Journal of Industrial Microbiology & Biotechnology (2002) 28, 291–296

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Effect of process parameters on the production and drying of *Leuconostoc mesenteroides* cultures

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Leuconostoc mesenteroides **BLAC was grown on MRS broth or on a carrot juice medium, and the effects of sugar concentration, type of pH control, aeration and fermentor size on viable counts were examined. The effect on viability of the type of centrifuge used to concentrate the bacterial culture was also examined. When the MRS broth had the traditional 110 mM glucose, pH control did not increase the final population. However, using a zone pH control mode, increasing the glucose content of MRS both from 110 to 220 mM almost doubled the population. In MRS broth, the amount of acetic acid produced was the same for all treatments, and was proportional to the amount of citrate consumed. There was a significantly lower cell yield in the carrot juice medium when the pH was not regulated. In the carrot juice medium, pH had a more pronounced effect on the final population level than did aeration, even though the quantity of viable cells was greater when the culture was aerated. In MRS broth, glucose was completely consumed during fermentation, but this was not the case in carrot juice medium. Aeration resulted in increased acetic acid content of the fermented medium. Viable counts were not affected by scaling the volume of the fermentation from 2 to 15 l ,or by the type of centrifuge used to concentrate the cells. Cells were concentrated by a factor of 10, but in both centrifuge types, viable counts showed only an eightfold average increase. However, freeze-dried powders obtained from the continuous pilot-plant-centrifuged cultures had, on the average, 33% lower populations than those obtained from the laboratory unit.**
Journal of Industrial Microbiology & Biotechnology (2002) **28,** 291–296 DOI: 10.1038/sj/jim/7000245

Keywords: starter; vegetable media; pH; aeration

Introduction

Leuconostoc cultures are used in the production of various useful products in the food industry. *Leuconostoc mesenteroides* ssp. *cremoris* and *L. mesenteroides* ssp. *dextranicum* are frequently used with other lactic acid bacteria as mesophilic starter cultures in dairy fermentations [11,13,19,23]. The ability of *Leuconostoc* cultures to produce acetoin and diacetyl has led to their widespread use as aroma producers in dairy products [4]. They also contribute to the production of openings in Gouda and Roquefort cheeses, helping the establishment of *Penicillium roqueforti* in the latter [12]. Lately, *L. mesenteroides* has gained attention as a producer of dextran, which can be used in plasma substitute formulations as a drug carrier and for chelating metals such as iron. In the food industry, dextran is used as an inhibitor of crystallisation of the sugars in syrups and candies [15,18]. *L. mesenteroides* is capable of using fructose as an alternative electron acceptor, reducing it to mannitol. Mannitol is widely used in the food industry and in the pharmaceutical formulation of chewable bars and tablets [20]. Some species of *L. mesenteroides* have also been involved in vegetable fermentations as they produce lactic acid and acetic acid [2]. They were also reported to produce bacteriocins [22] and to inhibit Gram-negative psychotrophic bacteria in milk [14].

Even though the use of *Leuconostoc* is well documented for the dairy industry [4,8-10], the scarce amount of information concerning the production of cultures on nondairy substrates, as well as the harvesting of *L. mesenteroides* on a larger scale, prompted the present work. Thus, the goal of this research was to measure the growth of *L. mesenteroides* on vegetable juices and to assess the effects of centrifugation and freeze-drying on cell viability.

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Received 9 July 2001; accepted 25 January 2002

Materials and methods

Biological material

L. mesenteroides ssp. *mesenteroides* BLAC was obtained from the culture collection of the Food Research and Development Centre. Reference cultures were prepared by adding fresh MRS-grown cultures to sterile nonfat milk solutions (20% wt/vol solids) and 5% (wt/vol) sucrose solutions in the ratio of 1:2:2. They were then freeze-dried. Powdered cultures were kept at 4°C until rehydrated. The powdered cultures were rehydrated by adding 1 g of powder to 4 ml of liquid medium containing 1.5% (wt/vol) peptone, 1.0% (wt/vol) tryptone and 0.5% (wt/vol) meat extract, and letting them stand at room temperature for 10 min. Subsequently, the cultures were transferred to MRS medium, incubated at 30°C for 24 h, then transferred a second time, using a 1% (vol/vol) inoculum, to MRS medium for use as starter cultures.

Fermentations in MRS medium

Bacteria were grown in 2-l New Brunswick fermentors (Bio-Flo 3000; Edison, NJ, USA) in MRS broth (Rosell, Montreal, Canada) with glucose concentrations adjusted to 110 or 220 mmol/l (mM). Two methods were used to control pH during the fermentations. The classical approach consisted of maintaining the pH constant at 6.0 with 5 N KOH with agitation at 100 rpm. The second method

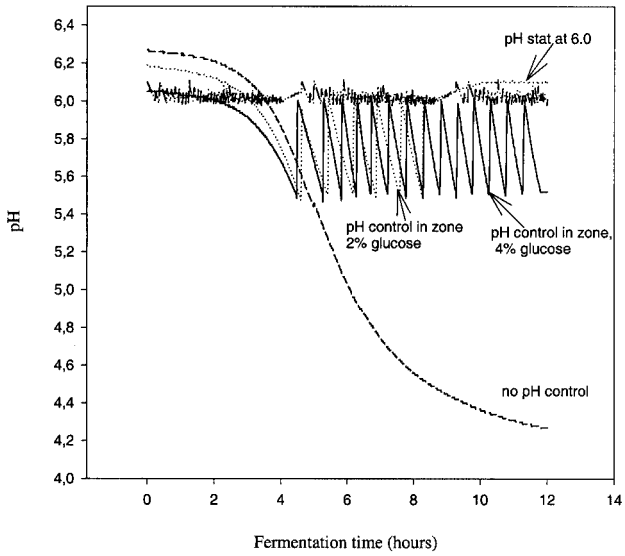

Figure 1 Acidification profiles of *L. mesenteroides* at various pH control modes in MRS medium.

consisted of starting the culture at pH 6.0 at 60 rpm and allowing the pH of the medium to decrease to 5.5, at which point it was returned to pH 6.0 (Figure 1). The agitation rate was increased to 100 rpm during base addition and resumed at 60 rpm when the pH reached 6.0. The cycle was repeated until the culture completely stopped its production of acid. This type of control will be referred to as the "zone pH control" mode. In some fermentations, the pH was not regulated and it was allowed to decrease to less than 5.0 (Figure 1). In this instance, fermentations were stopped after the pH had remained stable for 1 h. Agitation was carried out at 100 rpm even though no neutralization was performed, in order to enable the regulation of temperature in the fermentor. Data from fermentations are from three separate trials.

Fermentations in carrot juices

In trials involving vegetable juice, fermentations were conducted to evaluate the combined effects of pH control and aeration. The juice was reconstituted from a carrot concentrate (Lassonde, Rougemont, Canada) to 8° Brix in which 110 mM glucose, 344 mM NaCl and 42 mM sodium citrate were added. Juices were centrifuged to remove the pulp, heat-sterilised at 121°C for 10 min and filtered (Whatman 2, Maidstone, England, UK) to remove precipitates. The pH of the carrot juice was adjusted to 6.0 and controlled using zone pH control. In the aerated media, an agitation of 550 rpm was used, and sterile air (4.3 l/min) was added from the bottom of the 2-l vessel. Unless otherwise stated, cells were harvested at the end of fermentations using a laboratory-scale centrifuge operated at $10{,}000\times g$ for 10 min at 4°C (Beckman, Palo Alto, CA, USA). Fermentations in carrot juices were done in triplicate.

Scale-up of biomass production

Fermentations at the 2-l and the 15-l scale were compared. Cultures of 15 l were produced in a Bioengineering fermentor (NFL 19 L; Sagenraintrasse, Switzerland) using MRS medium supplemented with glucose to a final concentration of 165 mM. The pH was maintained at 6.0 with 5 N KOH. At the end of fermentation, media were cooled to 8°C and centrifuged to concentrate the cells. Two types of centrifuges were compared. The laboratory-scale centrifuge (Beckman) was operated at $10{,}000\times g$ in batch. The pilot-scale centrifuge Sharples T-1P (Alfa-Laval, Warminster) was operated at $30{,}000\times g$ in a continuous mode. The cell paste was recovered after 1 h of centrifugation. The pH of all fermented media was adjusted to 6.0 prior to centrifugation. Data from the scale-up fermentations are from three separate trials.

Freeze-drying

Cell pastes recovered from the laboratory or pilot plant centrifuges were suspended in a cooled (4°C) freeze-drying medium composed of 20% wt/wt skim milk powder and 146 mM sucrose. This medium was previously heated at 110°C for 10 min. Ascorbic acid was filtered-sterilised and added to the milk to a final concentration of 20 mM. Cells were allowed to stand in the cryoprotective solution for 30 min at 4°C. Afterward, the culture was dispensed in flat trays forming a 1-cm-thick layer and frozen at −70°C. It was then freeze-dried in a Lyo-San (Lachute, Quebec, Canada) freeze-drier for 48 h at 25°C and its viability was evaluated. Survival rate for *L. mesenteroides* was evaluated by multiplying the number of viable cells by the concentration factor from the centrifugation and the freeze-drying process (33×). The number of viable cells was then divided by the theoretical yield of cells and the result multiplied by 100.

Analyses

Substrates and products were analysed by HPLC using a Waters (Mississauga, Canada) system coupled to Millennium software. Samples were centrifuged and filtered prior to separation on an Aminex HPX 87H column (Bio-Rad, Mississauga, Canada) coupled to a refractive index monitor (Waters model 410) and a

Table 1 Cell yield in MRS and carrot juice media under various fermentation conditions and after freeze-drying

Fermentation conditions	Cell count at the end of fermentation	Cell count at the end of freeze-drying	% Survival
MRS 220 mM glucose, pH zone	7.0×10^{9}[a]	3.6×10^{10}	42
MRS 110 mM glucose, pH zone	3.6×10^{9}	4.7×10^{10}	40
MRS 110 mM glucose, pH 6.0	2.4×10^{9}	4.4×10^{10}	55
MRS 110 mM glucose, no pH control	5.7×10^{9}	5.6×10^{10}	30
Carrot, aeration, pH zone	6.9×10^{9}	5.6×10^{10}	25
Carrot, aeration, no pH control	3.4×10^{9}	1.5×10^{10}	13
Carrot, no aeration, pH zone	4.8×10^{9}	3.0×10^{10}	19
Carrot, no aeration, no pH control	2.8×10^{9}	3.7×10^{10}	40

[a]CFU/ml or CFU/g powder.

Table 2 Metabolites at the end of fermentation at various modes of pH control in MRS

	220 mM[a] glucose, pH zone	110 mM glucose, pH zone	110 mM glucose, pH 6.0	110 mM glucose, no pH control
Glucose	4[a]	2	3	2
Lactic acid	192	123	95	123
Acetic acid	70	70	70	70
Ethanol	174	109	133	54
Lactic/ethanol ratio[b]	1.1	1.1	0.7	2.3

[a]Concentration in mmol/l.
[b]On a molar basis.

photodiode array detector model 996 (Waters). The mobile phase was 8 mM H_2SO_4 at a flow rate of 0.4 ml/min.

Viable counts were obtained by plating the cells on MRS agar in duplicate for each analysis and incubating at 30°C for 48 h. MRS agar was shown to provide growth factors for culturing a variety of lactic acid bacteria and was used for all the plating. With the freeze-dried samples, 1 g of powder was added to 4 ml of a recovery medium as described above. Dilutions were carried out in sterile peptone (0.1% wt/vol) water and plated on MRS agar. Bacterial chains, in the first 100 ml dilution bottle, were broken with an homogeniser at 50,000 rpm for 1 min (Omni International, Gainsville, FL, USA). The overall standard deviation of the log values of the entire cell counts averaged 0.15. Statistical analyses were performed using the Instat software (GraphPad Software, San Diego, CA, USA).

Results

Fermentations in MRS (2-l scale)

Cultures in 110 mM glucose medium stopped producing acid after 12 h in the absence of pH control, and after only 8 h when pH was controlled by the zone method (Figure 1). With pH zone control, six neutralization cycles were required before the cultures in 110 mM glucose medium stopped producing acid. Fourteen neutralization cycles were required in the 220 mM glucose medium. Even though the number of neutralization cycles was more than double for the 220 mM glucose-containing medium, the fermentation time was much less than double. In the pH zone control mode, increasing the glucose content of MRS medium from 110 to 220 mM almost doubled the viable count obtained. Thus, the extended fermentation period seems to have translated into extended growth.

In the absence of pH control, the pH of the medium dropped to 4.3, but the low pH did not significantly affect the final viable count (Table 1).

The amount of acetic acid produced was the same for all treatments (Table 2). The MRS medium initially contained 62 mM acetate, which means that approximately 8 mM was produced by fermentation. This suggests that pH and agitation had no influence on the production of acetate. The final concentration of acetate depended on the initial amount of citrate in MRS medium and on its conversion to sodium acetate. Thus, the acetate concentrations obtained reached the theoretical values (70 mM), considering 1 mol of citric acid will generate 1 mol of acetic acid.

When MRS medium contained 110 mM glucose, the type of pH control had a more noticeable effect on the production of lactic acid and ethanol. The amount of lactic acid produced with the zone pH control mode was significantly higher than when it was controlled at 6.0, while the amount of ethanol produced was lower. When the pH was not regulated, the production of ethanol was much lower (Table 2). The lactic/ethanol ratio was two to three times lower when pH control was carried out, but the higher concentration of glucose did not affect the lactic/ethanol product ratios (Table 2).

Fermentation of carrot juice

Trials were conducted in vegetable juice to find an alternative medium to the MRS-type media. There were significantly lower cell populations in the carrot juice medium when the pH was not regulated. This is probably due to the presence of phosphates and citrate in the MRS medium producing a buffering action, which was not the case in the carrot juice. The pH had a more pronounced effect on the final biomass level than did aeration, even though the yield of viable cells was greatest when the culture was aerated (Table 1).

The production parameters affected the bioconversion processes. Aeration of the medium resulted in increased acetic acid in the fermented medium (Table 3). More lactic acid was produced in the fermented carrot juice when the pH was regulated (Table 3), which was not the case in MRS medium. The concentration of

Table 3 Metabolites at the end of fermentation in carrot juice medium under various pH control and aeration conditions

		Aeration, pH control	Aeration, no pH control	No aeration, pH control	No aeration, no pH control
Sucrose	110[a]	4	5	4	4
Glucose	108	85	108	97	107
Fructose	46	110	126	110	112
Citric acid	39	34	34	32	34
Lactic acid	–	113	65	91	79
Acetic acid	–	83	55	43	42
Ethanol	–	115	100	124	115

[a]Concentration in mmol/l.
Data in the first column are initial values.

Table 4 Effect of fermentor size and type of centrifuge on cell yield and harvesting of *L. mesenteroides* BLAC

Production steps	New Brunswick (2 l)		Bioengineering (15 l)	
End of fermentation[a]	4.1×10^{9}		4.1×10^{9}	
Type of centrifuge	Sorval	Sharples	Sorval	Sharples
Centrifugation	2.6×10^{10}	3.9×10^{10}	3.7×10^{10}	3.4×10^{10}
Freeze-dried powder	1.4×10^{11}	9.1×10^{10}	1.4×10^{11}	9.8×10^{10}

[a]CFU/ml or CFU/g powder.

carbohydrates left after the fermentation was important even when the pH was regulated. Glucose was in excess in the juice, as its concentration remained higher than 83 mM in all fermented media. The decrease in sucrose was accompanied by an increase in fructose. Molar transformations of data in Table 3 show that the glucose fraction obtained from sucrose hydrolysis was converted mainly to lactic acid and ethanol, while the fructose portion was excreted into the medium. Molar conversions of the data also showed a change in the lactate-to-ethanol ratios. Thus, with pH control in zone, there was 0.87 mol of lactate produced for every mole of ethanol, while without pH control this ratio was 0.67.

No trend could be found with respect to the effect of pH control or aeration on the cultures' subsequent survival upon freeze-drying. Cells produced in the carrot juice medium tended to have inferior survival rates than those obtained from MRS medium (Table 1), but the differences were judged to be not quite significant ($P=0.06$).

Fermentation and centrifugation scale-up

Increasing the scale of fermentation from 2 to 15 l did not significantly influence the viable populations obtained, nor did the type of centrifuge used (Table 4). The cells were concentrated by a factor of 10, but viable counts showed only an eightfold average increase. Furthermore, freeze-dried powders obtained from the Sharples-centrifuged cultures had, on the average, 33% lower populations than those obtained from the Sorvall laboratory unit. This is the only parameter tested that had a significant effect on survival after freeze-drying.

Discussion

Effect of pH control on cell counts

Acidification of the growth medium produced a drop in both internal and external cell pH, which ultimately inhibited growth. Two techniques have been used to reduce or prevent the detrimental effect of pH on lactic cultures: addition of buffers to the medium or external pH control.

Contrary to results in MRS medium, there were significantly fewer colony-forming units per milliliter in carrot juice medium when the pH was not regulated than when the pH was regulated. This probably resulted from the buffering action of phosphates and citrate, which were at higher concentrations in MRS medium than in carrot juice medium.

It has long been recognized that lactic acid cultures contain more cells when grown with pH control than without [1]. This was not the case in MRS medium with 110 mM glucose, but it was so in carrot juice as well as in MRS medium with 220 mM glucose. The buffering capacity of the MRS medium enabled the complete consumption of glucose (Table 2) when the medium contained only 110 mM glucose. Thus, in this instance, growth was not due to an inhibitory pH level but to depletion of the carbohydrate source. It is thus logical that pH control with an MRS medium containing 110 mM glucose did not improve yields. Increasing the glucose level to 220 mM enabled an increase in colony-forming units per milliliter, which was significant compared to the 110 mM glucose pH zone treatment, but only marginally higher than in 110 mM without pH control. At the same time, in the medium having 220 mM glucose, concentrations of lactic acid and ethanol increased in the medium. These may have limited growth to some extent. Thus, the maximal population under these conditions may have been reached before the culture stopped producing lactic acid. The literature is abundant on the effect of organic acids on growth of lactic cultures. Lactate inhibits growth of *Leuconostoc* even at low (28 mM) concentration [3], and at such a level, fermentation rates were stimulated by acetate. *Leuconostoc* is inhibited at pH levels between 5.3 and 5.7. Lactate was inhibitory at any concentration tested [3,6] and 277 mM lactate inhibited growth by 50%. This might be due to the fact that lactic acid is more effective than sodium acetate at decreasing the internal pH of *L. mesenteroides* cells [16].

Since increasing glucose from 110 to 220 mM was beneficial to growth, we attempted to determine the maximum concentration of metabolite that could be reached, and fermentations were conducted on carrot juice medium enriched with glucose. At first glance, results of fermentations in supplemented carrot juice suggest that excess metabolites could indeed stop growth and acidification, since significant amounts of residual glucose were found in the fermented carrot media under pH control. However, the lactic acid concentration did not exceed 113 mM in carrot juice medium, which was lower than that observed in MRS medium with either 110 or 220 mM glucose. This indicates that the accumulation of

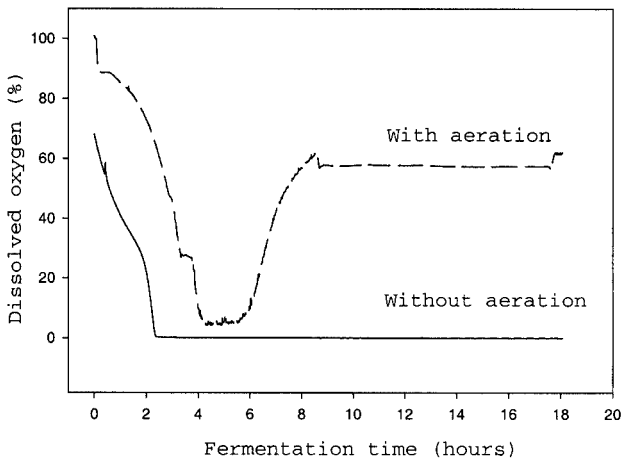

Figure 2 Dissolved oxygen in carrot juice under aerated and nonaerated conditions.

lactate was not the limiting growth factor; carrot juice medium might be a nutritionally poor medium. *Leuconostoc* cultures have variable requirements for vitamins and amino acids [7], and it could be hypothesized that the addition of yeast extracts or peptones would have enabled more extensive growth. More data are needed on the effect of medium composition and metabolite inhibition for the optimization of growth of this strain.

Controlling the pH in zone was carried out in order to limit agitation of the medium, since the agitation is only conducted when the base is added. Zone pH control is routinely conducted commercially for the production of lactic cultures. This is because many lactic acid bacteria are sensitive to oxygen [5] and the limited agitation during zone pH control reduces aeration of the medium. With this strain, constant agitation did not generate a reduced population in MRS medium containing 110 mM glucose. Since agitation did not seem detrimental to our strain, and since aeration had been shown to be beneficial for the growth of some *Leuconostoc* cultures [17], we decided to study the effect of aeration on viable counts.

Effect of aeration on cell yield

Growth occurred in nonaerated media, but oxygen was consumed under aeration and better growth was achieved. These results are in agreement with those of Whittenbury [24]. When the medium was sparged with air, the dissolved oxygen concentration fell to zero at the end of the lag phase (Figure 2), which is in agreement with the results obtained by Plihon *et al* [17]. In their experiment, by doubling or quadrupling the flow rate of air, only a slight increase in growth was observed. To avoid limitation by oxygen, pure oxygen can be used. Plihon *et al* [17] found that pure oxygen at 120 l/h increased growth and cell yields by over 50%. In our case, with pure oxygen, the effect of aeration could probably have been more pronounced. But even under the conditions used in this study, the final cell yield was improved by aeration in the carrot juice medium. A benefit of aerobic metabolism in lactic acid bacteria is the possibility of greater biomass yields at the expense of specific amounts of a carbohydrate energy source as observed by several authors [5,25]. Data from this study reveal a higher synthesis of acetic acid under aerated conditions. According to Plihon *et al* [17], oxygen acts on some enzymes involved in the metabolism of citrate in *L. mesenteroides*. Under aerated conditions, the activity of the acetate kinase is stimulated while the activity of the phosphotransacetylase is inhibited, bringing more acetate and less ethanol. These results agree with our observations.

The range of substrates that can be utilised under aerobic conditions is wider than under anaerobic conditions [5]. Results of this study do not show major shifts in substrate consumption. Greater yields were more pronounced at low sugar concentrations. It would be of interest to evaluate the effect of aeration on fermentation with media varying in carbohydrate concentrations.

Fermentor size

Scaling-up fermentations from laboratory to industrial scale generates differences in sterilization procedures, maintenance of aseptic conditions, aeration and temperature control as well as agitation units [21]. In this study, scaling-up from 2 to 15 l resulted in differences in sterilisation procedures as well as in aeration. In spite of these changes, scale-up did not affect the final population. Care was taken in carrying out laboratory assays, keeping in mind conditions that existed at larger scale. Thus, the autoclave sterilization was performed with a probe in the medium, and real retention times were applied, such as would occur in a larger fermentor. This may have contributed to laboratory results consistent with results expected for larger-scale fermentations.

Centrifuge type

Centrifugation was detrimental to viability with both centrifuges, since only 80% of the initial viable population was recovered after centrifugation. However, the high-speed ($30{,}000\times g$) Sharples centrifuge might have caused more damage to cells than the laboratory-scale centrifuge, as evidenced by reduced survival levels after freeze-drying of the Sharples-centrifuged cultures. Evidently, cells of *L. mesenteroides* BLAC are sensitive to the centrifugation process, and it will have to be determined if concentration by filtration would provide better survival.

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