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Short Communications

Effect of medium on growth and subsequent survival, after freeze-drying, of *Lactobacillus delbrueckii* subsp. *bulgaricus*

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SUMMARY

Growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*, grown at a constant pH (5.7) in papain- or pepsin-treated whey was superior to that in whey or whey permeate, but inferior to growth in milk; it was not significantly different from that found in a 1 : 1 whey : milk medium. When 2% sodium citrate was added to the fermented medium, the cell recovery levels (59–75%) upon centrifugation were not significantly different in papain-treated media compared to untreated substrates. Cultures obtained from papain-treated milk or whey showed similar mortality levels following freeze-drying (99%) to those grown on untreated milk or whey. Addition of Tween 80 to the growth medium improved survival rate by a factor of ten.

INTRODUCTION

There is growing interest in the freeze-drying (FD) of lactic acid cultures. Shipment of these cultures is easier than concentrated frozen starters, and shelf-life is longer [2]. They are promising for direct vat inoculation of cheesemaking milk [3] and they can be added easily to various dairy products such as ice cream or sweet acidophilus milk. Cultures such as *Bifidobacterium* or *Lactobacillus acidophilus* are also sold for their reputed health benefits [1].

Most studies on FD of lactic acid bacteria were performed on the effects of freezing media [5], freezing and drying conditions [9], modified atmosphere packaging [4] and rehydration and storage conditions [6,14]. There is little documentation, however, on the influence of growth media on subsequent survival of the cells during FD [7]. Tween and oleic acid [8] and calcium [16] influence survival of lactobacilli during FD. Although there have been studies on biomass production of lactic acid bacteria in whole [10] or enzyme-treated whey [13], there are no reports on the effects of culturing lactobacilli in these media on their subsequent survival to FD.

The aim of this work was to study biomass production and survival during FD of *Lactobacillus delbrueckii* subsp.

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MATERIALS AND METHODS

L. bulgaricus Y-12 was maintained in 12% rehydrated skim milk powder (12%) containing 0.25% $CaCo_3$ and heated at 112 °C for 10 min. The milk used for biomass production was prepared similarly, with the exception that carbonate was not added. The whey medium was rehydrated (6%) skimmed whey powder (Saputo, Québec). Whey permeate was obtained from Saputo (Saint-Hyacinthe) and frozen (−40 °C) until used. To prevent precipitation during sterilization (112 °C, 10 min), the pH of whey-based media was adjusted to 8.0 with 5N NaOH prior to heating. Some fermentations were performed on 1 : 1 mixes of milk, whey or permeate. Enzymatic hydrolysis was performed on some media prior to sterilization, using pepsin (Sigma P-7000, St-Louis, MO) or papain (American Chemicals Ltd., P-102, Montreal, Québec). Papain (0.5 g) was dissolved in 10 ml of water, prefiltered through Whatman No. 2 paper, filter-sterilized (0.22-μm pore size; Millipore, Montréal) and added to 1 l of milk or whey; cysteine was added at 0.2 g/l, and the medium was incubated 4 h at 37 °C. For pepsin hydrolysis, the whey medium was adjusted to pH 3 with phosphoric acid, 0.5 g/l pepsin was added and the

medium was incubated at 37 °C for 30 min. For one series of assays, 0.1% Tween 80 was added to papain-treated whey prior to inoculation. The FD medium consisted of 20% skim milk powder, 5% sucrose, 1% casein hydrolysate (Difco Casitone) and 0.35% ascorbic acid, which was filter-sterilized (0.22 μ) and added to the other ingredients following heat sterilization (112 °C, 10 min). The rehydration medium was that of Font de Valdez et al. [6].

Fermentations were performed in 2-l Braun Biostat M Fermentors (B. Braun Instruments; Melsungen, F.R.G.). After inoculation the medium contained 1.6×10^7 cells per ml. Temperature was maintained at 44 °C, agitation at 60 rpm and pH was maintained at 5.7 with 5N NH_4OH. The bacterial population was measured by plating on Elliker agar and incubating at 37 °C for 3 days. Results are presented in $\log_{10}$ cfu/ml.

For cell concentration, 2% sterile sodium citrate was added to the cooled (15 °C) fermented broth. The suspension (200 ml) was incubated at 15 °C for 10 min and centrifuged at 10000 × *g* for 10 min at a temperature of 4 °C. The pellet was resuspended in 20 ml of cold (4 °C) FD medium, resulting in a 10-fold concentration of the cells. The concentrated cell suspensions (2.5 ml) were distributed in 30-ml vials, frozen (−60 °C) in an air cabinet, and freeze-dried (FTS Systems Model TDS-4) 20 h at 23 °C under 70 μm vacuum. The vials were capped under atmospheric pressure, and were stored at 4 °C until used for thermal destruction curves [14]. Rehydration was performed by adding 2.5 ml of medium, and incubating the culture at 23 °C for 10 min. The viability was determined by plating on Elliker agar in which glucose was substituted by lactose.

Results are the average of three trials. Duncan multiple range formula was used for variance analysis (SAS software; Cary, NC, U.S.A.).

RESULTS

Whey permeate-based media were not appropriate for growth of *L. bulgaricus* (Fig. 1); cell counts were 20 times higher in milk as compared to whey permeate. Although higher than those in whey permeate media, cell counts obtained in whey media were inferior to those obtained in milk. Enzyme hydrolysis of whey did permit a slight (40%) increase in growth of *L. bulgaricus*. There were no significant differences in bacterial populations obtained in pepsin- or papain-treated media (Fig. 1). Cell counts obtained in enzyme-treated wheys were similar to that found in the milk whey medium, although they were lower (50%) than in milk alone.

Centrifugation of the milk medium resulted in a large deposit of casein, even with pH control. This was overcome by the addition of 2% sodium citrate. We recovered 75% of the viable cells from the whey medium and 59% from milk, percentages which were not significantly different ($P = 0.35$). Moreover, the papain treatment did not significantly ($P = 0.45$) increase the recuperation rates when citrate was added to the medium prior to centrifugation.

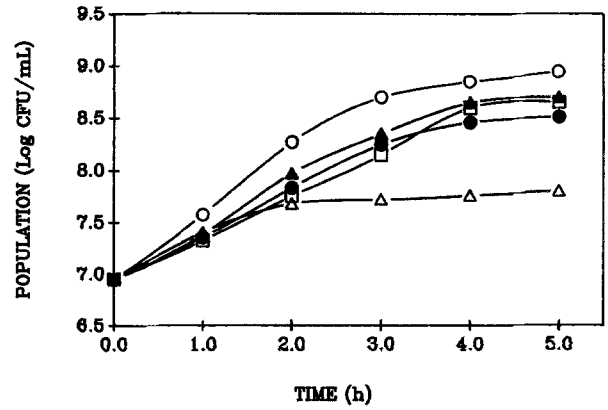

Fig. 1. Effect of medium composition on growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* Y-12. (○) Milk; (□) pepsin-treated whey; (▲) papain-treated whey; (●) whey; (△) whey permeate.

In all cases, the populations were reduced by more than two orders of magnitude following FD, regardless of the medium on which the cells were grown (Table 1). Most of the mortality occurred during drying, since on the average, 48% (0.42 log reduction) survived freezing. Papain hydrolysis alone did not influence the capacity of cells to survive FD, but addition of Tween 80 to papain-treated whey significantly reduced the mortality rate: when *L. bulgaricus* was grown in the Tween-supplemented whey, there was only a 1-log reduction in population.

The *D* values were obtained for each thermal destruction curve (37, 50 and 70 °C), and predicted conservations were determined [14]. Cultures grown on milk had the longest conservation potential (Table 2). Treatment of the substrates with papain seems to reduce the ability of cells

TABLE 1

Effect of growth medium on subsequent mortality of *L. bulgaricus* Y-12 upon freeze-drying

Medium	$\log_{10}$ mortality
Milk	2.62[a]
Milk-pap	2.23[a]
Whey	2.10[a]
Whey-pap	2.23[a]

[a] Values followed by the same letter are not significantly ($P < 0.05$) different.

TABLE 2

Relationship between D values and temperature of incubation for *L. bulgaricus* Y-12 grown on different media. Predicted D values at 4 and 20 °C were obtained from the regression equations

Medium	Regression equation	R	$D(4)$	$D(20)$
Milk	Log D = 2.89–0.035 T	0.98	562*	154*
Milk-pap	Log D = 2.37–0.021 T	0.87	193	89
Whey	Log D = 2.76–0.032 T	0.92	428	131
Whey-pap	Log D = 2.64–0.029 T	0.95	334	112

* D values (time required to obtain a 1-log reduction in viable cell counts) at 4 °C and 20 °C are in hours.

that have grown in these media to remain viable during subsequent storage.

DISCUSSION

Treatment of milk or whey with proteolytic enzymes significantly enhances the growth rates of lactic acid bacteria in these media [11]. This was the case for *L. bulgaricus* Y-12.

Recovery and concentration of cells is an important part of the process. Survival after FD was improved by concentrating the cell suspension [10]. Although protein hydrolysis can eliminate the necessity of using citrate for the concentration process [15], citrate was nevertheless added to the fermented broths prior to centrifugation. This was done to eliminate, as much as possible, particles of growth medium in the suspension. Thus, residual portions of the various growth media could not act as protective agents, and influence results.

The predicted D values at 4 °C were quite low, in view of the reports of stability for many months with these cultures [2]. This might be related to the presence of air in the vials. It has been shown that vacuum packed cultures are much more stable than those exposed to air during storage [4].

Whey treatment with proteases, prior to growth of the lactobacilli, increased cell counts, and did not adversely affect the resistance of cells to FD. Manufacturers of freeze-dried cultures should, however, pay close attention to the stability of such cultures upon storage.

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