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Survival, fermentation activity and storage stability of spray dried *Lactococcus lactis* starter cultures produced via different atomization regimes

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Abstract

Dried powders containing *Lactococcus lactis* ssp. *cremoris* were produced using laboratory and pilot scale spray dryers with lactose:whey protein isolate (3:1) as a protective medium. The effects of storage temperature (25, 4 and -18 ^{o}C) and time (30, 60 and 90 days) were studied. The survival and fermentation activity of the dried bacterial cells were significantly lower when the powders were stored at 25 ^{o}C compared to those stored at 4°C and -18°C; powders stored at 4°C and -18°C were statistically similar. The survival and fermentation activity of bacterial cells obtained from a laboratory scale two-fluid nozzle spray dryer were found to be higher than those of cells obtained from a pilot scale two-fluid spray dryer. A rotary wheel atomizer gave significantly higher survival and activity in the same dryer. These observations are consistent with cell damage due to high characteristic shear rates in the atomization process in nozzle type atomizers. The presence of ascorbic acid (oxygen scavenger) in the powder composition was found to improve both the survival and the fermentation activity of the dried bacterial cells significantly during storage. The survival and fermentation activity of dried bacterial cells in stored powders indicated that these parameters are system-specific and can be strongly affected by the storage temperature and presence or absence of antioxidant, and also by upstream processing conditions such as the mode of atomization and presence or absence of antioxidants in the dryer feed.

Keyword: *Lactococcus*, bacterial survival, fermentation activity, spray drying, storage temperature, storage time, atomization

1. Introduction

Drying of bacterial cultures for use in dairy fermentations and in other food and therapeutic applications is common commercial practice. There is continuing research interest in improving the application of drying techniques to various food-related bacteria, including *Lactobacillus paracasei* (Gardiner et al., 2000), *Lactobacillus curvatus* (Mauriello et al., 1999), *Lactobacillus rhamnosus* (Corcoran et al., 2005), *Brevibacterium linens* (To and Etzel, 1997b), *Lactobacillus helveticus* (Johnson and Etzel, 1995), and *Streptococcus thermophilus* and *Lactobacillus debrueckii* ssp. *bulgaricus* (Kim and Bhowmik, 1990). Investigations to improve their viability have examined various aspects such as intrinsic features of bacterial cultures (Bozoglu et al., 1987; Palmfeldt and Hahn-Hagerdal, 2000), the effect of growth media and growth conditions (Champagne and Gardner, 2001; Linders et al., 1997), cell harvesting conditions (Champagne and Gardner, 2002; Van De Guchte et al., 2002), stress adaptation (Broadbent and Lin, 1999; Prasad et al., 2003), application of protective agents (Koster et al., 2000; Leslie et al., 1994; Oldenhof et al., 2005), manipulating the rehydration conditions (Abadias et al., 2001; Zhao and Zhang, 2005) and improving the packaging and storage conditions (Brennan et al., 1983; Buitink et al., 2000; Castro et al., 1995).

Spray drying has advantages over other drying methods such as cost-effectiveness, relative ease in operation, easy scale-up for large throughputs and ready availability of suitable equipment at various scales (Gibbs et al., 1999; Horaczek and Viernstein, 2004). However, bacterial survival is generally poor. Storage conditions are considered to be one of the key factors which directly affect the cell viability and fermentation activity of spray-dried bacterial starter cultures (Peighambardoust et al., 2011). Several factors during storage affect survival and fermentation activity, including reaction with oxygen, moisture, light, microbial contamination and elevated temperature (Morgan et al., 2006). Survival decreases during storage, and survival is generally reported to be higher when lower storage temperatures are used (Boza et al., 2004; Corcoran et al., 2004; Desmond et al., 2002; Silva et al., 2002). Teixeria *et al*. (1996) suggested that oxidation of the fatty acids of membrane lipids is the most likely cause of death of microbial cells during storage. According to these authors, the lipid composition of the bacterial cell membrane changes due to the increased lipid oxidation during storage. Better viability of bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was reported when the spray dried bacterial cultures were stored under vacuum and in nitrogen-rich conditions compared to those stored in normal atmospheric air (Bozoglu

et al., 1987).

However, there are no reported studies comparing the survival and fermentation activity of bacterial cells obtained from laboratory- and pilot-scale spray drying operations as a function of storage temperature and storage time, and relatively few studies of the common cheese starter bacterium *Lactococcus lactis*. This study was aimed at investigating these issues as they affect preservation and use of a *L. lactis* ssp. *cremoris* dairy starter culture.

2. Material and methods

2.1. Materials

Lactococcus lactis subsp. *cremoris* strain ASCC930119 (Pillidge et al., 2009) from the Dairy Innovation Australia culture collection was cultured overnight in 15 ml of M17 broth (Oxoid, Australia) at 30°C under static conditions. The resulting culture was transferred to 1 L of M17 broth at 30°C under the same conditions. Cells were harvested by centrifugation at 2500×g for 5 minutes and then suspended in lactose:whey protein isolate (3:1) solution using gentle agitation at 25°C and adjusted to *ca.* 10^{10} cells/ml.

α-Lactose monohydrate (99.8% purity, Sigma-Aldrich, Australia), whey protein isolate (WPI, protein content 94.5%, obtained by courtesy of Murray Goulburn Cooperative, Australia) and L-ascorbic acid (Chem-Supply, Australia) were used as received.

2.2. Methods

2.2.1. Solution preparation

As described by Ghandi et al. (Ghandi et al., 2012b), lactose:WPI (3:1; total solids 35% w/w) in deionized water was prepared by heating at 45±1°C and gently agitating with a magnetic stirrer. A 10% (w/w) ascorbic acid solution was prepared, and added to the bacterial suspension (in lactose:WPI, pH=6.5±0.1) just before spray drying, to a concentration of 0.7% (w/w) and pH 5.5.

2.2.2. Powder production

Laboratory-scale spray drying was carried out using a Buchi 290 dryer (Buchi, Switzerland) with water evaporation capacity of 1 L/h. The bacterial suspension was pneumatically atomized using a two-fluid nozzle (nozzle diameter, inner D_i =1.2 mm; outer D_o=1.4 mm). The flow rate of the drying air was maintained at 35 m^3/h while the aspiration was at 100%.

Pilot-scale drying used a Drytec Spray Dryer (Drytec, England; water evaporation capacity: 8 L/h; exhaust fan capacity: 160 m^3/h) fitted with either a rotary wheel atomizer or a two-fluid nozzle (inner D_i =1.6 mm; outer D_o=2.4 mm; atomizing air pressure: 550 kPa).

Previous work had determined that inlet and outlet air temperatures of 130°C and 65°C, respectively, gave acceptable bacterial survival and residual moisture content not exceeding 5% (w/w) (Ghandi et al., 2012b). Powder was collected in a product container connected to the bottom of the cyclone separator and cooled using an electric fan.

2.2.3. Storage

The bacterial powders were stored for 90 days at three different temperatures (25ºC, 4ºC and -18ºC) in dark glass vials under nitrogen flush, with each vial vacuum sealed in a separate plastic bag. The storage duration was chosen based on the typical duration used in shelf life studies (Fonseca et al., 2000; To and Etzel, 1997b) and in many industrial settings. Bacterial survival was measured at intervals of 15 days across the 90 day storage trial. Acid production activity was evaluated at intervals of 30 days.

2.2.4. Bacterial survival during storage

Spray dried powder (0.1 g) was rehydrated in 9.9 mL sterile peptone water (1% w/v, pH=7.0±0.1) (Gardiner *et al.*, 2000). Live and dead cells were counted by a fluorescein diacetate (FDA) staining method (Lentini, 1993). The initial viable cell concentration of the fresh culture was determined at the time of drying. The results obtained from this method were cross-checked in some representative samples using a plate count method; differences in estimations were within 6%. Survival of bacteria was expressed as a percentage of the live bacterial cells immediately after spray drying (before the powders were stored = zero storage time).

2.2.5. Measurement of fermentation activity

Fermentation activity is described as the ability of the bacteria to produce lactic acid, and reflects the number of live cells and their health. Dried culture (0.7g of spray dried powder) was rehydrated in 35 mL sterile non-fat skim milk (10%, w/w) and stirred in a water-bath at 30°C for 2 minutes before starting the fermentation tests. These fermentation tests were carried out for 24 hours using a water bath maintained at 30°C. The change in pH of the suspension was taken as the measure of fermentation activity. The pH changes were monitored using a data logger (8-Channel Ion analyser, NICO 2000 Ltd., U.K.); pH *vs* time

data were recorded. Rates of pH change (dpH/dt) throughout the fermentations were estimated as described by Ghandi et al. for droplet mass loss analysis(Ghandi et al., 2012a).

2.2.6. Statistical analysis

Statistical analysis was performed using ANOVA. The mean values and standard deviations were calculated from triplicate experimental data. Differences were compared by Tukey test for all experiments and were considered significant at $p<0.05$. All statistical analyses were performed with Minitab 15 software (Minitab, Australia).

3. Results and discussion

It has previously been found (Ghandi et al., 2012b) that the extent of shear rate prevailing during the atomization stage of spray drying and the presence of an oxygen scavenger significantly affect bacterial survival. The extent of shear rate prevailing in both laboratory and pilot scale two-fluid nozzle atomizers and in a pilot scale rotary wheel atomizer and the corresponding survival of bacterial cells immediately after spray drying (0 day of storage) are presented in Table 1, along with information on bacterial survival and powder moisture content. The number of live bacterial cells in these samples is used as the basis (100% value) for comparison of survival over time (Fig. 1).

3.1. Laboratory scale study results

3.1.1. The survival of *L. lactis* upon storage

Survival data across the 90-day storage period at 15-day intervals are presented in Figure 1. Bacterial death was more rapid at higher temperatures. At 25°C (without ascorbic acid) survival fell below 50% within 30 days and by 90 days had fallen to only 3.3%. Addition of ascorbic acid slowed this decline, but only 6.2% survived at 90 days. Much slower death was observed in powders stored at 4°C and -18°C, with survival at 30 days of 88.3% (no acrobatic acid) and 88.7% (with acrobatic acid) at 4°C and 90.4% (no acrobatic acid) and 91.4% (with acrobatic acid) at -18°C and, by 90 days, 48.3% (no acrobatic acid) and 54.9% (with acrobatic acid) at 4°C and 61.6% (no acrobatic acid) and 65.5% (with acrobatic acid) at -18°C. The effect of addition of ascorbic acid became more pronounced after 30 days (at 25°C) and 45 days storage (at 4 and -18°C) (Fig. 1). Overall higher survival of bacteria was found with ascorbic acid than without.

3.1.2. The fermentation activity of spray dried *L. lactis*

Fermentation activity was measured through the change in pH due to production of lactic acid. The results are shown in Figures 2 and 3. A simple quantitative measure of relative acid production rate is the time taken to reach an arbitrary target pH; time taken to reach pH 5.5 (abbreviated here as $t_{5.5}$) is shown in Figure 4a.

An immediate difference can be seen on day 0, with acidification curves and $t_{5.5}$ showing the advantage of including ascorbic acid at the time of drying (Ghandi et al., 2012b). This effect persisted throughout storage. More than that, during storage at 4°C and -18°C ascorbic acid provided some protection against further loss of activity. The acidification curves in Figure 2 and the $t_{5.5}$ values (Figure 4a) show that loss of activity (increase in $t_{5.5}$) occurred more slowly in the presence of ascorbic acid. For example, at 4°C, $t_{5.5}$ increased by an average of 0.82 min per day of storage (estimated by linear regression analysis) in the presence of ascorbic acid but 2.26 min/day in its absence.

Powders stored at 25°C showed more rapid loss of fermentation activity than powders stored at 4 and -18°C. Perhaps the interaction of cellular moieties such as lipids with oxygen at higher temperature is the cause of the higher death at 25°C than the death at other two storage temperatures. At 25°C the presence of ascorbic acid conferred no apparent benefit in activity beyond its effects during drying. The $t_{5.5}$ increases at 25°C (6.08 min/day with ascorbic acid, 5.80 min/day without) do not show this protection, consistent with the results of Morgan et al. (2006).

Storage at -18°C showed little or no advantage over storage at 4°C (despite slightly higher live cell estimates) but, at both these temperatures, ascorbic acid conferred a major positive effect. These results emphasize the potential importance of the cold chain in storage and transport of cultures such as these and illustrate that, at least with the formulation used in this study, ascorbic acid provides no protection against loss of activity due to temperature abuse.

The fermentation activities of powders stored for 0, 30, 60 and 90 days in the presence of ascorbic acid are presented for ease of comparison in Figure 3. As can be seen from this figure and from Figure 4a, the fermentation activity, $t_{5.5}$ and time taken to achieve maximum dpH/dt decreased with storage time (especially after 90 days), consistent with the downward trend in bacterial survival (Fig. 1). For example, the survival values in

powder stored at 4°C in the presence of ascorbic acid were 88.7%, 74.9% and 54.9% at 30 days, 60 days and 90 days of storage, respectively, with corresponding to $t_{5.5}$ values of 8.5, 8.7 and 9.3 hours. Survival values suggested a small benefit from adding ascorbic acid but fermentation activity tests showed a major benefit, consistent with the expectation that both lethal and non-lethal oxidative damage can lead to loss of activity, and that ascorbic acid protects against both of these.

3.2. Survival and fermentation activity *L. lactis* in powders obtained from pilot scale spray drying

3.2.1. The survival of *L. lactis* during storage

Bacterial survival in powders obtained from pilot plant trials are presented in Figure 5 (panels a and b). Figure 5 (a) presents the data where a rotary wheel atomizer was used while panel (b) presents the data where a two-fluid nozzle was used for atomization.

The results are qualitatively and quantitatively similar to those obtained with the laboratory scale dryer, showing death of bacterial cells over time. Storage at 25°C resulted in very low survival values, storage at -18 ^{o}C gave the highest, and ascorbic acid enhanced survival.

At 4°C and at -18°C, the rotary wheel atomizer gave a higher survival trend than the two-fluid nozzle [Figure 5 panels (a) and (b)]. For example, when stored for 90 days at 4°C, the survival values were 25.4% (no ascorbic acid) and 41.9% (with ascorbic acid) in the powders produced using the two-fluid nozzle, while the corresponding values were 44.3% (no ascorbic acid) and 53.87% (with ascorbic acid) in powders produced through the rotary wheel atomizer. However, the rotary wheel atomizer showed no clear advantage when powder was stored at 25°C. Different characteristic shear rates prevail in these two atomization processes [(Ghandi et al., 2012b); Table 1]. The characteristic shear rate in the rotary wheel atomizer is much lower than in the two-fluid nozzle, leading to likely differences in the extent of physical, thermal and oxidative damage to cells during spray drying and different particle size distributions in the powders. The present study shows that these differences have potential long-term downstream effects during storage.

Ascorbic acid showed survival benefits at all storage temperatures tested. For instance, the survival of bacterial cells in powder produced by atomizing with the two-fluid nozzle and stored at 25°C for 45 days was 21% in the presence of ascorbic acid but only 10% in the absence of it [Figure 5 (b)].

3.2.2. The fermentation activity of *L. lactis* powders as a function of storage time

3.2.2.1. Fermentation activity of powders obtained using the rotary wheel atomizer

The fermentation activity data of powders obtained from rotary wheel atomized samples are shown in Figure 6 and Figure 4 (panels b and c). These powders showed higher activity than other powders on day 0 (the lowest initial $t_{5.5}$ values with and without ascorbic acid; Figure 4 (panels b and c)) but this advantage was largely lost on storage (*e.g.* at an average rate of 4.2min/day at 4°C in the presence of ascorbic acid). Consistent with the fermentation activity tests on powders obtained from laboratory scale spray drying, greatest deterioration took place at 25°C whereas 4°C and -18°C storage gave comparable results, and ascorbic acid showed initial benefits. However, the estimated rate of deterioration of $t_{5.5}$ was similar with and without ascorbic acid at all temperatures (with or without ascorbic acid, at 25°C, 4°C and -18°C respectively: 6.60, 7.18; 4.20, 3.60; 3.66, 3.96 min/day of storage). Even so, activity remained superior throughout the storage period to that of the powders produced in the same dryer using a two-fluid nozzle (see 3.2.2.2).

3.2.2.2. Fermentation activity of powders produced using the two-fluid nozzle

The fermentation activity values of powders produced using the two-fluid nozzle are shown in Figure 7 and Figure 4 (panels b and c). Consistent with the fermentation activity tests on powders obtained from laboratory scale spray drying, greatest deterioration took place at 25°C, 4°C and -18°C storage gave comparable results, and ascorbic acid showed benefits initially and during storage. Notably, these powders had the lowest initial activities (highest $t_{5.5}$ values) and they deteriorated rapidly (*e.g.* in the presence of ascorbic acid, 7.20 min/day at 25 °C or 2.50 min/day at 4 °C),

The fermentation activity of these powders was inferior to that of powders obtained in the same dryer using a rotary wheel atomizer. This can be attributed to higher bacterial survival [especially during the atomization stage (Ghandi et al., 2012b)] in the dryer with a rotary wheel atomizer. As shown in Table 1, the characteristic shear rates in the two-fluid nozzle are three orders of magnitude higher than the shear rates in the rotary wheel atomizer.

3.3. Powders obtained from laboratory and pilot scale dryers

The $t_{5.5}$ values for powdered cultures obtained from the laboratory and pilot-scale spray dryers using two-fluid nozzles are summarized in Figure 4. The presence of ascorbic acid

conferred greater benefit at laboratory scale. Powders prepared using the rotary-wheel atomizer showed lower $t_{5.5}$ values than powders prepared using a two fluid nozzle in the same dryer. Comparison of times taken to reach maximum dpH/dt (not shown) yielded equivalent conclusions to the simple pH curves and $t_{5.5}$ values. The observations are the results of differences in cell death and damage during drying and differences in the rate of activity loss during storage.

Lactococcus lactis is widely used in starter cultures for cheese manufacture. Starters are either freshly grown prior to cheese manufacture or pre-prepared as frozen or freeze-dried concentrates (Powell, 2007). Freeze-dried cultures are expensive to produce, in part due to the energy requirements of freeze-drying but also because of cell damage and death during the process. The surviving bacteria typically exhibit a long lag phase when added to the cheese vat (Sandine, 1996; To and Etzel, 1997a). Spray-dried cultures have not been commercially applied in this way because of even lower survival rates and poor storage stability (Peighambardoust et al., 2011). Although confirming the influence of drying conditions, storage temperature and the presence of antioxidants on bacterial survival and activity (Peighambardoust et al., 2011), the bacterial powders produced in the present work exhibited levels of fermentation activity too low to be useful in typical cheese manufacture. However, the high levels of survival achieved suggest that this approach could be useful as a source of viable bacterial biomass in industrial applications that do not require rapid recovery and acidification, such as cultures for enhancing cheese ripening or probiotic cultures.

4. Conclusions

The effects of storage condition (temperature and time) on the survival and fermentation activity of spray-dried *Lactococcus lactis* ssp. *cremoris* produced from both laboratory and pilot scale spray dryers were studied. Both the survival and fermentation activity of stored bacterial cells decreased significantly ($p<0.05$) with storage time in all the powders irrespective of type of atomizers or scale of dryers used for powder production. Both the survival and fermentation activity values decreased significantly more quickly ($p<0.05$) when powders were stored at 25 ^{o}C compared to the corresponding values at 4°C and -18 ^{o}C. The survival and fermentation activity values of powders stored at 4°C and -18 ^{o}C were not significantly different ($p>0.05$). The presence of ascorbic acid in the powder composition brought noticeable improvement in both the survival and fermentation activity of bacterial cells in powders stored up to 90 days at low temperatures. The survival and

fermentation activity histories of powders during storage indicated that both these parameters are system-specific and strongly depend on upstream processing conditions (mode of atomization used and presence and absence of antioxidants in feed) as well as on the temperature and presence of antioxidant during storage.

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Table 1. Survival of bacteria with Lactose:WPI (3:1) in the presence and absence of Ascorbic Acid (AA) and process parametes for laboratory and pilot scale studies. S_0 (%), mc (%) and a_w refer the bacterial survival after spray drying/before storage, moisture content and water activities of spray dried powders, respectively.

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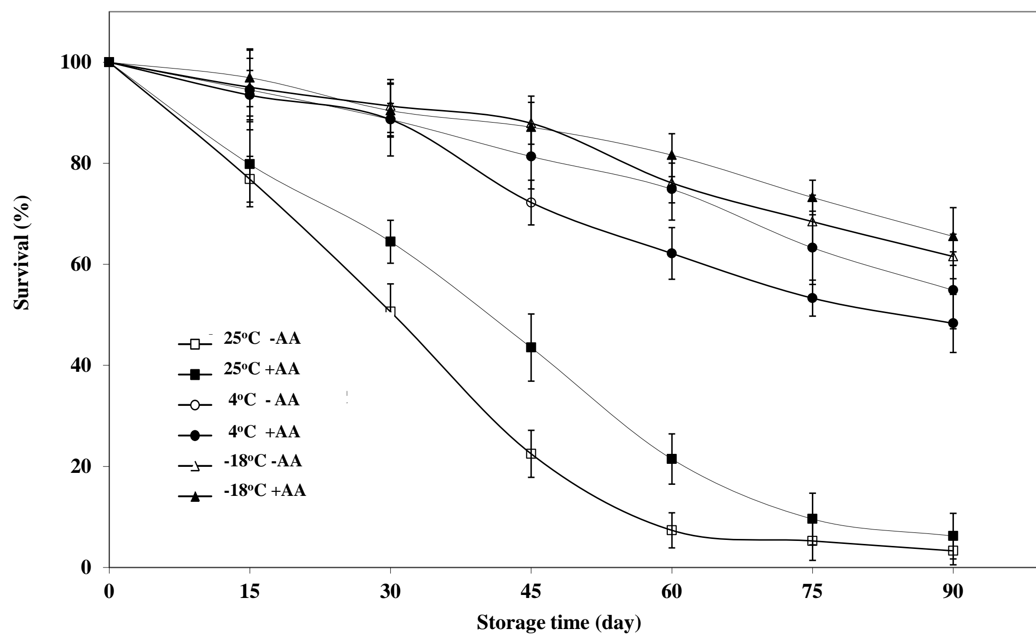
	Laboratory Scale spray dryer		Pilot Scale spray dryer			
	Two-Fluid nozzle		Rotary wheel atomizer		Two-Fluid nozzle	
Characteristic shear rate (s^{-1})	559×10^3		182×10^3		172×10^6	
Sample ID	-AA	+AA	-AA	+AA	-AA	+AA
Bacterial load in feed (cells/ml)	1.1×10^{10}	1.1×10^{10}	1.2×10^{10}	1.2×10^{10}	1.2×10^{10}	1.2×10^{10}
Bacterial load in powder (cell/ml)	2.8×10^9	4.3×10^9	3.5×10^9	6.5×10^9	1.2×10^9	2.5×10^9
S_0 (%)	25.5	38.7	29.9	54.2	9.8	20.8
mc (%)	4.890 ±0.027	5.190 ±0.092	4.250 ±0.041	4.630 ±0.182	3.910 ±0.192	4.110 ±0.074
a_w	0.252 ±0.001	0.281 ±0.005	0.220 ±0.002	0.250 ±0.002	0.220 ±0.003	0.240 ±0.002



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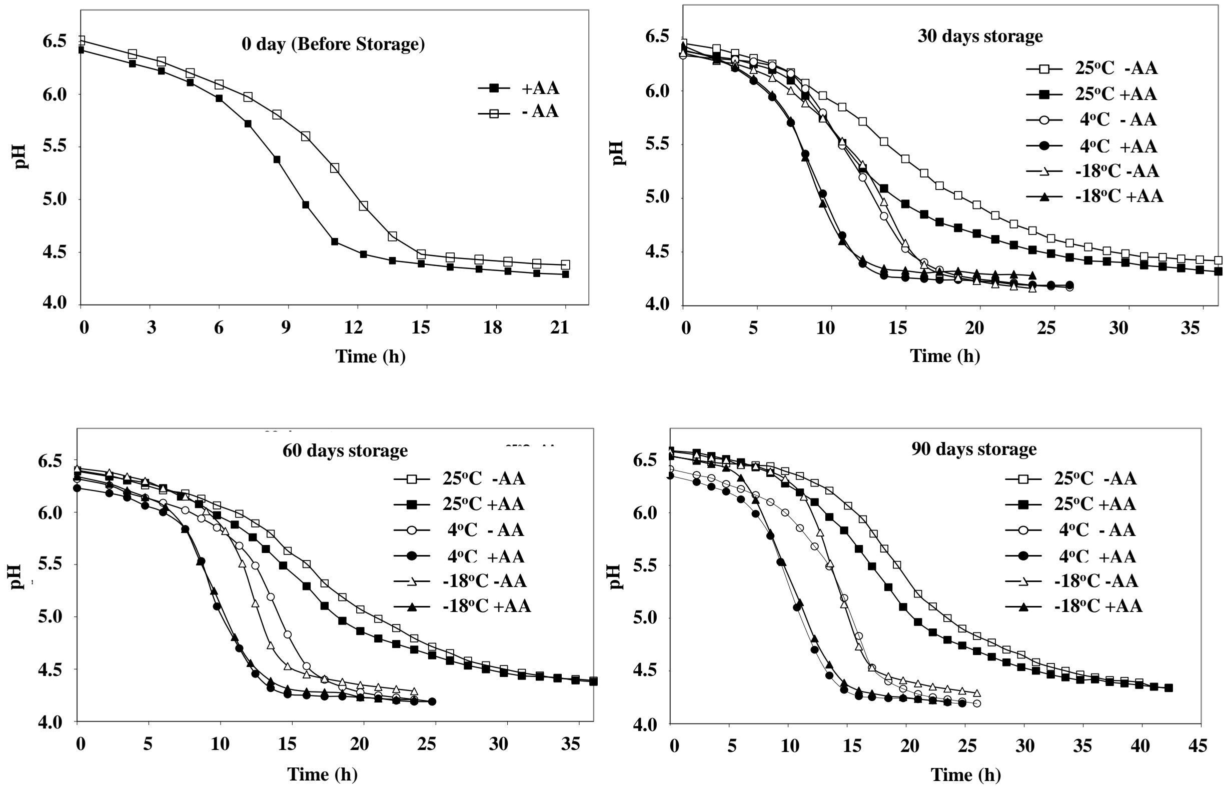

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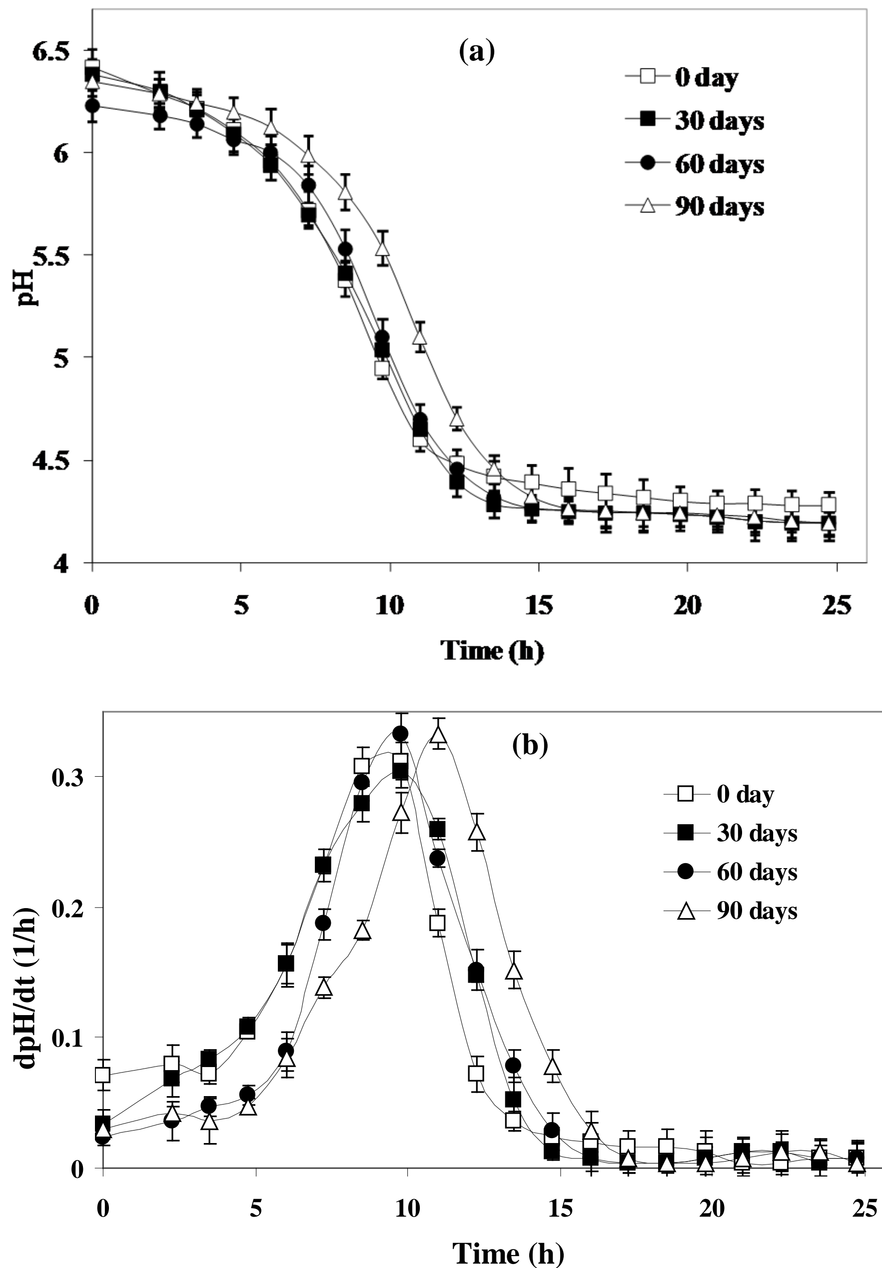


Fig. 3. The fermentation activity (a) and rates of pH change (dpH/dt) throughout the fermentations (b) of L. lactis powders stored at 4ºC at 0, 30, 60 and 90 days of storage. These samples contain ascorbic acid in formulation.

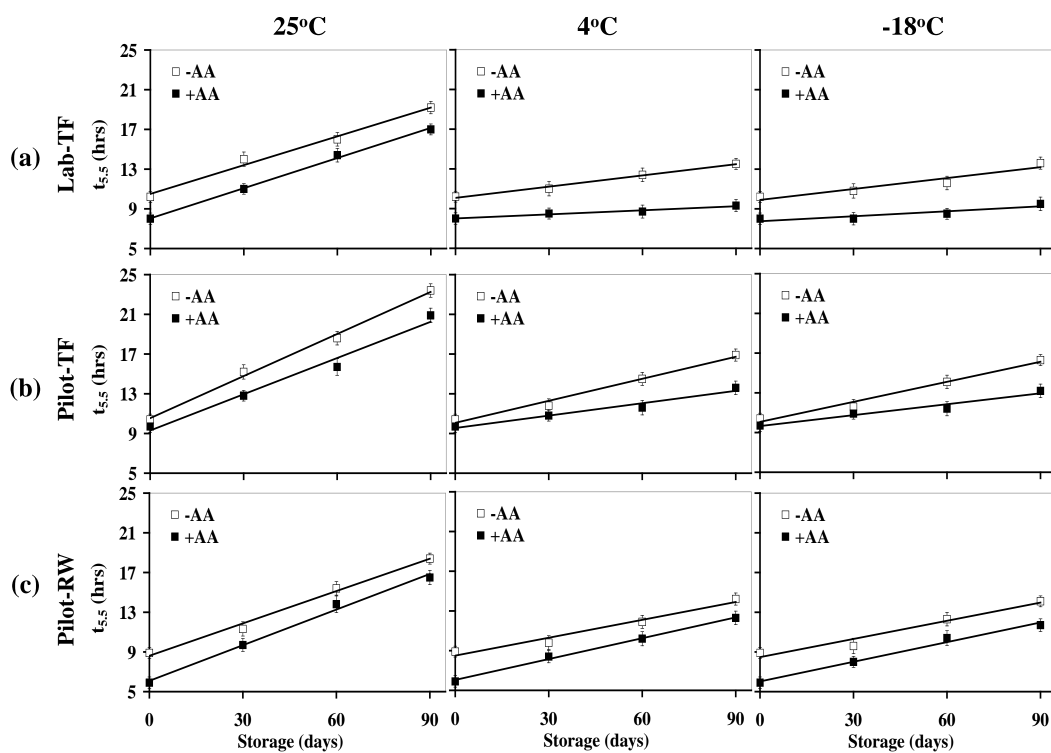
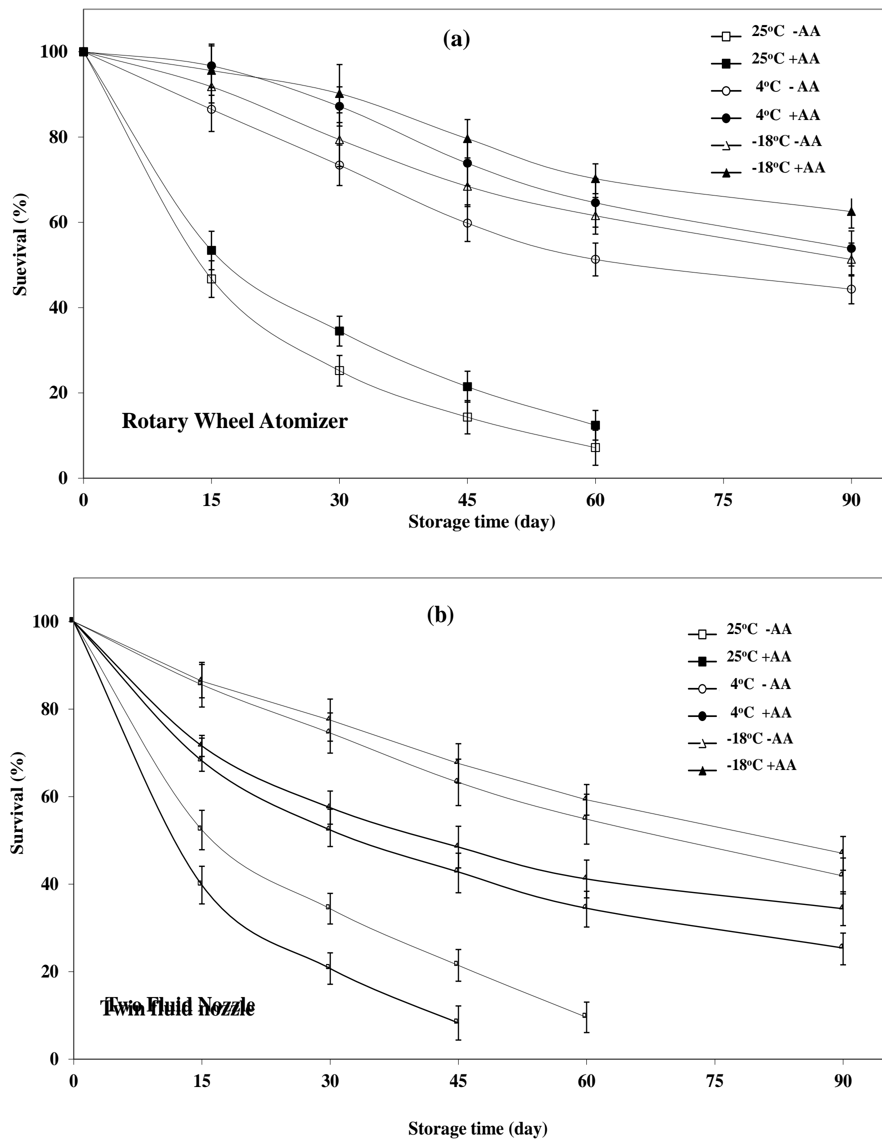


Fig. 4. The times required (hours) for powders stored for different length of time to reach a set pH value of 5.5 (TF refers to two fluid nozzle and RW refers to rotary wheel atomizer).



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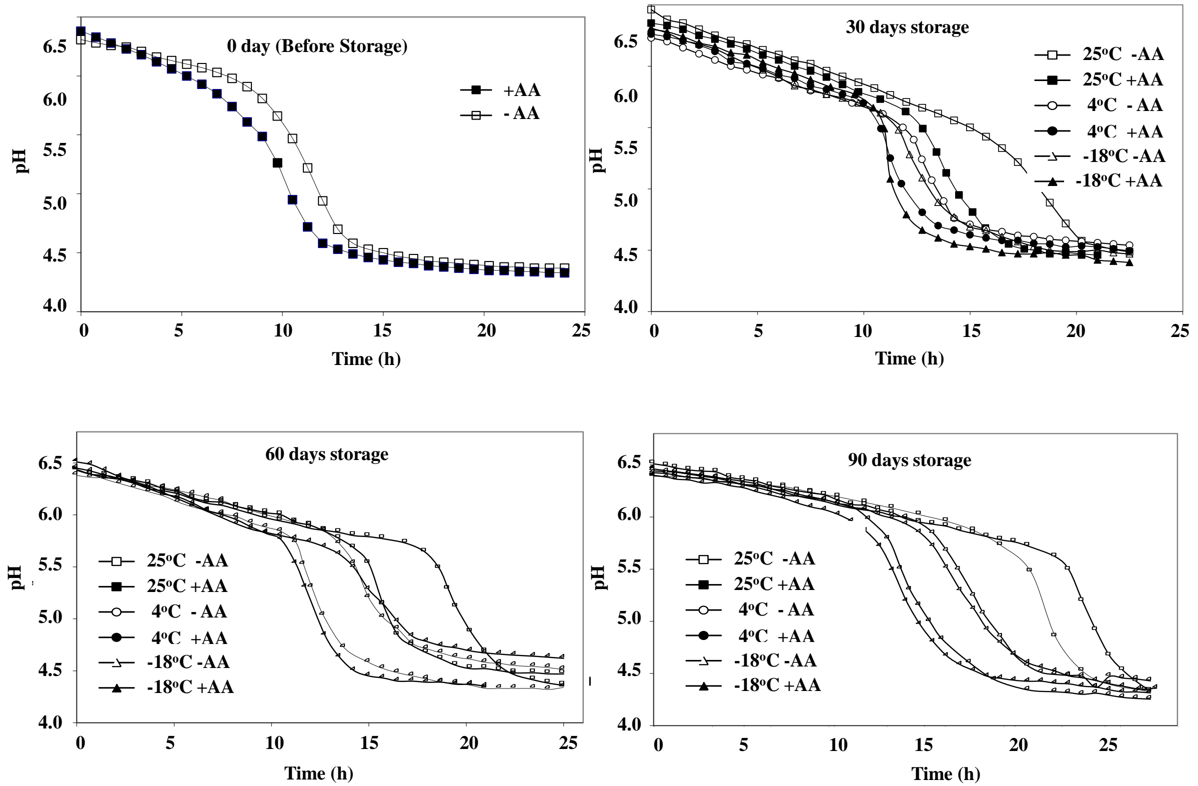

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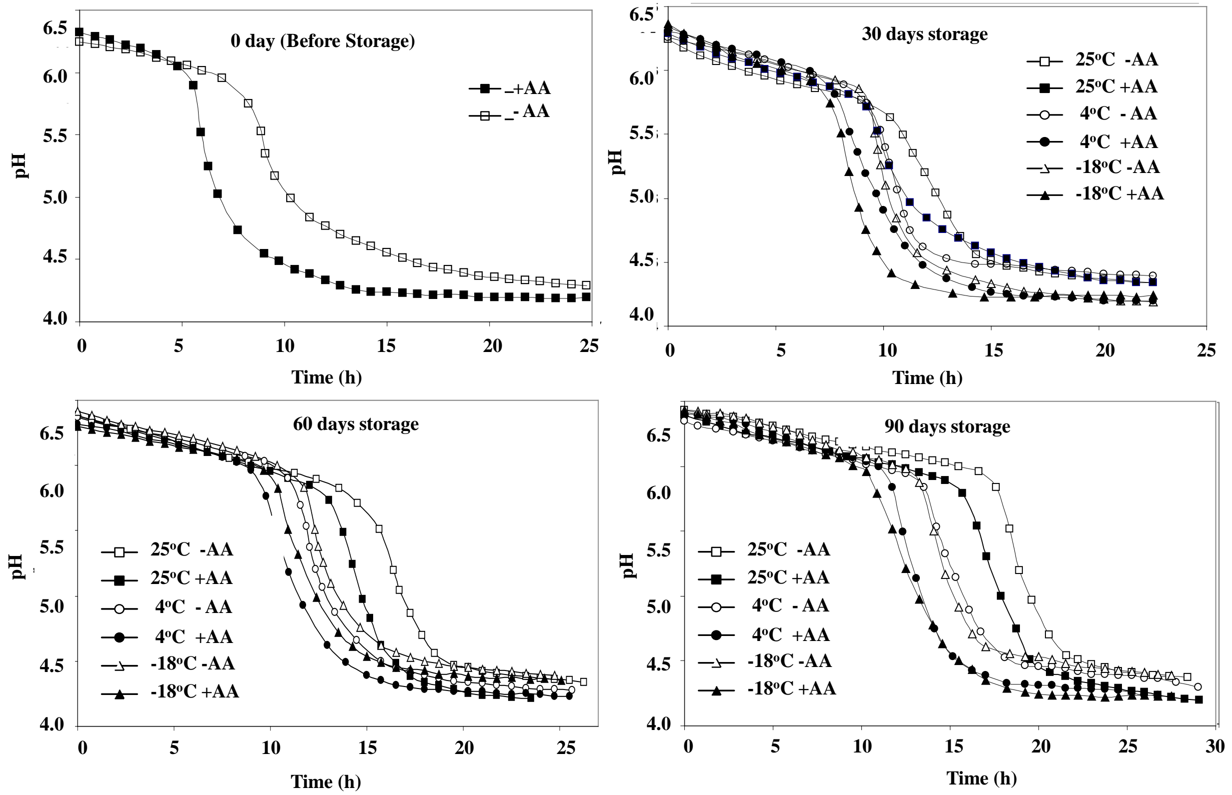

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