Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Influence of fermentation temperature and duration on survival and biocontrol efficacy of *Pseudomonas fluorescens* Pf153 freeze-dried cells

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Keywords
agriculture, bioprocessing, production, pseudomonads, shelf life.

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2019/0601: received 5 April 2019, revised 8 August 2019 and accepted 18 August 2019

doi:10.1111/jam.14458

Abstract

Aim: The aim of this paper was to determine whether the quality of formulated *Pseudomonas fluorescens* Pf153 can be influenced by changes in fermentation conditions. In this study, the influence of the fermentation temperature on the growth and its consequence on shelf life, viability and biocontrol efficacy of freeze-dried cells of *P. fluorescens* Pf153 was investigated.
Methods and Results: Cells of *P. fluorescens* Pf153 were grown at 20 and 28°C in flasks and fermenter and harvested in the mid-log and the beginning of the stationary phase. The survival during storage of freeze-dried cells was tested at 25°C. Cells fermented at 20°C survived in storage better than those grown at 28°C, irrespective of the harvesting time. Compared to the untreated control, in *in vitro* tests Pf153 was in all production temperature/duration combinations significantly effective against all tested *Botrytis cinerea* strains. But no differences between temperature/duration combinations were found. In bioassay on detached *Vicia faba* leaves, it was found that young cells, when fermented at 28°C had a significant positive influence on the biocontrol efficacy.
Conclusions: These results demonstrate that fermentation parameters have an influence on the performance and quality of a formulated product.
Significance and Impact of the Study: Only limited numbers of biocontrol products based on antagonistic pseudomonads are on the market. This can be attributed to the lack of suitable formulated products with high numbers of viable cells and a good shelf life. Currently, only limited information on the influence of the fermentation on subsequent downstreaming process is available. Within this study, we focused on the influence of the two important parameters fermentation temperature and harvest time on survival, shelf life and biocontrol efficacy of *P. fluorescens* Pf153.

Introduction

Although many micro-organisms have shown potential as biological control agents (BCAs) against plant pathogens in small scale experiments, their production for commercial use remains a major challenge. It requires not only a successful scale-up of the fermentation process but also the effective formulation of the organisms (Lee *et al.* 2006). In addition, the end product should be easy to handle and store (e.g. at room temperature) and to be easily applied (Angeli *et al.* 2016). *Pseudomonas* sp. are fast growing bacteria which can colonize plants roots and phyllosphere. They produce a large spectrum of bioactive metabolites like siderophores, volatiles and growth promoting substances, assist the plant in adapting to environmental stresses, are highly competitive with other micro-organism and can suppress soilborne pathogens. *Pseudomonas* sp. also produce antibiotic compounds

(Bhattacharjee and Dey 2014). Several strains of *P. fluorescens* have been shown to antagonize plant pathogens and have thus been considered BCAs (Manikandan *et al.* 2010; Jain and Das 2016). However, despite the proven potential as biocontrol agents, there are few commercial products based on *Pseudomonas* sp. on the market. Currently there are only two products in the European Union, (https://ec.europa.eu) and nine in the USA (https://www.epa.gov) that list *Pseudomonas* as active ingredients. One of the main reasons is the difficulty to formulate these bacteria for commercial use. *Pseudomonas* sp. are extremely sensitive to environmental stress factors and the available formulations rapidly loose viability (Paulitz and Belanger 2001; Corrêa *et al.* 2015).

Several variables affect survival: the fermentation medium, the physiological state at harvest time, the protective materials used during the formulation process and the type of drying technology (Bashan *et al.* 2014). The formulation process is designed to promote diverse BCA traits like survival in soil, production of antimicrobial compounds and disease suppression effectiveness. This influence can be positive or negative (Fuchs *et al.* 2000). To retain the biological traits of the formulated final product during extended storage is another major challenge for any formulation (Bashan *et al.* 2014). Fuchs *et al.* (2000) also considered that the cultivation conditions prior to formulation can influence the BCA traits positively or negatively.

Among the options for formulation of micro-organisms, freeze-drying is considered a gentle dehydration process, suitable to achieve a stable powder formulation. It is the most common technique for drying and storing bacteria and especially suitable for *P. fluorescens* (Mputu 2014; Stephan *et al.* 2016). By changing freezing rates, drying temperatures and cryo-protective agents, it was demonstrated that the viability of *P. fluorescens* after freeze-drying can be optimized (Stephan *et al.* 2016). However, due to intrinsic characteristics, like cell size and membrane composition, the process has to be optimized for each strain. Also, external conditions like presence of nutrients and freezing and drying parameters influence the resistance to lyophilisation and storage. Protective additives help to reduce cells damage during freeze-drying (Tanimomo *et al.* 2016). Fermentation parameters also influence the cell, its freeze-drying resistance and also the subsequent biocontrol efficacy. In previous studies, we had the first indications that growth temperature and a mild heat shock can influence the survival rate after freeze-drying. (Bisutti *et al.* 2015). But in that study we were not able to prove whether it was an effect of temperature or fermentation time.

Media composition, for example, influences biomass production (He *et al.* 2008; Angeli *et al.* 2016) and the production of secondary metabolites like antibiotics (He *et al.* 2008; Gao *et al.* 2016). Shelf life is one important factor influencing the acceptance of plant protection products. Shelf life of at least 1 year at room temperature is desired, and the final product should deliver a certain quantity in micro-organism cells or spores even after longer storage (Bashan *et al.* 2014).

During previous studies, it was found that freeze-dried *P. fluorescens* Pf153 (Pf153) cells reduced the infection symptoms of *Botrytis cinerea* on *Vicia faba* leaves. During those studies, we found that Pf153, fermented at 20°C for 16 h in KB medium in flasks and freeze-dried in the presence of lactose as lyoprotectant, showed increased efficacy against *B. cinerea* on detached broad bean leaves compared to the other tested fermentations parameters (Bisutti *et al.* 2015). In another study, fermentation media composition influenced the efficacy of Pf153 against *Phomopsis sclerotioides* mediated black root rot on cucumber (Fuchs *et al.* 2000). Based on the information that lower fermentation temperature positively influenced the biocontrol activity of Pf153 against *B. cinerea*, the aim of the present study was to assess the effect of fermentation duration and temperature on the efficacy and storage stability of freeze-dried cells of this bacterium.

Materials and methods

Preparation of precultures of *Pseudomonas fluorescens* Pf153

Strain Pf153 was provided by the ETH Zürich and routinely cultivated on tryptic soy agar plates (TSA; 30 g tryptic soy broth (Difco, Difco Laboratories, Detroit, USA) and 15 g agar-agar (Roth, Germany) in 1000 ml de-ionised water) at 25°C. To prepare the preculture inocula, cells were transferred by loop from TSA plates to 50-ml Erlenmeyer flasks containing 30 ml autoclaved King's medium B (KB; 20 g proteose peptone No. 3 (Difco), 10 ml glycerol (Merck, Darmstadt, Germany), 1·5 g KH_2PO_4 (Merck), 1·5 $MgSO_4 \times 7\ H_2O$ (Merck) (King *et al.* 1954) and incubated at 28°C for 24 h at 150 rev min^{-1} on a rotary shaker (Novotron, Infors, Switzerland).

Preparation of *Botrytis cinerea* inoculum

Botrytis cinerea provided by the JKI (Institute for Biological Control and Institute for Breeding Research on Fruit Crops: strains Bc111, 63444, 68731, 63451 and 62084) was grown on modified Czapek Dox agar (30 g skim milk powder (Saliter, Obergünzburg, Germany), 3 g $NaNO_3$, 1 g $K_2HPO_4 \times 3\ H_2O$ (Merck), 0·5 g $MgSO_4 \times 7\ H_2O$, 0·5 g KCl, 0·01 g Iron (II)-sulphate $\times$ 7 H_2O (Merck) and 18 g agar-agar brought up

to 1000 ml with de-ionised water) in Petri dishes (diameter 9 cm) for 3 weeks at 20°C. Conidial suspensions were prepared by flooding the plates with 0·1% malt extract solution (Merck), gently scraping the mycelium with a spatula and filtering the resulting suspension through three layers of muslin in a glass funnel. The concentration was adjusted to 5×10^5 conidia per millilitre after counting the conidia with a Thoma haemocytometer.

Cultivation and preparation of *P. fluorescens* Pf153

Strain Pf153 was cultivated in 100-ml Erlenmeyer flasks or in a 4-l fermenter. The flasks and the fermenter were filled with 30 ml and 3·5 l KB medium, respectively, and autoclaved at 121°C for 20 min. After cooling, precultures prepared as described above were added to the medium at a ratio of 1 : 100 and fermented at 20 or 28°C. At the end of the fermentation time, the cells were harvested and processed as described by Bisutti *et al.* (2015). Briefly, after repeated centrifugation, a cell suspension with optical density (OD_{595}) of 0·95 (±0·02) was mixed at a ratio of 1 : 1 with a 20% (w/v) autoclaved lactose solution. Three millilitres of the mixture was transferred to sterile glass vials and freeze-dried with a freezing rate of 0·04–0·12°C min^{-1} to −40°C and followed by drying for 18 h at −20°C and 0·15 mbar. At the end of the process, the vials were sealed under 0·15 mbar and kept in the freezer (−18°C) until use.

Determination of the number of viable cells

The freeze-dried product was made up to the original volume (3 ml) by adding sterile de-ionised water, and viable cells of Pf153 in fresh cultures and the freeze-dried preparation were enumerated using serial dilutions and determination of MPN as described in Bisutti *et al.* (2015).

Determination of the fermentation parameters

The experiment was performed in a 4-l fermenter (ISF-100; Infors) at 20 or at 28°C with 900 rev min^{-1} agitation and 3·70 Nl min^{-1} air supply. Formation of foam was avoided by adding Antifoam (Roth, Karlsruhe, Germany) in automatic mode. Samples were taken every 4 h. The cell density was measured as OD_{595} using a SPECTRA Mini AP (Tecan, Tecan GmbH, Crailsheim, Germany Switzerland). Samples were freeze-dried after the addition of a 20% (w/v) solution of lactose to the cell suspension in ratio of 1 : 1. Freeze-drying followed the protocol described above. The experiment was repeated in five independent runs for fermentation at 28°C and three runs for fermentation at 20°C.

Bioassay against *B. cinerea* on detached *Vicia faba* leaves

The design of the bioassay was as described by Bisutti *et al.* (2015). Briefly, three compound leaves of *V. faba* cv 'con Amore' with four leaflets were placed together on a steel wire mesh with soaked filter paper underneath, in 20 × 20 × 5 cm plexiglass boxes covered with a translucent lid (Gerda GmbH, Schwelm, Germany). Freeze-dried cells of Pf153 produced in flasks as described above, were suspended with de-ionised water to a concentration of 5×10^8 MPN per ml. One millilitre of this suspension was sprayed onto each leaf surface using an air-brush sprayer; pathogen inoculation followed immediately by spraying 1 ml conidial suspension of *B. cinerea* on the upper side of the leaf. The boxes were then incubated at 20°C with a day/night cycle of 16/8 h. As control treatments, water and a 0·2% (w/v) water suspension of Euparen (active ingredient: 50% Tolylfluanid, WG) were used. The disease severity was rated 5 days after inoculation by estimating the affected per cent leaf area using the following rating scale: 0 = No lesions, 1 = 0–1%, 2 = 2–5%, 3 = 6–10%, 4 = 11–25%, 5 = 26–50%, and 6 = 51–100%. A disease index (DI) was calculated as $DI = \frac{\sum(\text{rating value} \times \text{number of leaves in the rating})}{\text{total number of leaves} \times \text{highest rating value}} \times 100$. The experiment included four boxes per treatment and was repeated three times.

Shelf life of freeze-dried cells of *P. fluorescens* Pf153

The cells were produced in 100-ml Erlenmeyer flasks filled with 30 ml KB medium. The flasks were kept at 20 or 28°C on two identical rotary shakers at 150 rev min^{-1}. Samples were then harvested at different times: 16 and 28 h for incubation at 20°C and 8 and 16 h for incubation at 28°C. Afterwards the samples were freeze-dried as described above. For measuring the storage stability, samples were incubated at 25°C in the dark. Bacterial viability was determined once a week by analysing two vials per sample. The experiment was time-independent and was repeated four times.

Influence of incubation temperature on growth in the fermenter and survival after freeze-drying of *P. fluorescens* Pf153

The fermenter (Minifors, Infors) was kept at 20 or at 28°C, 900 rev min^{-1} agitation and 3·70 Nl min^{-1} air supply. Foam formation was avoided by adding Antifoam (Roth) in automatic mode. Five hundred millilitres of the cell suspension was harvested in the middle and at the end of the log phase corresponding at 16 and 28 h for the fermenter at 20°C and 8 and 16 h for the fermenter

at 28°C respectively. Thereafter, the samples were freeze-dried as described above and kept in the freezer at −18°C until use. The experiment was time independently repeated three times.

In vitro tests of the influence of fermentation temperature and duration on the efficacy of *P. fluorescens* Pf153 against different *B. cinerea* strains

The efficacy of freeze-dried cells of Pf153 produced in a fermenter at 20 or 28°C, respectively, was tested *in vitro* against five strains of *B. cinerea*. The test was performed on two media differing in carbon source: 1/10 PDA and PDFA (PDFA sugar content of fructose and glucose 2 mg ml^{-1} based on PDA (Hjeljord and Strømeg 2004)). One hundred microlitres of each cell suspension of Pf153 was prepared as described above, lactose solution (10% w/v) or Euparen (0·2% w/v) was pipetted in the middle of a 9 cm Petri plate and spread with a Drigalski spatula. Immediately thereafter, the plates were inoculated with *B. cinerea* by placing a 5 mm mycelial plug of a culture from modified Czapek Dox agar in the centre of the plates. The plates were then placed in an incubator at 20°C with a 16/8 h night and day cycle. Radial mycelial growth was assessed at 4, 7, 10 and 16 days after inoculation (DAI) by measuring the diameter along two perpendiculars. A ranking based on the efficacy $\left[\frac{\text{Mycelial growth with lactose} - \text{mycelial growth with Pf153}}{\text{Mycelial growth with lactose}} \times 100\right]$ of the four Pf153 fermentations was made with the relative performance index (RPI) calculated for each fermentation temperature/duration combination. RPI for efficacy is dimensionless and is between 0 and 100 because efficacy improves as disease rating decreases. The applied formula was $\text{RPI} = \left|\frac{x - x_{av}}{\sigma} - 2\right| \times 25$ where x is a single observation value for a strain, x_{av} is an average of all observations from all strains and σ is the standard deviation of all observations from all strains being ranked (Schisler and Slininger 1997). Three Petri dishes were used for each fermentation temperature and *B. cinerea* combination. The test was repeated three times.

Statistical analysis

Data were statistically analysed with the software SAS System for Windows ver. 9.3. Experiments with freeze-drying of Pf153 were analysed using the generalized linear model. The Shapiro–Wilk test was applied for testing for normality. The homogeneity of variance was proven/checked by the Levene test ($P < 0{\cdot}1$). For separation of the means, log10 transformed data were compared with the Student–Newman–Keuls test (SNK) ($P < 0{\cdot}05$). As no homogeneity of variance or normality was achieved in bioassays, the nonparametric test of Kruskal–Wallis was chosen. Following methods of the NPAR1WAY procedure (two-sided), the samples were compared in pairwise analysis (Wilcoxon, exact $P < 0{\cdot}05$).

Results

Determination of the fermentation parameters

Growth profiles of Pf153 fermented at 20 and 28°C measured as OD of the cultures are shown in Fig. 1 left. The exponential phase of the cells cultured at 20°C was time delayed by about 8 h in comparison to the cells fermented at 28°C. The beginning of the stationary phase was reached after 16 h for cells fermented at 28°C and after 28 h for cells fermented at 20°C. The experiment was stopped after 24 h for the fermentation at 28°C. The samples were also freeze-dried. The survival rates after freeze-drying are shown in Fig. 1 (right). Freeze-dried cells from the beginning of the log phase cultured at 20 and 28°C showed a reduction in survival. Directly after the beginning of the log phase, the survival after freeze-drying increased with a maximum survival at the stationary phase for cells fermented at 28°C, while for cells grown at 20°C the values fluctuated between 80 and 100%.

Influence of incubation temperature on growth of *P. fluorescens* Pf153 in the fermenter and survival after freeze-drying

When cells were cultivated in the fermenter, the cell yield was highest for the cells fermented for 28 h at 20°C followed by those fermented for 16 h at 28°C. Freeze-drying of cells caused a reduction in the number of viable cells (Table 1), statistically significant for the cells harvested at beginning of the stationary phase but not for those harvested in the middle of the log phase for both temperatures.

Shelf life of freeze-dried cells of *P. fluorescens* Pf153

The samples of freeze-dried cells were stored at 25°C for 12 weeks and MPN per millilitre was assessed each week (Fig. 2). After 10 weeks, the number of viable cells was reduced over time for all fermentation parameter combinations from about 10^9 to $<10^6$ MPN per millilitre for cells fermented at 28°C/8 h. The trend was for cells fermented at 20°C to show slightly better survival than those fermented at 28°C. Assessment at week 12 showed cell densities between 10^6 and 10^7 MPN per ml, with the exception of 28°C/8 h.

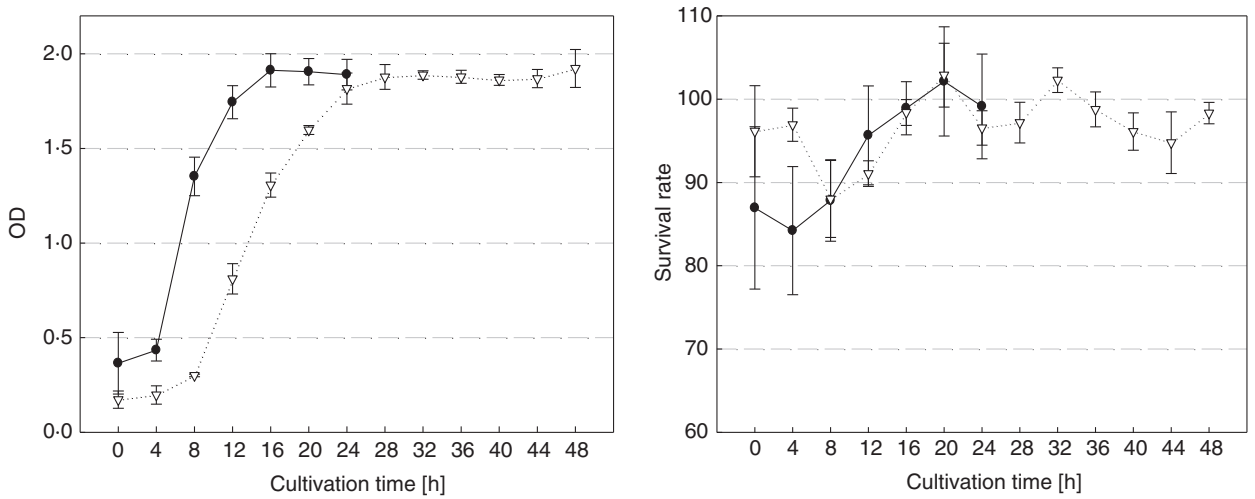

Figure 1 Growth curves for Pf153 grown at 20°C (triangle down) and 28°C (dots) in a fermenter measured as OD at 595-nm (left); Survival rates (calculated by dividing the log MPN after freeze-drying by the log MPN before freeze-drying, multiplied by 100).

Table 1 Number of viable cells direct after harvest and before and after the freeze-drying process of Pf153 fermented at different temperature for different times

Fermentation parameters	Yield*	Before freeze-drying*	After freeze-drying*
28°C/8 h	4·11 (±1·51) × 10^9 (B)	9·62 (±4·68) × 10^8 (a)	5·22 (±2·38) × 10^8 (a)
20°C/16 h	5·03 (±0·62) × 10^9 (B)	2·34 (±1·40) × 10^9 (a)	1·05 (±0·70) × 10^9 (a)
28°C/16 h	8·33 (±2·16) × 10^9 (B)	1·57 (±0·79) × 10^9 (a)	4·29 (±1·80) × 10^8 (b)
20°C/28 h	1·37 (±0·76) × 10^{10} (A)	1·13 (±0·34) × 10^9 (a)	5·64 (±4·84) × 10^8 (b)

Suspension prepared for freeze-drying had an OD of 0·95 ± 0·01.
Means of the yield data followed by the same letter are not significantly different following SNK test ($P < 0·05$).
Means of the fermentation parameters lines for before and after freeze-dried, followed by the same letter are not significantly different following SNK test ($P < 0·05$).
*Viability is expressed as MPN per millilitre and the reported data are mean (±SD) of three independent experiments.

In vitro tests of the influence of fermentation temperature and duration on the efficacy of *P. fluorescens* Pf153 against different *B. cinerea* strains

The assays for assessing the effect of co-inoculation of freeze-dried cells of Pf153 and *B. cinerea* were performed on the media PDFA and 1/10 PDA (Figs S1 and S2). On control (lactose) plates, the speed of mycelial growth was similar for the *Botrytis* strains tested except for strain 62084 that grew considerably faster than the other strains. Compared to the lactose controls, the radial growth of all strains was significantly reduced on plates co-inoculated with Pf153. The reduction pattern was similar on both media. A strong, significant reduction was recorded for the chemical Euparen (Figs S1 and S2; Table 2). The magnitude of the reducing effect of Pf153 on mycelial growth of the *B. cinerea* strains did not differ statistically between the different temperature/duration combinations used during fermentation of the bacterium (Table 2). At first sight (Figs S1 and S2; Table 2), no fermentation method showed an overall ability to reduce *B. cinerea* strains growth on both media. When calculating an RPI efficacy over all *B. cinerea* strains and media, the cells from the fermentation 20°C/28 h showed the highest and those from the fermentation 28°C/16 h the lowest efficacy (Table 3).

Bioassay against *B. cinerea* on detached *V. faba* leaves

Biocontrol efficacy of freeze-dried cells of Pf153 produced in Erlenmeyer flasks under different combinations of fermentation temperature and duration (20°C/16 h, 20°C/28 h, 28°C/8 h and 28°C/16 h) was assessed against *B. cinerea* on detached *V. faba* leaves (Fig. 3). All treatments reduced the disease severity significantly compared to the water control but not as effectively as the chemical Euparen (reduction respective to water control of 82·6%). The highest disease reduction was caused by cells harvested in the middle of the log phase (56·5% for 28°C and 48·5%

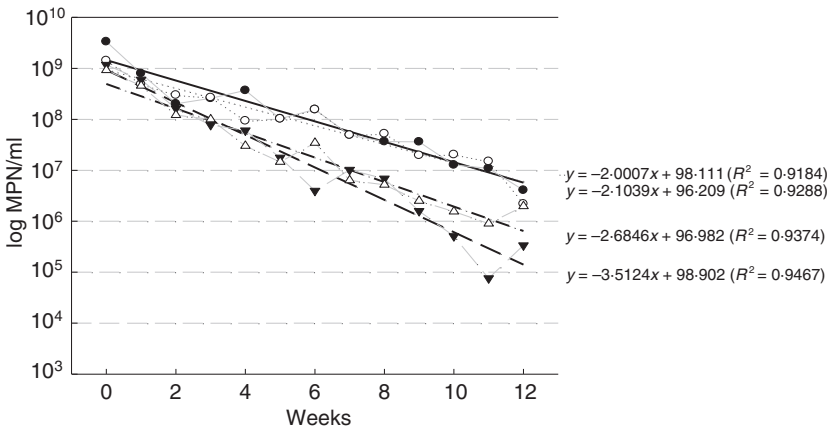

Figure 2 Influence of fermentation temperature and duration of Pf153 on viability (MPN per ml) during storage at 25°C for 12 weeks ((–•–) 20°C/16 h, (–○–) 20°C/28 h, (–▼–) 28°C/8 h, (–▲–) 28°C/16 h. (–○–) $y = -2{\cdot}0007x + 98{\cdot}111$ ($R^2 = 0{\cdot}9184$); (–•–) $y = -2{\cdot}1039x + 96{\cdot}209$ ($R^2 = 0{\cdot}9288$); (–▲–) $y = -2{\cdot}6846x + 96{\cdot}982$ ($R^2 = 0{\cdot}9374$); (–▼–) $y = -3{\cdot}5124x + 98{\cdot}902$ ($R^2 = 0{\cdot}9467$)).

Table 2 Reduction of radial mycelial growth of five strains of *Botrytis cinerea* on two different agar media in the presence of Pf153 or the chemical fungicide Euparen. Plates were inoculated with *B. cinerea* and freeze-dried cells of Pf153 from fermentations performed with different temperature/fermentation time combinations. Values are the percentage reduction of mycelial growth of *B. cinerea* relative to growth on control plates (lactose) determined after 10 days (strain 62084) or 16 days (all other strains) of co-cultivation

	Bc111		63444		68731		63451		62084	
	PDFA	*1/10 PDA*	*PDFA*	*1/10 PDA*	*PDFA*	*1/10 PDA*	*PDFA*	*1/10 PDA*	*PDFA*	*1/10 PDA*
Lactose	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)
28°C/8 h	56 (b)	53 (b)	40 (a)	30 (a)	28 (b)	31 (ab)	38 (b)	37 (b)	62 (b)	70 (b)
20°C/16 h	65 (b)	62 (b)	27 (a)	44 (a)	34 (b)	42 (b)	35 (b)	40 (b)	69 (b)	66 (b)
28°C/16 h	61 (b)	60 (b)	22 (a)	19 (a)	25 (b)	33 (ab)	38 (b)	32 (b)	64 (b)	69 (b)
20°C/28 h	65 (b)	61 (b)	31 (a)	38 (a)	38 (b)	37 (b)	46 (b)	43 (b)	71 (b)	71 (b)
Euparen	93 (c)	93 (c)	80 (b)	87 (b)	73 (c)	81 (c)	62 (c)	60 (c)	93 (c)	93 (c)

Statistics were made with the diametric mycelial growth values (means of three independent experiments), numbers within columns followed by the same letter are not significantly different according to the glimmix procedure.
No statistical differences were found between mycelial growth on 1/10 PDA and PDFA.

Table 3 Relative performance indices of Pf153 for reduction of radial mycelial growth of *Botrytis cinerea* (compare Table 2) on the media 1/10 PDA and PDFA

Fermentation parameters	1/10 PDA $RPI_{efficacy}$	PDFA $RPI_{efficacy}$	Combined $RPI_{efficacy}$
28°C/8 h	47·45	47·31	48·49
20°C/16 h	53·32	47·90	51·76
28°C/16 h	44·91	42·93	45·01
20°C/28 h	54·33	52·66	54·67

for 20°C grow temperature). Cells from the beginning of the stationary phase reduced disease at least by 37·9% (28°C/16 h).

Discussion

A standard industrial method for mass production of bacterial and fungal cells is liquid fermentation (Slininger and Shea-Wilbur 1995). In case of bacteria or fungi used in biocontrol, the aim was to obtain large amounts of viable micro-organism with high resistance to the formulation process, long shelf life and high efficacy (Ashofteh *et al.* 2009). In fact, when scaling up the production of BCAs, attention has to be paid to the efficacy because the process parameters yielding the best material for formulation are not necessarily optimal for producing biocontrol agents with a high efficacy (Spadaro and Gullino 2005; Angeli *et al.* 2016). Previously, a freeze-drying protocol was established specifically for Pf153 (Stephan *et al.* 2016) and the influence of different culture parameters on the process was studied. Additionally it was found that the level of biocontrol achieved was influenced by the fermentation temperature (Bisutti *et al.* 2015). In the present study, we therefore evaluate the influence of the fermentation parameters temperature and harvesting time on the efficacy against

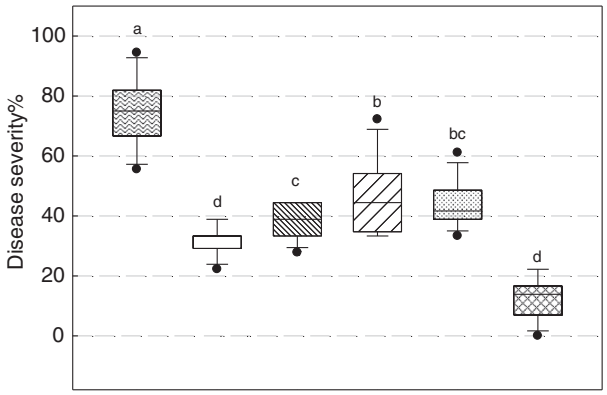

Figure 3 Disease severity of *Botrytis cinerea* in a bioassay on detached leaves of *Vicia faba* with freeze-dried preparations from Pf153 cultured at different combinations of the fermentation parameters temperature and duration of culture. The box plots represent the minimum, 25–75%, maximum and median values of disease severity. Fermentation parameters in the legend followed by the same letter are not significantly different following SNK test ($P < 0{\cdot}05$) ((▨) water, (▭) 28°C/8 h, (▧) 20°C/16 h, (▨) 28°C/16 h, (▨) 20°C/28 h, (▨) Euparen).

the pathogen *B. cinerea* of formulated cells of Pf153 and cell survival during storage.

Growth curves were determined to evaluate the effect of temperature on growth kinetics by taking samples every 4 h and plotting the measured OD against time. Cells grown at 28°C reached the stationary phase faster than cells grown at 20°C (Fig. 1). However, the yield in the mid-exponential phase at 20°C was 18·3% higher than at 28°C and at the beginning of the stationary phase this difference was even higher (39·2%) (Table 1). Increasing temperature increases cell growth but its influence on cell stability during formulation is not always positive. For example for lactic acid bacteria and other probiotics, an increase of the temperature to 37°C influenced positively their growth, however, this temperature was not the best to obtain cells resistant to freeze-drying (Liu *et al.* 2014; Tanimomo *et al.* 2016). In the present study, survival rates after freeze-drying changed slightly between experiments. Survival rate after freeze-drying for cells fermented at 20°C for 16 h was comparable with data reported by Bisutti *et al.* (2015) but for cells fermented at 28°C for 16 h the survival was lower than in that study (Table 1). When considering viability after freeze-drying through all experiments (also from data not shown), the harvesting time seemed not to have an influence on the survival of Pf153 (Fig. 1). Similar results were described by Saarela *et al.* (2005) for *Bifidobacterium animalis* ssp. *lactis*, however *Xanthomonas campestris* harvested during the early or late stationary phases showed a significantly higher survival than log phase-harvested cells (Jackson *et al.* 1998). In storage experiments, some *Pseudomonas* failed to survive storage, however, others lived up to 20 years. These different survival behaviours are attributed to the storage temperature: *Pseudomonas* survive better when stored at temperatures lower than 8°C as freeze-dried cells (Palmfeldt *et al.* 2003; Miyamoto-Shinohara *et al.* 2006). This better storage at standard refrigeration temperature was also reported for other Gram-negative bacteria like *X. campestris* (Jackson *et al.* 1998) and probiotic bacteria (Saarela *et al.* 2005; Tanimomo *et al.* 2016). Storage below freezing temperatures could be one major obstacle to the large-scale use of *Pseudomonas* formulations (Slininger *et al.* 1996; Stockwell and Stack 2007) because not all consumers can afford equipment to keep the product at low temperature. In our trials, we stored the vacuum sealed vials containing the freeze-dried Pf153 at 25°C for up to 12 weeks and tested each week the viability by MPN per millilitre (Fig. 2). The survival was reduced for all culture conditions while cells fermented at 28°C showed a faster reduction than the 20°C cultivated cells, especially after the fifth week of storage. At week 9, the survival rate of cells fermented at 28°C was as low as the 20°C fermented at week 12. For both temperatures, cells harvested at the beginning stationary phase showed better survival. For the commercially available bioproducts containing *Pseudomonas*, the reported storage temperature from −18 to 8°C resulted in a shelf life ranging from 8 months to weeks. At room temperature, the product containing *P. chlororaphis* is warranted for 3 weeks (Berninger *et al.* 2018). The formulate of *Arthrobacter chlorophenolicus* A6, which can be used for bioremediation, was stable and viability for 3 months when stored at 4°C, without efficacy reduction compared to the fresh cells (Bjerketorp *et al.* 2018). Pf153 was stable after 8 weeks at 25°C, with still at least $5{\cdot}2 \times 10^7$ MPN per millilitre. The shelf life of freeze-dried Pf153 is, therefore, in the given interval for other *Pseudomonas*. Various mechanisms, like culture physiology and environment, have been described to influence cell survival. Multiple cell protection mechanisms that occur at different times during culturing can result in differences in drying and storing survival characteristics of cells of different age (Slininger *et al.* 1996). This was shown for *P. putida* where culture conditions and physiological state notably influenced the bacterial tolerance to the freeze-drying process (Muñoz-Rojas *et al.* 2006). The authors proposed that this may be related to changes in membrane properties during growth. Temperature, pH, culture medium composition and the transition to the stationary phase alter the fatty acid content of the cell membrane of *P. fluorescens*. These modifications change the membrane viscosity and influence cellular functions including cell growth and stability (Fouchard *et al.* 2005). We presume that the different storage

behaviour is due to changes in the phospholipid fatty acids of the cells with increased ratio of unsaturated to saturated fatty acids making the cell more resistant to the freeze-drying conditions used and consequently for storage. An increase in unsaturated fatty acids in the cell membrane maintains fluidity and stability and decreases membrane leakage during rehydration (Liu *et al.* 2014).

The influence of the different fermentation procedures on the efficacy of Pf153 was tested against *B. cinerea* on *V. faba* leaves and against five different *B. cinerea* strains *in vitro*. In the experiments on detached leaves, a significant reduction of infection was shown for young cells especially when grown at 28°C (Fig. 3). Therefore, it could be shown for the first time that the cell age is influencing the survival after freeze-drying and the biological activity of freeze-dried cells. The *in vitro tests* were made with two media differing in sugar composition. The medium PFLA was proposed by Hjeljord and Strømeg (2004) when investigating BCAs against *B. cinerea* on strawberry blossoms. The authors developed the medium on the basis of the extract composition of the blossoms (Hjeljord and Strømeg 2004). In our case none of the temperature/duration combinations used with Pf153 was better against all *B. cinerea* strains and no statistical differences were found between the media used in the test (Table 2). When considering RPI, the cells grown at 20°C show better performance than the ones grown at 28°C despite the age (Table 3). The five *Botrytis* strains showed different growth rates on the two media and sensitivity to the chemical control Euparen (Figs S1 and S2). These different results between detached leaves and on plate assays can be caused by the test system, the diverse *B. cinerea* isolates but also from different growth rates. When the level of antagonism depends on the growth rate of the strain, this should be considered when ranking the potential antagonists by dual culture assays (Comby *et al.* 2017). Anyhow, our results were similar to the results of Bardin *et al.* (2013b) who showed that the efficacy of a BCA can vary, in certain conditions, depending on the *B. cinerea* strain. In test with *Microdochium dimerum* and Serenade Max® (*Bacillus subtilis* QST713) against different *B. cinerea* strains on tomato and lettuce plants, the protection provided by the BCAs was significantly influenced by the pathogen strain and its aggressiveness level (Bardin *et al.* 2013a; Bardin *et al.* 2013b). In other studies, variations in the sensitivity to antimicrobial compounds produced by BCAs to pathogens isolates were shown: resistance or tolerance to 2,4-diacetylphloroglucinol for *G. graminis* or *Fusarium oxysporum* and a wide range of sensitivity against pyrrolnitrin for *B. cinerea* (Bardin *et al.* 2015). *Botrytis cinerea* is a weak plant pathogen that can antagonize other micro-organisms on plant surfaces. For example, it produces botrydial and botrycine, active against Gram-positive bacteria and fungi or yeast respectively (Blakeman and Fokkema 1982). *Pseudomonas* can lyse fungal cells and degrade metabolites produced by pathogens that induce pathogenesis (Bhattacharjee and Dey 2014). Its ability to suppress growth and to modify hyphal structure was shown for *B. cinerea* (Blakeman and Fokkema 1982; Barka *et al.* 2002) and *Drechslera dictyoides* (Blakeman and Fokkema 1982). On the other hand, *Pseudomonas* can stimulate germination and infection of certain leaf-infecting pathogens on leaf and fruit surfaces (Blakeman and Fokkema 1982). It is reported that many phytopathogenic fungi are sensitive to volatile compounds produced by bacteria. Hydrogen cyanide, for example, is thought to be involved in root pathogen suppression (Pal and Gardener 2006) and fluorescent pseudomonads biocontrol activity and hydrogen cyanide production ability are hypothesized to have a close relationship with virulence (Jain and Das 2016). Pf153 synthesizes different antifungal compounds including hydrogen cyanide and an extracellular protease (Fuchs *et al.* 2000).

The performance of a biopesticide is enhanced by fermentation and formulation which are closely linked (Hynes and Boyetchko 2006). When optimizing the production of a *Pseudomonas*, the growth conditions need to be considered carefully (Fuchs *et al.* 2000). For quality control, it is also important to consider several target pathogen strains to obtain a better representation of the pathogen population (Bardin *et al.* 2013b) in the field. The improvement of BCAs during production allows the enhanced survival and activity under differing environmental conditions improving its potential as commercial product (Teixidó *et al.* 2005). Our experiments clearly demonstrate that by manipulating fermentation parameters it is possible to increase the storage survival of Pf153 (Fig. 2). In our case, fermentation at 20°C for 28 h showed the highest yield and storage survival at 25°C. Freeze-dried cells produced under these conditions showed the best performance in *in vitro* tests against different *Botrytis* strains when considering the RPI (Table 3) but not when tested on detached leaves (Fig. 3). This shows the importance of the efficacy testing system comprising pathogen diversity. However, only greenhouse and field trials could demonstrate if performance is also achievable in field conditions and if culture conditions have a stronger influence on field efficacy. To achieve a better quality and reliable efficacy of BCAs, optimization of production and formulation protocols are unavoidable.

Acknowledgements

This research was supported by SafeCrop Centre, funded by Fondo per la Ricerca, Autonomous Province of

Trento. We thank Eckhard Koch (JKI, Germany) and Travis Glare (Lincoln University, New Zealand) for proofreading the manuscript.

Conflict of Interest

The authors declare no conflict of interest on any of data published in this manuscript.

References

Angeli, D., Saharan, K., Segarra, G., Sicher, C. and Pertot, I. (2016) Production of *Ampelomyces quisqualis* conidia in submerged fermentation and improvements in the formulation for increased shelf-life. *Crop Prot* **97**, 135–144.

Ashofteh, F., Ahmadzadeh, M. and Fallahzadeh-Mamaghani, V. (2009) Effect of mineral components of the medium used to grow biocontrol strain UTPF61 of *Pseudomonas fluorescens* on its antagonistic activity against *Sclerotinia* wilt of sunflower and its survival during and after formulation process. *Journal of Plant Pathology* **91**, 607–613.

Bardin, M., Comby, M., Lenaerts, R. and Nicot, P.C. (2013a) Diversity in susceptibility of *Botrytis cinerea* to biocontrol products inducing plant defence mechanisms. *IOBC-WPRS Bull* **88**, 45–49.

Bardin, M., Comby, M., Troulet, C. and Nicot, P.C. (2013b) Relationship between the aggressiveness of *Botrytis cinerea* on tomato and the efficacy of biocontrol. *IOBC-WPRS Bull* **86**, 163–168.

Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillot, B., Siegwart, M. and Nicot, P.C. (2015) Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Frontiers in Plant Science* **6**, 1–13.

Barka, E.A., Gognies, S., Nowak, J., Audran, J.-C. and Belarbi, A. (2002) Inhibitory effect of endophyte bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. *Biol Control* **24**, 135–142.

Bashan, Y., de-Bashan, L.E., Prabhu, S.R. and Hernandez, J.-P. (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant Soil* **378**, 1–33.

Berninger, T., González López, Ó., Bejarano, A., Preininger, C. and Sessitsch, A. (2018) Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microb Biotechnol* **11**, 277–301.

Bhattacharjee, R. and Dey, U. (2014) An overview of fungal and bacterial biopesticides to control plant pathogens/diseases. *Afr J Microbiol Res* **8**, 1749–1762.

Bisutti, I.L., Hirt, K. and Stephan, D. (2015) Influence of different growth conditions on the survival and the efficacy of freeze-dried *Pseudomonas fluorescens* strain Pf153. *Biocontrol Sci Tech* **25**, 1269–1284.

Bjerketorp, J., Röling, W.F., Feng, X.-M., Garcia, A.H., Heipieper, H.J. and Håkansson, S. (2018) Formulation and stabilization of an *Arthrobacter* strain with good storage stability and 4-chlorophenol-degradation activity for bioremediation. *Appl Microbiol Biotechnol* **102**, 2031–2040.

Blakeman, J.P. and Fokkema, N. (1982) Potential for biological control of plant diseases on the phylloplane. *Annu Rev Phytopathol* **20**, 167–190.

Comby, M., Gacoin, M., Robineau, M., Rabenoelina, F., Ptas, S., Dupont, J., Profizi, C. and Baillieul, F. (2017) Screening of wheat endophytes as biological control agents against Fusarium head blight using two different in vitro tests. *Microbiol Res* **202**, 1120.

Corrêa, E., Sutton, J. and Bettiol, W. (2015) Formulation of *Pseudomonas chlororaphis* strains for improved shelf life. *Biol Control* **80**, 50–55.

Fouchard, S., Abdellaoui-Maâne, Z., Boulanger, A., Llopiz, P. and Neunlist, S. (2005) Influence of growth conditions on *Pseudomonas fluorescens* strains: a link between metabolite production and the PLFA profile. *FEMS Microbiol Lett* **251**, 211–218.

Fuchs, J.G., Moënne-Loccoz, Y. and Défago, G. (2000) The laboratory medium used to grow biocontrol *Pseudomonas* sp Pf153 influences its subsequent ability to protect cucumber from black root rot. *Soil Biol Biochem* **32**, 421–424.

Gao, X.N., He, Q.R., Jiang, Y. and Huang, L.L. (2016) Optimization of nutrient and fermentation parameters for antifungal activity by *Streptomyces lavendulae* Xjy and its biocontrol efficacies against *Fulvia fulva* and *Botryosphaeria dothidea*. *J Phytopathol* **164**, 155–165.

He, L., Xu, Y.-Q. and Zhang, X.-H. (2008) Medium factor optimization and fermentation kinetics for phenazine-1-carboxylic acid production by *Pseudomonas* sp M18G. *Biotechnol Bioeng* **100**, 250–259.

Hjeljord, L.G. and Strømeg, G.M. (2004) *Biological Control of Botrytis*: Searching for a Realistic Screen. Book of Abstracts XIII International Botrytis Symposium, Antalya, Turkey 63.

Hynes, R.K. and Boyetchko, S.M. (2006) Research initiatives in the art and science of biopesticide formulations. *Soil Biol Biochem* **38**, 845–849.

Jackson, M., Frymier, J., Wilkinson, B., Zorner, P. and Evans, S. (1998) Growth requirements for production of stable cells of the bioherbicidal bacterium *Xanthomonas campestris*. *J Ind Microbiol Biotechnol* **21**, 237–241.

Jain, A. and Das, S. (2016) Insight into the interaction between plants and associated fluorescent *Pseudomonas* spp. *Int J Agron*. http://dx.doi.org/10.1155/2016/4269010

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demostration of pyocyanin and fluorescin. *J Lab Clin Med* **44**, 301–307.

Lee, J.P., Lee, S.W., Kim, C.S., Son, J.H., Song, J.H., Lee, K.Y., Kim, H.J., Jung, S.J. *et al.* (2006) Evaluation of formulations of *Bacillus licheniformis* for the biological

control of tomato gray mold caused by *Botrytis cinerea*. *Biol Control* **37**, 329–337.

Liu, X., Hou, C., Zhang, J., Zeng, X. and Qiao, S. (2014) Fermentation conditions influence the fatty acid composition of the membranes of *Lactobacillus reuteri* I5007 and its survival following freeze-drying. *Lett Appl Microbiol* **59**, 398–403.

Manikandan, R., Saravanakumar, D., Rajendran, L., Raguchander, T. and Samiyappan, R. (2010) Standardization of liquid formulation of *Pseudomonas fluorescens* Pf1 for its efficacy against *Fusarium* wilt of tomato. *Biol Control* **54**, 83–89.

Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T. and Nakahara, T. (2006) Survival curves for microbial species stored by freeze-drying. *Cryobiology* **52**, 27–32.

Mputu, K.J.-N. (2014) Impact du séchage sur la viabilité de *Pseudomonas fluorescens* (synthèse bibliographique). *Biotechnol Agron Soc Environ* **18**, 134–141.

Muñoz-Rojas, J., Bernal, P., Duque, E., Godoy, P., Segura, A. and Ramos, J.L. (2006) Involvement of cyclopropane fatty acids in the response of *Pseudomonas putida* KT2440 to freeze-drying. *Appl Environ Microbiol* **72**, 472–477.

Pal, K.K. and Gardener, B.M. (2006) Biological control of plant pathogens. *Plant Health Instr* **2**, 1117–1142.

Palmfeldt, J., Rådström, P. and Hahn-Hägerdal, B. (2003) Optimisation of initial cell concentration enhances freeze-drying tolerance of *Pseudomonas chlororaphis*. *Cryobiology* **47**, 21–29.

Paulitz, T.C. and Belanger, R.R. (2001) Biological control in greenhouse systems. *Annu Rev Phytopathol* **39**, 103–133.

Saarela, M., Virkajärvi, I., Alakomi, H.L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T. and Mättö, J. (2005) Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of *Bifidobacterium animalis* ssp *lactis* cells produced without milk-based ingredients. *J Appl Microbiol* **99**, 1330–1339.

Schisler, D.A. and Slininger, P.J. (1997) Microbial selection strategies that enhance the likelihood of developing commercial biological control products. *J Ind Microbiol Biotechnol* **19**, 172–179.

Slininger, P.J. and Shea-Wilbur, M.A. (1995) Liquid-culture pH, temperature, and carbon (not nitrogen) source regulate phenazine productivity of take-all biocontrol agent *Pseudomonas fluorescens* 2–79. *Appl Microbiol Biotechnol* **43**, 794–800.

Slininger, P.J., VanCauwenberge, J.E., Bothast, R.J., Weller, D.M., Thomashow, L.S. and Cook, R.J. (1996) Effect of growth culture physiological state, metabolites, and formulation on the viability, phytotoxicity, and efficacy of the take-all biocontrol agent *Pseudomonas fluorescens* 2–79 stored encapsulated on wheat seeds. *Appl Microbiol Biotechnol* **45**, 391–398.

Spadaro, D. and Gullino, M.L. (2005) Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop Prot* **24**, 601–613.

Stephan, D., Da Silva, A.P.M. and Bisutti, I.L. (2016) Optimization of a freeze-drying process for the biocontrol agent *Pseudomonas* spp. and its influence on viability, storability and efficacy. *Biol Control* **94**, 74–81.

Stockwell, V.O. and Stack, J.P. (2007) Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* **97**, 244–249.

Tanimomo, J., Delcenserie, V., Taminiau, B., Daube, G., Saint-Hubert, C. and Durieux, A. (2016) Growth and freeze-drying optimization of *Bifidobacterium crudilactis*. *Food Nutr Sci* **7**, 616–626.

Teixidó, N., Cañamás, T.P., Usall, J., Torres, R., Magan, N. and Viñas, I. (2005) Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. *Lett Appl Microbiol* **41**, 248–252.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mycelial growth of five different *Botrytis cinerea* isolates on PDFA medium in the presence of formulated Pf153 cultivated at 20 or 28°C and harvested at different growth phases (mid-exponential and at the beginning of the stationary phase).

Figure S2. Mycelial growth of five different *Botrytis cinerea* isolates on 1/10 PDA medium in the presence of formulated Pf153 cultivated at 20 or 28°C and harvested at different growth phases (mid-exponential and at the beginning of the stationary phase).