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ORIGINAL ARTICLE

Metabolism of *Klebsiella pneumoniae* freeze-dried cultures for the design of BOD bioassays

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Abstract

Aims: The survival rate of freeze-dried cultures is not enough information for technological applications of micro-organisms. There could be serious metabolic/structural damage in the survivors, leading to a delay time that can jeopardize the design of a rapid biochemical oxygen demand (BOD) metabolic-based bioassay. Therefore, we will study the metabolic activity (as ferricyanide reduction activity) and the survival rate (as colony-forming units, CFU) of different *Klebsiella pneumoniae* freeze-dried cultures looking for stable metabolic conditions after 35 days of storage.

Method and Results: Here, we tried several simple freeze-drying processes of *Kl. pneumoniae*. Electrochemical measurements of ferrocyanide and survival rates obtained with the different freeze-dried cultures were used to choose the best freeze-drying process that leads to a rapid metabolic-based bioassay.

Conclusions: The use of milk plus monosodium glutamate was the best choice to obtain a *Kl. pneumoniae* freeze-dried culture with metabolic stable conditions after storage at −20°C without the need of vacuum storage and ready to use after 20 min of rehydration. We also demonstrate that the viability and the metabolic activity are not always directly correlated.

Significance and Impact of the Study: This study shows that the use of this *Kl. pneumoniae* freeze-dried culture is appropriate for the design of a rapid BOD bioassay.

Introduction

Freeze-drying, many times also called lyophilization, is a common method for the preparation and preservation of biological materials used for research, commercial and industrial applications offering many advantages in terms of storage, handling and use, as well as producing large amounts of concentrated dried material (Perry 1995).

However, freeze-drying may be lethal to a large fraction of a given population leading to negative effects on the viability of many cell types (Morgan *et al.* 2006). Such viability loss is influenced by the cryoprotectant used, the initial cell density and the physiological state of the cells (Leslie *et al.* 1995; Morgan *et al.* 2006). To reduce deleterious effects, different substances such as sugars (lactose, maltose and trehalose) or amino acids (monosodium glutamate) have been assayed for their protective effect during the drying and storage of different bacterial strains (Leslie *et al.* 1995; Martos *et al.* 2007).

But not all additives are effective during both freeze-drying and storage steps, and all depend on the strain used. Skim milk is selected as drying medium usually for *Lactobacillus*, but also for many other bacteria. Supplementing skim milk with other proposed protective agents may enhance its intrinsic protective effect during storage (Leslie *et al.* 1995; Martos *et al.* 2007). The effectiveness of some of these cryoprotectants may be related to natural metabolites produced by each strain in stressing environments.

A common cellular mechanism of osmotic-stress adaptation is the intracellular accumulation of inorganic and/or organic solutes in the cytosol to restore turgor and avoid the plasmolysis caused by hyperosmotic shock (Csonka 1989; Madkour *et al.* 1990).

Nevertheless, viability is not a wholly reliable criterion for evaluating the success of freeze-drying, as survivors might be metabolically or structurally damaged. The preservation of the microbial material is also essential for the performance of microbial bioassays or biosensors because their reliance on the ability of cells to produce a detectable signal that can serve as a reporter of a particular environmental condition. The current industrial standard for preserving micro-organism to be used in biosensors and bioassays is freeze-drying (Bjerketorp *et al.* 2006).

Numerous whole-cell sensing systems have been developed for environmental monitoring purposes. The utility of whole-cell sensing systems could further be enhanced by an appropriate packaging that increases their shelf life and improves their effective use for on-site monitoring. Portability, preservation and long-term storage of the sensor bacteria are crucial features for on-site applications (Farré *et al.* 2005; Bjerketorp *et al.* 2006). This would be of particular importance in developing countries, which often experience difficult environmental conditions and have poor storage and transportation facilities, or in the challenging environments of a disaster.

Data have reported an alternative method of preservation, storage and transport of the sensor bacteria for toxicity determination based on the use of bacterial spores that despite the advantages, it is unsuitable for on-site monitoring given the time-consuming process of spore germination (an overnight revitalization; Date *et al.* 2007).

Microbial biosensors or bioassays, whose difference lies in the disposition of the biological recognition element (Farré *et al.* 2005), have also been proposed to overcome the major problem of the standard method for evaluating biochemical oxygen demand (BOD) (BOD_5 test), the 5-day determination time (APHA 1995). Immobilized micro-organisms have been proposed for BOD determinations (Liu and Mattiasson 2002; Sakaguchi *et al.* 2007); however, they often result in a decreasing performance of the biosensor or bioassay along time leading to nonreproducible results given the vital role played by their physiological state and the concentration on the immobilization support (Bjerketorp *et al.* 2006). It also has been shown that mixed community biosensors change their properties in time, yielding nonreproducible results (Liu and Mattiasson 2002).

A ferricyanide-mediated BOD_{st} (short term) technique has been reported as an approach in which O_2 was replaced by potassium hexacyanoferrate (III) (ferricyanide; Pasco *et al.* 2000). Ferrocyanide is accumulated, while organic compounds are microbially degraded producing CO_2. The ferrocyanide quantification, conducted by amperometry or coulometry, has been used as an indirect method to determine BOD_5 values (Pasco *et al.* 2000; Catterall *et al.* 2003), while glutamic acid solution (GGA) or OECD standards are used as carbon sources (Pasco *et al.* 2000; Yoshida *et al.* 2000; Catterall *et al.* 2003). A standard glucose–GGA is frequently used to verify the methodological protocols and the viability of indigenous populations in the BOD_5 test; the OECD standard (OECD 1993) is usually employed to assess the biodegradability or the removal of substances in activated sludge systems.

We have previously reported a BOD_{st} bioassay based on the ferricyanide reduction (Bonetto *et al.* 2011). Now, we present improvements for this bioassay, the use of a freeze-dried *Kl. pneumoniae* culture with a stable metabolism at least for 35 days after storage at −20°C. This would allow the design of a portable long shelf-life bioassay improving the feasibility of commercialization.

Materials and methods

Solutions and culture media

The LB broth contained (g l^{-1}) bacto-tryptone (10), NaCl (10) and yeast extract (5). For agar plate count, 1·5% (w/v) of agar was added. The minimum medium (MM) contained (g l^{-1}) Na_2HPO_4 (6), KH_2PO_4 (3), NH_4Cl (1), NaCl (0·5), $MgSO_4{\cdot}7H_2O$ (0·12) and $CaCl_2{\cdot}2H_2O$ (0·01), with pH adjusted to 7. The GGA solution contained (g l^{-1}) glutamic acid (0·15) and glucose (0·15) with a measured BOD_5 value of 198 ± 31 mg l^{-1}. The OECD standard ($OECD_{std}$) contained (g l^{-1}) peptone (15), meat extract (11), urea (3·0), NaCl (0·7), $CaCl_2$ anhydrous (0·3), $MgSO_4{\cdot}7H_2O$ (0·2) and K_2HPO_4 3 H_2O (3·7) with a measured BOD_5 value of 17·000 mg l^{-1} (OECD 1993). The potassium ferricyanide, $OECD_{std}$ and GGA solutions were prepared in MM. The other solutions were prepared in double osmosis water. The $OECD_{std}$, GGA, trehalose and skim milk added with sodium glutamate solutions were sterilized by membrane filtration (0·22 μm). The concentration of the formaldehyde stock solution was 40% (w/v), and it was used as the inhibitor compound (Bonetto *et al.* 2011).

Freeze-drying process of *Klebsiella pneumoniae*

A *Klebsiella pneumoniae* strain was isolated from a freeze-dried product containing 4 bacterial strains and stored in freeze-dried cultures at −20°C. This strain presented a similarity level of 98% with *Kl. pneumoniae* strain K30 and K8 (GenBank accession numbers EU661377 and EU661374), both originally isolated from rhizosphere. The detailed isolation and identification of the strain have been published in Bonetto *et al.* (2011).

Klebsiella pneumoniae was grown under aeration conditions at 29 ± 1°C either in LB broth until the late exponential phase was reached (an optical density at 600 nm,

OD_{600} of 2·5 ± 0·2, corresponding to a $2{\cdot}9 \times 10^8$ CFU ml^{-1} concentration; culture *A*) or in LB broth supplied with 20% (v/v) of glycerol for 6 days until an OD_{600} of 0·6 ± 0·1 was reached ($2{\cdot}3 \times 10^8$ CFU ml^{-1}, culture *B*). Bacteria cells were harvested afterwards by centrifugation at 900 ***g*** for 6 min.

The pellets from cultures *A* and *B* were washed twice with MM or not. Different protectant solutions or none were added to the pellets (either washed or not) from culture *A*. After the addition of these solutions to the corresponding pellets, the suspensions were homogenized, left for 20 min at room temperature and frozen at −20°C for 4 h before freeze-drying. No protectant solution was added to the pellets from culture *B*.

The protectant solutions used (in final concentrations) were skim milk added with monosodium glutamate, MSG (10% w/v and 5% w/v, respectively) or trehalose (100 mmol l^{-1}). Cultures with the addition of skim milk plus MSG (milk-MSG) have been called A_3 and A_4, and those with the addition of trehalose, A_5 and A_6.

Each sample was first frozen to −20°C and then placed in the freeze-dried chamber placed at −40°C. The vacuum was applied from 10 mbar up to 0·1 mbar, and the final temperature after 12 h of freeze-drying was 25°C.

Survival rates after freeze-drying

The recovery of each freeze-dried culture was made in MM and left for rehydration for 20 min (these suspensions were also used to study the metabolic activity). Appropriate dilutions were plated in agar LB and incubated at 37 ± 1°C for 24 h to calculate the survival rate as an average of three plates ($n = 3$) for each dilution.

The arithmetical survival rate (ASR) has been expressed as:

$$\mathrm{ASR} = \frac{(\mathrm{CFU\,ml^{-1}})_{\mathrm{f-d}}}{(\mathrm{CFU\,ml^{-1}})_{\mathrm{lc}}} \times 100,$$

where f-d: freeze-dried culture and lc: liquid culture before freeze-drying.

A logarithmic survival rate (LSR) has also been calculated as:

$$\mathrm{LSR} = \frac{[\mathrm{Log_{10}\,(CFU\,ml^{-1})}]_{\mathrm{f-d}}}{[\mathrm{Log_{10}\,(CFUml^{-1})}]_{\mathrm{lc}}} \times 100$$

Metabolic activity of the freeze-dried cultures

Amperometric assays were performed with 2 ml samples after incubating bacteria and ferricyanide at 37 ± 1°C for 2 h in the presence of different final concentrations of either GGA or $OECD_{std}$ solutions (see details in Data S1).

Equipment

OD was measured with a Kontron Uvikon 710 Spectrophotometer (Zurich, Switzerland). Bacterial pellets were obtained with a Sorvall RC-5B Centrifuge, GMI Inc. (Minnesota, USA) at 900 ***g*** and freeze-dried in a Rificor L-05 freeze-drier (Buenos Aires, Argentina).

Results

Survival rate of *Kl. pneumoniae* after freeze-drying

Survival rates of each freeze-dried culture were measured 1 day after freeze-drying and 20 or 35 days after storage at −20°C (Table 1). As the percentage survival of a given treatment can be given a misleading positive skew if for instance a percentage loss of viability is reported on actual arithmetic counts compared to log cell counts (Morgan *et al.* 2006), we have calculated both after 1 day of each freeze-drying process.

In Table 1, it can be seen that the washed pellets had higher survival rates than the nonwashed pellets. It also can be seen that the best ASR was obtained with the freeze-dried cultures obtained from culturing *Kl. pneumoniae* in LB broth in aerated conditions (culture *A*). The best protectants to obtain a high *Kl. pneumoniae* survival rate are the milk-MSG (only if LB is removed from the pellet before the addition of the protectant solution, A_4) and the trehalose (either added to washed or nonwashed pellets, A_6 or A_5, respectively) solutions. As some survival rates had higher SDs (over 20%), their ASR values have been informed as nonconclusive.

Some authors have also proved that stress culture conditions promote the synthesis of natural protectant products (Csonka 1989; Madkour *et al.* 1990). As glycerol can freely diffuse across the membrane (Csonka 1989), perhaps it can promote the synthesis of some natural protectant in *Kl. pneumoniae* if the strain is cultured in high glycerol concentrations. Thus, we cultured *Kl. pneumoniae* in LB broth added with 20% (v/v) of glycerol under aerated conditions (culture *B*) before freeze-drying to study plausible improvements in the survival rate and/or the metabolism of the strain. However, after freeze-drying the pellets from the culture *B*, we only obtained a freeze-dried product if the pellet was washed; the B_1 'culture' was a dense paste instead of a powder, so it was useless.

As the assay to study the metabolic condition of the freeze-dried cultures consisted in calibration curves with either GGA or $OECD_{std}$ made by amperometry and needed to be compared with the results obtained in calibration curves using fresh cultures, no carbon source was used in the rehydration solution (MM) of the freeze-dried cultures.

Table 1 Arithmetic and logarithmic survival rates (as defined in Materials and methods) of different *Klebsiella pneumoniae* freeze-dried cultures ($n = 3$)

Liquid culture	Protectant solution	Pellets (freeze-dried culture)	ASR, %*	LSR, %*	ASR, %†	ASR, %‡
A	None	Nonwashed (A_1)	0·17 ± 0·01	73·8	ND	ND
		Washed (A_2)	6·2 ± 0·1	86·7	3·3 ± 0·1	ND
	Milk-MSG	Nonwashed (A_3)	9·8 ± 0·2	89·0	3·3 ± 0·2	ND
		Washed (A_4)	35·1 ± 4·1	95·5	27·5 ± 2·3	21·2 ± 0·6
	Trehalose	Nonwashed (A_5)	31·0 ± 1·8	92·0	NC	28·0 ± 1·8
		Washed (A_6)	35 ± 1	95·0	NC	31 ± 1
B	None	Nonwashed (B_1)	No freeze-dried product was obtained			
		Washed (B_2)	4·9 ± 0·5	86	ND	ND

ASR values are shown as mean ± standard deviation (SD). Only the mean of each LSR value is informed.
ASR, arithmetical survival rate; ND, not determined; NC, not conclusive.
Values were calculated after *1, †20 or ‡35 days of freeze-drying.

Metabolic activity of *Kl. pneumoniae* after freeze-drying

The freeze-dried culture is going to be used as biological material for a BOD bioassay, thus it is essential that the metabolic activity remains stable after the storage time. Given that survivors may be metabolically damaged depending on the freeze-drying process chosen, we assayed the metabolic activity of each freeze-dried culture after 1 day of freeze-drying.

We observed that the A_1, A_4 and A_5 freeze-dried cultures can employ GGA as carbon source better than the rest of the cultures assayed (Fig. S1), leading to assays where the sensitivity (the amperometric curve slope, Fig. S1) barely decays (1·66, 1·88 and 1·80 nA l mg^{-1}, respectively) compared with the sensitivity of the assay with a *Kl. pneumoniae* fresh culture (2·11 nA l mg^{-1}).

But GGA may not be a good standard to evaluate the metabolic activity of a freeze-dried culture for BOD_{st} determinations because even when glucose is the easiest carbon source to use, it is not usually present in wastewater or real water samples. So, we assayed the metabolic activity of some of these cultures (also after 1 day of freeze-drying) incubated with the $OECD_{std}$. As the A_1 culture had a different result in a previous metabolic assay with GGA (with a sensitivity of 0·55 nA l mg^{-1}, and a smaller linear range, up to 200 mg l^{-1}), we decided not to study the A_1 metabolic activity with $OECD_{std}$. The B_2 culture was also not assayed with $OECD_{std}$ as it was not able to metabolize glucose.

Cultures A_4 and A_5 were able to use the $OECD_{std}$ as a carbon source and ferricyanide as the electron acceptor within a linear range similar to that of the fresh culture (from 20 to 200 mg l^{-1}; Fig. S2). It also can be seen that the assay maintains a similar sensitivity when the fresh culture (1·06 nA l mg^{-1}) is replaced by A_4 (1·37 nA l mg^{-1}) or A_5 (0·94 nA l mg^{-1}) freeze-dried cultures. The A_2 and A_3 freeze-dried cultures were no adequate for the design of our bioassay; they presented decreased sensitivity (30 or 50%, respectively) using the $OECD_{std}$ and comparing it with the fresh culture.

To ease the comparison of the metabolic viability of either a fresh culture or a freeze-dried culture, we present a metabolic/CFU index (M/C) calculated as:

$$\mathrm{M/C} = \frac{[\mu\text{g ferrocyanide} \times (\text{CFU} \times \text{mg BOD5})^{-1}]_{\text{f-d}}}{[\mu\text{g ferrocyanide} \times (\text{CFU} \times \text{mg BOD5})^{-1}]_{\text{fc}}},$$

where f-d: freeze-dried culture and fc: fresh culture. To calculate this index, we used the slope values from the linear range of a μg ferrocyanide *vs* CFU × mg BOD_5 of each freeze-dried culture curve and the slope of the fresh culture curve (Table 2). The compared metabolic activity corresponds to the assays made the first day after freeze-drying.

As Table 2 shows, results obtained with the A_4 culture were better than those obtained with the A_5 culture. The M/C indexes, obtained using the A_4 culture, either with GGA or with $OECD_{std}$ are closer to 1, so this freeze-dried culture and the fresh culture have similar metabolic viability. This freeze-drying process keeps almost intact

Table 2 M/C index using either glutamic acid solution (GGA) or $OECD_{std}$ as the carbon source

Freeze-dried culture	M/C index. GGA. (linear range in mg BOD_5 l^{-1})	M/C index. $OECD_{std}$. (linear range in mg BOD_5 l^{-1})
A_1	6·4 (25–250)	ND
A_2	2·4 (25–250)	1·1 (25–125)
A_3	1·5 (25–250)	1·1 (25–85)
A_4	0·9 (25–330)	1·3 (25–220)
A_5	4·6 (60–450)	0·38 (25–220)
A_6	2·2 (60–200)	ND

ND, not determined.

the bacteria metabolic state. The highest M/C value informed corresponding to the A_1 culture is not reliable because the previous freeze-dried culture made under the same conditions presented an M/C index of 0·88.

Given the M/C index results obtained after a day of freeze-drying, only the best freeze-dried cultures, A_4 and A_5, were assayed to compare the metabolism of the freeze-dried cultures after 35 days of storage at −20°C. Table S1 presents normalized sensitivities {[f-d sensitivity × (fc sensitivity)$^{-1}$] × 100} of the assays with A_4 or A_5 cultures either using GGA or $OECD_{std}$ as carbon source after a day or after 35 days of freeze-drying.

Table S1 shows that the use of milk-MSG is better than the use of trehalose to obtain a *Kl. pneumoniae* freeze-dried culture with stable metabolic conditions up to after 35 days of storage. The bioassay also maintains the linear range either using the A_4 or the A_5 freeze-dried culture after 35 days of storage despite some lost of the bioassay sensitivity.

Discussion

The differences in cell survival and metabolic viability obtained in this study indicate that not all the protectants used are effective during both freeze-drying and storage steps. The pellets washing step was made to study the impact that LB could have in the freeze-dried cultures. Washing the pellets always improved the survival rate of the freeze-dried cultures (Table 1). We have not enough data to explain this phenomenon, but maybe this improvement is related to the removal of accumulated potentially toxic metabolites before obtaining the *Kl. pneumoniae* freeze-dried cultures, as it has been shown with some bacteria in Landwall and Holme (1977). The washing procedure may also cause structural damage and/or stress to the cells leading to different metabolic assay performances (Table 2 and Figs S1 and S2).

The best example to show that the freeze-drying process may lead to survivors with high damage in the metabolic state is the B_2 freeze-dried culture that, despite having an ASR of 5% (Table 1), showed almost no capacity to metabolize the glucose present in the GGA standard, at least in 2 h and after 20 min of rehydration in MM (Fig. S1). Instead, the A_2 culture presented a similar ASR after 1 day of freeze-drying and rather good metabolizing capacities. Therefore, we prove that after rehydration, the survivors can have serious metabolic damages (depending on the freeze-drying process) leading to a delay time that can compromise the performance of a rapid metabolic-based bioassay or biosensor.

Based on the assumption that after freeze-drying some enzymatic activity from nonsurvivors may be present in the final product even if the ASR decreases (Tan and Wu 1999), we used a ratio of 1 (freeze-dried weight/fresh culture dry weight) as microbial concentration in the metabolic assays using freeze-dried cultures and used the M/C index to study the metabolic efficacy of the freeze-dried cultures.

The M/C index obtained with the A_5 freeze-dried culture is 4 when GGA is used as carbon source; this may be associated to a remaining enzymatic activity in the freeze-dried culture capable of using GGA and reduce ferricyanide, beyond the CFUs capacity to metabolize GGA. In the A_1 freeze-dried culture, a metabolic activity seems to remain after freeze-drying even when the ASR is <1% and would be able to use GGA as carbon source and reduce ferricyanide, thus increasing the M/C index up to 6 (Tables 1 and 2). Despite this, other freeze-dried culture has been previously obtained under same conditions as the A_1 culture and had significant differences in the survival rate and the GGA metabolic activity, so we considered that this freeze-drying process leads to nonreproducible cultures.

Instead, the use of the $OECD_{std}$ seems to require complex enzymatic machinery. The A_5 freeze-dried culture index using $OECD_{std}$ as carbon source is lower than 1; thus, the enzymatic machinery able to metabolize GGA cannot use $OECD_{std}$, nor can many of the CFU present in this freeze-dried culture (Table 2).

The loss of viability in freeze-dried cultures may not be critical if the issue is to preserve a strain, but it is very important for the design of a rapid metabolic-based bioassay or biosensor. The development of bioassays to be used in *in situ* measurements requires an adequate bacteria disposition. Usually toxicity bioassays using luminous bacteria, either wild type or engineered bacteria, employ freeze-dried biological material after at least 15 min and no more than 2-h rehydration time (Gu *et al.* 2001; ISO 11348-3 (E) 2007), but we have found no reported results using freeze-dried bacteria for BOD_{st} determinations.

We have already proved that a *Kl. pneumoniae* fresh culture can be used successfully as biological material for a BOD_{st} bioassay (Bonetto *et al.* 2011). Now, we present a BOD determination bioassay based on a *Kl. pneumoniae* freeze-dried culture obtained after the addition of milk-MSG to a washed pellet (culture A_4) that works in stable conditions for 35 days of storage at −20°C and showed reproducible results along the different assays. This amperometric bioassay can be used in *in situ* measurements.

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Supporting Information

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

Data S1 Materials and methods.

Figure S1 Calibration curves using GGA as carbon source.

Figure S2 Calibration curves using $OECD_{std}$ as carbon source.

Table S1 Normalized sensitivities from A_4 and A_5 freeze-dried cultures using either GGA or $OECD_{std}$ as carbon sources ($n = 3$).

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