this genus and in anaerobic situations choose the fermentative process.

Disease suppression efficacy of biocontrol strains varies with the medium used in the fermentation for production of biomass, and strains can vary with regard to their amenability to liquid culture fermentations suppression (Roberts & Lohrke, 2003). For example, carbon and nitrogen sources that slightly increased ascospore production of *T. flavus* reduced efficacy of biocontrol of *Verticillium* wilt compared with ascospores produced on potato dextrose agar (Engelkes *et al.*, 1997). For this reason the effect of growing medium, produced with different by-products and wastes, on the biocontrol capability of *M. andauensis* was assayed using efficacy trials on pome fruits against blue mould. The results shown that *P. expansum* decay was significantly higher on untreated apples when compared with fruit treated with *M. andauensis* PBC-2 produced in any of the tested media, without significant differences between substrates. Yánez-Mendizábal *et al.* (2012) also demonstrated in fruit trials that cell and cell free supernatant from CPA-8 grown in the low cost optimized medium maintained biocontrol efficacy against *Monilinia fructicola* on peaches and it was the same as treatments from CPA-8 grown in 863 and MOLP media. In contrary, in efficacy trials on pome fruits, the greatest reduction in disease incidence was obtained with *M. pulcherrima* cells grown onYEMS (Spadaro *et al.*, 2010).

M. andauensis PBC-2 grew well in wounds, like the occurred with *P. agglomerans* CPA-2 (Nunes *et al.*, 2001; Teixidó *et al.*, 2001), *Candida sake* CPA-1 (Viñas *et al.*, 1998; Usall *et al.*, 2001), *Pseudomonas syringae*, *Sporobolomyces roseus* (Janisiewicz, 1995) and *Rahnella aquatilis* (Calvo *et al.*, 2007). However, and unlike those biocontrol agents, *M. andauensis* PBC-2 also grew well on the surface presented an excellent ability to colonize the surface of the fruit, with a notorious population during and after cold storage. This may represent a competitive advantage to further reinfection or new damages and might be related with a possible mode of action, competition for space and nutrients, of this biocontrol agent. On the other hand, the ability to grow on the surface could raise some reluctance to use a product with this characteristic so close to human consumption, nevertheless Manso & Nunes (2011) showed that *M. andauensis* at 37 ºC, the human body temperature, did not grow on simulated gastric fluid, indicating that it might not grow in the human stomach.

The commercial development of biocontrol agents is a challenging but critical stage in the development of potentially useful products. The acceptability of biological control product by consumers will be influenced by price, efficacy and convenience. The commercial use requires a high cell production at a low cost, the optimization of microorganism growth must be based on development of a low cost medium composed by inexpensive raw materials that provide maximum growth while maintain its biocontrol efficacy.

This study has shown that the biocontrol agent *M. andauensis* PBC-2 can be produced in different media, using by-products and waste from food industry, particularly s from citrus industry juice, while maintaining the efficacy. Waste from citrus can serve as an economic culture medium, as suggested by the encouraging results obtained.

Future research will concentrate on optimization growth conditions, as mentioned before, the pH control, the oxygen supply and stirring are same aspects to probe.

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Chapter 7

Study of modes of action of the biocontrol agent *Metschnikowia andauensis* PBC-2

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STUDY OF MODES OF ACTION OF THE BIOCONTROL AGENT *METSCHNIKOWIA ANDAUENSIS* PBC-2

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Abstract

Metschnikowia andauensis NCYC 3728 (PBC-2) is an effective antagonist against the postharvest pathogens *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolofiner* on pome fruits, however its mode of action is unknown. The ability of this strain to produce inhibitory compounds in 4 distinct media (PDA, NYDA, YPDA, CJA) at 3 temperatures (1, 25, 30 ºC), was investigated. It was also assayed the competition for iron in media with different iron concentrations and characterized the capability of PBC-2 produce and secrete fungal cell wall lytic enzymes, like chitinase, protease, and glucanase in a culture media with fungal pathogen cell wall as unique carbon source. *M. andauensis* PBC-2 did not show any inhibition zone to cope pathogens in any of the tested media. The results obtained in this study suggest that the production and secretion of lytic enzymes is not the main or more important mode of action of the new biocontrol agent PBC-2, since the production of chitinase was observed only past 5 and 7 d of incubation, and the production of β-1.3-glucanases and proteases was not observed, which mean that the biocontrol agent PBC-2 have more than one mechanism of action.

Keywords: biocontrol agent, inhibitory compounds, iron competition, lytic enzymes, mode of action

Introduction

Fruits and vegetables are highly perishable products, especially during the postharvest phase, when considerable losses, due to microbiological diseases, disorders, transpiration and senescence, can occur. Traditionally, postharvest diseases are often controlled by the application of synthetic fungicides, however and during the last decade the application of microorganisms for the biocontrol of postharvest diseases has received increasing attention (Droby 2006; Nunes *et al.* 2009). Several yeasts and bacteria have been shown to protect against a number of postharvest pathogens on a variety of harvested commodities (Janisiewicz *et al.* 1994; Chand-Goyal & Spots 1996; Ippolito *et al.* 2000; Kurtzmann & Droby 2001; Nunes *et al.* 2001; Vero *et al.* 2002). Antagonistic yeasts have received particular attention, as their activity usually does not depend on the production of antibiotics or other toxic secondary metabolites, which could have a negative environmental or toxicological impact (Sipiczki 2006). Different species of genus *Metschnikowia* have been described as an effective biocontrol agents (Kurtzmann & Droby 2002; Spadaro *et al*. 2002; Karabulut *et al* 2004; Kinay & Yildz 2008). The yeast *Metschnikowia andauensis* NCYC 3728 (PBC-2) (Manso & Nunes, 2010), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer*, in pome and *Penicillium digitatum* and *Penicillium italicum* citrus fruits.

The knowledge of the mechanisms involved in biological control play an essential role in the development and registration of a biocontrol agent and in maximizing the efficacy of this control system (Janisiewicz *et al.* 2001). Attempts to characterize those mechanisms has resulted in a variety of studies like antibiosis with volatile and non-volatile compounds, competition for nutrients and space, induction of resistance in fruits (Calvente *et al.* 1999; Janisiewicz *et al.* 2000; Poppe *et al.* 2003; Santos & Marquina, 2004; Saravanakumar *et al.* 2008). The direct interaction of the biocontrol agent with the pathogen, for example, by the involvement of fungal cell wall degrading enzymes, like chitinase, protease and glucanase, is also suggested to play a role in the mechanisms of action (Berto *et al.* 2001; Castoria 1997; Mahadevan *et al*. 1997; Saravanakumar *et al.* 2009).

The aim of this study was to determine the mechanisms of biocontrol activity by *M. andauensis* PBC-2. The ability of this strain to produce *in vitro* antagonism, siderophes and produce and secrete fungal cell wall lytic enzymes was investigated.

Material & Methods

Biocontrol Agent

Metschnikowia andauensis NCYC 3728 (PBC-2) previously isolated from the carposphere of 'Bravo de Esmolfe' apples from Portugal and characterized as a biocontrol agent for postharvest diseases of apples and citrus was used in this work. It was stored as a cell suspension in 20% (v/v) glycerol at -80 ºC. When required, *M. andauensi*s PBC-2 was streaked on NYDA medium (8 g L^{-1} nutrient broth; 6 g L^{-1} yeast extract; 10 g L^{-1} glucose; 15 g L^{-1} agar) and incubated at 25 ºC.

Pathogens

P. expansum, *R. stolonifer* and *B. cinerea* strains used were isolated from decayed pome fruits, and selected for their high level of aggressiveness on 'Red Delicious' apples. All the strains were maintained on PDA medium (Potato Dextrose Agar) at 4 °C.

In Vitro Antagonism

The ability of the yeast to inhibit growth of *P. expansum*, *B. cinerea*, *R. stolonifer* was tested on dual cultures on different culture media, at different temperatures. A mycelial disk (diameter 5 mm) from 10 days (d) old cultures of each pathogen, was placed at the center of Petri dishes containing the following media, PDA, NYDA, YPDA (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} glucose; 15 g L^{-1} agar), and CJA (20 g L^{-1} citrus juice by-products with 15 g L^{-1} agar). For each medium tested, the yeast, produced in the respective liquid media, was streaked at two sides of the pathogen. Plates were incubated in different temperatures, 1, 25 and 30 ºC. Control plates without antagonist were performed for each pathogen, in each media, at all temperatures. After 15 d of incubation diameters of pathogens colonies towards the antagonist were measured in each case and compared to colony diamenters in control plates. Three plates constituted a single replicate and the experiment was replicated twice.

Competition for Iron

Petri dishes containing a medium for siderophore production (sucrose 25 g L^{-1}; ammonium sulphate g L^{-1}; potassium dibasic phosphate 3 g L^{-1}; citric acid 1 g L^{-1}; magnesium sulphate 0.08 g L^{-1}; zinc sulphate 0.002 g L^{-1}; agar 20 g L^{-1}) were amended with different concentration of iron (0, 10, 100 and 500 μM of ferric chloride). An agar disk (diameter 5 mm) from 10-d old cultures of *P. expansum* or *B. cinerea* was placed at 30 mm from the edge plate. A single streak of *M. andauensis* PBC-2 was inoculated 40 mm from the pathogen. Plates were incubated at 25 ºC and the development of both organisms was observed.

Fungal Cell Wall Production and Induction of Lytic Enzymes

P. expansum strain was grown on YES broth (sucrose 150 g L^{-1}; yeast extract 20 g L^{-1}) at 25 ºC, for 7 d. Obtained mycelium was dried with sterile filter paper and ground in a sterile mortar in the presence of liquid nitrogen to get a fine powder. The mycelial powder was suspended in 5 M NaCl, sonicated for 5 min and centrifuged at 5724×*g* for 10 min. The supernatant was discarded and the pellet was washed 3 times with distilled water. Fungal cell wall were dried in Petri dishes at 60 ºC for 3 h.

Flasks containing yeast nitrogen base medium (YNB) supplemented with previously prepared fungal cell wall at 1 g L^{-1}, were inoculated with PBC-2 fresh cells at an initial concentration 1×10^5 cfu mL^{-1} and incubated at 25 ºC, 150 rpm. Samples were withdrawn aseptically at different times and filtrated by 0.45 μm (Millipore). Filtrates were stored at -20 ºC, until enzymes determination. Experiment was conducted in three replicates.

Enzymes Assays

β-1,3 glucanase activity was assayed in 62.5 μL of culture filtrate and 62.5 μL of 0.05 M acetate buffer, pH 5, containing 1% laminarin. The reaction was stopped by adding dinitrosalicylic reagent (DNS) and heating the tubes for 5 min at 100 ºC. The net increase of reducing sugar in the reaction mixture was determined by comparing the measured optical densities (540 nm) with those on a standard curve prepared with glucose. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 μmol reduction group min^{-1}.

Chitinase activity was assayed in 90 μL of culture filtrate and 10 μL potassium phosphate buffer, pH 6.1, containing 0.18 mM nitrophenyl N-acetyl β-D glucosamide. The reaction was stopped by adding 10 μL NaOH 1 M. The formation of *p*-nitrophenol was followed by absorbance at 405 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protease activity was measured in a reaction mixture contained 100 μL of culture filtrate, 100 μL 0.05 M acetate buffer, pH 5 and 100 μL azocasein 1%. The reaction was stopped adding 400 μL TCA 10% and after centrifugation 500 μL of NaOH 525 mM was added to the supernatant and the optical density was measured at 450 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protein quantification was determinate using the kit BCA from Pierce, following the fabricant instructions.

Results

In Vitro Antagonism

M. andauensis NCYC 3728 (PBC-2) did not show any inhibition zone to cope pathogens. In YPDA and CJA media, pathogens developed without sporulation. Optimal growth of pathogens, in the absence or in the presence of the biocontrol agent, was observed in PDA medium (Fig 1). Growth rate of all pathogens was higher at 25 ºC, when compared to 1 and 30 ºC. Although with some differences in growth between the media, the pathogens in the presence of the biocontrol agent, developed without inhibition, when compared with the pathogen inoculated separately.

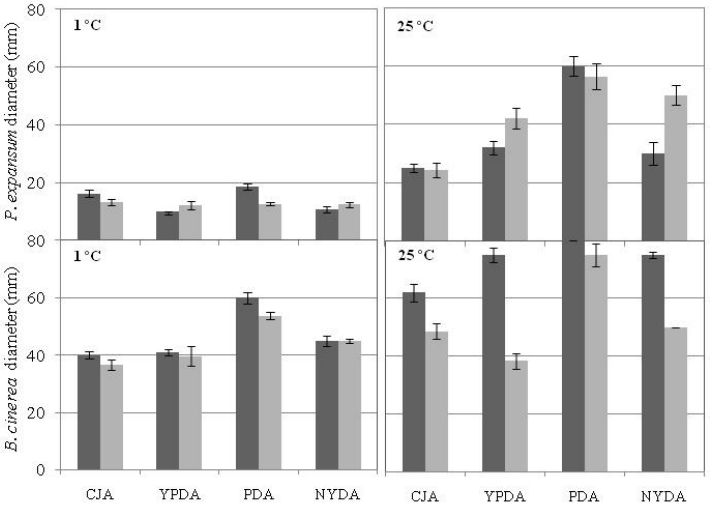

Fig 1. Diameter development of *P. expansum* and *B. cinerea* grown in 4 different culture media, *in vitro* assays, incubated at 1 and 25 ºC. ■ Control treatment, inoculation only the pathogen. ■ Simultaneous inoculation of the pathogen and *M. andauensis* PBC-2. Bars represent standard deviation.

The high diffusion of pulcherrim produced by the biocontrol agent was observed in PDA and NYDA (data not shown).

Competition for Iron

At the tested conditions no inhibition zones were observed in the presence or in the absence of iron. *M. andauensis* PBC-2 produced pale pink to dark red colonies under different iron conditions. The intensity of the colour increased with increasing iron concentrations. The antagonistic yeast produced wider pigmented halos in the medium without iron and an increase in ferric chloride concentration decreased the pigmented halo (Fig 2).

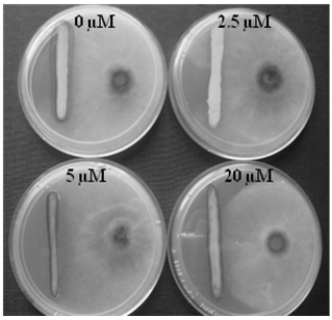

Fig 2. Dual cultures of *M. andauensis* PBC-2 and *B. cinerea* in media with different concentrations of ferric chloride (0, 2.5, 5, 20 μM).

Fungal Cell Wall Production and Induction of Lytic Enzymes

The biocontrol agent PBC-2 showed a slight growth in the YNB media supplemented with *P. expansum* cell wall. Growth was more evident after 5 d of incubation even so no more than 10 fold of the initial population was achieved.

Enzymes Assays

The production of glucanase and protease was not observed at the tested conditions. The maximal level of protein was reached after 5 d of incubation (Fig 3), reaching 113 μg mL^{-1}, after which the production of chitinase increased until the 7th day (Fig 4).

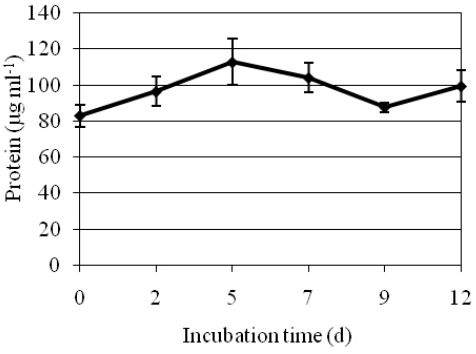

Fig 3. Time course of changes in concentration of protein during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

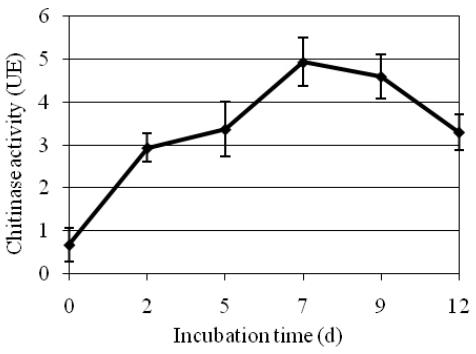

Fig 4. Time course of changes in chitinase activity during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

Discussion

Metschnikowia andauensis NCYC 3728 (PBC-2), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer*, in pome and *P. digitatum* and *P. italicum* IN citrus fruit. However the mechanism by which this yeast reduces decay is not known. In the present work, *in vitro* antagonism, competition for iron and production of extracellular lytic enzymes, was investigated as possible modes of action. No inhibition of any of the tested pathogens was observed in the 4 different media, at the 3 temperatures studied. Results obtained suggest that in our experimental conditions, the production of inhibitory compounds is not the mode of action of this biocontrol agent. Numerous reports in the literature describe the inhibition of postharvest diseases by antibiotics-producing microorganisms, especially in the case of bacteria. *Bacillus subtilis* and *Pseudomonas cepacia* are known to kill pathogens by producing the antibiotic iturin (Gueldner *et al.* 1988). *Pseudomonas cepacia* inhibited the growth of postharvest pathogens like *B. cinerea* and *P. expansum* in apple by producing an antibiotic, pyrrolnitrin (Janisiewicz *et al.* 1991). *Aureobasidium pullulans* produces aureobasidin A, an antifungal cyclic depsipeptide antibiotic that inhibit the development of *P. digitatum*, *P. italicum*, *P. expansum*, *B. cinerea* and *Monilinia fructicola* (Liu *et al.* 2007). However, at present, antibiotic-producing antagonists are not likely to be registered for postharvest use on food products (Nunes *et al.* 2009; Sharma *et al.* 2009). Since the antagonistic activity of *M. andauensis* PBC-2 did not seem to be related to inhibitory substances, competition for iron was tested as a possible mechanism. Iron is essential for the fungal growth and pathogenesis and iron sequestration by non-pathogenic microbes, could be exploited in novel systems for biological control of postharvest pathogens (Calvente *et al.* 1999; Zhang *et al.* 2007). The yeast *M. andauensis* PBC-2 produced a pigmented zone around them, in the media without iron or with low concentrations of this micronutrient. Increasing the ferric chloride concentration the pigmented halo diminished, but the pale pink colour of the colonies became red. Contrasting with the observed by Sipiczki (2006), Saravanakumar *et al.* (2008) and Vero *et al.* (2009), in the present work, no inhibition in the pathogens development was observed, in the presence or in the absence of iron in the media (Fig 2). In the mentioned studies, higher inhibition halos by the antagonist strains in front of the pathogens were observed in lower iron amendments, suggesting the depletion of the micronutrient by the biocontrol agent under low iron conditions. Nevertheless, the colour change of the *M. andauensis* PBC-2 colonies observed with the increase of iron concentration is in agreement with previous findings of Sipiczki (2006), who demonstrated the iron competence between strains of *M. pulcherrima* and pathogenic fungi, suggesting the iron immobilization from the medium and the formation of a red, insoluble pigment called pulcherrimin. By the formation of this insoluble complex, it was suggested that iron remained in the medium but was inaccessible.

Microbial antagonists produce lytic enzymes such as glucanases, chitinases, and proteases that help in the cell wall degradation of the pathogenic fungi (Castoria *et al.* 2001). In the present study, the production of proteases and β-1,3-glucanase was not observed at the tested conditions. The highest level of protein was reached after 5 d of incubation and 2 d later the chitinase activity achieved its maximum level. Chitin, the unbranched homopolymer of N-acetyl glucosamine in a β-1,4 linkage, is a structural component of cell walls in most of the fungi, chitinases hydrolyze this polymer and have been implicated in biocontrol processes (Castoria *et al.* 2001). Although reported in many works, as an important role in the biocontrol activity, the chitinase productivity differs greatly between biocontrol agents. Saravanakumar *et al.* (2009) found a higher production of chitinases by yeast *M. pulcherrima* strain MACH1 in PDB and YPD in the presence of pathogen cell wall indicated the induction of chitinases by biocontrol yeast. Vero *et al.* (2009) demonstrated that *A. pullulans* strain ApB produced both chitinase and β-1,3-glucanase at 5 ºC, in the presence of *P. expansum* cell walls in minimal medium and in apple juice. Production of these enzymes at 25 ºC, by an *A. pullulans* isolate has previously been reported by Castoria *et al.* (2001).

The current study demonstrated that *M. andauensis* PBC-2 did not inhibit pathogens development, through the production of inhibitory substances or by iron competition. The determination of lytic enzymes

revealed that this strain, at the tested conditions, did not produced and secrete β-1,3-glucanase and proteases, nevertheless it showed a modest chitinase activity, which can be consider an advantage, once the biocontrol agent PBC-2 have more than one mechanism of action.

Further studies should be performed in order to clarify the modes of action involved in the antagonistic activity of the biocontrol agent *M. andauensis* PBC-2. Competition for nutrients, other than iron, as well as, the ability of the biocontrol agent, to induce host plants to produce antioxidants and synthesis of pathogenesis-related proteins, should be examined.

Acknowledgements

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Discussion

DISCUSSION

Postharvest diseases cause considerable losses to harvested fruit and vegetables during transportation and storage (Sharma *et al.*, 2009). Much of these losses are due to the attack of several fungi and bacteria because of the high amount of nutrients and water content and as after harvest fruit have lost most of the intrinsic resistance that protects them while they are attach to the plant (Droby *et al.*, 1992)

On citrus, *Penicillium digitatum* and *Penicillium italicum* causing respectively green mold and blue mold, are responsible for 80% of decay that occur from harvest to commercialization (Eaks & Eckert, 1989). In pome fruit, the most important pathogenic species is *Penicillium expansum*, responsible for blue mold (Viñas *et al.*, 1998), however the fungi belonging to the genera *Botrytis*, *Rhizopus* are also critical.

Fungicides are the primary means to control losses, however, the global trend appears to be shifting towards reduced the use of fungicides on produce, for that reason there is a strong public and scientific desire to search for safer and eco-friendly alternatives for reducing the decay loss in the harvested commodities (Mari *et al.*, 2007). The use of microbial agents is quite promising and gained popularity in the last 2 decades worldwide.

In this investigation, one of the main objectives was to search for new biocontrol agents to control postharvest diseases on citrus and pome fruits.

To find a biocontrol agent, numerous microorganisms must be collected and tested, since only a small fraction of them meet the stringent selection criteria.

Some authors assumed that the microorganisms naturally present in the plant or in the surrounding area to protect, show advantages over others, (Chalutz & Wilson, 1990; Wilson & Wisnieski, 1994) and it is accepted that antagonists should be isolated from the environment where they will be applied (Janisiewicz & Korsten, 2002; Teixidó *et al.*, 2011). In postharvest, fruit microbiota have been the richest source of antagonists against fruit decay and the active ingredient in all currently available commercial biocontrol products (Janisiewicz *et al.*, 2013), nevertheless some argue that there is no relationship between efficiency and the isolation source (Jijakli *et al.*, 1999). For example Cheah & Tran (1995) and Zhou *et al.* (2008) used with success an unconventional strategy to develop biological control agents, screening industrial yeasts and food microbes for controlling postharvest rots caused by *P. digitatum* on citrus and by *Monilinia fructicola* on peaches, respectively.

In the present work more than 1800 microorganisms were isolated from the fructoplane and phylloplane of different pome and citrus orchards, 80% were screened for inhibitory activity against *P. expansum* on apple and pear, and *P. digitatum* on oranges. From the screened microorganisms only 7.6% reduced incidence and severity by more than 25%, less than 3% reduced incidence and severity by more than 50%.

Many authors refer as the selection criteria an incidence reduction by at least 50%, (Janisiewicz, 1997; Viñas *et al.*, 1998; Barkai-Golan, 2001). In the present study the selection criteria was narrowed, (reduction of disease incidence and severity by 75% or more), face to this restrictive conditions, 4 microorganisms fulfilled the minimum criteria (3 yeasts and 1 bacterium strain). Studies performed by Nunes *et al.* (2001), reported that from 247 microorganisms, 2% reduced incidence by more than 50% and severity by more than 75%. In similar studies performed with clementines and oranges, for the control of *P. digitatum*, only 1.4% of the tested microorganisms reduced the incidence in 50% (Palou, 2002). Likewise, Viñas *et al.* (1998) and Nunes *et al.* (2007) on pome fruits; Karabulut & Baykal (2003) and Zhang *et al.* (2009) on peaches and Wilson & Chalutz (1989) and Taqarort *et al.* (2008) on citrus, reported similar results. Different results were obtained by Jijalki & Lepoive (1993) in apples, with only 0.61% of the isolates.

Potential biocontrol agents often have a narrow range of activity, because they act on specific hosts against well-defined pathogens under particular environmental conditions which represent some significant limitations. To avoid this restriction it is preferably a method to select antagonists with a broader spectrum of activity, including efficacy tests for various pathogens, different hosts and conditions (Wilson *et al.*, 1993; Lima *et al.*, 1999). The antagonistic ability of the 4 microorganisms that fulfill the selection criteria was evaluated in efficacy experiments with different varieties of pome and citrus fruit, against different pathogens, broadening the spectrum of action. The consistent and higher control of the pathogens development was achieved by one bacterium and one yeast.

The bacterium *Pantoea agglomerans* PBC-1 was identified by the Centraalbureau Voor Schimmelcultures, (CBS), (Delft, The Netherlands), is a facultative anaerobic gram-negative bacterium that belongs to the family Enterobacteriaceae. The strain PBC-1 isolated from the surface of 'Valencia late' oranges, has yellow colonies, round, well-defined with smooth edges.

Pantoea agglomerans occurs commonly on nectarines (Janisiewicz & Buyer, 2010) and has been also isolated from pome fruits (Nunes *et al.*, 2002; Bonaterra *et al.*, 2003). It has been reported to be a very effective antagonist against blue mold, gray mold and *Rhizopu*s rot on pome fruits (Nunes *et al.*, 2001, 2002; Morales *et al.*, 2008), green and blue mold on citrus (Plaza *et al.*, 2001; Teixidó *et al.*, 2001; Torres *et al.*, 2007) and brown decay and *Rhizopus* rot on nectarine and peaches (Bonaterra *et al.*, 2003). A commercial product of this species with strain PBC-2 of *Pantoea agglomerans* is already in the market. Is a freeze-dried formulation, called Pantovital, commercialized by BioDURCAL S.L. (Teixidó *et al.*, 2011).

The other biocontrol agent, the yeast was isolated from the surface of 'Bravo de Esmolfe' apples, a typical portuguese apple cultivar, classified as a PDO. The yeast was identified by the CBS and the National Collection of Yeast Cultures (NCYC) (UK) as *Metschnikowia andauensis*. The strain *M. andauensis* PBC-2 was deposited with the number NCYC3728T and a patent of use as a biocontrol agent has been conceded (national patent nº 105210).

Colonies of *M. andauensis* PBC-2 are white to cream, well-defined with smooth edges. The maroon pulcherrimin pigment was produced (Molnár & Prillinger, 2005). Cells propagate by multilateral budding.

Grown on YM liquid medium (yeast extract, malt extract, peptone and glucose), after 2 days, cells of *M. andauensis* PBC-2, have oval shape, (3-6) × (5-8) µm, single, in pairs or in groups.. In YM Agar after two days the cells are round to oval, (3-9) × (5-9) µm, single, in pairs, or in groups. No produces pseudohyphae or true hyphae.

The ascomycetous yeast species of the genus *Metschnikowia* isolated from terrestrial habitats are typically associated with flowers and fruits (Phaff & Starmer, 1987; Lachance *et al.*, 2001) and transmitted to new niches by insects (Miller & Phaff, 1998). *Metschnikowia* species have been tested as biocontrol agent against postharvest diseases, mainly *Botrytis cinerea* and *Penicillium* spp. on apples, table grapes, grapefruit, cherry tomatoes, strawberries, nectarines, apricot (De Curtis *et al.*, 1996; Schena *et al.*, 2000; Janisiewicz *et al.*, 2001; Kurtzman & Droby, 2001; Spadaro *et al.*, 2002, 2004; Karabulut *et al.*, 2003, 2004; Grebenisan *et al.*, 2006; Ferrari *et al.*, 2007).

One species of the genus *Metschnikowia* was commercially developed and is already available on the market. The product with the commercial name Shemer, is a fluidized bed-dried formulation, distributed by Bayer Cropscience, has on is base *M.*

fructicola and is recommended for citrus, pome grapes, stone fruits, strawberries and sweet potatoes (Teixidó *et al.*, 2011).

It is noted that only biocontrol agents from a very narrow range of genera have been isolated (*Aerobasidium*, *Bacillus*, *Candida*, *Cryptococcus*, *Metschnikowia*, *Pantoea*, *Pichia*, *Pseudomonas*, *Rhodontorula* and *Trichoderma*) since the method of screening used by the most researchers is similar. To overcome this shortcoming, Droby *et al.* (2009) suggested the idea of using a new variety of screening procedures to increase the range of new microbial species.

Postharvest biocontrol agents are applied to consumable products, therefore they need to have strict requirements including non-production of toxic metabolites, thus although there are numerous bacteria known as biocontrol agents, much interest has been given to yeasts. Several important properties of yeasts make them useful for biocontrol purposes, yeasts do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotic metabolites as possibly produced by bacterial antagonists. Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time. They rapidly utilize available nutrients and can sustain many of the pesticides used in the postharvest environment. Yeasts can grow rapidly on low cost substrates in fermentors and are therefore easy to produce in large quantities. The suggested modes of action of biocontrol yeasts are not likely to constitute any hazard for the consumer (Grebenisan *et al.*, 2006).

Most of the potential biocontrol agents showed a typical gradual increase in control decay as the concentration of the pathogen decreased (Nunes *et al.*, 2007; Janisiewicz *et al.*, 2013). On the other hand some reports have demonstrated a direct relationship between the population density of an antagonist and its effectiveness as a postharvest biological control treatment (Karabulut *et al.*, 2004; Manso *et al.*, 2004; Zhang *et al.*, 2009). The mentioned sustains the reported by Wilson & Wisniewski (1994), the existence of a quantitative balance at the wound site between the number of antagonist and pathogen propagules, which affects the outcome of the interaction and determines whether the wound becomes the site of infection.

In the screenings in pome fruits, the pathogen concentration used was 1×10^4 conidia/ml, which corresponds to the pathogen inoculums pressure in the packinghouses (Janisiewicz, 1988). In primary screening in citrus it was used a pathogen concentration of 1×10^5 conidia/ml, less than that used by Palou (2002), who chose a high concentration (1×10^6 conidia/ml), but higher than the one chosen by

Chalutz & Wilson (1989), who applied less stringent conditions (1×10^4 conidia/ml) and only select 3.3% of the microorganisms, which shows that most bacteria and yeasts naturally present in fruits, applied as postharvest treatment, has a null or a limited capability to control the development of postharvest pathogens. Therefore in a biocontrol agent development program it is important to isolate and test a large number of microorganisms.

The potential for using lower inoculums doses for achieving control is very important from a cost perspective. From a practical point of view, the limit for yeasts is about 5×10^7 cfu/ml and for bacterium is 1×10^9 cfu/ml (Janisiewicz, 1998).

The fresh biomass of *P. agglomerans* PBC-1 applied at 1×10^8 cfu/ml on apples and on citrus against *P. expansum* and *P. digitatum*, respectively, promoted a significant reduction of 86% of each pathogen. Reported concentrations of *P. agglomerans* to control postharvest diseases, varied from 8×10^7 cfu/ml on apples (Nunes *et al.*, 2002) and pears (Nunes *et al.*, 2001) against *P. expansum*, *Rhizopus* and *Botrytis* to 2×10^8 cfu/ml on citrus (Teixidó *et al.*, 2001; Torres *et al.*, 2007).

M. andauensis PBC-2 applied at 1×10^7 cfu/ml reduced incidence of *P. digitatum* and *P. italicum* by 73 and 77% on mandarins inoculated with 10^5 conidia/ml. On oranges inoculated with both pathogens at 10^6 conidia/ml, reductions achieved 74 and 83%, respectively. The same concentration allowed on pome fruits a reduction of incidence and severity of 95% and 90% respectively, of *P. expansum* inoculated with10^4 conidia/ml.

Piano *et al.* (1997) stated that the antagonistic activity of *M. pulcherrima* was dependent on the concentration of the antagonist, at 10^5 and 10^6 cfu/ml no satisfactory level of control was reached, at 10^7 and 10^8 cfu/ml the biocontrol agent was effective.

Both potential biocontrol agents provided a satisfactory level of control which is low enough to be considered feasible for commercial use and is lower than that recommended for yeasts and for bacteria.

Commercial use and application of biocontrol agent have been slow mainly due to their variable efficacy, inconsistency performance under different environmental conditions (Wang *et al.*, 2003; Fravel, 2005) and limited tolerance to fluctuating environmental conditions (Teixidó *et al.*, 2011). In part, this could be related to the fact that most of screening assays have been conducted only at laboratory scale and have not been tested in different environments and commodities. Therefore the efficacy of *M. andauensis* PBC-2 was proved during four seasons, in semi-commercial trials, on apples

"Golden Delicious" and pears "Rocha", simulating the conditions normally used in packinghouse during cold storage. Fruits were wounded and the treatment was applied dipping fruits for 30 s in a *M. andauensis* aqueous suspension at 10^7cfu/ml. After 1 h, fruits were submerged for 30 s in a conidial suspension of the pathogen 10^4 conidia/ml. Fruits were stored during 3 moths, at at 1±0.5 ◦C and 80±5% RH and 7 days at 20±1 ◦C.

The antagonist efficacy was compared with the efficacy of the fungicide Imazalil at a recommended concentration for standard postharvest treatment (0.5%) and no differences were observed between the application of the antagonist and the commercial fungicides. In semi-commercial trials in controlled atmosphere conditions, *M. andauensis* PBC-2 maintained its efficacy and in some cases its efficiency was also improved (data not shown).

Comparable incidence reductions to those presented by fungicides were achieved with *Metschnikowia pulcherrima.* Dipping boxes of apples in a suspension at 10^7 cfu/ml and storing for 8 months in controlled atmosphere at 1 °C, showed levels of control against *B. cinerea* and *P. expansum* similar to those from thiabendazole (Spadaro *et al.*, 2001).

Most of the pome fruits are stored in cold storage. Thus, for an effective control, a microbial antagonist should have the ability to grow, multiply and suppress the pathogen at low temperature. The disease control and population dynamics of *M. andauensis* PBC-2 show that this biocontrol agent has the features described above, a good indication of their commercial potential.

Studies of population dynamic show that *M. andauensis* cells were able to maintain a stable population in wounds and that population reached a level above than the mentioned by McGuire (2000), 1×10^4 cfu/cm^2. Janisiewicz *et al.* (2001) demonstrate that strains of *M. pulcherrima*, increased populations by approximately two log units in apple wounds during 1 month of storage at 1 ºC followed by 5 days at 24 ºC, concluding that this species is an excellent colonizer of apple wounds and can thrive on apple as a substrate.

The ability to colonize wounds of *M. andauensis* PBC-2 was expected given the results of *M. pulcherrima* and *M. fructicola,* nevertheless *M. andauensis* PBC-2 was also able to grow on the fruit surface, with a notorious viable population during and after cold storage.

Figure 8.1a and 8.2b shown an apple surface completely colonized by cells of *M. andauensis* PBC-2, after 7 days at 20 ºC.

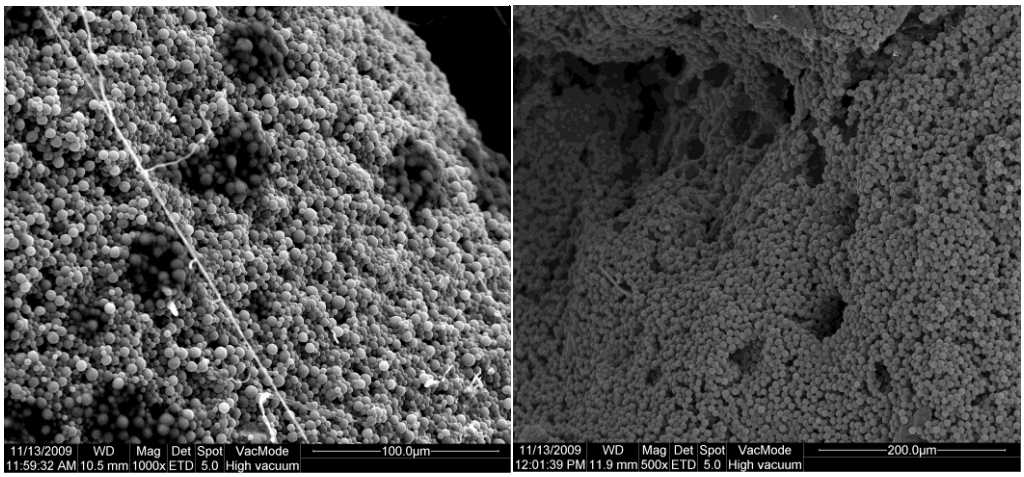

Figure 8.1 a, b. Micrographs of scanning electron microscope of apple surface colonized by cells of *M. andauensis* PBC-2, after 7 days at 20 ºC.

Fokkema (1996) stated that in order to prevent the establishment of the pathogen on the plant, it is important for the biocontrol agent to be present on the fruit surface before the arrival of the propagules of the pathogen. For this point of view, the ability to colonize the fruit surface may represent a competitive advantage to further reinfection or new damages and might be related with a possible mode of action, competition for space and nutrients, of this biocontrol agent. On the other hand, the ability to grow on the surface could raise some reluctance to use a product with this characteristic so close to human consumption. Nonetheless, *M. andauensis* PBC-2 at 37ºC, the human body temperature did not grow on simulated gastric fluid, indicating that it might not grow in the human stomach, which may represent a guarantee to consumers and less an obstacle to its registration.

To be considered as a feasible alternative to fungicides, production, one of the most important steps in the multifaceted development process of a biocontrol agent, must be sustainable. 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. The purpose of production is to produce the greatest quantity of efficacious biomass in the shortest period of time (Fravel *et al.*, 2005).

Practical skills in microbiology and kinetic analysis are required, small-scale culture is mostly carried out using shake flasks of 250 ml to 1000 ml capacity. Medium composition, pH, temperature and other environmental conditions allowing optimal growth and productivity have to be determined. Calculated parameters such as specific growth rate, productivity and biomass yield are used to describe performance of the

organism (Doran, 1995). Once known the biocontrol agent growth profile, scale-up production studies are initiated, first in biological bioreactor at laboratorial scale and then to pilot and industrial scale.

The first step in the optimization of the strategy is the development of a defined or semi-defined culture medium which supports a good growth of the biocontrol agent. After that the optimized defined medium serves as a nutritional framework, from which a production medium can be formulated, replacing the nutritional components of the defined medium for alternative low cost subtracts (Wraight *et al.*, 2001).

In the present study 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; Zhang *et al.*, 2009; Singh *et al.*, 2012), were tested in the production of the potential biocontrol agent *M. andauensis* PBC-2.

The YPD medium was the one with the highest viable population after 40 h of incubation and was chosen as the nutritional framework in this study, from which the optimization studies are conducted, including studies of pH, temperature, nature and concentration of sources nitrogen and carbon.

The most favorable pH for growth was around 6.5, although it was observed growth in the others pH tested, showing that to growth PBC-2 has a large range of pH, which can be advantageous in a situation of stress or to improve colonization ability. No significant differences were found between the growth at 25 or 30 ºC, which revealed that there is no need of heating, turning the process more economical. Moreover, the temperature of 25 ºC is reported in many works in the production of species belonging to *Metschnikowia* genus (Piano *et al.*, 1997; Schena *et al.*, 2000; Spadaro *et al.*, 2002; Zhang *et al.*, 2010).

In nitrogen source optimization, the maximum population achieved was 4.2×10^8 cfu/ml with peptone and with yeast extract 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.

The effect of the nature of the carbon source was evaluated, replacing the usual carbon source, glucose, by others like sucrose or fructose. As expected by the assimilation features of *M. andauensis* PBC-2 (data not shown), this yeast can assimilate the tested sugars. The highest specific growth rate was observed with sucrose (0.107 1/h), however no significant differences were registered between this carbon

source and glucose (0.098 1/h). On the other hand, difference in biomass productivity was observed among sucrose and the others two sources tested. 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 1.2×10^8, 5.3×10^8 and 1.3×10^8 cfu/ml with glucose, sucrose and fructose, respectively.

The assays of to optimize the carbon source, showed no differences on specific growth between the three tested concentrations (5, 12.5, 20 g/l). Concerning to biomass yield and productivity, higher sucrose 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 production of *M. andauensis* PBC- in STR.

Sucrose was also the best carbon source for biomass growth of *Metschnikowia koreensis*, a microorganism reported for its bioreduction capability. The yeast grew poorly on starch, sodium citrate, sodium succinate and sodium potassium tartrate, but grew well on glucose, sucrose and mannitol (Singh *et al.*, 2012). Nonetheless, in the case of the mentioned study, the aim was to develop a culture media and investigate operational conditions to maximize specific enzyme activity, what occurred with glucose instead of sucrose. Likewise, biomass growth was maximized in a medium with an initial pH of 5. In contrast, a neutral initial pH was optimal for attaining the maximum biomass specific enzyme activity.

The multiplicity of carbon sources liable to be used by yeast species of the *Metschnikowia* genus, was reported by Spadaro *et al.* (2010) in the production of *M. pucherrima* and by Nozaki *et al.* (2003) in the production of D-arabitol by *Metschnikowia reukaufii* AJ4787. In that study the yeast produce the desired product from fructose or sucrose with almost the same yield as from glucose, suggesting that the strain AJ14787 can convert fructose via pentose pathway, and might also have an invertase catalyzing the hydrolysis of sucrose.

This means that depending on the intended, with the same organism, it is possible to achieve different purposes, by simply changing the medium and production conditions.

To optimize the conditions for aerobic production of the biocontrol agent *Pantoea agglomerans* PBC-1, fermentations were performed in shake flasks and 2-L mechanically stirred reactor. The effect of different carbon sources (sucrose, glucose

and fructose 10 g/l) for the growth optimum of the biocontrol agent was studied. As expected by assimilation features of *P. agglomerans*, PBC-1 was able to grown in all carbon sources tested. Slight specific growth rate, biomass productivity and viable populations were reached with sucrose and fructose. After 20 h of incubation the viable population was 3.9×10^9, 3.9×10^9 and 1.4×10^9 cfu/ml in sucrose, fructose and glucose, respectively. Sucrose at 5 g/l was selected for the production of *P. agglomerans*, PBC-1 in STR.

Costa *et al.* (2002) studied the best nitrogen and carbon source that provide maximum biomass production of *P. agglomerans* CPA-1. Fructose, sucrose, trehalose and maltose were the carbon sources that provided the highest viable population, combined with inorganic or organic nitrogen source. Comparatively to the other carbon sources, glucose showed poor results, when yeast extract was used as the nitrogen source, biocontrol agent growth was lower than in the nitrogen source alone.

A study of the effect of carbon and nitrogen on insoluble phosphate solubilization by *P. agglomerans* R-42, shown that glucose was the best carbon source, the one that promotes the higher growth and $Ca_3(PO_4)_2$ solubilization (Son *et al.*, 2006).

Some authors considered glucose as the main carbon source by all microorganisms because of its size, rapid uptake, utilization and cellular energy conversion, nonetheless in the present study, sucrose was selected for further experiments in the production of *P. agglomerans* PBC-1 and *M. andauensis* PBC-2, since its feasibility and accessibility.

In a typical batch culture, the dissolved oxygen concentration decreases in the media along with the growth time, until the microorganism reaches the stationary phase and then the dissolved oxygen concentration increases (Gomez *et al.*, 2006). In many aerobic fermentations, oxygen is an important nutrient that is used by microorganisms for growth, maintenance and metabolite production, and scarcity of oxygen affects the process performance and productivity (Lima-Costa *et al.*, 1997; Garcia-Ochoa *et al.*, 2009). Oxygen has a low solubility in water (7–9 mg/l) and during growth the oxygen uptake by the growing cells reduces the dissolved oxygen tension in the medium, reaching, in many cases, critical dissolved oxygen concentrations (Rocha-Valadez *et al.*, 2007). Therefore, it is important to ensure an adequate supply and delivery of oxygen from aeration flux to the culture broth, which is achieved with a good mixture.

STRs commonly used in industrial scale fermentation processes, provide high values of mass and heat transfer rates and excellent mixing (Seigel *et al.*, 1992; Garcia-

Ochoa *et al.*, 2009). In these systems, a high number of variables affect the mass transfer and mixing, but the most important are stirrer speed, type and number of stirrers and gas flow rate used. A common approach to increasing the gas liquid mass transfer rate in a STR is to increase the speed of the impeller, which increases the gas liquid interfacial area. However to increase the speed of the impeller, additional power are required, which can be a problem in industrial-scale, (Liu & Wu, 2006), could create foam and could cause cell damage due to increasing shear stresses (Bredwell *et al.*, 1998; Cascaval *et al.*, 2006). Alternatives to overtake this problem, led to extensive hydrodynamics studies with advancements in impeller design, on sparger and baffle designs.

The optimization of the production of *P. agglomerans* PCB-1 in STR was also carried out by subjecting the microorganism to different mixing conditions. The comparison between different geometry evidences the relevance of a radial flux for better broth homogenization, achieved with the use of Rushton turbine and L-sparger. The specific growth rate and biomass yield were significantly higher than what was obtained with marine propeller and porous sparger.

Although correlations for volumetric oxygen transfer coefficient, K_La, are extensive in the literature, there are still considerable problems concerning the accuracy of the estimated values and their validity in different bioreactors or different scales and also under different operational conditions (Badino *et al.*, 2001; Raposo, 2004). In the present work the volumetric oxygen transfer in the stirred tank bioreactor was measured using the dynamic method (Atkinson & Muvituna, 1991). Thus, during the fermentation, the air flow was switched off so that the concentration of dissolved oxygen declined linearly because of consumption by the yeast. The slope of this part of the dissolved oxygen concentration profile was used to estimate the oxygen consumption rate OUR (g/l.h). The air supply was then switched on and the increase in the concentration of dissolved oxygen was recorded. . However, in the culture of *M. andauensis* PBC-2 or in the culture *P. agglomerans* PBC-1 in any aeration and agitation conditions tested, the dissolved oxygen concentration decreases dramatically in an early phase of growth, approaches 0, and does not increase even when the microorganism reaches the stationary growth phase. Under these conditions, it is impossible to measure the OUR and k_La during fermentation by the classic dynamic method. Thus, the search for other methods to improve oxygenation in the reactor should be included in future studies.

In the case of *M. andauensis* PBC-2, additional studies to investigate the effect of different hydrodynamic conditions, should be performed. Still, in the production of this biocontrol agent in STR, the aeration rate chosen was 1 vvm, precisely the aeration rate that afforded the highest maximum biomass concentration of *M. koreensis* (Singh *et al.*, 2012).

In *P. agglomerans* PBC-1 production in STR, the effect of the initial inoculum was studied at 1×10^6 and 1×10^7 cfu/ml, and the main difference between experiments was the duration of the lag phase. At 1×10^6 cfu/ml the lag phase was 8 h and at 1×10^7 cfu/ml, was shortened to 5 h.. This may represent a reduction in the biocontrol production cost. Abadias *et al.* (2003) also shortened the growth time with higher initial inoculum. A lower initial inoculum (1.5×10^5 cfu/ml) gave the lowest *Candida sake* growth, obtaining the maximum population after 30 h of culturing (3.7×10^8 cfu/ml), meanwhile similar counts were obtained when the inoculum used was 9×10^5 cfu/ml or 5.8×10^6 cfu/ml with the maximum growth obtained after 26-30 h of culturing 6-7.4 $\times10^8$ cfu/ml.

Fed-batch culture allows the maintenance of high cell viability for longer periods of time in the stationary growth phase. In the production of *P. agglomerans* PBC-1, fed-batch technology permitted a longer exponential phase of 30 h and higher cell viability, compared to batch cultures. This high value remained unchanged during the stationary growth phase (90 h).

The use of fed-batch technology in the production of biocontrol agents was also investigated by other researchers. In the production of *Trichoderma viride*, the final spore yield obtained in fed-batch culture was two times higher than the apparent spore-carrying capacity of batch culture (Onilude *et al.*, 2012). Similarly, high cell density and maximum inhibitory activity of antifungal in supernatant was achieved in fed-batch cultures of *Bacillus subtilis*, circumventing the adverse effects of acetate formation registered in batch mode (Matar *et al.*, 2009). In contrast, in the production of *C. sake* growth in fed-batch conditions the biomass production was not improved compared with that obtained in batch conditions (Abadias *et al.*, 2003).

The industrial waste, once generated, cannot be accumulated indefinitely. Besides creating potential environmental problems, the waste represents loss of raw materials and energy, requiring significant investments in treatments to control pollution (Pelizer *et al.*, 2007). The waste can contain many substances with high added-value . If appropriate technology is employed, this material can be converted to commercial

products or raw materials for secondary processes. Likewise, many food industry by-products may be useful as sources of nutrients giving the opportunity to obtain added-value products.

Based in these principles and with the intent to produce biomass at low cost, an important factor in the implementation of a biological control system, the possibility of use food industry by-products and wastes, was investigated.

Carob industry by-products and citrus juice industry waste and by-products were studied as alternative nutrients, due to the wide expression and economical importance of those industries in the region where the main part of this work was developed.

Sugar extractions were performed from carob kibbles and then the extracts were used as carbon source in the production of *P. agglomerans* PBC-1 in shake flasks and in STR. Optimal sugar extraction was achieved at a solid/liquid ratio of 1:10 (w/v), at 25 ºC afforded a good yield of sugar extraction (94.17%), Our results are in agreement with those of Petit & Pinilla, who studied the production and purification of syrup from carob pulps and saw that the efficiency of sugar extraction increased when the pulp/water ratio decreased. At the same time, the sugar concentration measured decreased due to dilution. With a 1:10 ratio (w/v) of pulp water, at room temperature, neutral pH, for 1 h, it was possible to achieve a sugar extraction yield of 94.4%.

The growth profiles of *P. agglomerans* PBC-1, observed in carob sugar extracts resembled those observed with sucrose. At 5 g/l of sugar, the biomass productivity (0.280 g/l.h), biomass yield (1.303 g/g) and viable populations (9×10^9 cfu/ml) reached in a short period of time, evidences the high potential of this by-product as a carbon source to produce high amounts of biomass at low cost. In STR, the biomass productivity and biomass yield were 0.163 g/l.h and 1.527 g/g in the bioreactors inoculated at 10^6 cfu/ml, and 0.181 g/l.h and 1.468 g/g in the bioreactors inoculated at 10^7 cfu/ml, respectively.

The great potential of carob pod aqueous extract was disclosed as a source of second-generation bioethanol. Extract can be fermented at high sugar concentration (250 g/l) with an excellent ethanol/glucose conversion yield (0.47–0.50 g/g) when supplemented with small amounts of C and N sources (Lima-Costa *et al.*, 2012).

In the search for alternative sources, in order to reduce the production costs, *M. andauensis* PBC-2 was able to growth in different media, using by-products and waste from food industry, particularly from citrus juice industry, while maintaining the efficacy.

A liquid waste from citrus industry that causes serious environmental problems was used with success. The transition from shake flasks production to STR, was made using the YL optimized medium, composed by 10 g/1 of yeast extract and citrus liquor performing a sugar concentration of 12.5 g/l. After 40 h of incubation a viable population of 3.4×10^8 cfu/ml was achieved and a biomass productivity of 0.435 g/l.h and yield of 1.502 g/g, were observed, similar to productivity (0.439 g/l.h) and yield (1.416 g/g) obtained with sucrose. The use of citrus liquor represents a feasible alternative of a waste with high pollution load, difficult to treat, that can compromise the continuity of this industrial activity, with devastating effects for the local economy.

Cheese whey, another by-product from food industry, was equally used with success by other species of *Metschnikowia* genus, *M. pulcherrima*, however with a different propose, the production of citric acid (Singh *et al.*, 2004).

It should also be noted that the biomass produced in different medium with waste or food industry by-products, was tested *in vivo* assays, in semi-commercial conditions, showing that the effectiveness of two agents were maintained, and in some cases has even improved. Moreover, *P. agglomerans* PBC-1 was even more effective when produced in medium with extracts of carob by-products (data not shown).

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Concluding remarks and future

Considerações Finais e Futuro

CONCLUDING REMARKS

The main objectives of this study, the isolation and production of biocontrol agents, major postharvest diseases of citrus and pome fruit, were successfully achieved. Among the results obtained, the following conclusions must be highlighted:

From the total of 1465 microorganisms tested, approximately 7.6% diminished severity and incidence by more than 25% and less than 3% reduced incidence and severity by more than 50%, 4 microorganisms fulfilled the selection criteria of reduction in severity and incidence by 75%;

The consistent and higher control of the pathogens development was achieved by the bacterium *Pantoea agglomerans*, strain PBC-1 and the yeast *Metschinikowia andauensis*, strain PBC-2;

The minimal effective concentration of the antagonists proved to be reasonable for the commercial development of the two microorganisms:

M. andauensis PBC-2 applied at 1×10^7 cfu/ml allowed a reduction of incidence and severity of 90 and 95%, respectively. *P. agglomerans* PBC-1 applied at 1×10^8 cfu/ml on apples and on citrus promoted a reduction of 86% of *P. expansum* and *P. digitatum*, respectively;

M. andauensis PBC-2 has a broad spectrum of action being effective in the control of *Rhizopus stolonifer*, *Penicillium expansum* and *Botritys cinerea*, on 'Rocha' pears and on different apple cultivars and against *Penicillium digitatum* and *Penicillium italicum* on mandarins and oranges;

M. andauensis PBC-2 may use different nutrients in its growth. The standard medium that provides the greatest growth of PBC-2 was YPD medium;

The carbon source that increase the specific growth rate (0.107 1/h), biomass productivity (0.669g/l.h) and biomass yield (1.487g/g) of *M. andauensis* PBC-2 was sucrose at a concentration of 12.5 g/l. The medium YPS (10 g/l yeast extract; 20 g/l peptone; 12.5 g/l sucrose) was selected for production in bioreactor, with the optimized conditions of pH 6.5 at 25 ºC. 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.

P. agglomerans PBC-1 can also used the three tested carbon sources, fructose, glucose and fructose and also as *M. andauensis* PBC-2, the high biomass was achieved with sucrose;

The medium yeast extract at 5 g/l and sucrose at 5 g/l was selected for production of *P. agglomerans* PBC-1 in a STR;

In the design of the reactor aeration/mixing to growth *P. agglomerans* PBC-1, the Rushton turbine and L-sparger allow to achieve higher specific growth rate and biomass yield when compared to with marine propeller and porous sparger, suggesting the relevance of a radial flux for better broth homogenization;

The initial inoculum of 1×10^6 cfu/ml was selected for the production of *P. agglomerans* PBC-1 in STR since provided similar population to that obtained with initial inoculum of 1×10^7 cfu/ml (9×10^9 cfu/ml), however with a 5 h longer lag phase ;

The fed-batch cultivation of P. *agglomerans* permitted a longer exponential phase of 30 h and higher cell viability that remained unchanged during the stationary growth phase (90 h), however the growth profile and kinetic parameters was similar to batch cultures;

Carob by-products exhibit appreciable concentration of soluble sugars, extractable with water. A solid/liquid ratio of 1:10 (w/v), at 25 ºC, afforded a good yield of sugar extraction (94.17%);

Carob by-products were demonstrated to be eligible as raw material for *P. agglomerans* PBC-1 production. The media using carob byproducts selected for production in STR was yeast extract at 5 g/l and extract from carob kibbles, at a concentration of 5 g/l of sugar concentration, and provided similar population to that achieved on flasks trials (4×10^9 cfu/ml). The specific growth rate, biomass productivity and biomass yield were 0.110 (1/h), 0.163 (g/l.h) and 1.527 (g/g), at an inoculums concentration of 10^6 cfu/ml, and 0.119 (1/h), 0.181 (g/l.h) and 1.468 (g/g) at 10^7 cfu/ml;

M. andauensis PBC-2 can be produced in different media, using by-products and waste from food industry, particularly from citrus juice industry. The medium that provided the greatest biomass of *M. andauensis* PBC-2 was YL (10 g/l of yeast extract and citrus liquor at a sugar concentration of 12.5 g/l), and was selected for production in STR provided similar population to that achieved on flasks trials;

The production of *M. andauensis* PBC-2 in STR using the YL medium, achieved a viable population of 3.4×10^8 cfu/ml, after 40 h of incubation. The biomass productivity and biomass yield were 0.435 g/l.h and yield of 1.502 g/g, respectively;

Citrus liquor, a waste from citrus juice industry can be used as the carbon source of an economic culture medium. Its use represents a feasible alternative of a waste with high pollution charge, difficult to treat, that can compromise the continuity of this industrial activity, with devastating effects for the local economy;

The antagonistic efficacy of the cells of both biocontrol agents grown using by-products as carbon sources was similar to that obtained with cells grown in defined medium

The efficacy of *M. andauensis* PBC-2 was confirmed during four seasons, in semi-commercial trials, simulating the temperature normally used during cold storage, and results were similar to the efficacy of the fungicide imazalil at a recommended concentration;

M. andauensis PBC-2 presented an excellent ability to colonize wounds and fruit surface, with a notorious population during and after cold storage;

M. andauensis at 37 ºC, the human body temperature, did not grow on simulated gastric fluid, indicating that it might not grow in the human stomach;

Production of lytic enzymes, such β-1,3 glucanase, proteases and chitinase, and iron competition is not the mode of action of *M. andauensis* PBC-2.

CONSIDERAÇÕES FINAIS

Os principais objetivos deste trabalho, ou seja, o isolamento e produção de agentes biocontrolo, das principais doenças de pós-colheita de citrinos e pomóideas foram alcançados com sucesso. De entre os resultados obtidos convém destacar as seguintes conclusões:

De 1465 microrganismos testados, cerca de 7,6% diminuíram a severidade e a incidência em mais de 25%, menos de 3% reduziram a incidência e a severidade em mais de 50%, 4 microrganismos satisfizeram os critérios de seleção, uma redução da severidade e incidência de pelo menos 75%;

O maior e mais consistente controlo do desenvolvimento dos agentes patogénicos, foi observado com a aplicação da bactéria *Pantoea agglomerans* PBC-1 e da levedura *M. andauensis* PBC-2;

A concentração mínima eficaz dos dois potenciais antagonistas provou ser razoável para o desenvolvimento comercial dos dois microrganismos:

M. andauensis PBC-2, aplicada a 1×10^7 ufc/ml permitiu uma redução da incidência e da severidade de 90 e 95%, respectivamente. *P. agglomerans* PBC-1 aplicada a 1×10^8 ufc/ml em maçãs e em citrinos no controlo de *P. expansum* e *P. digitatum*, respectivamente, propiciaram uma redução significativa de cerca de 86% de cada um dos agentes patogénicos;

O amplo espectro de ação de *M. andauensis* PBC-2 foi aprovado, tendo-se observado o controlo efetivo de *Rhizopus stolonifer*, *Penicillium expansum* e *Botritys cinerea*, em pêra 'Rocha' e em diferentes cultivares de maçã e contra *Penicillium digitatum* e *Penicillium italicum* em tangerinas e laranjas;

M. andauensis PBC-2 pode usar diferentes nutrientes em seu crescimento. O meio definido YPD, foi aquele que promoveu o maior crescimento;

A fonte de carbono, que garantiu a maior a taxa específica de crescimento (0.107 1/h), produtividade de biomassa, (0.669g/lh) e rendimento de biomassa (1.487g/g) de *M. andauensis* PBC-2, foi a sacarose a uma concentração de 12.5 g/l. O meio YPS (10 g/l de extracto de levedura, 20 g/l de peptona, 12.5 g/l de sacarose) foi seleccionado para a

produção em reactor biológico mecanicamente agitado, com as condições optimizadas de pH 6.5 a 25 º C. Após 44 h uma população viável de 3.1×10^8 cfu/ ml foi alcançado, a produtividade de biomassa e rendimento foi de 0.439 g/l.h e 1.416 g/g, respectivamente;

P. agglomerans PBC-1 também tem capacidade de utilizar as três fontes de carbono testadas, frutose, glucose e sacarose e tal como *M. andauensis* PBC-2, foi a sacarose a fonte que garantiu maior biomassa;

O meio composto por extracto de levedura a 5 g/l e sacarose a 5 g/l foi selecionado como meio de produção de *P. agglomerans* PBC-1, em reactor biológico mecanicamente agitado;

No dimensionamento das condições de arejamento/mistura de crescimento *P. agglomerans* PBC-1, a turbina "Rushton" e o dispersor em "L", permitiram obter uma maior taxa específica de crescimento e rendimento de biomassa, quando comparados com a hélice marinha e dispersor poroso, sugerindo a importância de um fluxo radial para melhor homogeneização meio de cultura.

O efeito da concentração do inoculo inicial foi estudado 1×10^6 e 1 x 10^7 ufc/mL. A principal diferença entre as concentrações testadas foi a duração da fase de latência, que foi reduzida em 5 h no reactor inoculado a 1×10^7 ufc/mL, tal pode representar uma redução nos custos de produção do agente de biocontrolo;

A produção de *P. agglomerans* PBC-1, em modo "fed-batch" permitiu uma prolongada fase exponencial de mais de 30 h e uma viabilidade celular superior, que se manteve inalterada durante a fase estacionária de crescimento (90h), contudo, o perfil de crescimento e os parâmetros cinéticos, foram semelhantes ao modo descontínuo.

Os subprodutos da indústria de alfarroba contêm apreciáveis concentrações de açúcares solúveis, extraíveis com água. A razão sólido líquido de 1:10 (p/v), a 25 ºC, permitiu obter um elevado rendimento de extração de 94.17%.

Os subprodutos da indústria de alfarroba demonstraram ser elegíveis como matéria-prima na produção de *P. agglomerans* PBC-1. O meio com de subprodutos da indústria de alfarroba, selecionado para produção de *P. agglomerans* PBC-1, em reactor biológico mecanicamente agitado, composto por 5g/l de extracto de levedura e por extratos de subprodutos de alfarroba perfazendo uma concentração de inicial açúcar de 5 g/l, permitiram obter populações similares, às obtidas nos ensaios em balão (4×10^9 ufc/ml). A taxa específica de crescimento, produtividade de biomassa e o rendimento de biomassa foram 0.110 (1/h), 0.163 (g/l.h) e 1.527 (g/g), no reactor inoculado a 10^6 ufc/ml e 0.119 (1/h), 0.181 (g/l.h) e 1.468 (g/g) at 10^7 cfu/ml, no reactor inoculado a 10^7 ufc/ml;

M. andauensis PBC-2 pode ser produzido em diferentes meios, utilizando subprodutos e resíduos provenientes da indústria alimentar, em particular da indústria de sumo de citrinos. O meio que proporcionou a maior biomassa de *M. andauensis* PBC-2 foi o meio YL (10 g/l de extracto de levedura e licor cítrico a uma concentração de açúcar de 12.5 g/l), e foi escolhida para a produção em STR população semelhante à fornecida obtida em ensaios de frascos;

A produção de *M. andauensis* PBC-2 em reactor mecanicamente agitado, usando o meio YL, permitir obter uma população viável de 3.4×10^8 ufc/ml, após 40 h de incubação. A produtividade de biomassa e o rendimento de biomassa foram 0.435 g/l.h e 1.502 g/g, respectivamente;

O licor, resíduo líquido da indústria de sumos de citrinos, pode ser usado como fonte de carbono de um meio de cultura económico. A sua utilização representa uma alternativa para um resíduo, com elevada carga poluente, de difícil tratamento, e que pode comprometer a continuidade desta actividade industrial, com efeitos devastadores para a economia local;

A actividade antagonista das células de ambos os agentes de biocontrolo produzidas com subprodutos, como fontes de carbono, foi semelhante ao obtido com células produzidas em meio padrão;

A eficácia da *M. andauensis* PBC-2 foi confirmada durante quatro campanhas, em ensaios semi-comerciais, simulando as condições vulgarmente utilizadas durante o armazenamento destes frutos. Os resultados obtidos com a aplicação do potencial agente

de biocontrolo foram comparáveis à eficácia obtida com a aplicação do fungicida Imazalil, aplicado na concentração recomendada;

M. andauensis PBC-2 apresentou uma excelente capacidade de colonizar feridas, bem como de crescer na superfície do fruto, com uma população apreciável durante e após o armazenamento refrigerado;

A 37 º C, a temperatura do corpo humano, *M. andauensis* não cresceu no fluido gástrico simulado, indicando que este potencial agente de biocontrolo não tem capacidade de crescer no estômago humano;

A produção de enzimas líticas, tais como β-1,3 glucanases, proteases e quitinases, bem como, competição por ferro, não são o modo de ação de *M. andauensis* PBC-2.

FUTURE PERSPECTIVES

This thesis opens a series of possibilities concerning the development of two novel biological control agents, *M. andauensis* PBC-2 and the strain PBC-1 of *Pantoea agglomerans*.

Ongoing and future work includes:

- Optimize design operation conditions for semi-industrial and industrial biomass production,
- Increase biomass productivity and biomass yield with the by-products already used in this work and search for new alternative sources. Study the combination of different by-product, at higher concentrations, in order to avoid the premature source depletion,
- Economical analysis of production bioprocesses for both biocontrol agents, considering different modes and scales production scenarios,
- Study the effect of stress conditions, like shock temperature and pH modifications, on biomass production and its effect on antagonist activity
- Establish and optimize the BCA production in Fed-batch ,
- Development of molecular tools and a quality control system that allows to monitor the genetic stability over time of the biocontrol agent as a commercial product,
- Combination with other methods in an integrated vision of disease management, specifically reduced dosages of fungicides, chemical and physical methods
- Enhancement of BCA effectiveness with addiction of some salts to the culture media,
- Improve the knowledge on which kind of mode of action against pathogens could be involved ,
- Study the effect of BCA application with different nutrients, sources of carbon or nitrogen, to understand which of them are involved in the mechanism of competition,
- Additional safety tests, such as the acute oral toxicity which is important from a toxicological point of view and BCA registration

- BCA formulation studies to extend the shelf-life of the product, facilitating the storage for periods ranging from 6 months to 2 years and permit the product access to the market

PERSPETIVAS FUTURAS

Esta tese abre uma série de possibilidades, relativa ao desenvolvimento de dois novos agentes de controlo biológico, *M. andauensis* PBC-2 e de uma estirpe PBC-1 de *Pantoea agglomerans*.

Os trabalhos em curso e futuros incluem:

- Otimizar as condições de produção de biomassa dos agentes de biocontrolo, a nível semi-industrial e industrial,
- Aumentar a produtividade e o rendimento de biomassa com os subprodutos já utilizados neste trabalho e procurar novas fontes alternativas. Estudar o feito combinado de diferentes subprodutos e a concentrações mais elevadas de forma a evitar o esgotamento nas primeiras horas,
- Estudo económico do processo de produção, para ambos os agentes de biocontrolo, considerando diferentes modos e escalas de produção,
- Estudar o efeito de condições de stress, como choques térmicos alteração do pH, na produção de biomassa e de seu efeito sobre a actividade antagonista,
- Estabelecer e otimizar a produção dos agentes de biocontrolo em modo "Fed-batch" ,
- Desenvolvimento de ferramentas moleculares e de um sistema de controlo de qualidade, que permita monitorizar a estabilidade genética ao longo do tempo, como um produto comercial,
- Combinação dos agentes de biocontrolo com outros métodos, numa visão integrada da gestão da doença, nomeadamente combinação com métodos físicos, químicos e doses reduzidas de fungicidas autorizados,
- Melhorar a eficácia dos agentes de biocontrolo com introdução de sais no meio de produção,
- Aprofundar o estudo dos possíveis modos de ação envolvidos ,
- Estudar o efeito da aplicação do agente de biocontrolo com diferentes nutrientes, fontes de carbono ou azoto, para compreender se estão envolvidos no mecanismo de competição,
- Realizar testes adicionais de segurança, tais como a toxicidade oral aguda, de extrema importância do ponto de vista toxicológico e de registo

- Estudos de formulação do agente de biocontrolo, para prolongar a validade do produto, facilitando o armazenamento por períodos de 6 meses a 2 anos e assim permitir o acesso do produto ao mercado