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Process Biochemistry 40 (2005) 411–416

PROCESS BIOCHEMISTRY

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Lactobacillus casei cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production

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Received 4 August 2003; received in revised form 4 December 2003; accepted 15 January 2004

Abstract

Lactobacillus casei cells were immobilized on fruit (apple and quince) pieces separately and the immobilized biocatalysts were used for 15 successive fermentation batches of whey immediately after their preparation and for fermentations of milk immediately after their preparation and after a long storage period at low temperature. The immobilized biocatalysts used for successive fermentation batches of whey proved to be very effective and suitable for food grade lactic acid production, while no loss of activity at all temperatures (30, 37, 45 °C) tested was observed, as very high lactose conversion was reported. Cell immobilization of *L. casei* on apple and quince pieces was also shown by electron microscopy. In addition, apple and quince supported biocatalysts were used for milk fermentation immediately after their preparation, and after storage for 15, 98, and 129 days at 4 °C. No infection was reported during the storage periods. After storage, the immobilized biocatalysts were reactivated very quickly and produced milk fermentation products with a fruity, distinctive aroma which remained during all storage period.

Keywords: *Lactobacillus casei* immobilization; Apple pieces; Quince pieces; Whey fermentation; Lactic acid production; Fermented milk; *Lactobacillus casei* survival; Probiotic additive

1. Introduction

Nowadays, a new trend is the development of novel fermented milks, which contain microorganisms, called probiotics such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and others. Probiotic lactic acid bacteria act beneficially in human health, and therefore, a wide variety of lactic acid bacteria strains are available to consumers in both traditional fermented foods and in supplement form [1,2]. Generally, the production of probiotic foods that will contain specific probiotic strains at suitable levels of viable cells during their shelf life is a technological challenge [3].

The shelf life of probiotics should be controlled in order to manufacture products with adequate live bacteria (at least 10^7 CFU/g) to obtain the health promoting benefits of probiotic cultures [4].

The viability of probiotic bacteria can be improved by methods such as immobilization, appropriate selection of acid, and bile resistant strains, use of oxygen impermeable containers, stress adaptation, and others [2].

Lactic acid is also an important chemical used in a wide variety of applications, being used primarily in the food industry as an acidulant, preservative, and for the production of emulsifying agents [5].

Lactobacillus casei cells have been immobilized in some supports for lactic acid production. Agar was more effective than polyacrylamide for *L. casei* entrapment for lactic acid production from whey [6]. Also, calcium pectate gel and chemically modified chitosan beads were used as supports for *L. casei* cell immobilization [7]. Alginate has so far been a popular matrix for immobilization of lactic acid bacteria [8–11]. Other supports used for immobilization include porous foam glass particles [12], ceramic beads or porous glass [13], poraver beads [14], and gluten pellets [15].

However, cell immobilization on a food-grade support is essential for food production. In addition, aroma and taste play a significant role in customer acceptance.

Apple [16,17] and quince pieces [18,19] have been proposed as immobilization supports of yeast strains for room-

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doi:10.1016/j.procbio.2004.01.029

and low-temperature wine-making. Apple and quince pieces are of food-grade purity, cheap, and abundant in nature, acid resistant supports, while the immobilization technique is simple and easy and showed high operational stability and a significant increase in productivity in alcohol production. The products (wine) were of very good quality with a distinctive aromatic potential. In addition, apple pieces proved to be very effective supports for the survival of apple-immobilized yeast cells, as the immobilized biocatalyst was able to reactivate after storage of 120 days [20].

In order to increase probiotic viability, production of apple and quince pieces supported *L. casei* is necessary due to the suitability of the supports as food ingredients. The produced biocatalysts have to be also examined for their suitability for lactic acid production. Therefore, the aims of this investigation were the increase of probiotic viability through fruit supported probiotic organism and use of the produced biocatalysts for lactic acid production.

2. Materials and methods

Lactobacillus casei isolated from a commercial dairy product was used in the present study. It was grown on synthetic medium containing (%, w/v): yeast extract 0.5%, K_2HPO_4 0.1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.5%, and lactose 2% in distilled water. This medium was sterilized at 121 °C for 15 min. Flasks were incubated at 37 °C without agitation.

Whey was produced in the laboratory after milk coagulation using the enzyme rennet. It had a pH 6.4–6.6 and contained 50 g lactose/l.

2.1. Cell immobilization

Apple and quince pieces were used as supports for immobilization. For the immobilization of cells, pieces of apple or quince (∼500 g) were introduced in 1 l of liquid culture of *L. casei* and allowed overnight at 37 °C without agitation. When immobilization was complet, the fermented liquid was decanted and the supported biocatalysts were washed twice with 250 ml of whey. The biocatalysts were then used for lactic fermentation.

2.2. Successive fermentation batches of whey

One hundred and seventy grams of apple or quince supported biocatalyst, prepared as described above, were introduced into 400 ml of whey and 15 successive fermentation batches were carried out at different temperatures: 30, 37, and 45 °C. All fermentations were performed under stationary conditions. During fermentation the pH was adjusted in the range 5.5–6.0 by addition of a saturated solution of Na_2CO_3. When the fermentation was completed, the liquid was decanted and the supports were washed twice with 250 ml of whey. At the end of every batch, samples were collected and analyzed for lactic acid, ethanol, and residual sugar.

Six successive fermentation batches of whey using initially 12.5 g/l wet weight free cells were carried out at 30, 37, and 45 °C. The exact quantity of immobilized cells was impossible to estimate and an indicative quantity of free cells was used.

2.3. Probiotic fermented milk production

Ninety grams of apple or quince *L. casei* supported biocatalyst were introduced in 400 ml of milk previously heated at 95 °C for 5 min and then cooled at 45 °C. Fermentations were also carried out using free cells (12.5 g/l). All fermentations were carried out at 45 °C. During fermentations, pH values were measured. At the end of each fermentation, the biocatalysts were allowed to remain successively in the fermented mixture at 4 °C for increasing time periods of 15, 98, and 129 days each time, after which the liquid was decanted and the immobilized biocatalysts were collected, washed twice with 250 ml of milk and tested again for milk fermentation. Samples of the fermented milk were collected after the end of each storage period and analyzed for lactic acid, ethanol, and lactose.

2.4. Analyses

Lactic acid, ethanol, and residual sugar were determined by high performance liquid chromatography, using a Shimadzu chromatograph with a SCR-101N stainless steel column, a LC-9A pump, an CTO-10A oven at 60 °C and a RID-6A refractive index detector. Three times distilled water was used as mobile phase with a flow rate of 0.8 ml/min and butanol-1 was used as an internal standard. Samples of 0.5 ml of the product and 2.5 ml of a 1% (v/v) solution of butanol-1 were diluted to 50 ml and 40 μl were injected directly to the column. The lactic acid, ethanol, and residual sugar concentrations were calculated using standard curves.

Lactic acid productivity was calculated as grams of lactic acid per litre liquid volume produced per day. Lactic acid production yield was expressed as grams of lactic acid produced/100 g of sugar and conversion was calculated by the following equation:

$$\frac{\text{Initial sugar concentration} - \text{Residual sugar concentration}}{\text{Initial sugar concentration}} \times 100.$$

2.5. Scanning electron microscopy

The immobilization of *L. casei* on apple and quince pieces was monitored by scanning electron microscopy. Pieces of the immobilized biocatalysts were washed with whey and dried overnight at 30 °C. The dried samples were

coated with gold in a Balzers SCD 004 Sputter coater for 2 min and examined in a JSM-6300 scanning electron microscope.

3. Results and discussion

The experimental part of this investigation was organized in order to show (i) cell immobilization of *L. casei* on fruit pieces; (ii) survival of fruit pieces supported *L. casei*; and (iii) efficiency of the produced biocatalysts in probiotic fermented milk and lactic acid production.

Lactobacillus casei cells were immobilized on apple and quince pieces separately and then 15 successive whey fermentation batches for each immobilized biocatalyst were carried out at 30, 37, and 45 °C.

In order to achieve adaptation of cells, biomass was produced in synthetic media containing lactose, since lactose is contained in milk and whey, which were used as raw materials in this study. When biomass was produced, it was mixed with apple or quince pieces for cell immobilization and after washing of the support, successive fermentation batches were carried out in order to study the biocatalysts' operational stability, and therefore, survival of cells. Results are summarized in Table 1. Cell immobilization was confirmed by the stability of lactic acid productivity in 15 successive fermentation batches of about 50 days duration for each immobilized biocatalyst. Fig. 1 presents electron micrographs of *L. casei* cells immobilized on apple and quince pieces. Therefore, electron microscopy and the series of successive fermentation batches show that *L. casei* was immobilized on the aforementioned fruit pieces. Although the immobilized biocatalysts were available for further fermentation, this was considered unnecessary.

Furthermore, lactic acid fermentation of whey resulted in high substrate conversion at all temperatures studied, while a part of the remaining lactose in whey was converted to alcohol by-product ranging between 0.6 and 1.6% (v/v). Lactic acid productivity and yield increased with temperature increase. At 45 °C, the values obtained for yield and lactic acid productivity were higher compared to process performance at lower temperatures. *Lactobacillus casei* free cells showed a fermentation time increase in the second batch in relation to the first batch at all fermentation temperatures. In contrast, the immobilized cells showed a decrease and/or stabilization of the fermentation time. Apple and quince pieces proved to be very protective supports to bacterium cells for repeated operation under moderately acidic conditions. Fruit volume was gradually reduced, probably due to apple and quince sugar utilization by the lactobacillus, leaving the residual nonfermentable cellulose. Volume reduction was higher in apple supported biocatalyst than in quince supported biocatalyst during the whole experiment, probably due to apple sugar content which is higher than quince. A similar volume reduction of apple and quince biocatalysts was observed in alcoholic fermentation [16–20]. Therefore, periodical addition of apple and quince pieces in the bioreactor is proposed in industrial use.

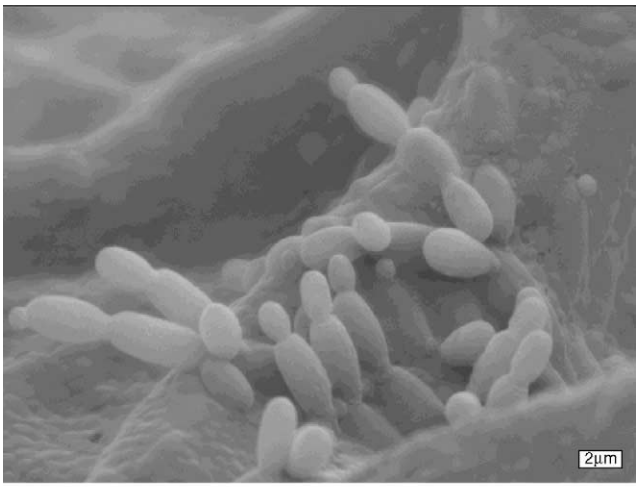

Fig. 1. Electron micrographs showing immobilized cells of *L. casei* on (a) apple and (b) quince pieces.

3.1. Probiotic fermented milk production

Apple and quince supported biocatalysts were also used for lactic fermentation of milk and possible storage for a long period. The biocatalysts were introduced separately into milk and allowed to ferment at 45 °C. The fermentation process was monitored by pH reduction and the results are summarized in Fig. 2a. Preliminary sensory tests carried out in the laboratory ascertained the fruity distinctive aromatic potential of the products even after storage for 129 days. Apple and quince supported *L. casei* remained active after storage of the fermented products at 4 °C for 15 days in usual containers and were reused for lactic fermentation of milk (Fig. 2b). The immobilized biocatalysts were reactivated and the pH started to decrease in about 1.5–2 h.

Table 1
Effect of temperature on lactic acid production by successive fermentation batches of whey using immobilized cells of *Lactobacillus casei* on apple and quince pieces and free cells

Support	Fermentation temperature (°C)	Successive fermentation batches	Fermentation time (h)	Lactic acid (g/l)	Ethanol (%, v/v)	Lactose (g/l)	Lactic acid production yield (g/100 g)	Daily lactic acid productivity (g/l)	Conversion (%)
Apple	30	1	118	15.1	1.6	Tr	30.2	3.1	100
		2	122	19.3	1.1	Tr	38.6	3.8	100
		3	107	18.2	0.8	2.9	36.4	4.1	94
		4	88	15.3	1.2	6.7	30.6	4.2	87
		5	101	17.4	0.9	2.7	34.8	4.1	95
	37	6	89	25.4	0.7	2.5	50.8	6.8	95
		7	102	24.7	0.9	0.3	49.4	5.8	99
		8	69	21.4	0.8	7.8	42.8	7.4	84
		9	91	25.5	0.7	1.1	51.0	6.7	98
		10	86	25.4	0.6	1.5	50.8	7.1	97
	45	11	72	32.9	0.6	2.6	65.8	11.0	95
		12	63	32.0	0.7	9.0	64.0	12.1	82
		13	56	30.3	0.7	0.7	60.6	13.0	99
		14	65	31.7	0.7	2.8	63.4	11.7	94
		15	59	29.8	0.8	0.6	59.6	12.1	99
Quince	30	1	157	16.7	1.5	9.9	33.4	2.6	80
		2	122	17.2	1.1	8.9	34.4	3.4	82
		3	114	18.4	1.1	3.2	36.8	3.9	94
		4	111	18.5	0.9	1.5	37.0	4.0	97
		5	110	18.1	0.9	4.5	36.2	3.9	91
	37	6	81	22.3	0.8	4.5	44.6	6.6	91
		7	90	20.2	0.7	0.2	40.4	5.4	100
		8	94	21.7	0.9	0.3	43.4	5.5	99
		9	96	23.2	0.8	3.9	46.4	5.8	92
		10	98	22.6	0.9	0.2	45.2	5.5	100
	45	11	72	36.1	0.6	4.9	72.2	12.0	90
		12	70	34.5	0.7	2.8	69.0	11.8	94
		13	78	35.5	0.8	1.0	71.0	10.9	98
		14	75	36.0	0.6	1.0	72.0	11.5	98
		15	72	34.9	0.8	1.1	69.8	11.6	98
Free cells	30	1	130	12.7	1.3	8.6	25.4	2.3	83
		2	165	13.9	1.1	6.8	27.8	2.0	86
	37	3	92	15.9	1.0	Tr	31.8	4.1	100
		4	114	19.7	0.9	Tr	39.4	4.1	100
	45	5	78	33.6	0.7	2.0	67.2	10.3	96
		6	106	31.6	0.7	2.1	63.2	7.2	96

Tr: traces.

In the case of quince supported *L. casei*, the final pH was lower after storage. The immobilized biocatalysts survived and reactivated quickly after storage at 4 °C for 98 (Fig. 2c) and 129 days (Fig. 2d) without any contamination observed (Tables 2 and 3). In contrast, free cells were contaminated after storage for 98 days. Contamination was determined visually and by sensory evaluation. According to Vedamuthu [21] a maximum of 1.5% acidity expressed as lactic acid can be attained and only about 30% of the lactose content of milk is used in lactic acid fermentations. The results presented in this work agree with the aforementioned data. The

Table 2
Fermentation parameters obtained in probiotic fermented milk production with *L. casei* immobilized cells after storage for 98 days

Support	State	Lactose (g/l)	Lactic acid (g/l)	Ethanol (%, v/v)
Apple	After storage	19.1	13.3	0.2
	After reactivation	23.8	11.2	0.2
Quince	After storage	16.2	12.6	0.3
	After reactivation	25.8	11.2	0.3
Free cells	After storage	22.1	10.1	0.3
	After reactivation	24.6	10.9	0.2

Table 3
Fermentation parameters obtained in probiotic fermented milk production with *L. casei* immobilized cells after storage for 129 days

Support	State	Lactose (g/l)	Lactic acid (g/l)	Ethanol (%, v/v)
Apple	After storage	28.81	10.79	1.91
	After reactivation	27.53	10.83	1.50
Quince	After storage	28.56	11.26	1.45
	After reactivation	28.60	10.55	1.50

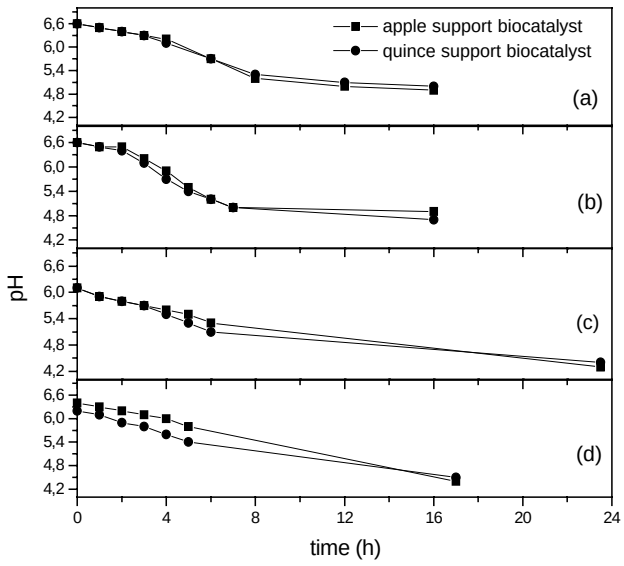

Fig. 2. Kinetics during milk fermentation using immobilized *L. casei* cells on apple and quince pieces before (a) and after storage at 4 °C for 15 days (b), for 98 days (c), and for 129 days (d).

results clearly showed that *L. casei* cells survived for extended storage time periods and were adapted to the acidic environment, which usually has an inhibitory effect on cells survival. The fact that no loss of activity in successive fermentation batches after various storage time periods at 4 °C was observed, strengthens further the possibility for survival of immobilized *L. casei* on fruits pieces for a long period.

Some researchers [3] used porous potato starch granules as carrier of probiotic bacteria to ensure their viability in the large intestine. Gut flora plays an important role in human health. Because fruit pieces contain cellulose, which is not digested, they reach the colon. Moreover, we strongly believe that future clinical tests will support the beneficial effects of fruits based probiotics. Fruit pieces are promising carriers of probiotic bacteria such as *L. casei* and may be used in the production of a large variety of probiotic milk fermented food products and/or other probiotic food products as well as in the prolongation of their shelf life. The improved aroma of the fermented milk product by immobilized *L. casei* cells on fruit pieces also encourages further research and constitutes the subject of a future study.

3.2. Technological consideration

According to the results presented in this work, the fruit pieces supported *L. casei* can be produced using whey as raw material, which is a liquid effluent with negligible cost charging the receiver water with high amounts of organic load. Therefore, the production cost of the probiotic microorganism will be very low and cost effective in comparison with the use of other raw materials based on starch. The results of this investigation related with the production of a fruit supported *L. casei* can be useful for production of (i) probiotic additive in foods; (ii) biocatalyst for probiotic fermented milk production; and (iii) lactic acid production from whey using immobilized cells. Immobilized cells of *L. casei* on apple and quince pieces after freeze drying could be added to various solid foods, such as breakfast cereals to provide probiotic properties. A freeze dried product could also be used in baking. Finally, the biocatalysts presented in this work could be used to produce probiotic fermented milk, providing aroma, and taste arriving from fruits.

In the case of fermented milk production, the biocatalyst could be stored for a long period at 4 °C. As a result, when there is a possibility for cooling, emptying, and filling of the bioreactor and preparation of new biocatalyst could be avoided when the factory is halted, increasing extremely the operation stability. Likewise, this is also validated for lactic acid production using whey. The same bioreactor could also be used successively in the same factory either for fermented milk production or lactic acid production using whey.

The experience acquired enables proposition of a multi stage fixed-bed tower (MFBT) bioreactor [22], in order to achieve support division in at least three floors. The bioreactor should be designed to be of relatively low volume (about 10,000 l) and cell immobilization could be performed in the bioreactor. Taking into consideration the above discussion of technical problems, the scale-up of the above technology seems feasible.

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