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ORIGINAL PAPER

Optimization of lactic acid production by immobilized *Lactococcus lactis* IO-1

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Abstract Production of lactic acid from glucose by immobilized cells of *Lactococcus lactis* IO-1 was investigated using cells that had been immobilized by either entrapment in beads of alginate or encapsulation in microcapsules of alginate membrane. The fermentation process was optimized in shake flasks using the Taguchi method and then further assessed in a production bioreactor. The bioreactor consisted of a packed bed of immobilized cells and its operation involved recycling of the broth through the bed. Both batch and continuous modes of operation of the reactor were investigated. Microencapsulation proved to be the better method of immobilization. For microencapsulated cells at immobilized cell concentration of 5.3 g l^{-1}, the optimal production medium had the following initial concentrations of nutrients (g l^{-1}): glucose 45, yeast extract 10, beef extract 10, peptone 7.5 and calcium chloride 10 at an initial pH of 6.85. Under these conditions, at 37 °C, the volumetric productivity of lactic acid in shake flasks was 1.8 g l^{-1} h^{-1}. Use of a packed bed of encapsulated cells with recycle of the broth through the bed, increased the volumetric productivity to 4.5 g l^{-1} h^{-1}. The packed bed could be used in repeated batch runs to produce lactic acid.

Keywords Lactic acid · Taguchi method · *Lactococcus lactis* · Immobilization · Packed bed bioreactor

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Introduction

Lactic acid (2-hydroxypropanoic acid), $CH_3CHOHCOOH$, is an important organic acid that is used in various food and non-food applications [5, 43]. Both fermentation and chemical synthesis are used for producing lactic acid. Lactic acid is of particular interest as a starting material for producing biodegradable poly(lactic acid) plastics [18, 21]. Substantial commercial interest exists in producing these plastics from renewable resources such as starch-derived glucose via fermentation, because of increasing emphasis on sustainable production processes [8]. There is, therefore, a need to develop fermentation processes that can provide lactic acid at a greatly reduced cost compared to existing processes. Fermentation methods for producing lactic acid have been reviewed by Litchfield [20] and Wasewar et al. [46].

This paper reports on an optimized fermentation process for producing lactic acid using immobilized cells of the bacterium *Lactococcus lactis* IO-1. The fermentation process was optimized using the well known Taguchi method [33, 34] that has been effectively used

for optimizing various other fermentations [4, 29, 30]. This optimization method has been previously applied to production of lactic acid by a different microorganism (*Lactobacillus amylovorus*) and the entirely different production scheme of solid state fermentation [23].

A packed bed bioreactor with immobilized cells was used for the fermentation, in attempts to devise an inexpensive process. The high cell densities that can be attained by immobilization offer important advantages including the following: ability to reuse the immobilized cells repeatedly and therefore reduce processing time [28]; elimination of the need to remove the bacterial cells from the final fermentation broth; a high density of cells resulting in an enhanced productivity [24, 26, 31], conversion of the substrate and final concentration of lactic acid; and reduced risk of contamination because of a high concentration of the desired cells [28]. Production of lactic acid by fermentation with *L. lactis* does not require oxygen and therefore this fermentation is specially suited to using immobilized cells in packed bed bioreactors. Advantages of using a packed bed recycle system have been previously recognized [27, 37, 38], but such a system has not been evaluated with *L. lactis*. While high-cell density fermentations with suspended cells can be used to enhance productivity, this mode of cultivation necessitates continuous separation and recycling of the biomass, significantly adding to the cost of operation [1].

Major factors that influence the cost of production using immobilized cells are the expense of the immobilization methodology [6, 47] and the cost of the fermentation medium [2, 7, 10, 11]. Metabolic engineering of the producing microorganisms has been recognized as a likely major future contributor to reducing the cost of production of lactic acid [39].

Materials and methods

Microorganism and culture medium

Lactococcus lactis IO-1 (TISTR 1401) [12], maintained in MRS medium at 4 °C was used. The MRS medium contained the following components (per liter of distilled water): glucose 10 g, peptone 10 g, beef extract 10 g, yeast extract 5 g, K_2HPO_4 2 g, sodium acetate 5 g, tri-ammonium citrate 2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $MnSO_4 \cdot 4H_2O$ 0.2 g and Tween 80 1 ml.

Cell preparation

Batches of *L. lactis* IO-1 cells were prepared by inoculating sterilized and cooled MRS medium with a 5% v/v inoculum and incubation at 37 °C under static conditions for 24 h. The cells were harvested by centrifugation at 13,700*g* for 10 min (Sorvall RC-28S centrifuge, GS3 rotor). The cell paste was washed by resuspending it in sterile distilled water followed by a second centrifugation step under conditions noted above.

Immobilization

Cells were immobilized by microencapsulation in a membrane capsule and entrapment in a gel matrix, for use in different experiments. For microencapsulation, the cell paste (20.9 g wet wt) was mixed (150 ml beaker) with 54 ml of a solution that contained 20% w/v polyethylene glycol (PEG 6000; Sigma, St. Louis, MO, USA) and 2% w/v aqueous calcium chloride. The resulting suspension was extruded dropwise through a injection needle (0.7 mm hole diameter) on the surface of a sterile solution (1,000 ml) of 0.5% w/v sodium alginate that contained 0.1% v/v Tween 80 in a 2,000 ml agitated beaker (4.5 cm magnetic stirrer, 700 rpm) [19]. This procedure produced a dispersion of liquid droplets surrounded by a membrane of alginate. The alginate-membrane capsules were recovered by filtering it through a wire mesh screen. The capsule diameter was 2.7–3.1 mm. (Thirty capsules taken from several different batches were measured with vernier calipers.) The screened microcapsules were washed with sterile distilled water and resuspended for 30 min in a gently stirred solution of 1% $CaCl_2$, pH 6.0, for hardening [19]. This procedure provided a total microcapsule wet weight of 110.6 g, corresponding to an estimated 5,500 capsules. The available viable cell concentration in the capsules ranged between 1.73×10^{10} and 6.72×10^{10} CFU per ml of capsule volume. These capsules were used for producing lactic acid as explained in the next section.

For immobilization by matrix entrapment, the harvested cell paste (20.9 g wet wt) was mixed with a 4% sodium alginate solution (360 ml) and sterile water (360 ml) in a 1-l beaker. The mixture was then added dropwise to a 3% solution of $CaCl_2$ (1,000 ml) while stirring continuously (2,000 ml beaker, 4.5 cm magnetic stirrer, 700 rpm). The gel beads produced were further hardened in 3% $CaCl_2$ solution by allowing them to stand for 2 h. The beads were then recovered by screening and washed with sterile distilled water. The beads ranged in diameter from 3.0 to 3.2 mm. The viable cell concentration in the beads was 1.30×10^{10} CFU per ml of bead.

Fermentations

Fermentation optimization was first conducted in 500-ml Erlenmeyer flasks that contained 100 ml MRS medium. Variations were prepared in accordance with the experimental design identified in Tables 1 and 2. All experiments were carried out at 37 °C. The flasks were held on an orbital shaker at 100 rpm. Samples were withdrawn periodically during the 12 h duration of fermentations and analyzed for glucose and lactic acid.

In a batch operation, lactic acid production was carried out using immobilized cells in a 250-ml packed bed reactor with broth recycle to a stirred tank (Fig. 1). The broth in the tank was held at 37 °C, 400 rpm agitation rate and a controlled pH of 6.85. The stirred tank was initially filled with 1 l of MRS medium. From the tank the medium was pumped to the top of the packed bed. The medium emerging from the bed was returned to the stirred tank. The flow rate of the medium in the bed was a constant 16 ml min^{-1}. Once all the glucose in the medium had been consumed, the packed bed and the tank were drained and thoroughly washed with sterile distilled water. The packed column was then used for producing the next batch of lactic acid.

For experiments involving continuous production of lactic acid, the reactor system was started in the batch mode exactly as explained above. Once all the glucose had been consumed, the operation was switched to continuous mode in which a fresh batch of glucose-containing medium was fed to the stirred tank at a preset flow rate. The medium from the tank was continuously withdrawn at the same rate at which the tank was fed, so that the volume in the stirred tank remained constant. The dilution rate in the stirred tank was 0.5 h^{-1} and this required a constant fresh medium feed rate of 8.33 ml min^{-1}. A constant recycle rate of 16 ml min^{-1} was maintained through the packed bed. A steady state was eventually attained in which the concentration of the lactic acid in the harvest stream was constant for the specified optimal conditions.

Analyses

Glucose and lactic acid concentrations were determined by colorimetric methods of Miller [22] and Barker and Summerson [3], respectively, as well as by high-performance liquid chromatography (HPLC). For the latter, the chromatography column was Aminex HPX-87H column (Biorad, USA) operated at 50 °C. The mobile phase flow rate was 0.40 ml min^{-1}. The mobile phase was 5 mM sulfuric acid [40].

Cell concentration was determined gravimetrically by filtering a sample through a 0.45 μm pore size membrane filter, drying the solids at 105 °C overnight, and weighing them. Cell viability was measured by dissolving 30 microcapsules or beads of known average volume in 5 ml of sterile 1.0% tri sodium citrate solution [45], agar plate inoculation at various levels of dilution, and counting of the number of colonies formed.

Experimental design

The following eight factors were selected for optimization of the lactic acid production with immobilized cells: (1) type of immobilization (i.e., entrapment and microencapsulation, or two levels); (2) concentration of the immobilized cells in the matrix or microcapsule; (3) initial concentration of glucose; (4) initial concentration of yeast extract; (5) initial concentration of beef extract; (6) initial pH; (7) calcium chloride concentration; and (8) initial concentration of peptone. Each factor (except the first) was assessed at three levels. The factors and their levels are shown in Table 1. Following the Taguchi method, these factors were optimized by orthogonal arrays (OA) of 18 experiments. The factors and their levels for each experiment are shown in Table 2. All 18 experiments were carried

Table 1 Experimental design for optimizing lactic acid fermentation. Experimental factors and their levels

No.	Factors	Levels		
		1	2	3
1	Type of immobilization (A) (only 2 levels by design)	Entrapment	Encapsulation	–
2	Cell concentration of immobilized cells (g l^{-1}) (B)	3.96	5.28	6.60
3	Initial glucose concentration (g l^{-1}) (C)	25.0	35.0	45.0
4	Initial yeast extract concentration (g l^{-1}) (D)	2.5	5.0	10.0
5	Initial beef extract concentration (g l^{-1}) (E)	5.0	7.5	10.0
6	Initial pH (F)	6.0	6.5	6.85
7	Initial calcium chloride concentration (g l^{-1}) (G)	2.5	5.0	10.0
8	Initial peptone concentration (g l^{-1}) (H)	5.0	7.5	10.0

Table 2 Experimental design for optimizing lactic acid fermentation. Layout of the L_{18} ($2^1 \times 3^7$) orthogonal arrays

Exp.	Factors							
	A	B	C	D	E	F	G	H
1	1	1	1	1	1	1	1	1
2	1	1	2	2	2	2	2	2
3	1	1	3	3	3	3	3	3
4	1	2	1	1	2	2	3	3
5	1	2	2	2	3	3	1	1
6	1	2	3	3	1	1	2	2
7	1	3	1	2	1	3	2	3
8	1	3	2	3	2	1	3	1
9	1	3	3	1	3	2	1	2
10	2	1	1	3	3	2	2	1
11	2	1	2	1	1	3	3	2
12	2	1	3	2	2	1	1	3
13	2	2	1	2	3	1	3	2
14	2	2	2	3	1	2	1	3
15	2	2	3	1	2	3	2	1
16	2	3	1	3	2	3	1	2
17	2	3	2	1	3	1	2	3
18	2	3	3	2	1	2	3	1

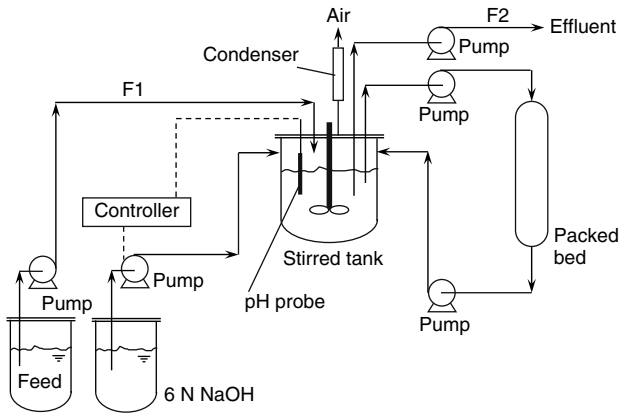

Fig. 1 Packed bed bioreactor system with broth recycle for producing lactic acid using immobilized cells. The flow streams F1 and F2 did not exist in the batch mode of operation

out in duplicate in shake flasks. The flasks were sampled every 2 h for the 12-h duration of fermentation. The optimal conditions with respect to the factors tested were assessed by plotting the signal-to-noise (*S*/*N*) ratios of the factor averages at each factor level, against all factor levels.

Results and discussion

Optimization of production in shake flasks

The final lactic acid concentration (C_P) and the volumetric productivity (Q_P) of lactic acid are shown in Table 3 for the various experiments. The maximum and minimum values of the effects of the various factors are shown in Table 4, for the final concentration of lactic acid and its productivity. The main effect of each factor and the percent main effect are indicated (Table 4). The values in Table 4 were calculated following the well known methodology, as documented by Roy [33], for example. The ANOVA results for the various factors affecting final concentration of lactic acid and its productivity are shown in Table 5a, b, respectively.

The signal-to-noise (*S*/*N*) ratio (Table 3) is the principal criterion for identifying optimal conditions in Taguchi's method [34]. A high *S*/*N* value is used as an indicator of optimality. Among the 18 experimental trials, both the highest lactic acid concentration and productivity were obtained under the culture conditions of treatment 9 (Table 3). The highest lactic acid concentration and productivity were 13.8 g l^{-1} and 1.73 g l^{-1} h^{-1}, respectively. These values were about sevenfold greater than the lowest values of these variables. The trial 9 conditions were as follows: cell immobilization by entrapment; immobilized cell concentration of 6.6 g l^{-1} in the matrix; a production medium composed of 45 g l^{-1} glucose, 2.5 g l^{-1} yeast extract, 10 g l^{-1} beef extract, 2.5 g l^{-1} calcium chloride and 7.5 g l^{-1} peptone, and initial pH 6.5. The last seven of the factors listed, had a strong effect on production of lactic acid. The type of immobilization method used had relatively small effect on concentration and productivity of lactic acid (Table 4).

Concentration of yeast extract was the most important factor affecting production, as this factor had a percent main effect value of 18% (Table 4). This observation is consistent with a similar finding for production of lactic acid using *Lactobacillus amylovorus* NRRL B-4542 in solid-state fermentation [23]. Production was maximized at yeast extract concentration of 30 g l^{-1} [23]. Lactic acid production by *Lactobacillus delbrueckii* [17] and immobilized cells of *Lactobacillus helveticus* [36] has also been reported to be positively influenced by a high concentration of yeast extract. The analysis of variance (ANOVA) in Table 5 confirmed that the factors tested had significant effects (i.e., $p < 0.05$) on concentration and productivity of lactic acid.

The effect of changes in factor values on *S*/*N* ratio is plotted in Fig. 2a, b for lactic acid concentration and volumetric productivity, respectively. The factor values that provided highest concentration and productivity of lactic acid were identical (Fig. 2). The factor values for optimality were: cell immobilization by encapsulation; immobilized cell concentration of 5.28 g l^{-1} in the mi-

Table 3 Shake flask fermentation results for final lactic acid concentration (C_P) and volumetric productivity (Q_P)

Exp.	C_P (g l^{-1})					Q_P (g l^{-1} h^{-1})				
	1	2	Average	SD	*S*/*N* ratio (dB)	1	2	Average	SD	*S*/*N* ratio (dB)
1	1.21	2.75	1.98	1.092	0.439	0.15	0.34	0.25	0.134	–8.625
2	6.95	6.94	6.94	0.009	6.911	0.87	0.87	0.87	0.000	–2.110
3	12.90	13.31	13.11	0.295	9.668	1.61	1.66	1.64	0.035	0.629
4	7.72	8.05	7.88	0.233	7.459	0.97	1.01	0.99	0.028	–1.552
5	11.74	11.47	11.60	0.188	9.140	1.47	1.39	1.43	0.057	0.043
6	12.49	11.05	11.77	1.020	9.177	1.56	1.38	1.47	0.127	0.144
7	8.70	8.30	8.50	0.283	7.784	1.09	1.04	1.07	0.035	–1.235
8	12.52	12.46	12.49	0.043	9.460	1.56	1.56	1.56	0.000	0.426
9	13.94	13.67	13.81	0.188	9.896	1.74	1.71	1.73	0.021	0.862
10	8.28	7.05	7.67	0.874	7.298	1.11	0.88	1.00	0.163	–1.614
11	10.16	10.00	10.08	0.117	8.528	1.32	1.3	1.31	0.014	–0.333
12	7.36	7.22	7.29	0.096	7.123	0.92	0.9	0.91	0.014	–1.915
13	13.00	11.08	12.04	1.354	9.261	1.63	1.39	1.51	0.170	0.243
14	10.46	10.11	10.28	0.248	8.614	1.31	1.26	1.29	0.035	–0.419
15	9.15	9.80	9.48	0.458	8.253	1.14	1.22	1.18	0.057	–0.794
16	10.61	12.44	11.53	1.294	9.071	1.33	1.56	1.45	0.163	0.052
17	4.84	4.58	4.71	0.189	5.219	0.61	0.57	0.59	0.028	–3.804
18	8.67	9.33	9.00	0.462	8.029	1.08	1.17	1.13	0.064	–1.004

Note: The signal-to-noise ratio (*S*/*N* ratio) was calculated as –10 $\log_{10}$ (MSD) where the mean square deviation (MSD) was $(1/y_1^2 + 1/y_2^2 + 1/y_3^2 + ...)/n$ [33] (y = experimental result of C_P or Q_P). Each of the 18 experimental trials was carried out in duplicate

Table 4 Analysis of the factors affecting lactic acid fermentation

Levels	A	B	C	D	E	F	G	H
(a) Lactic acid concentration (C_P)								
1	7.770	6.661	6.885	6.632	7.095	6.780	7.381	7.103
2	7.933	8.651	7.979	8.041	8.046	8.035	7.440	8.807
3	–	8.243	8.691	8.881	8.414	8.741	8.734	7.645
Min	7.770	6.661	6.885	6.632	7.095	6.780	7.381	7.103
Max	7.933	8.651	8.691	8.881	8.414	8.741	8.734	8.807
Main effect	0.163	1.989	1.805	2.249	1.318	1.961	1.354	1.704
% Main effect	1.30%	15.86%	14.39%	17.93%	10.51%	15.64%	10.79%	13.59%
(b) Volumetric productivity (Q_P) of lactic acid								
1	–1.268	–2.328	–2.122	–2.374	–1.912	–2.255	–1.667	–1.928
2	–1.065	–0.389	–1.033	–0.996	–0.982	–0.973	–1.569	–0.190
3	–	–0.784	–0.347	–0.130	–0.607	–0.273	–0.265	–1.383
Min	–1.269	–2.328	–2.122	–2.374	–1.912	–2.255	–1.667	–1.928
Max	–1.065	–0.389	–0.347	–0.130	–0.607	–0.273	–0.265	–0.190
Main effect	0.203	1.939	1.775	2.244	1.305	1.982	1.402	1.738
% Main effect	1.62%	15.40%	14.10%	17.82%	10.37%	15.75%	11.14%	13.80%

Note: The factor averages at each factor level were obtained by adding the *S*/*N* ratio results (C_P or Q_P) of all trial conditions at the level considered and then dividing by the numbers of data points added (9 and 6 for factor A and factors B–H, respectively). The main effect of each factor was the difference between the maximum and minimum values of the factor averages at each factor level (Main effect = max – min), while the percent main effect of each factor was calculated as the percentage of its main effect divided by the sum of the main effects of all factors; thus, percent main effect = (main effect × 100)/Σ all main effects [33]

crocapsules; 45 g l^{-1} glucose, 10.0 g l^{-1} yeast extract, 10 g l^{-1} beef extract, 10.0 g l^{-1} calcium chloride, 7.5 g l^{-1} peptone; and initial pH 6.85. Under this combination of optimal conditions, three factors had different values compared with the screening experiment trial no. 9 mentioned above. Under optimal conditions, the effect of concentration of immobilized cells decreased to the second level, while the effect of concentrations of yeast extract and calcium chloride increased to their highest levels of 10 g l^{-1} from 2.5 g l^{-1}. Furthermore, the initial pH optimum was slightly higher at 6.85 compared with the previous value of 6.5.

Table 5 Analysis of variance (ANOVA) of factors affecting lactic acid fermentation

Factors	Sum of squares	DOF	Variance	*F*-ratio	Confidence (%)	Significance level
(a) Lactic acid concentration (C_P)						
A	4.00	1	4.00	9.82	99.4	$p < 0.01$
B	48.09	2	24.04	58.96	100.0	$p < 0.001$
C	36.94	2	18.47	45.29	100.0	$p < 0.001$
D	60.43	2	30.22	74.09	100.0	$p < 0.001$
E	22.00	2	11.00	26.98	100.0	$p < 0.001$
F	33.37	2	16.68	40.91	100.0	$p < 0.001$
G	40.27	2	20.13	49.37	100.0	$p < 0.001$
H	44.68	2	22.34	54.78	100.0	$p < 0.001$
Other	29.55	2	14.77	36.23	100.0	$p < 0.001$
Error	7.34	18	0.41			
Total	326.67	35				
(b) Volumetric productivity of lactic acid (Q_P)						
A	0.05	1	0.05	6.18	97.7	$p < 0.05$
B	0.68	2	0.34	46.18	100.0	$p < 0.001$
C	0.54	2	0.34	36.61	100.0	$p < 0.001$
D	0.94	2	0.47	63.87	100.0	$p < 0.001$
E	0.33	2	0.17	22.55	100.0	$p < 0.001$
F	0.54	2	0.27	36.36	100.0	$p < 0.001$
G	0.64	2	0.32	43.64	100.0	$p < 0.001$
H	0.74	2	0.37	50.28	100.0	$p < 0.001$
Other	0.50	2	0.25	34.00	100.0	$p < 0.001$
Error	0.13	18	0.01			
Total	5.09	35				

Note: The sum of squares of factors (SS_{Factor}) and error (SS_{Error}) were calculated by using $SS_{Factor} = \sum_{k=1}^{L} \frac{\left(\sum_{i=1}^{n} y_{ki}\right)^2}{n} - \frac{T^2}{N}$ and $SS_{Error} = \sum_{j=1}^{M} (SD)_j^2 (r-1)$, respectively, while the factors variance (V_{Factor}) and *F*-ratio (F_{ratio}) were obtained from $V_{Factor} = SS_{Factor}/DOF_{Factor}$ and $F_{ratio} = V_{Factor}/V_{Error}$, respectively. Here L, k, n, i, y, T, N, M, j and r are level number, factor level, number of experimental results at each factor level, each experimental trial considered at factor level, experimental result, sum of all experimental results, total number of experimental results, number of experimental trials, each experimental trial and number of tests at each experimental trial, respectively [33, 34]. Analysis of variance (ANOVA) was performed using a Microsoft Excel worksheet

Although immobilization by encapsulation can theoretically provide a higher cell density than is attainable through entrapment [6, 47], the immobilization methodology had a relatively minor impact on lactic acid production in this work (Fig. 2a, b).

The optimal concentration of calcium chloride was fourfold the value that was initially tested in the culture medium. A high concentration of calcium chloride apparently helped in stabilizing the immobilizing alginate matrix [6] by countering the calcium-chelating effect of the lactate. In the absence of an elevated concentration of calcium, the lactate produced can effectively chelate the calcium ion in the alginate gel to soften the immobilizing matrix and make it susceptible to disintegration by dissolution.

Confirmation experiments and estimated result

The statistical optimization method used provides a model for predicting the optimal performance (Y_{opt}) as influenced by the significant factors, as follows:

$$Y_{opt} = \bar{T} + \sum (\bar{F}_i - \bar{T}), \quad (1)$$

where $\overline{T}$ and $\bar{F}_i$ represent the grand average of performance and the significant factor averages at each factor level, respectively [34]. For the eight factors that significantly influenced the concentration and productivity of lactic acid, the expected lactic acid concentration and productivity at the optimal conditions identified in Fig. 2 could be estimated using the following equation:

$$Y_{opt} = \bar{T} + (\bar{A}_2 - \bar{T}) + (\bar{B}_2 - \bar{T}) + (\bar{C}_3 - \bar{T}) + (\bar{D}_3 - \bar{T}) + (\bar{E}_3 - \bar{T}) + (\bar{F}_3 - \bar{T}) + (\bar{G}_3 - \bar{T}) + (\bar{H}_2 - \bar{T}). \quad (2)$$

The expected values of lactic acid concentration and productivity were 18.0 g l^{-1} and 2.30 g l^{-1} h^{-1}, respectively.

Confirmatory experiments were carried out in duplicate under the above identified optimal conditions. The lactic acid concentration and productivity obtained were 10.6 and 1.76 g l^{-1}, respectively. Both concentration and productivity of lactic acid were substantially lower than the expected optimal values estimated with Eq. 2; nevertheless, the measured pro-

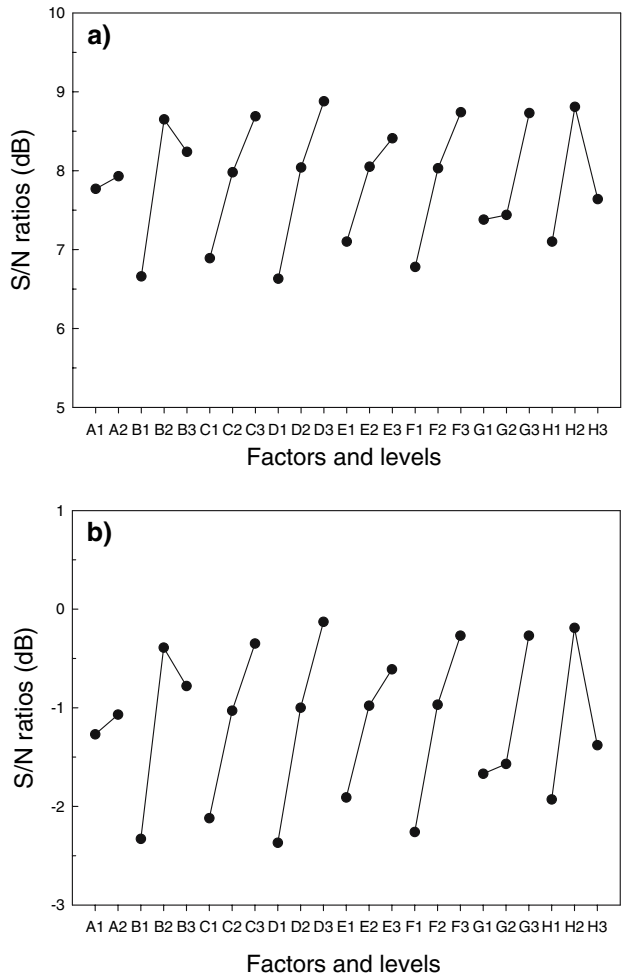

Fig. 2 **a** Sum of signal-to-noise (*S*/*N*) ratios for the various factors and levels relating to lactic acid concentration; **b** sum of *S*/*N* ratios for the various factors and levels relating to volumetric productivity of lactic acid. Data are shown in Table 4

ductivity was quite comparable to the value previously attained in the experiment trial 9 (Table 3). Inability to predict performance closely with Eq. 2 was associated with a lack of pH control in shake flasks. Production of lactic acid lowed pH and this affected its further production [6]. This aspect is discussed further in the next section where production of lactic acid is carried out in a packed bed reactor under conditions of controlled pH.

Under optimal conditions, the concentration of lactic acid in the microcapsules was quite high at 49.1 g l^{-1}. Based on simulations using a published kinetic model for production of lactic acid by *L. lactis* IO-1 [40], a lactic acid concentration of just 42 g l^{-1} would seriously inhibit its production especially at low pH values at which lactic acid occurs predominantly in its undissociated form. In studies with several other lactobacilli, both bacterial growth and lactic acid production have been found to be inhibited by accumulation of undissociated lactic acid in the medium [2, 35, 38]. A high initial concentration of the carbon source can also inhibit lactobacilli but this effect generally occurs at concentrations exceeding 100 g l^{-1} [32].

Production of lactic acid in the packed bed reactor

Batchwise and repeated batch operation

The results for batch production of lactic acid using alginate-membrane encapsulated *L. lactis* IO-1 in the packed bed reactor with broth recycled at a constant rate of 16 ml min^{-1}, controlled pH of 6.85, and the above specified optimal conditions, are shown in Fig. 3a. For two consecutive runs, most of the glucose had been converted to lactic acid within 12 h of operation. At 12 h, the yield of lactic acid on glucose was 89 and 98% for the first and second consecutive batch runs, respectively. The final concentrations of lactic acid were 24.9 and 29.8 g l^{-1}, for the first and second consecutive batches, respectively. These final lactic acid concentrations were substantially greater than the values achieved in shake flask under optimal conditions.

Repeated batch fermentations (Fig. 3b) demonstrated the feasibility of using immobilized cells for multiple fermentation cycles. The rates of consumption of glucose and production of lactic acid increased in going from batch run 1 to batch run 3 (Fig. 3b). This was because of cell growth in the microcapsules that resulted in increased biocatalytic activity in the reactor. The values of the final concentration of lactic acid, the yield of lactic acid on glucose, the specific rate of glucose consumption, the specific rate of lactic acid formation, and the volumetric productivity of lactic acid, are summarized in Table 6 for batch and repeated batch fermentations. Comparing the batch run in the packed bed reactor with shake flask batch fermentations, both the final lactic acid concentration and productivity were substantially greater in the packed bed primarily because of controlled pH. In addition, better mass transfer of glucose to the microcapsules and of lactic acid from the microcapsules to the surrounding medium, likely contributed to the improved performance of the packed bed. Diffusion limited transport of lactic acid from microcapsules of immobilized cells has indeed been reported [6].

Under controlled pH conditions of the packed bed, the productivity of the lactic acid was within ±6% of the expected value (Eq. 2) of 2.30 g l^{-1} h^{-1} and the final concentration of lactic acid exceeded the expected value of 18.0 g l^{-1} (Eq. 2) by 65% (Table 6).

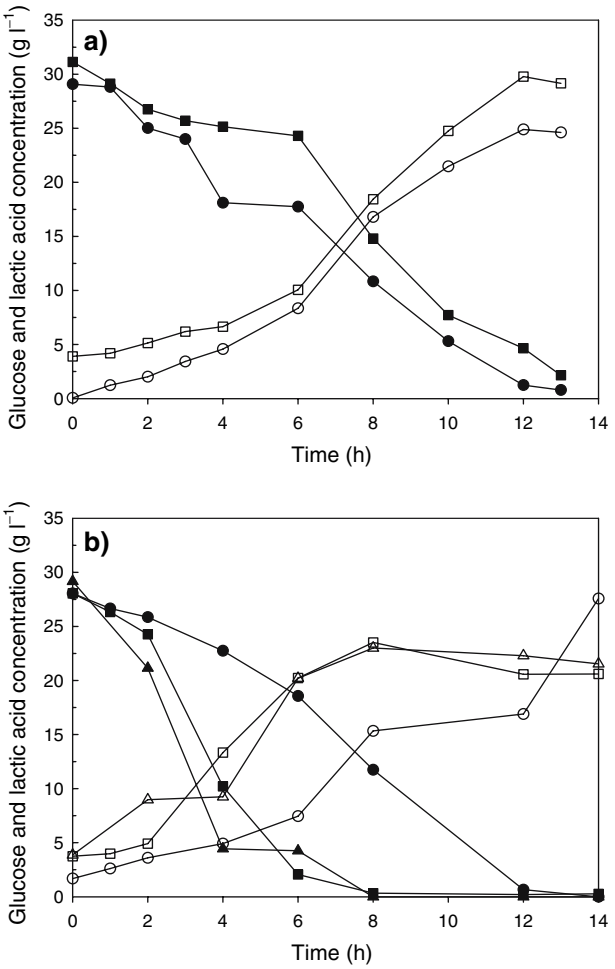

Fig. 3 **a** Lactic acid production and glucose consumption in two batch runs with alginate-membrane encapsulated *L. lactis* IO-1 in the packed bed reactor with broth recycle under specified optimal conditions: run 1 (*filled circle*, *open circle*) and run 2 (*filled square*, *open square*). **b** Repeated batch production of lactic acid using alginate-membrane encapsulated cells in the packed bed reactor with broth recycle under specified optimal conditions: batch 1 (*filled circle*, *open circle*), batch 2 (*filled square*, *open square*), batch 3 (*filled triangle*, *open triangle*). In all cases, *hollow symbols* represent lactic acid and *filled symbols* represent glucose

Preventing inhibition of the cells by lactic acid requires continuous removal of the produced lactic acid from the culture medium. This is the rational for the continuous mode of operation discussed next.

Continuous production

In continuous lactic acid production, fresh medium was fed continuously to the reservoir that supplied the packed bed bioreactor and an equal amount of the spent medium was continuously harvested (Fig. 1) in attempts to dilute the lactic acid and reduce its inhibitory effect [37].

Concentration of glucose and lactic acid during the startup batch phase and subsequent continuous operation of the packed bed bioreactor are shown in Fig. 4 as a function of time. The data shown are for a dilution rate (D) of 0.5 h^{-1} for alginate-membrane encapsulated *L. lactis* IO-1 operated at a controlled pH of 6.85 and the earlier specified optimal conditions (Fig. 2). At steady state, the continuous operation achieved a lactic acid concentration of 8.9 g l^{-1} and a residual glucose concentration of 21.2 g l^{-1}. Increased consumption of glucose can be achieved by reducing the dilution rate, but this will increase the steady state concentration of lactic acid and consequent inhibition.

Continuous operation did not greatly affect the yield of lactic acid on glucose when compared with batch operation (Table 6). However, continuous operation increased the specific substrate conversion rate by more than twofold compared with batch operation. The specific rate of production of lactic acid was increased by nearly twofold by switching from batch to continuous operation (Table 6). The lactic acid productivity of the continuous reactor was twofold greater than in the batch mode of operation.

Clearly, continuous operation successfully reduced product inhibition of the bacterial cells compared with operation in the batch mode. The lactic acid yield in

Table 6 Comparison of fermentation parameters for batch, repeated batch and continuous production of lactic acid in packed bed bioreactor

Parameters[a]	Batch production[b]	Repeated batch production			Continuous production (D = 0.5 h^{-1})
		First	Second	Third	
C_P (g l^{-1})	29.78	27.58	23.52	23.00	8.91
$Y_{P/S}$ (g g^{-1})	0.98	0.92	0.71	0.66	0.89
q_S (g g^{-1} h^{-1})	0.42	0.44	0.66	0.69	0.95
q_P (g g^{-1} h^{-1})	0.41	0.41	0.47	0.45	0.84
Q_P (g l^{-1} h^{-1})	2.16	2.16	2.47	2.39	4.46

[a] C_P, $Y_{P/S}$, q_S, q_P, Q_P represent concentration of lactic acid, yield of lactic acid on glucose, specific rate of glucose consumption, specific rate of lactic acid formation, and the volumetric productivity of lactic acid, respectively

[b] Taken from the second batchwise production of lactic acid (Fig. 3a)

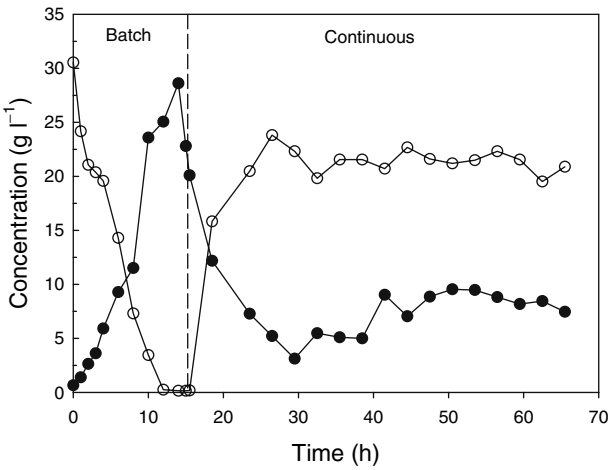

Fig. 4 Continuous production of lactic acid using alginate-membrane encapsulated *L. lactis* IO-1 in the packed bed reactor with broth recycle under specified optimal conditions at a dilution rate of 0.5 h^{-1}

continuous operation was 0.89, or 90% of the theoretical maximum. At a dilution rate of 0.81 h^{-1} the volumetric productivity of lactic acid in a suspension of free cells was 4.42 g l^{-1} h^{-1} [41], or nearly the same as the productivity at the dilution rate of 0.5 h^{-1} (Table 6). This was because in continuous operation volumetric productivity is the product of dilution rate and the product concentration in the effluent stream. Increasing the dilution rate reduces the concentration of the product in the effluent. Clearly, there is an optimal dilution rate, i.e., the minimum value of the dilution rate that would still ensures a productivity of around 4.46 g l^{-1} h^{-1}. This optimal dilution rate appears to be only slightly greater than 0.81 h^{-1}.

Comparison with other work

Various aspects of microbial production of lactic acid have been reviewed by Hofvendahl and Hahn-Hägerdal [11], Wasewar et al. [46] and Singh et al. [39]. More than 60 different lactobacilli and other microorganisms have been used to produce lactic acid from various carbon sources [11]. In comparison with lactobacilli, the genus *Lactococcus* has been used infrequently. For example, only ten of the 63 lactic acid producers noted in Table 2 of Hofvendahl and Hahn-Hägerdal [11] are lactococci. Fewer than a dozen [12–16, 24, 25, 40–42, 44] of the more than 190 studies cited in the literature [11, 39] have used *L. lactis* IO-1, the microorganism used in the present study. Most of the previous work with *L. lactis* IO-1 focused on taxonomic studies [12], use of electrodialysis in reducing product inhibition [13, 25, 44], and carbon sources other than glucose [14, 15, 24, 40], as used in this work.

A consideration of yield of lactic acid on carbon source is important in determining economics of production. With the exception of an apparently erroneous yield coefficient of 1.5 g g^{-1} lactose reported in the literature (Table 2 of [11]), the lactic acid yield coefficients on carbon sources in conventionally conducted *L. lactis* fermentations have ranged from 0.21 to 0.88 g g^{-1} (Table 2 of [11]). In comparison with this, our highest yield coefficients were substantially greater at 0.98 and 0.92 g g^{-1} for batch and repeated batch production, respectively (Table 6).

According to the literature (Table 2 of [11]), the highest reported yield coefficient of lactic acid on glucose using any microorganism is 0.98. This value was obtained with entirely different microorganisms (*Lactobacillus zeae* ATCC 393 and *Lactobacillus coryniformis* sp. *torquens* ATCC 25600) than we used. Unfortunately, these high-yielding lactobacilli gave a low productivity of lactic acid at 4.0 g l^{-1} h^{-1}, compared with our highest productivity value of 4.46 g l^{-1} h^{-1} (Table 6). The final average concentration of lactic acid achieved with the two high-yielding lactobacilli was 38 g l^{-1} (Table 2 of [11]). Using *L. lactis* IO-1, we achieved a comparable concentration of 30 g l^{-1}.

The highest reported final concentration of lactic acid in batch fermentation appears to be 120 g l^{-1}. This value was obtained with *Lactobacillus casei* NRRL B-441 grown on a complex substrate (barley flour hydrolysate) (Table 2 of [11]). The productivity of this fermentation was only 1.5 g l^{-1} h^{-1}, or 34% of the productivity we obtained in continuous operation (Table 6) and 69% of our batch productivity (Table 6). Furthermore, the yield coefficient attained with *L. casei* NRRL B-441 was only 0.67 g g^{-1}, or nearly 32% lower than our batch culture value of 0.98 g g^{-1}. In view of these results, *L. lactis* IO-1 cultured using the methodology of this work, is clearly one of the superior producers of lactic acid.

Concluding remarks

The production of lactic acid with immobilized *L. lactis* IO-1 was optimized using Taguchi method. The optimal conditions obtained were verified with the production of lactic acid using both repeated batch and continuous modes of operation in a packed bed reactor. With controlled pH during the fermentation, the productivity of lactic acid increased greatly in comparison with data obtained in shake flasks. The continuous mode of operation of the packed bed gave the

highest lactic acid productivity, as observed also by Senthuran et al. [37] for *Lactobacillus casei*. Under the conditions used, a large quantity of residual glucose remained in the effluent of the continuous mode of operation. Potentially, the amount of residual glucose may be reduced by reducing the dilution rate. Alternatively, glucose-containing effluent can be recycled to the reactor after removing lactic acid by, for example, adsorption on an ion exchange resin [9]. As another option for preventing glucose loss, glucose may be fed using a pH-dependent feeding system [16], or by using an online glucose concentration analyzer to control the feeding.

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