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Improvement of Lactic Cell Production

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ABSTRACT

The production of *Lactobacillus brevis* has been improved by changing the medium composition and the physiological conditions. The cellular concentration reaches 8.2×10^{10} cells/mL in 21 h with a fed-batch technique in MRS medium. Freeze-drying has been used as technology for drying the cells and survival rates have been improved with different additives such as glycerol, $CaCO_3$, and skimmed-milk powder up to 70%. A model has been developed to predict the stability of freeze-dried cultures during long-term conservation. This model, based on Arrhenius equation, has been confirmed by experimental data.

Index Entries: *L. brevis*; freeze-drying; fermentation; accelerated storage test.

INTRODUCTION

Lactic acid bacteria are largely employed in the food industry, especially for the fermentation of milk, meat, fruit, vegetables, and bread products *(1)*. Culture concentrates of microorganisms are then used.

The preparation of those culture concentrates requires production and maintenance techniques that maximize the storage stability, viability, and activity of the bacterial cells *(2–4)*. The most common technologies used for cell conditioning are fluidization, spray-drying, freezing, and freeze-drying. The choice depends on technological views, but also economical and practical constraints. In literature, lyophilization is frequently reported in preserving and distributing lactic starter cultures *(5,6)*.

Several factors, including the nature of the suspending solution, influence the ability of lactic organisms to survive after lyophilization *(5)*. Different cryoprotectors are commonly used to improve this stability. Glycerol, adonitol, dimethyl sulfoxide, carbohydrates, milk and its pro-

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ducts (nonfat dry solids of milk for example), serum, peptone, dextran, sodium glutamate, and yeast extract are some of them *(7,8)*.

The storage stability of a lactic starter is a very important factor for its industrial use. Optimal residual moisture of conservation differs in function of the culture medium composition, the protective compounds, the drying technique, and the condition of conservation used *(9)*. De Valdez et al. *(10)* proposed a tool for predicting the long-term conservation of freeze-dried powder of *Lactobacillus reuteri*. It is an accelerated storage test based on the Arrhenius' law.

Among lactic acid bacteria found in industry, *Lactobacillus brevis*, a heterofermentative microorganism, appeared to be very important because of its acidification property and, especially, for its production of flavors. It is therefore used in sausage as a fermentative agent. It also improves organoleptic qualities of bread. This microorganism is then interesting to study.

The purpose of this study is to improve the production of a starter culture of *L. brevis* and to develop a predicting model for long-term conservation of this powder at low temperature. The optimization of the production involves the optimization of the growth of the lactic bacteria in fermentor and of the drying procedure by lyophilization.

MATERIALS AND METHODS

Source and Maintenance of Microorganism

Lactobacillus brevis ATCC8287 was obtained from the culture of the Laboratory of Microbiology of Gent (Belgium). The strain was maintained on MRS agar at 4°C after incubation at 30°C for 48 h.

Media

MRS broth contains 10 g of casein peptone, 5 g of yeast extract, 5 g of meat extract, 20 g of glucose, 1 mL Tween-80, 5 g of CH_3COONa, 2 g of diammonium citrate, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot H_2O$ and 2 g of K_2HPO_4 in 1 L distilled water *(11)*. MRS agar was prepared by adding 16 g of agar, 5 g of $CaCO_3$ and 0.5 mL of an ethylic solution of bromocresol purple per liter. CSL broth contains 50 g corn-steep liquor, 21 g of yeast extract, 50 g of glucose, 0.0264 g of $MgSO_4 \cdot 7H_2O$, 0.0264 g of $MnSO_4 \cdot H_2O$ and 0.01264 g of $Fe_2(SO_4)_3$ per L of distilled water. The culture medium is adjusted to pH 6.8 with KOH 6 *N* before sterilization (121°C for 20 min).

Improvement of *L. brevis* Growth

Assays in Flasks

Assays were performed in flasks (300 mL) containing 150 mL broth, using a 1% inoculum and stirring at 140 rpm. Different temperatures of

incubation were tested (25, 30, and 36°C). Before experimental use, microorganisms were developed once in MRS broth. The microorganism growth was followed by measurement of the optical density at 540 nm (spectrophotometer Beckman, Fullerton, USA). Viable cell count was estimated by spreading 0.1 mL of appropriate dilutions over the surface of MRS agar plates. Dilutions were performed in peptone water containing 1 g of casein peptone, 2 g of Tween-80, and 5 g of NaCl per L distilled water.

Assays in Fermentor

Bacteria were propagated twice in MRS broth at 30°C with stirring at 140 rpm (in 100 mL for 16 to 18 h, and then in 500 mL for 8 h using 2% inoculum). Bacteria were next produced in a 20-L (Biolaffite Poissy, France) fermentor containing 12 L of culture medium, using 5% inoculum (stirring at 150 rpm, aeration rate of 0.2 v/v/min).

Initial cell concentration was approx 5×10^7 CFU/mL in all cases. Evolution of the bacterial growth was evaluated by an optical density measurement at 540 nm (spectrophotometer, Beckman) and an MRS agar count. Glucose concentration was also measured by enzymatic method (Biochemistry Analyzer YSI, OH).

Freeze-Drying

After the fermentation, cells were harvested by centrifugation at 14,000*g* (Sharpless MV15521IHC, UK). The pellet was suspended in peptone water (ratio of the mixture 1:1). Experiments were carried out with the following protective media:

1. 10% (w/v) nonfat dry milk solids and glycerol at different concentrations (2, 3, 4, and 5% [v/v]).
2. 10% (w/v) nonfat dry milk solids, 5% (v/v) glycerol, and 0.1% (w/v) $CaCO_3$.
3. 10% (w/v) nonfat dry milk solids, 5% (v/v) glycerol, and 0.1% (w/v) $MnSO_4$.
4. 10% (w/v) nonfat dry milk solids and 5% (w/v) maltose.
5. 10% (w/v) nonfat dry milk solids and 5% (w/v) saccharose.
6. 10% (w/v) nonfat dry milk solids and 5% (w/v) sodium glutamate.

After homogenization, concentrated samples were frozen at −20°C for 14 to 16 h and placed at −50°C for 24 h. Samples were then freeze-dried at +30°C, at a vacuum of 0.5 atm for 40 h (CHRIST, alpha I-21, Osterode am Harz, Germany).

Powders obtained were grinded, distributed in plastic bags and stored at +4°C. Survival rate of *L. brevis* was estimated by MRS agar count after centrifugation, lyophilization, and during storage.

Accelerated Storage Test

Samples were incubated at 30, 46, and 60°C. Viability assays were performed on each sample removed at constant intervals of time by MRS agar count. Samples were removed every 24 h at 30°C, every 3 h at 46°C, and every 1 h at 60°C.

Calculation of the results was done with the equation used in the fundamental studies of Greiff and Rightsel *(12)*. The method is resumed below. Thermal degradation of the microorganisms should follow the logarithmic form of the Arrhenius equation with respect to absolute temperature.

$$\log k = -(\Delta Ha/2303R)\cdot 1/T \quad (1)$$

where k is the specific rate of degradation (h^{-1}), ΔHa is the heat of activation (J/mole), R is the gas constant (8.32 J/mole.°K), and T is the absolute temperature (°K).

This equation indicates that any value proportional to the specific rate k would permit calculation of the slope of the log k vs $1/T$, and therefore the heat of activation. If such a relation among several values determined at high temperature is reasonably linear, the degradation rate at storage temperature can be calculated from the experimentally determined degradation rates at high temperature. The equation for a pseudo-order reaction to determine the rate of degradation *(k)* appeared appropriate.

$$\log N = \log N_o - k\cdot t \quad (2)$$

where N_o is the initial cell concentration (CFU/mL), N is the cell concentration at any time *(t)* (CFU/mL), k is the specific rate of degradation (h^{-1}) and t is the time (h).

Determination of k for each temperature tested (30, 46, and 60°C) is performed using Eq. 2. The construction of Arrhenius plot (k vs $1/T$) allows the estimation of k at lower temperatures. Cell concentration after long-term storage at low temperature can then be estimated.

Freeze-dried samples were also kept at 4°C to determine the percentage of survival after a few months and verify the model.

RESULTS

Improvement of *L. brevis* Growth

Assays in Flasks

Optimal temperature and initial pH of the culture medium have been first determined in MRS broth. Cellular growth has been followed by turbidimetric measurement (optical density at 540 nm). A correlation of

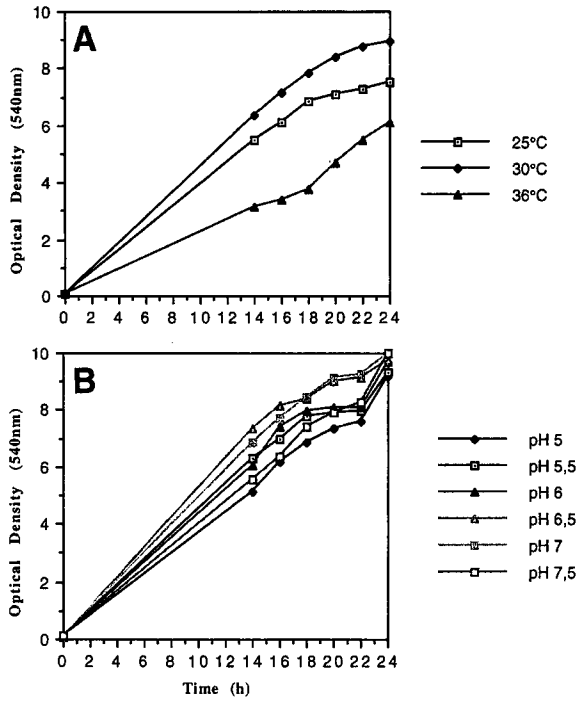

Fig. 1. **A** Effect of temperature on *L. brevis* growth in flask (MRS broth, 140 g-rpm of stirring). **B** Effect of initial pH on *L. brevis* growth in flask (MRS broth, 30°C, 140 rpm of stirring).

$N = -1.6 \times 10^9 + 9.6 \times 10^8 \cdot OD$ ($r = 0.97$) was obtained. Figures 1A and B show results obtained.

Optimal temperature for *L. brevis* growth is 30°C. *L. brevis* growth is more important when initial pH of the medium is greater than 6.0. It also seems that the growth is slower for initial medium of pH 7.0 and 7.5. A pH of 6.5 will therefore be retained as optimal initial pH medium for *L. brevis* growth.

The effect of the concentration of glucose, meat extract, and yeast extract was also tested. The final biomass is higher with increasing initial glucose concentrations (15–20 g/L). The optimal concentration of meat and yeast extracts is 3 and 5 g/L, respectively in MRS broth (data not shown).

The same final cell concentration has also been observed in MRS broth and CSL broth (6.9×10^9 CFU/mL in MRS broth after 23 h fermentation vs 6.3×10^9 CFU/mL in CSL broth).

Assays in Fermentor

During its growth, *L. brevis* generates lactic acid, which causes a diminution of the pH medium (from 6.5 at the beginning of fermentation to 4.0 at the end of the microorganism's growth). As *L. brevis* is sensitive to low pH, it is preferable to regulate this parameter. This regulation is not

Table 1
Influence of pH on the Growth of *L. brevis* in Fermentor

pH	Neutralizer used	Final cell concentration (cfu/ml)*10^{10}	Duration of fermentation (h)
6	KOH 6N	1,4 ±0,08	19h50'
6,2	KOH 6N	1,8 ±0,12	18h30'
6,5	KOH 6N	1,7 ±0,11	19h00
6,8	KOH 6N	1,5 ±0,16	21h00

CSL, 30°C, 150 rpm of agitation, 0.2 v/v/m in aeration rate.

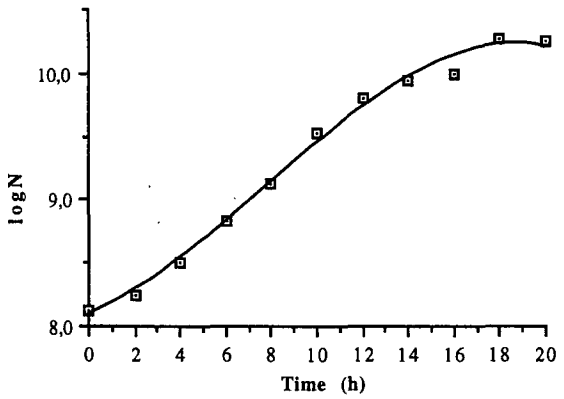

Fig. 2. Evolution of the growth of *L. brevis* in a 20-L fermentor. (MRS broth, 30°C, pH 6.5 regulated by KOH 6 N, 150 rpm of agitation, 0.2 v/v/min aeration rate).

possible in a flask but is in a fermentor. So, the first assays in the fermentor will concern detection of optimal pH. Fermentation is followed by glucose concentration determination and was stopped when glucose concentration was lower than 2 g/L. Cell concentration is then estimated by MRS agar count. Table 1 shows results obtained in CSL broth.

Final cell concentrations are similar in all cases. As we refer to fermentation time, it appears that optimal pH is ranging between 6.2 and 6.5.

Figure 2 shows the evolution of the growth curve of *L. brevis* in a 20-L fermentor (MRS broth, 30°C, pH 6.5, 150 rmp of agitation, aeration rate of 0.2 v/v/min). As assays in flasks have shown an identical growth of *L. brevis* in MRS and CSL broth, and as CSL broth is a turbid medium, growth curve was established in MRS broth to permit an optical density measurement.

The generation time (g = 1 h 50 min) and the maximal growth rate (μ_m = 0.39 h^{-1}) have been calculated from this growth curve.

The next step was the improvement of the final cell concentration, so two different media (CSL broth and modified CSL broth) were tested. Modified CSL broth (CSLm1 broth) consisted of CSL broth with a supple-

Table 2
Influence of the Medium on the *L. brevis* Growth

Medium	Duration of fermentation (h)	Final cell concentration (cfu/ml)*10^{10}
MRS	19	1,8 ±0,10
CSL	18	1,5 ±0,15
$CSLm_1$	18	1,7 ±0,12

30°C, 150 rpm of agitation, 0.2 v/v/m in aeration rate, pH 6.2 regulated by KOH 6 *N*.

ment of meat extract (2 g/L). Fermentation was followed by glucose concentration measurement and was stopped when glucose concentration became lower than 2 g/L. Final cell concentration was then measured by MRS agar count. Results are shown in Table 2. A fermentation in MRS broth is given for comparison.

In all cases, final cell concentrations reached the same value. Differences appeared in terms of medium cost. CSL broth is cheapest. Adding meat extract to this medium does not significantly improve *L. brevis* growth.

In order to improve the production of biomass, batch and fed-batch techniques were compared using MRS broth, CSL broth, and CSLm2 broth (containing 37.5 g/L of glucose instead of 50 g/L in CSL broth). Final cell concentration was determined at the end of the fermentation, when glucose concentration was lower than 2 g/L. The different assays and results are summarized in Table 3.

Fed-batch technique with CSL broth leads to a final cell concentration higher than batch technique (approx 2 times).

In the assays with MRS broth, a fed-batch technique with the whole medium gives no more growth than with the glucose alone.

Freeze-Drying

Assays of freeze-drying were performed with cells produced in CSL broth, at 30°C, 150 rpm of agitation, 0.2 v/v/min of aeration rate and pH regulated at 6.5 by KOH 6 *N*. Fermentation was stopped when glucose concentration was lower than 2 g/L. Under these conditions, the final cell concentration reached 1.5×10^{10} CFU/mL after 18 h of fermentation.

Loss of cells can occur at three steps, i.e., the centrifugation, the lyophilization, and the conservation. Cellular loss coming from the centrifugation process ranges from 10 to 20% (data not shown). The effect of the different protective media on the percentage of survival after lyophilization and the residual moisture content of the powders are shown in Table 4.

Table 3
Influence of Method of Carbohydrate Addition on the Growth of *L. brevis*

Medium used	Carbohydrates adding technique used	Amount of glucose added at the begining of the fermentation (g/l)	Amount and moment of the second glucose addition	Final cell concentration (cfu/ml) $*10^{10}$
CSL broth	batch	50	none	1,5 ±0,12
$CSLm_2$ broth	batch	37,5	none	1,9 ±0,3
CSL broth	fed-batch	25	25g/l after 11h of fermentation	2,8 ±0,18
MRS broth	fed-batch (glucose)	20	20 g/l after 12h of fermentation	8,2 ±0,8
MRS broth	fed-batch (MRS*)	20	addition of a modified MRS broth* after 12 h of fermentation (*this modified medium contains the same elements than MRS broth but at different concentration (1/2) excepted for glucose)	8,8 ±0,9

MRS and CSL broth, 30°C, 150 rpm of agitation, 0.2 v/v/m in aeration rate, pH 6.2 regulated by KOH 6 *N*.

Survival rates are relatively high (67–86%) and the best results concern the assay with nonfat dry milk solids, glycerol, and $CaCO_3$ (85.9%). Good percentages are also observed with glycerol and maltose (respectively, 73–82 and 79.6%).

Samples were stored at 4°C. MRS agar counts were performed over a few months. Results are shown in Fig. 3.

Percentage of survival rapidly decreases after the first weeks of storage. The decrease is less important after 1 month, and the survival concentration tends to stabilize.

Among the different cryoprotective media used, glycerol gives the best results in terms of survival rate (from 62 to 70% after 4 months of storage at 4°C).

Accelerated Storage Test

In order to elaborate a model of the stability of *L. brevis* during long-term storage, specific rate of degradation *(k)* of *L. brevis* freeze-dried in

Table 4
Effect of Cryoprotective Media on the Survival Rate and the Percentage of Dry Matter of Lyophilized *L. brevis*.

Cryoprotective media	Survival rate (%)	Dry matter content (%)
non fat dry milk solids (NFDMS) (10%) + glycerol (5%)	79,6	87
NFDMS (10%) + glycerol (4%)	82	90
NFDMS (10%) + glycerol (3%)	79	93
NFDMS (10%) + glycerol (2%)	73	95
NFDMS (10%) +glycerol (5%) + $CaCO_3$ (0,1%)	85,9	87
NFDMS (10%) +glycerol (5%) + $MnSO_4$ (0,1%)	74,2	88
NFDMS (10%) + maltose (5%)	79,6	95
NFDMS (10%) + saccharose (5%)	67,4	93
NFDMS (10%) + sodium glutamate (5%)	67,1	92

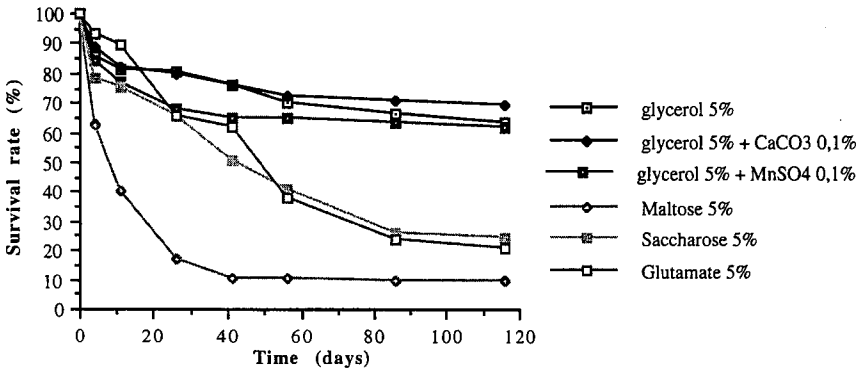

Fig. 3. Influence of cryoprotective media on the viability of *L. brevis* freeze-dried during storage at 4°C.

presence of nonfat dry milk solids (10%) and glycerol (5%) was first determined according to Eq. 2 as described in the subheading "Materials and Methods," for three high temperatures. The viability of freeze-dried cells during short storage at 30, 46, and 60°C was evaluated by MRS agar count. The value of k was calculated for each temperature on basis of a linear regression of the different points measured (Fig. 4).

The specific rate of degradation is given by the slope of the straight lines obtained (Table 5).

Table 5
Specific Rates of Degradation at 30, 46, and 60°C of Freeze-Dried *L. brevis*.

Storage temperature (°C)	Specific rate of degradation (h^{-1})
30	0,019 (k_{30})
46	0,32 (k_{46})
60	1,66 (k_{60})

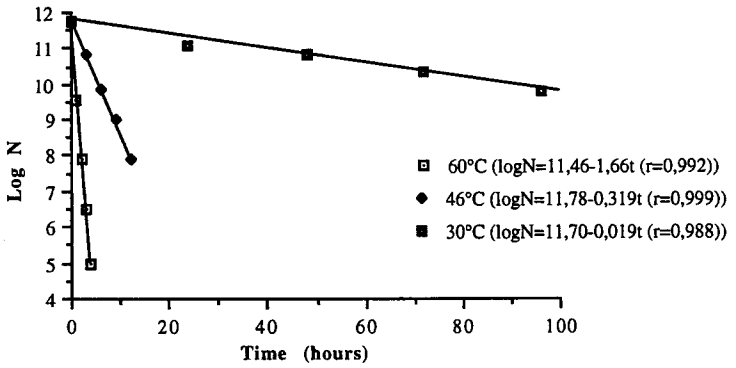

Fig. 4. Mortality of freeze-dried *L. brevis* stored at 30, 46, and 60°C in function of time (with nonfat dry milk solids [10%] and glycerol [5%] as cryoprotectors).

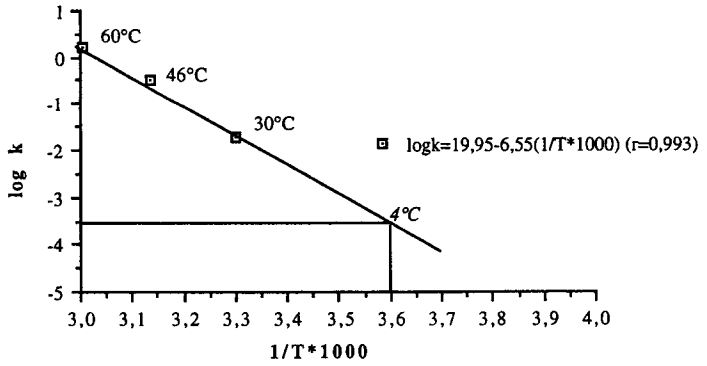

Fig. 5. Arrhenius plot of freeze-dried *L. brevis* (with nonfat dry milk solids [10%] and glycerol [5%] as cryoprotectors).

The specific rates of degradation above allow us to plot the Arrhenius graph, i.e., log k vs 1/T as shown in Fig. 5.

The specific rate of degradation at 4°C ($k_4 = 1.97\ 10^{-4}\ h^{-1}$) was estimated from this Arrhenius plot.

Table 6
Comparison of the Estimated and the Experimentally Measured Survival Rates of Freeze-Dried *L. brevis*.

During of storage at 4°C (days)	Survival rate estimated by the accelerated storage test (%)	Survival rate experimentally measured (%)
50	58	59
137	23	16

With nonfat dry milk solids 10% and glycerol 5% as cryoprotectors.

The cell concentration of the freeze-dried *L. brevis* stored at 4°C is estimated by using Eq. 2 given in the subheading "Materials and Methods".

The survival rate of freeze-dried *L. brevis* after 50 and 137 d estimated by the accelerated storage test and experimentally measured are represented in Table 6.

After 50 days storage, *L. brevis'* survival rate estimated by accelerated storage test is quite similar to survival rate experimentally measured. After 137 d storage at 4°C, results present a difference of 30%.

DISCUSSION

Improvement of the *L. brevis* Growth

A culture medium providing an optimal growth with maximum economy would be of great interest for industrial use *(13)*.

Using the optimal operating conditions in MRS broth (30°C, pH ranging from 6.2 to 6.5 regulate with KOH 6 *N*, agitation of 150 rpm, aeration rate of 0.2 v/v/min), the generation time of *L. brevis* was fixed at 110 min. Yildiz and Westhoff *(14)* found a generation time of 130 min in filtered cabbage juice for *L. brevis*.

MRS broth is an expensive medium comparatively to CSL broth, which is based on corn steep, a byproduct of corn industry. Corn steep contains proteins, mineral salts (especially oligo-elements), and molecules with vitaminic function (phytic acid, inositol, and so on). CSL broth appeared to be a good substitute of MRS broth. It provides the same final cell concentration and costs less (it is 48% less expensive). It will be noticed than CSL broth contains more glucose (50 vs 20 g/L) and more yeast extract (21 vs 5 g/L) than MRS broth. These differences might also explain the good growth of *L. brevis* in CSL broth.

Fed-batch technique provides an appreciable gain of cell production (2.8×10^{10} CFU/mL instead of 1.5×10^{10} CFU/mL in CSL broth). A fed-batch performed in MRS broth gives a higher final cell concentration than

a fed batch in CSL broth (8.2×10^{10} CFU/mL vs 2.8×10^{10} CFU/mL). As CSL broth is 48% less expensive than MRS broth, a fed-batch in MRS broth is lightly more interesting (because it allows a production of cells three times more important than CSL broth). A fed-batch realized in MRS broth with a complete medium gives no gain of cell production compared with glucose addition in two steps. This first type of fed-batch is therefore not economically interesting and won't be retained.

Some assays have been performed by replacing casein peptone in MRS broth by vegetable protein (potato protein). This modified medium gives an identical final cell concentration than MRS broth but is cheaper (data not shown). Other experiments will be needed to confirm this interesting result. Fed-batch technique with this medium had also to be performed.

Freeze-Drying

The additives for the freeze-drying procedure are very important and affect the cell survival and residual water content. The efficiency of a cryoprotective agent in the prevention of cell death during lyophilization, does not necessarily reflect its capacity to prevent loss of viability during storage *(10)*.

Among various cryoprotective agents used in this study, glycerol exhibits the best percentage of survival after lyophilization (82 and 86%).

Addition of glycerol (5%) implies a high residual moisture of the powders (12%) compared to other cryoprotectors used (5–8%). The differences in the content of residual water observed here could be explained by a higher degree of affinity of this cryoprotective agent for water *(15)*.

A high residual moisture content of the powders could be negative for long-term preservation. Nevertheless it had been proven that a reduction of glycerol concentration, which decreases the residual moisture, is not beneficial for the survival rate (from 85 to 73% for a glycerol concentration from 5 to 2%).

Moreover, it has been experimentally pointed out that the best conservation of the freeze-dried cells is obtained when using glycerol (from 62 to 70% after 4 months storage at 4°C).

Despite a higher residual moisture content, glycerol exhibits the best conservation results. This residual water is probably retained by glycerol and then is not available for cells. Other authors mentioned the use of 5% glycerol to decrease lethality during the drying process *(18,6)*. Viability during storage obtained with other cryoprotectors is quite small (from 10 to 30%).

The curves of the evolution of the viability at 4°C shows a tendency for rapid death of dried cells in the early period of storage followed by a stabilization. This phenomenon has been reported by other authors *(6,17–20)*.

It is assumed that a part of the surviving cells might be injured immediately after freeze-drying which leads to an initial postdrying lethality. Then the cell population, which is less susceptible to this postdrying inactivation mechanism might be selected to display an apparent stability in viability during the subsequent prolonged period *(20)*.

Use of $CaCO_3$ with glycerol allows a slight improve of long-term conservation.

Accelerated Storage Test

Accelerated storage test is an acceptable extrapolation tool of the stability at 4°C of freeze-dried *L. brevis* after 50 d. Indeed, comparison of predicted and experimental survival rates did not show any significant differences. Other experiments will be needed to confirm results obtained for longer storage period.

This method is advantageous because of its rapidity, with degradation rates determined at high temperatures requiring a relatively short time for their evaluation (from several hours to a couple of days). There is one restriction concerning the selection of the temperatures used for accelerated storage test, i.e., that the differences in the rates of degradation associated with each temperature must be statistically significant *(12)*.

This test can also provide a good comparison tool of efficiency of different cryprotectors for freeze-drying (in term of survival after lyophilization and stability during the storage).

It will be interesting to extend this kind of model to other cultures and other drying techniques. This model can also be applied to other fields. It has already been used to estimate thermal degradation of vitamin preparations and the measles virus *(21,12)*.

But, as the storage stability of dried cells depends on the residual moisture content, Arrhenius plot will always be associated with the residual moisture content of the sample tested.

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