Enhanced Production of L-(+)-Lactic Acid in Chemostat by *Lactobacillus casei* DSM 20011 Using Ion-Exchange Resins and Cross-Flow Filtration in a Fully Automated Pilot Plant Controlled via NIR

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Abstract: Due to the lack of suitable in-process sensors, on-line monitoring of fermentation processes is restricted almost exclusively to the measurement of physical parameters only indirectly related to key process variables, i.e., substrate, product, and biomass concentration. This obstacle can be overcome by near infrared (NIR) spectroscopy, which allows not only real-time process monitoring, but also automated process control, provided that NIR-generated information is fed to a suitable computerized bioreactor control system. Once the relevant calibrations have been obtained, substrate, biomass and product concentration can be evaluated on-line and used by the bioreactor control system to manage the fermentation. In this work, an NIR-based control system allowed the full automation of a small-scale pilot plant for lactic acid production and provided an excellent tool for process optimization. The growth-inhibiting effect of lactic acid present in the culture broth is enhanced when the growth-limiting substrate, glucose, is also present at relatively high concentrations. Both combined factors can result in a severe reduction of the performance of the lactate production process. A dedicated software enabling on-line NIR data acquisition and reduction, and automated process management through feed addition, culture removal and/or product recovery by microfiltration was developed in order to allow the implementation of continuous fermentation processes with recycling of culture medium and cell recycling. Both operation modes were tested at different dilution rates and the respective cultivation parameters observed were compared with those obtained in a conventional continuous fermentation. Steady states were obtained in both modes with high performance on lactate production. The highest lactate volumetric productivity, 138 g L^{-1} h^{-1}, was obtained in continuous fermentation with cell recycling. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* **67**: 147–156, 2000.

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INTRODUCTION

Development and optimization of fermentation processes are strongly dependent on accurate, real-time control of chemical and physical process variables. Computer control of such processes is in turn dependent upon continuous, automated data acquisition. While several novel methods of in situ measurement of chemical species inside bioreactors are currently under development, a fundamental problem remains to be solved, i.e., the potentially large numbers of chemical parameters that would be desirable to measure simultaneously on-line. Approaches based on specific sensors, while solving specific analytical problems do not address this basic requirement of fermentation monitoring and control. Near infrared (NIR) spectroscopy offers considerable promise in this respect, but it has only recently been considered as a possible tool in fermentation technology. This is surprising in consideration of its well-proven versatility, rapidity of response, and non-invasivity.

The absorption of near-infrared radiation (700–2500 nm) by organic molecules is related to the overtone and combination bands of the -CH, -NH, and -OH fundamental molecular stretching and bending vibrations that are observed in the mid-IR region of the electromagnetic spectrum. NIR absorptions are generally 10–100 times weaker in intensity than the fundamental mid-IR absorption bands. The weakness of the absorptions is actually a benefit, providing direct analysis of samples without dilution or the requirement of short optical pathlengths or dispersion in non-absorbing matrices used in traditional sampling techniques in UV/vis and mid-IR spectroscopies. NIR absorption bands are also much

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broader and tend to be highly overlapped. Despite the intuitive disadvantage of broad and overlapping absorption bands, sophisticated chemometric techniques can extract meaningful information from the complex NIR spectrum (Brimmer and Hall, 1993).

Nishinari et al. (1989) described on-line control of glucose concentration during starch hydrolysis; Cavinato et al. (1990) used an optical-fibre NIR instrument to monitor the alcoholic fermentation; Picque et al. (1993) reported monitoring of the alcoholic and the lactic acid fermentations; Brimmer and Hall (1993) and Yano and Harata (1994) described the use of NIR to monitor nutrient concentrations; Ge et al. (1994) described the monitoring of biomass concentration by NIR; Macaloney et al. (1994, 1997) used NIR to monitor the glycerol fermentation and multiple components in a high cell-density recombinant *Escherichia coli* production process; and Hall et al. (1996) indicated direct NIR-bioreactor interfacing as the way ahead in fermentation process monitoring and control.

Such an approach has already been advocated by the authors as the solution of choice for monitoring glucose to lactic acid fermentation by *Lactobacillus casei* subs. *casei* DSM 20011 (Dosi et al., 1996; Vaccari et al., 1994). Calibration curves for glucose, lactic acid and biomass were obtained, and the time-course of the fermentation was successfully followed by interfacing the NIR instrument to the bioreactor. The logical development of such study, namely the direct control of the fermentation by means of NIR interfacing, is reported in this work.

The growth-inhibiting effect of lactic acid present in the culture broth has been previously reported (Gadgil and Venkatesh, 1997; Cachon and Diviès, 1994; González-Vara et al., 1996; Nikolajsen et al., 1991; Ohara et al., 1992a; Srivastava et al., 1992). Moreover, the growth-inhibiting effect is enhanced when the substrate, glucose, is also present at concentrations higher than about 40 g L^{-1} (Åkelberg et al., 1998; Gonçalves et al., 1991; González-Vara et al., 1996; Vaccari et al., 1993; Venkatesh et al., 1993). Both combined factors can result in a severe reduction of the performance of the lactate production process.

Therefore, different strategies have been proposed to improve the lactate production process: changes in medium composition (Åkerberg et al., 1998; Goksungur and Guvenc, 1997; Venkatesh et al., 1993), immobilization of the producing strain (Goksungur and Guvenc, 1999; Roukas and Kotzekidou, 1996), use of particular bioreactors, like filter-bed-type (Ohara et al., 1993), continuous membrane (Tejayadi and Cheryan, 1995), or hollow fiber (Vick Roy et al., 1982) bioreactors, extraction of lactic acid using ion-exchange resins (Srivastava et al., 1992; Vaccari et al., 1993), and continuous processes with cell-recycling (Aeschlimann and von Stockar, 1991; Hjörlefsdottir et al., 1990; Kulozic and Wilde, 1999; Major and Bull, 1989; Vick Roy et al., 1983; Xavier et al., 1995).

A combination of these two last techniques was used in this work. A dedicated software enabling on-line NIR data acquisition and reduction, and automated process management through feed addition, culture removal, and/or product recovery by microfiltration was developed in order to allow the implementation of a continuous fermentation process with recycling of culture medium (MRF) and a continuous fermentation process with cell recycling (CRF). In the MRF operation mode the fermentation broth was continuously fed back into bioreactor after microfiltration and lactate extraction through ion-exchange columns, thus decreasing the lactate concentration in the culture broth in order to prevent its growth-inhibiting effect. In the CRF operation mode the biomass was partially retained within the bioreactor to increase its concentration, thereby increasing glucose consumption. This allows to obtain, even at high dilution rates, low residual concentrations of non-consumed substrate which are desirable for minimizing feedstock and product recovery costs. A high glucose concentration in the feed was used in both operations modes.

The goals of this work were to test the proposed NIR-based control system allowing the full automation of the pilot plant and to value its usefulness for process optimization by comparing the results obtained in MRF and CRF operation modes with those observed in a conventional continuous fermentation.

MATERIALS AND METHODS

Microorganisms

The homofermentative strain *L. casei* subsp. *casei* DMS 20011 (ATCC 393), an L-(+) lactate producer, was used throughout.

Cultivation Media

Stock cultures were stored in glycerol (20%) at −80°C. The fermentation experiments were performed in MRS Broth (Merck) and slightly modified GS Medium (González-Vara et al., 1996) whose composition was as follows (gL^{-1}): glucose, 100; yeast extract, 30; $MgSO_4 \cdot 7H_2O$, 0.6; sodium acetate, 1; $FeSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.03; KH_2PO_4, 0.5; K_2HPO_4, 0.5; pH 6.5 after sterilization. The media were sterilized in continuous mode by filtration through membrane filters (0.2 μm).

Fermentation Equipment and Conditions

A BM-PPS3 3000 bioreactor (Bioindustrie Mantovane, Italy) having a working volume of 2 L was used under chemostat conditions in the following operation modes: conventional continuous fermentation (CF), culture medium recycling (MRF), or complete cell recycling (CRF). The bioreactor was thermostated at 36°C, and pH was maintained at 6.4 by automatic titration with 4 *N* NaOH. Constant stirring (120 rpm) was kept during the fermentation. Anaerobic conditions were maintained by means of nitrogen that was sparged into the culture and used to flush the head-

space of the bioreactor before inoculation. No antifoaming agents were used. The bioreactor was inoculated with a 10% (v/v) inoculum from a 16 h flask culture grown in MRS broth. Glucose, lactic acid, and biomass concentration in the culture were monitored on-line by NIR spectroscopy. Steady state was considered to be attained when all these parameters remained constant for at least two residence times. As a minimum, four residence times were allowed to elapse following a change in the cultivation conditions. Samples were collected during steady state and analyzed conventionally to test the reliability of NIR measurements. For each dilution rate, the mode of operation was changed following the sequence: CF $\rightarrow$ MRF $\rightarrow$ CRF. Changeover to a new mode was effected once the steady state had been attained with the previous mode.

In MRF mode the fermentation broth was continuously filtered and the cell-free permeate was completely fed back into the bioreactor after lactate extraction through ion-exchange resins. The loss of biomass or glucose and other nutrients present in the medium as a consequence of recycle proved to be negligible. In other words, the cell-free stream being recirculated to the bioreactor represented a source of dilution of lactic acid but not of other culture components, including biomass. When saturated, the columns containing the ion-exchange material were replaced while maintaining aseptic conditions in the feed-back loop. The cell-free stream being recirculated to the bioreactor represented a source of dilution of lactic acid but not of other culture components, including biomass. The ratio of the recirculation flow to the total culture volume was therefore regarded as a dilution rate with respect to lactic acid, indicated as *d*. The value of *d* was adjusted automatically by the NIR-based control system in order to reduce the lactic acid concentration to a preset value. This was achieved by automatic adjustment of the flow rate of peristaltic pump Pp_2 (Fig. 1), determining the permeate flow rate.

In CRF mode, a fraction of the outflow from the bioreactor was continuously filtered and the retentate was fed back to the culture to increase the biomass concentration in the bioreactor. The cell-free stream leaving the system, representing a fraction *c* of the total outflow *F*, was percolated through ion-exchange resins to recover lactate, and was then discarded. Figure 2 shows a schematic view of the stream flow rates in the CRF mode. The value of *c* was automatically set by the NIR-based control system in order to reduce—as a consequence of the increased biomass concentration and, thus, of the increased substrate consumption—the residual concentration of nonconsumed glucose in the culture to a preset value. This was achieved by automatic adjustments of the flow rate of peristaltic pump Pp2 (Fig. 1), determining the permeate flow rate.

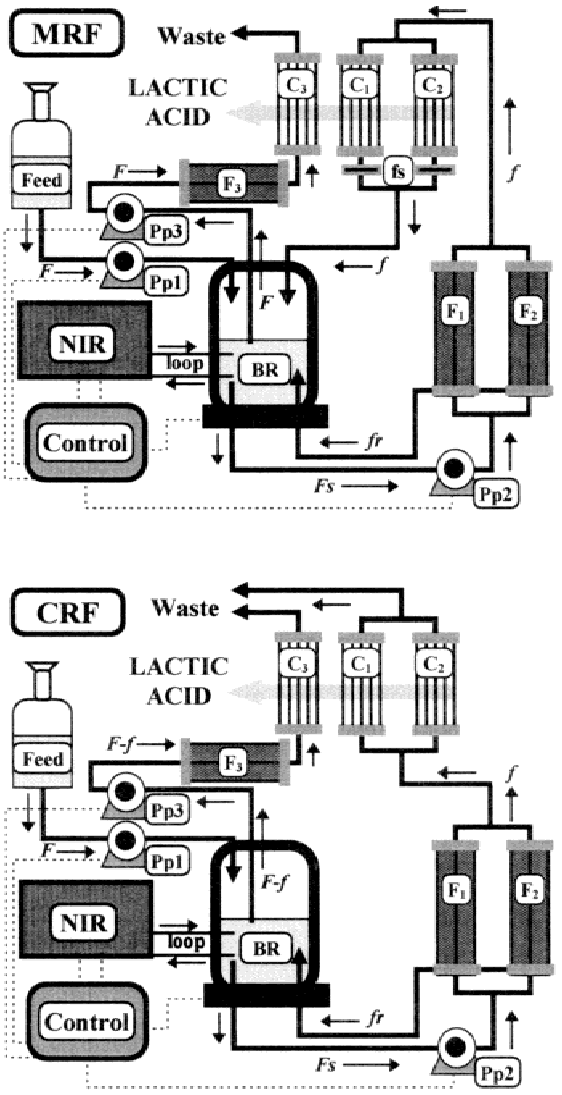

Figure 1. Scheme of the pilot plant. (BR) bioreactor, (C_i) ion-exchange resin columns, (Control) computerized control system, (CRF) continuous fermentation with cell recycling, (F_i) microfiltration units, (Feed) fresh medium reservoir, (fs) safety sterilizing filters, (Lactic acid) product recovery, (loop) measurement loop, (MRF) continuous fermentation with medium recycling, (NIR) NIR spectroscopy central unit, (Pp_i) perislaltic pumps, (Waste) production discard; (*F*) feed flow rate, (*f*) permeate flow rate, (*fr*) retentate flow rate, (*Fs*) flow rate out of bioreactor to microfiltration units F_1 and F_2; (solid lines) piping, (dotted lines) interfacing, (solid arrows) flow direction, (large gray arrows) product recovery from ion-exchange resin columns.

Microfiltration

The high-efficiency micro-filtration system was composed of two tangential cross-flow units, in situ sterilizable by steam (121°C), containing ceramic modules (pore size of 0.2 μm) with a total filtering surface of 0.24 m^2 and equipped with a counter-current cleaning system to prevent fouling due to biomass accumulation (Bioindustrie Mantovane; Mantova, Italy). The two units were installed in parallel and were used alternatively to allow simultaneously the normal filtration function and the operations of cleaning and sterilization. The flow of fermentation broth was diverted from one to the other filtration unit when an inlet gauge pressure higher than 0.6 bar was detected. The maximum recirculation flow was approximately 10 L min^{-1}, which corresponds to a tangential velocity of about 4 m s^{-1}. The

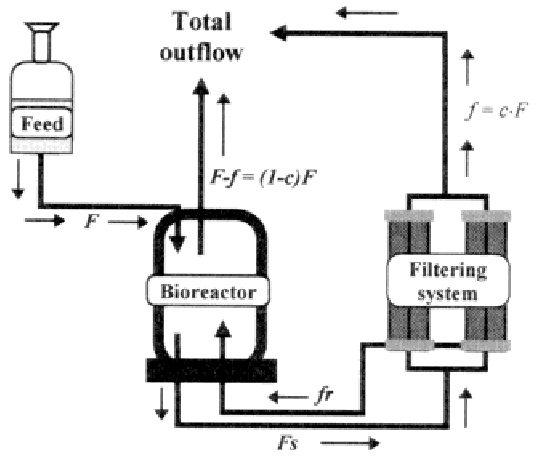

Figure 2. Schematic view of the stream flow rates in the pilot plant running in CRF mode. (*F*) feed flow rate, (*f*) permeate flow rate, (*c*) fraction of outflowing culture stream filtered or recycling factor, (*fr*) retentate flow rate, (*Fs*) flow rate out of bioreactor to microfiltration system; (solid lines) piping, (solid arrows) flow direction.

volume of culture contained in the recycling loop was 0.24 L for CRF mode and 2.28 L for MRF mode (of which, 2 L in the ion-exchange column), respectively.

Ion-Exchange Resins

The extraction of lactic acid from the filtered culture stream was carried out by using a strong anion exchange resin bed (Amberlite IRA-420, Rohm and Haas, USA) in the carbonate form (Vaccari et al., 1993). The culture filtrate was percolated through suitable columns containing 10 L of resin. The amount of broth treated per litre of resin depended on the concentration of lactic acid present in the broth. Saturation of the resin was monitored by testing the resin effluent for the presence of lactic acid. The lactic acid contained in saturated resin columns was recovered, after washing with water, by elution with a solution of 5% ammonium carbonate at a flow rate of 30 L/h. This solution regenerated at the same time the resin which, after washing with water, was ready to be reutilized.

NIR Apparatus

The NIR instrument used was an InfraAlyzer 450 (Bran & Luebbe Co., Norderstedt, Germany) equipped with a cell for liquid samples (diameter, 27 mm; 0.2 mm thick, 114 mm^3 volume, gold-plated bottom). After calibration curves were prepared for glucose, lactic acid, and biomass, the sample cell was connected with the bioreactor through a loop (Dosi et al., 1996; Vaccari et al., 1994). Data acquisition and reduction were carried out every 3 min and the results were transferred, through an interface, to the bioreactor control system.

NIR-Bioreactor Interface

Interfacing of the NIR spectrometer with the bioreactor was achieved by means of a RS232 serial communication link. At the end of each measurement cycle, the NIR spectrometer transmitted the absorbance values obtained at a number of selected wavelengths to the bioreactor control system. The control system then converted this set of absorbance measurements to corresponding values of concentration of biomass, glucose, and lactic acid utilizing sets of coefficients entered by the operator at the end of calibration runs. The calculated values of process variables were used by the control system to implement the control strategies previously described.

Conventional Analyses

Samples were taken from the bioreactor at irregular intervals and analyzed by conventional methods to check the reliability of NIR measurements. Glucose and lactic acid were determined by HPLC under isochratic conditions using an Aminex HPX-87H column (300 × 7.8 mm) at 25°C with 0.01 *N* H_3PO_4 as eluent (flow 0.6 mL min^{-1}) and a refractive index detector. Biomass dry weight was determined by collecting the cells contained in 10 mL of the fermentation broth onto membrane filters (0.45 μm), washing them with distilled water, drying at 100°C for 24 h and weighing. The net dry cell weight was obtained subtracting the weight of the empty filters.

Calculations

The overall lactate volumetric productivity (R), the lactate specific productivity (q_p) and the specific consumption rate of glucose (q_s) were calculated as follows.

CF Mode

In the steady state of a CF,

$$\mu = D \tag{1}$$

thus

$$R = q_p\, x \tag{2}$$

and, in accordance with the Luedeking-Piret's model for lactic fermentation (Leudeking and Piret, 1959):

$$q_p = y_{p/x} \cdot D + m_p \tag{3}$$

$$q_s = \frac{D}{y_{x/s}} + m_s \tag{4}$$

where D is the dilution rate (h^{-1}), x the biomass concentration in the steady state (g L^{-1}), $y_{p/x}$ and $y_{x/s}$ are, respectively, the lactate/biomass and the biomass/consumed glucose yields (g g^{-1}), m_p is the non-growth associated term for product formation (g g^{-1} h^{-1}), and m_s is the substrate consumption term for maintenance (g g^{-1} h^{-1}).

MRF Mode

In the MRF mode used, the mass balance for product was

$$\frac{dP}{dt} = y_{p/x}\, x\, \mu + m_p\, x - d\, p - D\, p \tag{5}$$

where p is the product concentration ($g \cdot L^{-1}$), t is the fermentation time (h), and d is the dilution rate due to recycling loop (h^{-1}).

At the steady state,

$$\frac{dP}{dt} = 0 \tag{6}$$

thus:

$$q_p = \frac{p}{x}(D + d) \tag{7}$$

$$D = \frac{F}{V}; \qquad d = \frac{f}{V} \tag{8}$$

where p and x are, respectively, the lactate and biomass concentrations in the culture during the steady state ($g\ L^{-1}$), F is the input feed flow rate ($L\ h^{-1}$), V is the culture volume (L), and f is the permeate flow rate ($L\ h^{-1}$). Overall R can be calculated by means of Eqs. (2) and (7). The loss of glucose in broth as a consequence of recycle though ion-exchange resins was proved to be negligible. Thus, q_s could be estimated by Eq. (4).

CRF Mode

In the steady state of a CRF,

$$\mu = D\,(1 - c) \tag{9}$$

thus

$$q_p = D\,(1 - c) \cdot y_{p/x} + m_p \tag{10}$$

$$q_s = \frac{D\,(1 - c)}{y_{x/s}} + m_s \tag{11}$$

$$c = \frac{f}{F} \tag{12}$$

where c is the fraction of outflowing culture stream filtered or recycling factor. Overall R can be calculated by means of Eqs. (2) and (3).

RESULTS

The real-time data acquisition and reduction of the proposed NIR-based control system permitted the design of two operational strategies for both MRF and CRF. The NIR system controlled the flow of culture broth filtered, according to data of glucose (s), biomass (x), and lactate (p) concentrations in the culture. The dilution rate with respect to lactic acid achieved in MRF mode as a consequence of recycling (d) was automatically set by the NIR-based control system in such a way as to maintain lactic acid concentration in the culture below growth-inhibitory levels (González-Vara et al., 1996). To achieve this the set-point of p was adjusted to $8.5 \pm 0.2\ g\ L^{-1}$.

When in CRF mode, the cell recycle factor, c, was modulated in order to reduce the residual concentration of non-consumed glucose in the culture (set-point $s = 0.2 \pm 0.1\ g\ L^{-1}$) as a consequence of the increased biomass concentration and, thus, of the increased substrate consumption. In other words, biomass feedback through cell recycle increased the biomass concentration in the culture, while the substrate input rate and, thus, the total outflow rate, F, was kept constant. The resulting decrease in the biomass specific growth rate, μ, allowed the virtually complete utilization of glucose as long as the specific growth rate did not exceed about $0.1\ h^{-1}$ (Table I). The parameters of the PID algorithm used to modulate permeate flow were tuned to provide a stable control. In addition, the value of the biomass concentration was used to modulate the output of the controller in order to prevent biomass wash-out. The phase to attain the steady-state was longer in MRF than in CRF mode. The reason was presumably the doubled residence time of the former due to the increased total liquid volume resulting from the presence of recycled medium contained in the ion-exchange column, viz. 2 L.

The results obtained in the steady states at different dilution rates (D) for CF, MRF, and CRF modes are shown in Fig. 3. At D higher than $0.2\ h^{-1}$ a progressive decrease in x and p and a concomitant increase in s were observed in CF mode, whereas a similar effect was evident for MRF mode

Table I. Values of specific growth rate calculated by Eq. (9) in the experiments conducted in continuous culture with cell recycling.[a]

D (h^{-1})	c	μ (h^{-1})
0.05	0.00	0.05
0.10	0.00	0.10
0.15	0.40	0.09
0.20	0.56	0.09
0.24	0.64	0.09
0.29	0.72	0.08
0.35	0.76	0.08
0.41	0.79	0.09
0.46	0.82	0.08
0.49	0.83	0.08
0.55	0.84	0.09
0.60	0.84	0.10
0.65	0.84	0.10
0.69	0.84	0.11
0.75	0.84	0.12
0.91	0.84	0.15
1.21	0.84	0.19
1.41	0.84	0.23
1.62	0.84	0.26
1.84	0.84	0.29
2.12	0.84	0.34

[a]Nomenclature: D, dilution rate; c, fraction of culture stream filtered; μ, specific growth rate.

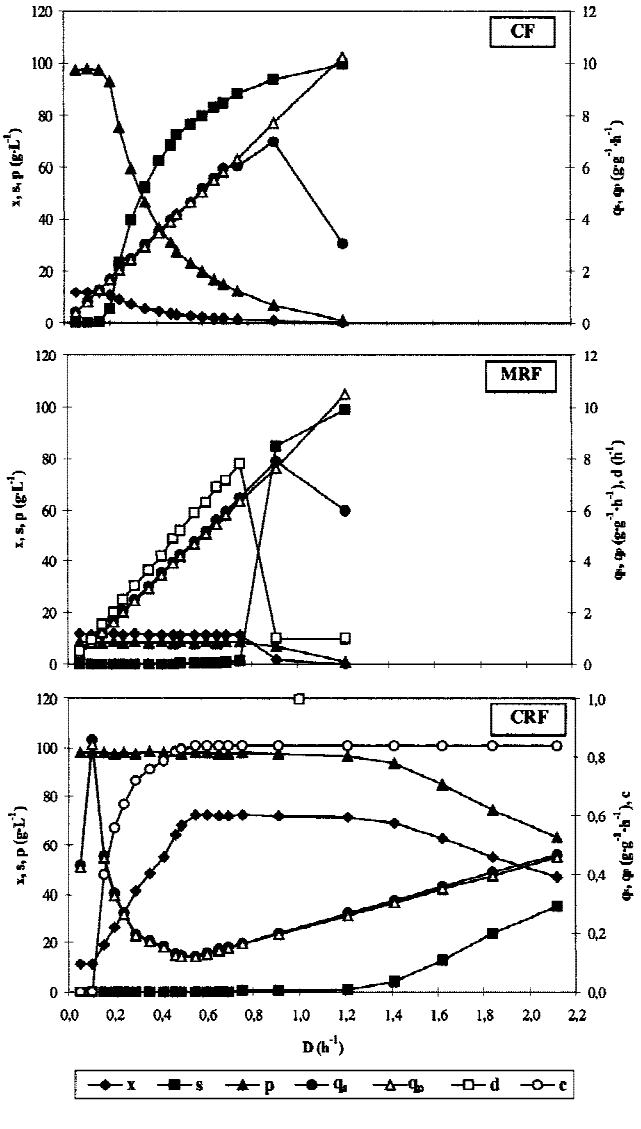

Figure 3. Results of the experiments conducted in continuous culture: (CF) chemostat; (MRF) recycling of culture medium; (CRF) cell recycling; (D) dilution rate (h^{-1}); (x) biomass concentration in the culture at steady state (g L^{-1}); (s) glucose concentration on broth at steady state (g L^{-1}); (p) lactic acid concentration in the culture at steady state (g L^{-1}); (q_s) specific glucose consumption rate (g g^{-1} h^{-1}); (q_p) lactic acid specific productivity (g g^{-1} h^{-1}); (d) dilution rate with respect to lactic acid due to extraction through ion-exchange resins (h^{-1}); (c) fraction of culture stream filtered (dimensionless).

only at values of D close to wash-out point ($D > 0.91$ h^{-1}). However, it should be pointed out that d was not allowed to drop below at a minimum value of 1 ($f = 2$ L h^{-1}) in the MRF experiments carried out at $D = 0.91$ h^{-1} and $D = 1.21$ h^{-1}. Such minimum value of d was imposed because the lactate concentration measured on broth for these values of D was lower than the set point of 8.5 ± 0.2 g L^{-1} and NIR-based control system would have stopped the recycling, excluding from the bioreactor the medium contained in recycling loop.

Regarding the CRF mode, the maximum value of x was obtained in the range $D = 0.55$–1.21 h^{-1}. Appreciable amounts of nonconsumed glucose were detected in the culture when $D > 1.21$ h^{-1}. In this region the product concentration, p, decreased, after remaining at a constant value of about 98 g L^{-1} for lower values of D. It is worth noting that c was not allowed to exceed a maximum value of 0.84 in order to prevent biomass fouling of filtering unit. This is the reason why the concentration factor c remained constant at this value for D higher than 0.65 even if the substrate concentration, s, had exceeded the set point of 0.2 ± 0.1 gL^{-1}.

Constant yield factors of produced lactate/consumed glucose ($y_{p/s}$) and produced biomass/consumed glucose ($y_{x/s}$) were observed in all operation modes throughout the range of D explored, with the exception of $y_{x/s}$ in CRF mode. Average values of $y_{p/s}$ and $y_{x/s}$ were calculated by linear regression of q_p against q_s and x against ($s_r - s$), respectively, where s_r is the substrate concentration in the feed. The correlation coefficients were ≈0.99 in all cases. The results were as follows: CF, $y_{p/s} = 0.99$ g(lactate) g(glucose)$^{-1}$ and $y_{x/s} = 0.12$ g(biomass) g(glucose)$^{-11}$; MRF, $y_{p/s} = 0.98$ g(lactate) g(glucose)$^{-1}$ and $y_{x/s} = 0.12$ g(biomass) g(glucose)$^{-1}$; CRF, $y_{p/s} = 0.98$ g(lactate) g(glucose)$^{-1}$. The value of $y_{p/s}$ calculated for the MRF mode was also confirmed by an overall mass balance of lactic acid produced (direct determination of total lactic acid present in the system at the end of the experiment, $p \cdot V_{tot}$, plus lactic acid recovered from the ion-exchange resins) and of total glucose consumed. A value of $y_{p/s} = 0.98$ g(lactate) g(glucose)$^{-1}$ was obtained, that was in agreement with the value calculated by linear regression over the D range explored. Regarding the value of $y_{x/s}$ in the CRF mode, it decreased by increasing D. This effect was purely a consequence of the physical concentrating effect of CRF. Hence, if $y_{x/s}$ is calculated as growth yield, i.e., taking into account the recycling factor by fitting of a linear regression of $x(1 - c)$ vs ($s_r - s$)—where s_r is the glucose concentration in the feed—an average value of 0.12 g g^{-1} is obtained, with a correlation coefficient of 0.99.

The trends of the specific consumption rate of glucose (q_q) and of the lactate specific production rate (q_p) throughout the range of D explored were nearly identical in CF and MRF modes, whereas in CRF mode the values of both variables were lower than those obtained in CF and MRF modes for a given D, because of the increased biomass concentration (Fig. 3).

Figure 4 shows the overall volumetric productivities of lactic acid (R) obtained in the different operation modes. The maximum value of R (137.6 g L^{-1} h^{-1}) was obtained in CRF mode with $D = 1.62$ h^{-1}. In CF and MRF modes, maximum R values of 18.2 g L^{-1} h^{-1} ($D = 0.20$ h^{-1}) and 72.2 g L^{-1} h^{-1} ($D = 0.75$ h^{-1}), respectively, were obtained.

DISCUSSION

The usefulness of the proposed system was particularly evident when growth inhibition effects were present, i.e., at D higher than 0.15–0.2 h^{-1}. As shown in Fig. 3, in CF mode x and p decreased and s increased gradually as D was in-

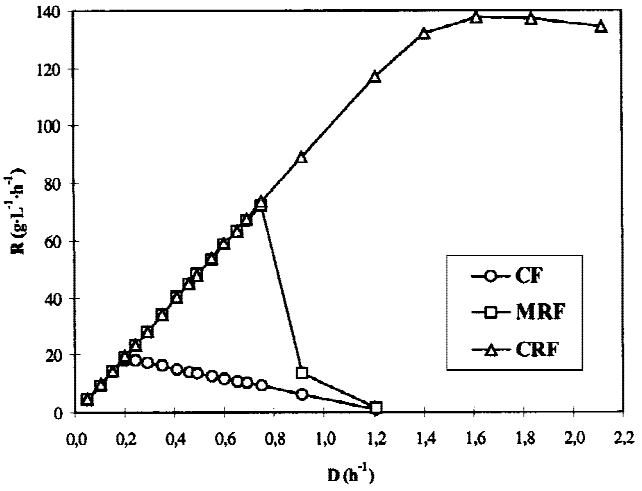

Figure 4. Volumetric productivities (*R*) calculated for *L. casei* fermentations carried out at different dilution rates (*D*) in the operation modes: conventional continuous fermentation (*CF*), continuous fermentation with recycling of medium (*MRF*), and continuous fermentation with cell recycling (*CRF*).

creased above 0.2 h^{-1}. Such a trend on *x*, *p*, and *s* is in accordance with the characteristic profile of noncompetitive growth inhibition, clearly different from the ideal behaviour of the simple Monod model (González-Vara et al., 1996; Pirt, 1975). In non-competitive product inhibition, if product formation is growth-linked (i.e., $y_{p/x}$ is constant irrespective of μ) a plot of steady-state *x* vs D^{-1} should give a straight line at high values of *D*, and the intercept with the abscissa corresponds to the critical dilution rate, D_{crit}, while the intercept with the ordinate is equal to the quantity, $-y_{x/s}K_i y_{p/x}^{-1}$, where K_i is the growth inhibition constant (Pirt, 1975). The plot is shown in Fig. 5, and the parameters calculated are $D_{crit} = 1.27\ h^{-1}$; $K_i = 146.4\ g\ L^{-1}$. The validity of the underlying assumption of a constant $y_{p/x}$ (i.e., growth-linked product formation) is shown in Fig. 6, where

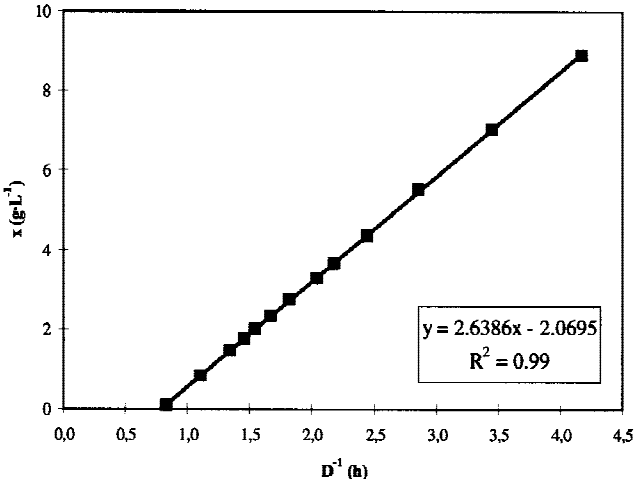

Figure 5. Non-competitive product inhibition by lactic acid in chemostat culture (mode CF). Calculated values: $D_{crit} = 1.27\ h^{-1}$; $K_i = 146.42\ g\ L^{-1}$.

the specific rate of lactic acid formation, q_p, is plotted against the dilution rate *D* ($= \mu$ in the CF mode at steady state). The excellent linearity of the plot, in conjunction with the absence of a significant intercept, show that in this system lactic acid production is totally growth-linked. At low dilution rates, i.e., $D < 0.2\ h^{-1}$, the values of *x*, *p*, and *s* were similar in the three operation modes (except of course the increased *x* in CRF mode due to biomass feedback) showing that product inhibition was not apparent in this region. On the other hand, the profiles of *x* and *s* observed in MRF mode were similar to those predicted by the Monod model throughout the entire range of *D* explored, clearly as a consequence of the decreased concentration of the inhibitory product brought about by the dilution of the culture by the lactate-free recycle stream obtained after ion-exchange extraction.

With regard to the CRF mode, the increased lactic acid volumetric productivity can be ascribed to the increased biomass concentration, and thus, to the decreased μ, brought about by cell recycling. As a consequence of the increasing values of the recycling factor, *c*, the specific growth rate was kept below 0.1 h^{-1} up to $D = 0.65\ h^{-1}$ (Table I), but increased thereafter because the recycling factor was not increased above 0.84. As a result, the concentrations of residual glucose and of lactic acid in the CRF mode at values of *D* up to 0.65 h^{-1} were comparable to those obtained in the CF mode at $D = 0.1\ h^{-1}$. In conclusion, the product inhibition exerted by lactic acid was eliminated, or limited, in the MRF mode through removal of the product, whereas it was circumvented in the CRF mode through the decrease in the specific growth rate.

To investigate further the growth-inhibitory effect of lactic acid an experiment was carried out in the MRF mode at several values of *D*, with the imposed operational condition: $s \leq 0.2 \pm 0.1\ g\ L^{-1}$, i.e., maintaining *s* near to total consumption. The action of the NIR-based control system to maintain such set point was the same as in the previous experiments in MRF mode, i.e., by varying *d*. The results are summarized in Table II. When $D > 0.6\ h^{-1}$, the requirements for *d* exceeded the capability of both the microfiltration unit and the ion-exchange columns. For $D < 0.16\ h^{-1}$, no recycling was required to obtain the practically complete consumption of glucose. On the other hand, the values of *p* observed in steady state for $D > 0.16\ h^{-1}$ were the concentration of the inhibitory product corresponding to the set values of *s* and *D*. This was confirmed by pulse-experiments with lactate imposed on the steady state, but maintaining constant the value of *d* (Table II). In all the cases, a slight increase (<2%) in *p* resulted in an almost instantaneous decrease (significant difference by Student's *t*-test) of *x* as well as in an increase in *s* (data not shown).

Several factors have been described as limiting for the application of CRF to lactate fermentation. For instance, the specificity on the production of optically pure L-(+)-lactate isomer, which is of great importance for the synthesis of polylactic acid polymers (González-Vara et al., 1996; Vaccari et al., 1993), could be affected by *D* or by the operation

Table II. Results of the experiment carried out in the MRF mode at several values of *D*, with the imposed operational condition $s \leq 0.2 \pm 0.1$ g L^{-1}.[a]

(h^{-1})	($g\ L^{-1}$)			($g\ g^{-1}\ h^{-1}$)		($g\ L^{-1}\ h^{-1}$)	(h^{-1})
D	*x*	*s*	*p*	q_s	q_p	*R*	*d*
0.02	11.56	0.02	97.69	0.17	0.17	1.95	0.00
0.04	11.52	0.04	97.67	0.35	0.34	3.91	0.00
0.06	11.58	0.06	97.66	0.52	0.51	5.86	0.00
0.08	11.55	0.12	97.62	0.69	0.68	7.81	0.00
0.12	11.54	0.30	97.42	1.04	1.01	11.69	0.00
0.16	11.55	0.16	43.36	1.38	1.35	15.61	0.20
0.20	11.59	0.12	32.56	1.72	1.74	19.52	0.42
0.24	11.56	0.12	18.86	2.07	2.04	23.42	1.01
0.30	11.52	0.16	19.51	2.60	2.54	29.27	1.20
0.40	11.54	0.20	12.00	3.46	3.34	39.01	2.81
0.52	11.54	0.24	7.80	4.50	4.49	50.69	6.12
0.60	11.52	0.32	6.82	5.19	5.12	58.44	8.04

[a]Nomenclature: *D*, dilution rate; *x*, biomass concentration in the culture at steady state; *s*, glucose concentration on broth at steady state; *p*, lactic acid concentration in the culture at steady state; q_s, specific glucose consumption rate; q_p, lactic acid specific productivity; *R*, overall volumetric productivity; *d*, dilution rate with respect to lactic acid due to extraction through ion-exchange resins; *c*, fraction of culture stream filtered.

mode (Aeschlimann and von Stockar, 1991; Hjörlefsdottir et al., 1990; Steffen et al., 1973). This effect was not observed in this study because the strain used does not possess a racemase (González-Vara et al., 1996). On the other hand, Major and Bull (1989) have described the unfavourable influence of cell recycling on lactate production performances. They showed that increasing the recycling factor for a given *D* led to decrease of yield factors $y_{x/s}$ (expressed as growth yield), and $y_{p/x}$, as well as a shift in product profile, with an increased production of minor products such as acetate and ethanol. The reduction in growth yield was described by the authors in terms of a specific rate of consumption of glucose for non-anabolic purposes, namely maintenance requirements. The reason proposed for this effect as well as for the change in product profile was the stringent glucose limitation imposed by the increase of the recycling factor. They concluded that while low residual substrate concentrations are desirable for minimizing feedstock and product recovery costs, such stringent glucose limitation of lactic fermentation must be avoided if maximum reactor performance is to be achieved in the CRF process (Major and Bull, 1989). In the present study, the plot of q_s against *D* can be interpolated with a straight line passing through the origin (Fig. 3), which suggests that the substrate consumption term for maintenance is negligible, in discrepancy with the classic model of Luedeking–Piret for lactic fermentation (Luedeking and Piret, 1959). This is also confirmed by the constant slope and negligible intercept of the plot in Fig. 6, showing negligible non-growth linked energy production by lactate fermentation. Similar results were obtained by Åkerberg et al. (1998), Major and Bull (1989), Ohara et al. (1992b), Parente et al. (1994), and Venkatesh et al. (1993), among others. In the present experiments, the decrease in $y_{x/s}$ was a consequence of the purely physical concentrating effect of CRF, maintaining the growth yield at a constant value throughout the entire range of *D* assayed. In fact, the NIR-based control system avoided the stringent glucose limitation and this resulted in the optimization of CRF process, allowing the exploitation of the full genotypic potential of *L. casei* for lactate production.

The highest lactate productivity obtained, 138 g L^{-1} h^{-1} (CRF mode, $D = 1.84\ h^{-1}$), was greater than those reported by other authors (Goksungur and Guvenc, 1997, 1999; Kulozik and Wilde, 1999; Roukast and Kotzekidou, 1996; Ohara et al., 1993; Tejayadi and Cheryan, 1995; Vick Roy et al., 1982, 1983; Xavier et al., 1995). By comparing the values of *R* obtained in CF, MRF, and CRF modes, it is possible to conclude that the results obtained were very similar in all three modes at $D \leq 0.20\ h^{-1}$, very close in

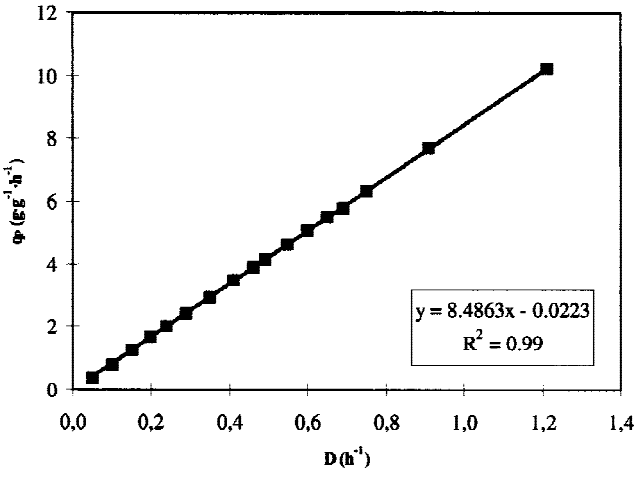

Figure 6. Relationship between the specific rate of lactic acid production, q_p, and the dilution rate, *D*, in chemostat culture (CF mode). Calculated value: $y_{p/x} = 8.49$ g(lactate) g(biomass)$^{-1}$.

MRF and CRF but lower in CF at $0.20\ h^{-1} \leq D \leq 0.75\ h^{-1}$, and higher in CRF only at $D > 0.75\ h^{-1}$, where wash-out effects become serious in the CF and MRF modes. Hence, the choice of the operation mode in terms of R should be done taking into account the value of D. Within the range $0.20\ h^{-1} \leq D \leq 0.75\ h^{-1}$, MRF could be the mode of choice because the recycling flow required is lower than in the CRF mode, decreasing the fouling of the microfiltration system. Nevertheless, saturation of the ion-exchange columns would impose periodic substitutions, which would prevent extension of the process to a long fermentation period maintaining the steady state. In an industrial environment this problem could be easily solved by means of proper automatic systems for diverting the permeate stream from saturated to regenerated columns as well as for lactate recovering.

To conclude, the NIR-based control system utilized in this work has allowed the full automation of a small-scale pilot plant and set the ground for further process optimization. High performances of lactate production were obtained employing control strategies based on fundamental process variables measured in real-time. The stability imparted the fermentation process by NIR-based control system and the reliability of NIR measurements are remarkable features that should allow the development of more advanced control system such as those based on expert control protocols and fuzzy controllers.

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References

Aeschlimann A, von Stockar U. 1991. Continuous production of lactic acid from whey permeate by *Lactobacillus helveticus* in a cell-recycle reactor. Enzyme Microb Technol 13:811–816.

Åkerberg C, Hofvendahl K, Zacchi G, Hahn-Hägerdal B. 1998. Modelling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetics of lactic acid production by *Lactococcus lactis* ssp. *lactis* ATCC 19435 in whole-wheat flour. Appl Microbiol Biotechnol 49(6):682–690.

Brimmer PJ, Hall JW. 1993. Determination of nutrient levels in a bioprocess using near-infrared spectroscopy. Can J Appl Spectrosc 38(6): 155–162.

Cachon R, Diviès C. 1994. Generalized model of the effect of pH on lactate fermentation and citrate bioconversion in *Lactococcus lactis* ssp. *lactis* biovar. diacetylactis. Appl Microbiol Biotechnol 41(6):694–699.

Cavinato AG, Mayes DM, Ge Z, Callis JB. 1990. Noninvasive method for monitoring ethanol in fermentation processes using fiber-optic near-infrared spectroscopy. Anal Chem 62:1977–1982.

Dosi E, Vaccari G, Campi AL, Mantovani G, González-Vara y R A, Trilli A. 1996. Control of fermentations by means of on-line near infrared spectrometry. NIR Publications 249–254.

Gadgil CJ, Venkatesh KV. 1997. Structured model for batch culture growth of *Lactobacillus bulgaricus*. J Chem Technol Biotechnol 68:89–93.

Ge Z, Cavinato AG, Callis JB. 1994. Noninvasive spectroscopy for monitoring cell density in a fermentation process. Anal Chem 66: 1354–1362.

Goksungur Y, Guvenc U. 1997. Batch and continuous production of lactic acid from beet molasses by *Lactobacillus delbrueckii* IFO 3202. J Chem Technol Biotechnol 69:399–404.

Goksungur Y, Guvenc U. 1999. Production of lactic acid from beet molasses by calcium alginate immobilized *Lactobacillus delbrueckii* IFO 3202. J Chem Technol Biotechnol 74:131–136.

Gonçalves LMD, Ramos A, Almeida JSA, Xabier MRB, Carrondo MJT. 1997. Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. Appl Microbiol Biotechnol 48(3):346–350.

González-Vara y RA, Pinelli D, Rossi M, Fajner D, Magelli F, Matteuzzi D. 1996. Production of L-(+) and D(−) lactic acid isomers by *Lactobacillus casei* subsp. *casei* DSM 20011 and *Lactobacillus coryniformis* subsp. *torquens* DSM 20004 in continuous fermentation. J Ferment Bioeng 81(6):548–552.

Hall JW, McNeil B, Rollins MJ, Draper I, Thompson BG, Macaloney G. 1996. Near-infrared spectroscopic determination of acetate, ammonium, biomass, and glycerol in an industrial *Escherichia coli* fermentation. Appl Spectrosc 50(1):102–108.

Hjörlefsdottir S, Seevaratnam S, Holst O, Mattiasson B. 1990. Effects of complete cell recycling on product formation by *Lactobacillus casei* ssp. *rhamnosus* in continuous cultures. Curr Microbiol 20:287–292.

Kulozic U, Wilde J. 1999. Rapid lactic acid production at high cell concentrations in whey ultrafiltrate by *Lactobacillus helveticus*. Enzyme Microbial Technol 24:297–302.

Luedeking R, Piret EL. 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. J Biochem Microbiol Technol Eng 1:393–405.

Macaloney G, Hall JW, Rollins MJ, Draper I, Anderson KB, Preston J, Thompson BG, McNeil B. 1997. The utility and performance of near-infrared spectroscopy in simultaneous monitoring of multiple components in a high cell-density recombinant *Escherichia coli* production process. Bioproc Eng 17(3):157–167.

Macaloney G, Hall JW, Rollins MJ, Draper I, Thompson BG, McNeil B. 1994. Monitoring biomass and glycerol in an *Escherichia coli* fermentation using near-infrared spectroscopy. Biotechnol Tech 8(4): 281–286.

Major NC, Bull AT. 1989. The physiology of lactate production by *Lactobacillus delbreuckii* in a chemostat with cell recycle. Biotechnol Bioeng 34:592–599.

Nikolajsen K, Nielsen J, Villadsen J. 1991. Modeling of a microbial system. III. Growth on mixed substrates. Biotechnol Bioeng 38:24–29.

Nishinari K, Cho RK, Iwamoto M. 1989. On-line control of glucose concentration during starch hydrolysis using near infrared spectroscopy. Starch/Stärke. 41(3):110–112.

Ohara H, Hiyama K, Yoshida T. 1992a. Non-competitive product inhibition in lactic acid fermentation from glucose. Appl Microbiol Biotechnol 36:773–776.

Ohara H, Hiyama K, Yoshida T. 1992b. Kinetics of growth and lactic acid production in continuous and batch culture. Appl Microbiol Biotechnol 37:544–548.

Ohara H, Hiyama K, Yoshida T. 1993. Lactic acid production by a filter-bed-type reactor. J Ferment Bioeng 76:73–75.

Parente E, Ricciardi A, Addario G. 1994. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. Appl Microbiol Biotechnol 41:388–394.

Picque D, Lefier D, Grappin R, Corrieu G. 1993. Monitoring of fermentation by infrared spectrometry: Alcoholic and lactic fermentations. Anal Chim Acta 279:67–72.

Pirt SJ. 1975. Effect of chemical inhibition and activation of growth. In: Principles of microbe and cell cultivation. Oxford: Blackwell Scientific Publications. p 170–185.

Roukas T, Kotzekidou P. 1996. Continuous production of lactic acide from deproteinized whey by coimmobilized *Lactobacillus casei* and *Lactobacillus lactis* cells in a packed-bed. Food Biotechnol 10:231–242.

Srivastava A, Roychoudhury PK, Sahai V. 1992. Extractive lactic acid fermentation using ion-exchange resin. Biotechnol Bioeng 39: 607–613.

Steffen C, Nick B, Blanc BH. 1973. Konfiguration der Milchsäure ver-

schiedener Milchsäurebakterienstämme in Abhängigkeit fabrikationstechnischer Bedingungen. Schweiz Milchw Forsch 2:37–52.

Tejayadi S, Cheryan M. 1995. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. Appl Microbiol Biotechnol 43:242–248.

Vaccari G, Dosi E, Campi A, Mantovani G, González-Vara y RA, Matteuzzi D. 1994. A near-infrared spectroscopy technique for the control of fermentation processes: An application to lactic acid fermentation. Biotechnol Bioeng 43:913–917.

Vaccari G, González-Vara y RA, Dosi E, Brigidi P, Campi A, Matteuzzi D. 1993. Fermentative production of L-lactic acid by *Lactobacillus casei* DSM 20011 and product recovery using ion exchange resins. Appl Microbiol Biotechnol 40:23–27.

Venkatesh KV, Okos MR, Wankat PC. 1993. Kinetic model of growth and lactic acid production from lactose by *Lactobacillus bulgaricus*. Process Biochem 28:231–241.

Vick Roy TB, Blanch HW, Wilke CR. 1982. Lactic acid production by *Lactobacillus delbreuckii* in a hollow fiber bioreactor. Biotechnol Lett 4:483–488.

Vick Roy TB, Mandel DK, Dea DK, Blanch HW, Wilke CR. 1983. The application of recycle to continuous fermentative lactic acid production. Biotechnol Lett 5:665–670.

Xavier AMRB, Gonçalves LMD, Moreira JL, Carrondo MJT. 1995. Operational patterns affecting lactic acid production in ultrafiltration cell recycle bioreactor. Biotechnol Bioeng 45:320–327.

Yano T, Harata M. 1994. Prediction of the concentration of several constituents in a mouse-mouse hybridoma culture by near-infrared spectroscopy. J Ferment Bioeng 77(6):659–662.