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ORIGINAL PAPER

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

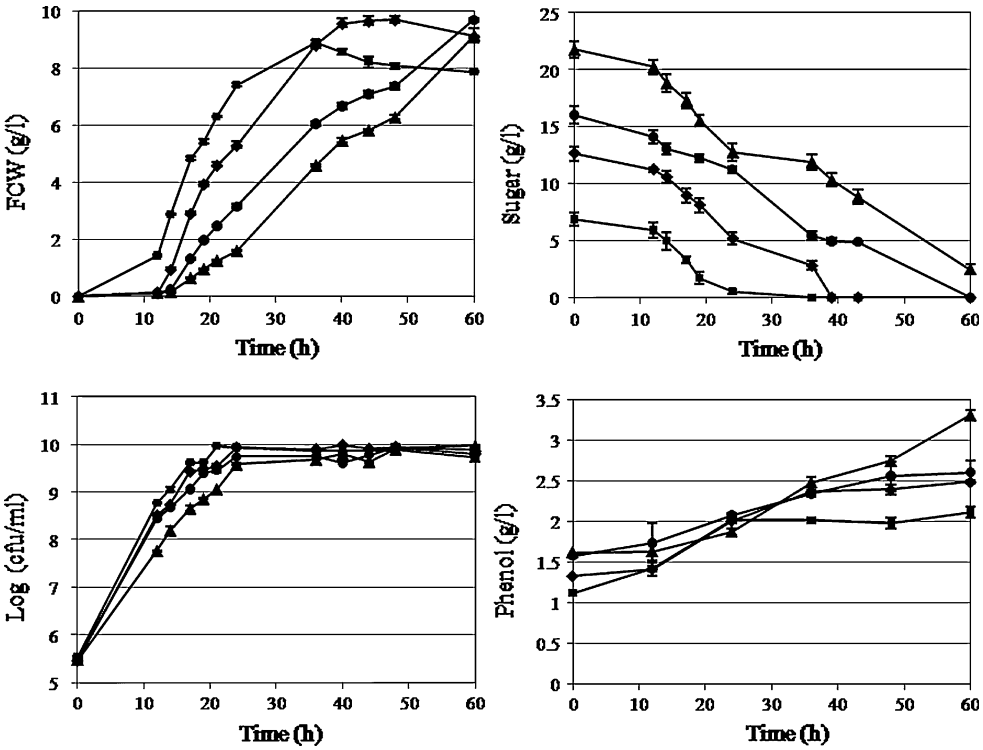

Fig. 1 Growth of *P. agglomerans* PBC-1 with different sugar concentration 5 g l^{-1} (*squares*), 10 g l^{-1} (*diamonds*), 15 g l^{-1} (*circles*), 20 g l^{-1} (*triangles*) from carob extracts. **a** Fresh cell weight (*FCW*), g l^{-1}; **b** sugar depletion, g l^{-1}; **c** viable cells, expressed 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

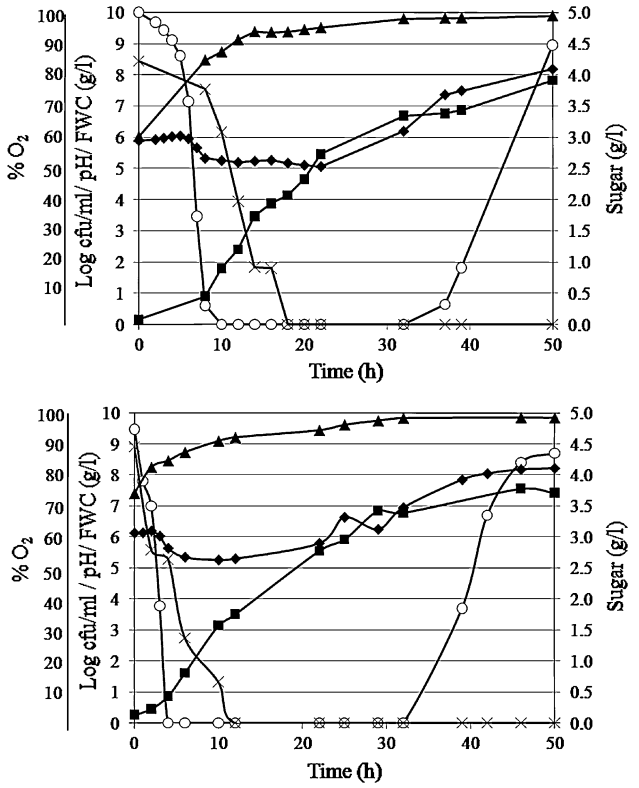

Fig. 2 Time course of viable cells cfu ml^{-1} (*triangles*), fresh cell weight (*squares*), pH value (*diamonds*), oxygen concentration (*circles*), consumption of sugar (*times*) in batch cultivation of *P. agglomerans* PBC-1, performed with aeration flux of 100 l h^{-1}, L-shaped sparger, 250 rpm, 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_La 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_La 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.

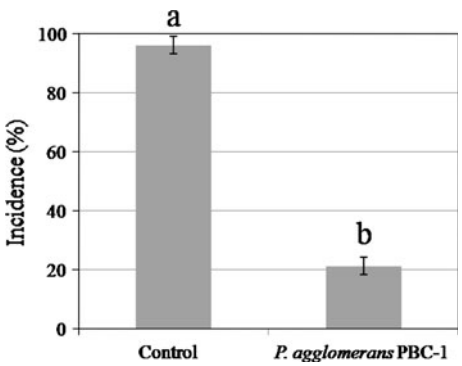

Fig. 3 Incidence of blue mould on wounded 'Golden Delicious' apples treated with *P. agglomerans* PBC-1 at 10^8 cfu ml^{-1}, followed by inoculation with the pathogen, *Penicillium expansum*, at 10^4 spores ml^{-1} after 7 days of incubation at 20 ± 1°C and 80 ± 5% RH. Each fruit was wounded twice. Ten 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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