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Food Additive Lactic Acid Production by Immobilized Cells of *Lactobacillus brevis* on Delignified Cellulosic Material

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Improvements in yield and productivity in lactic acid fermentation by *Lactobaccilus brevis* cells immobilized on delignified cellulosic (DC) material are reported. The system proved to be more efficient in comparison with the work reported by other workers. Yields of 80 and 100% conversion using glucose were obtained at 30 °C in 1 day of fermentation time. Lactic acid fermentation using whey as substrate was obtained at 30 °C in 1−1.5 days, resulting in 70% yield, whereas the remaining lactose in whey was converted to alcohol byproduct, leading to a 90% lactose exploitation and 100% conversion. Cell immobilization of *L. brevis* on DC material was proved by its reuses in repeated batch fermentations and through electron microscopy. A series of 10 repeated batch fermentations without any loss in cell activity showed a tendency for high operational stability. The presence of DC material resulted in a drastic drop of the fermentation time from 48 to 13 h.

KEYWORDS: Lactic acid production; *Lactobacillus brevis*; delignified cellulosics; whey fermentation

INTRODUCTION

Lactic acid is an important chemical used in a wide variety of applications, being used primarily in the food industry as an acidulant and preservative and for the production of emulsifying agents (*1*). Other applications of lactic acid are for cosmetics, pharmaceuticals, metal etching, and textile-finishing operations and as a precursor for biodegradable polylactic acid production.

Lactic acid can be obtained by either chemical or biotechnological means. The biotechnological procedures for lactic acid production are based on the bioconversion of sugar solutions by microorganisms. Recently, efforts have been made for lactic acid production using various sugar sources such as wood (*2*) and wheat straw hemicellulose hydrolysate (*3*).

Given the low productivity of batch processes for lactic acid production, recent research has focused on increasing the cell concentration in the reactor. Cell immobilization is one of the most attractive methods in maintaining high cell concentration in the reactor. Production of lactic acid with immobilized cells on alginates has been reported (*4*−*7*), showing better results than with free cells. However, gels are considered to be inconvenient because they are chemically unstable and can easily be disrupted by lactate. The use of more stable supports such as porous foam glass particles (*8*), ceramic beads or porous glass (*9*), poraver beads (*10*), and gluten pellets (*11*) hardly offered a better alternative as they are relatively expensive materials. Even though cell immobilization results in increased productivity compared to free cells, the industrialization of immobilized cells has not been achieved. This can be attributed to the fact that industrialization needs a low cost, easily handled, food-grade purity support with high operational stability.

Delignified cellulosic (DC) material has been proposed as an immobilization support of yeast strains for wine-making (*12*), low-temperature brewing (*13*), and continuous and high-temperature alcoholic fermentation of whey (*14*, *15*). DC material is a support of food-grade purity, cheap, and abundant in nature, and the immobilization technique is simple and easy and showed high operational stability and a significant increase in productivity in alcohol production.

Cell immobilization of *Lactobacillus brevis* on DC material was carried out to determine possible advantages on lactic acid productivity and yield, similar to those observed in the alcoholic fermentation (*12*−*16*), and to compare the results with those reported by other researchers. The strategy adopted was to use *L. brevis* immobilized on DC material in contrast to *Lactobacillus casei* immobilized on gluten pellets (*11*) and to examine the behavior of the biocatalyst during glucose initially and then lactose fermentation. Finally, the aim of this study was to proceed to fermentations using whey, knowing that it is a very polluting liquid of the dairy industry with negligible cost. Results concerning glucose fermentation could be useful for glucose-containing raw materials.

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MATERIALS AND METHODS

Cell Cultivation. *L. brevis* used in the present study was obtained from the Pasteur Institute. It was grown on synthetic medium consisting of brain−heart base, 3.7%; yeast extract, 0.5%; cysteine−HCl, 0.05%; K_2HPO_4, 0.1%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.5%; and glucose or lactose, 2% in distilled water. This complete medium was sterilized at 121 °C for 15 min. The flasks were incubated at 30 °C. After fermentation, bacterial cells were separated by centrifugation and pressed wet cells (15−20 g) were prepared. Whey was produced in the laboratory after milk coagulation using the enzyme rennet, and it contained ~50 g/L lactose.

Cell Immobilization. DC material was used as the support for immobilization. The preparation of wet DC was carried out as described in a previous study (*12*). In brief, DC material was produced from wood sawdust after treatment with 1% NaOH solution and heating for 3 h at the boiling point for removal of lignin.

For the immobilization of cells, 55 g of wet DC material, 250 mL of synthetic medium, and 5 g of wet cells of *L. brevis* were placed in a 500 mL flask. The synthetic medium contained glucose or lactose, 5%; yeast extract, 0.5%; K_2HPO_4, 0.1%; $(NH_4)_2SO_4$, 0.1%; and $MgSO_4 \cdot 7H_2O$, 0.5% in distilled water. The flask was incubated at 30 °C and allowed to ferment overnight. During the immobilization, the pH was maintained at 5.0−6.0 by the addition of a saturated solution of Na_2CO_3. The immobilization process was monitored by estimating the residual sugar and the production of lactic acid using an HPLC instrument, as described under Analyses. When the immobilization was completed, the fermented liquid was decanted and the supported biocatalyst was washed twice with 250 mL of liquid that was used for the next fermentation batch. The biocatalyst was then used for lactic acid production.

Repeated Batch Fermentations of Glucose, Lactose, and Whey. Fifty-five grams of wet weight of DC material-supported *L. brevis*, prepared as described above, was introduced in 250 mL of synthetic medium containing glucose, lactose, or whey. For glucose and lactose, repeated batch fermentations were carried out at different temperatures: 30, 20, 15, 10, and 5 °C. Fermentations of whey were carried out at 30 °C. All fermentations were performed without agitation under stationary condition. During fermentation the pH was adjusted in the range of 5.0−6.0, as described above. Completion of fermentation was monitored in an analogous way as described for immobilization process. When the fermentation was completed, the liquid was decanted and the support was washed twice, with 250 mL of synthetic medium. At the end of every batch, samples were collected and analyzed for lactic acid, ethanol, and residual sugar.

To obtain comparison of the results, four repeated batch fermentations of whey using 10 g L^{-1} wet weight free cells were carried out at 30 °C.

Analyses. Lactic acid, ethanol, and residual sugar were determined by high-performance liquid chromatography, using a Shimadzu chromatograph with an SCR-101N stainless steel column, an LC-9A pump, a CTO-10A oven at 60 °C, and an 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 was injected directly to the column. The lactic acid, ethanol, and residual sugar concentrations were calculated using standard curves.

Lactic acid yield was expressed as grams of lactic acid produced per 100 g of sugar; conversion was calculated by using the following equation:

$$(\text{initial sugar concn} - \text{residual sugar concn})/\text{initial sugar concn} \times 100$$

Lactic acid productivity was calculated as grams of lactic acid per liter of liquid volume produced per day.

Scanning Electron Microscopy. The immobilization of *L. brevis* on DC material was monitored by scanning electron microscopy. Pieces of the immobilized biocatalyst were washed with a synthetic medium of glucose or lactose and dried overnight at 30 °C. The dried sample was coated with gold in a Balzers SCD 004 sputter coater for 2 min and examined in a JSM-6300 scanning electron microscope.

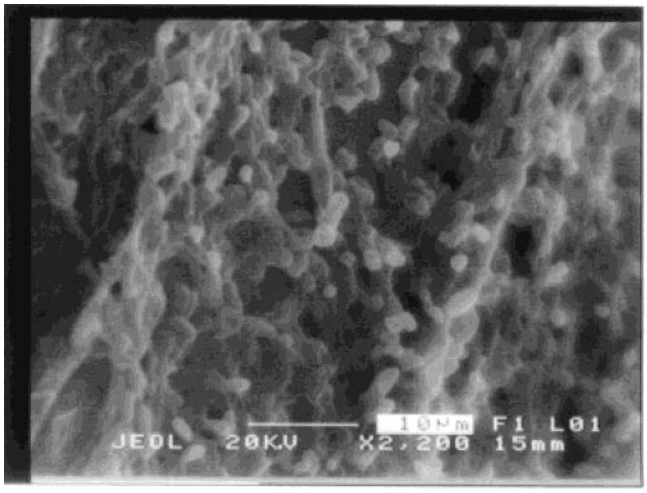

Figure 1. Electron micrograph showing immobilized cells of *L. brevis* on DC material.

RESULTS AND DISCUSSION

DC material is a very attractive support for industrial application and could lead to an improvement of lactic acid productivity during food-grade lactic acid production. Therefore, a study of lactic acid production by immobilized *Lactobacillus* on DC material is also necessary.

L. brevis is a heterofermentative lactic acid bacterium, which produces lactic acid, carbon dioxide, and ethanol from hexoses.

To use adapted cells, biomass was produced using the same sugar that was employed for fermentation. When biomass was produced, it was mixed with DC material for cell immobilization and, after washing of the support, repeated batch fermentations were carried out to study the biocatalyst's operational stability. To investigate the possibility of lactic acid production in cold climates, low-temperature fermentations were carried out, whereas to examine possible exploitation of byproducts, ethanol formation was also examined. The results are summarized in **Figures 1** and **2** and **Tables 1**−**3**.

Figure 1 presents an electron micrograph of *L. brevis* cells immobilized on DC material. Cell immobilization was also confirmed by the reproducibility of lactic acid productivity in repeated batch fermentations (**Tables 1**−**3**) at every temperature. Lactic acid fermentation of glucose resulted in high substrate conversion and yield. Lactic acid production was achieved up to 80% with conversion up to 100% (**Table 1**). Lower initial glucose concentration (4%) resulted in higher yield and conversion but lower productivity compared to higher initial glucose concentration (9%). Generally, higher lactic acid production yields and conversions were reported using 4% initial glucose concentration compared to 9% (**Table 1**). On the contrary, final lactic acid concentrations using 9% initial glucose concentration were almost double compared to those using 4%, leading to higher lactic acid productivities. At 30 °C the values obtained for yield, lactic acid productivity, and conversion were higher compared to process performance at lower temperatures. A significantly higher concentration of ethanol was formed at all temperatures studied when the initial glucose concentration was 9%. Lactic acid fermentation of lactose led to lower yield, lactic acid productivity, and conversion compared to fermentations carried out using glucose. The highest yield reported was ~19%, lower than that of glucose (**Table 2**), whereas about the same amounts of ethanol were detected.

Table 1. Effect of Temperature on Kinetic Parameters and Product Concentrations during Lactic Acid Production in Fermentations with Immobilized Cells of *L. brevis* on DC Material Using Glucose

initial glucose concn (%)	temp (°C)	no. of repeated batch fermentations	fermentation time[a] (h)	lactic acid concn[a] (g L^{-1})	ethanol concn[a] (% vol)	residual glucose[a] (g L^{-1})	lactic acid production yield[a] (g/100 g)	daily lactic acid productivity[a] (g L^{-1})	conversion %[a]
4	30	5	22.8	30.4	0.3	0.1	76.0	33.2	98.6
	20	3	81.3	29.0	0.3	1.3	72.5	8.6	96.8
	15	3	112.7	25.9	0.2	1.5	64.8	5.5	96.2
	10	3	320.0	25.1	0.3	5.2	62.8	1.9	86.9
	5	3	516.0	23.5	0.3	5.5	58.9	1.1	86.4
9	30	4	41.5	63.6	0.9	4.4	70.6	36.9	95.1
	20	4	110.0	61.0	0.8	8.5	67.7	13.4	90.6
	15	4	153.8	53.8	1.0	10.2	59.8	8.5	88.6
	10	4	448.5	50.7	1.0	10.3	56.3	2.7	88.6
	5	3	665.3	48.6	0.97	12.2	54.0	1.7	86.4

[a] Average values.

Table 2. Effect of Temperature on Kinetic Parameters during Lactic Acid Production in Fermentations with Immobilized Cells of *L. brevis* on DC Material Using Lactose

initial lactose concn (%)	temp (°C)	no. of epeated batch fermentation	fermentation time[a] (h)	lactic acid concn[a] (g L^{-1})	ethanol concn[a] (% vol)	residual lactose[a] (g L^{-1})	lactic acid production yield[a] (g/100 g)	daily lactic acid productivity[a] (g L^{-1})	conversion %[a]
4	30	3	38.0	25.1	0.3	0.1	62.9	15.9	98.6
	20	2	105.0	24.1	0.2	1.6	60.3	5.5	97.1
	15	2	147.0	22.2	0.2	6.1	55.5	3.6	84.8
	10	1	421.0	20.0	0.3	8.9	50.0	1.1	77.8
	5	1	679.0	18.1	0.3	10.9	45.3	0.6	72.8
9	30	3	54.7.	58.0	1.4	1.0	64.4	25.5	97.4
	20	3	179.3	49.2	0.9	11.2	54.7	6.6	87.6
	15	1	260.0	47.2	0.8	17.8	52.4	4.4	80.2
	10	1	728.0	44.0	1.2	19.0	48.9	1.5	78.9

[a] Average values.

Table 3. Fermentation Kinetic Parameters Observed at 30 °C during Lactic Acid Production by Repeated Batch Fermentations with Immobilized Cells on DC and Free Cells of *L. brevis* Using Whey (Lactose Content = 50 g/L)

support	repeated batch fermentation	fermentation time (h)	lactic acid concn (g L^{-1})	ethanol concn (% vol)	residual lactose (g L^{-1})	lactic acid production yield (g/100 g)	daily lactic acid productivity (g L^{-1})	conversion %
DC	1	38	33.2	0.4	tr[a]	66.4	21.0	100.0
	2	35	31.8	0.4	0.5	63.6	21.8	99.0
	3	34	30.3	0.6	0.5	60.6	21.4	99.0
	4	34	32.3	0.4	0.8	64.6	22.8	98.4
	5	33	31.7	0.5	0.4	63.4	23.1	99.2
	6	36	35.6	0.3	tr	71.2	23.7	100.0
	7	31	31.8	0.5	1.5	63.6	24.6	97.0
	8	30	33.8	0.6	0.6	67.6	27.0	98.8
	9	29	35.1	0.5	tr	70.2	29.0	100.0
	10	32	30.5	0.4	1.3	61.0	22.9	97.4
free cells	1	48	21.2	0.6	6.1	42.4	10.6	87.8
	2	4	20.6	0.5	5.7	41.2	11.0	88.6
	3	47	21.5	0.7	7.6	43.0	11.0	84.8
	4	44	24.4	0.6	4.8	48.8	13.3	90.4

[a] tr, traces.

As the best results were obtained using glucose and lactose at 30 °C, repeated batch fermentations of whey were carried out at this temperature. The results clearly indicated improved productivities, yield, and conversion in comparison with lactose fermentation. Ethanol formation was in the range of 0.4−0.6% (v/v), whereas highest yield and conversion reported were 71.2 and 100%, respectively (**Table 3**).

Comparison of Kinetic Parameters of the Immobilized *L. brevis* Cells on DC Material with Those of Free Cells. This new immobilized biocatalyst showed improved results compared to free cells (**Table 3**). Yield was ∼49% higher and lactic acid productivity was increased >100% in comparison with free cells.

Effect of the Presence of DC Material on Lactic Acid Fermentation. The effect of the presence of DC material in lactose fermentation was also studied. Equal amounts of *L. brevis* free cells were placed in two different flasks, one of which contained a quantity of DC material. The fermentation kinetics presented in **Figure 2** indicated that fermentation using

Table 4. Productivities and Conversion Obtained (Estimated from Reported Data) for Batch Lactic Acid Production by Various Researchers

	microorganism	raw material	support	lactic acid productivity (g L^{-1} day^{-1})	conversion (%)	ref
immobilized cells	*L. casei*	lactose	polyacrylamide	13.0	73	*18*
	L. casei	lactose	agar	16.8	80	*18*
	L. delbrueckii	glucose	alginate	36.5	90	*19*
	L.casei	glucose	calcium alginate	38.4	99	*4*
	L. casei	whey	calcium alginate	18.2	85	*20*
	L. lactis	whey	calcium alginate	13.3	53	*20*
	coimmobilization of *L. casei* and *L. lactis*	whey	calcium alginate	20.6	94	*20*
	L. casei	glucose	alginate capsules	65.4	100	*5*
	L. casei	glucose	poraver beads	100.8	NR[a]	*10*
	L. casei	lactose	poraver beads	67.2	NR	*10*
	L. casei	glucose	gluten	84.4	NR	*11*
	L. casei	sucrose	gluten	64.8	NR	*11*
	L. brevis	glucose	DC	45.1	98	this work
	L. brevis	lactose	DC	16.5	100	this work
	L. brevis	whey	DC	29.0	100	this work
free cells	*L. delbrueckii* IFO 3534	glucose		37.0	NR	*19*
	L. delbrueckii NRRL B-445	wood		124.8	NR	*2*
	L. casei	deproteinized whey		16.0	80	*6*
	L. lactis	deproteinized whey		10.5	63	*6*
	mixture of *L. casei* and *L. lactis*	deproteinized whey		22.3	93	*5*
	L. casei	glucose		21.6	NR	*10*
	L. casei	lactose		48.0	NR	*10*
	L. casei	lactose/glucose (1:19)		67.2	95	*10*
	L. casei subsp. *rhamnosus*	date juice		NR	90	*21*
	L. casei	glucose		55.2	NR	*11*
	L. casei	fructose		29.5	NR	*11*
	L. casei	sucrose		52.4	NR	*11*
	L. casei	lactose		19.1	NR	*11*
	L. brevis	whey		13.3	90	this work

[a] NR, not reported.

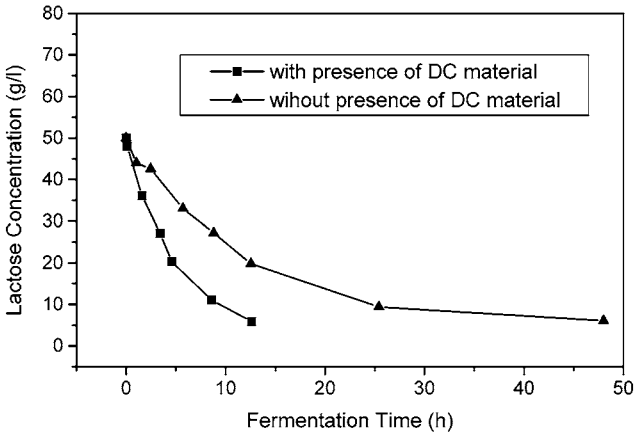

Figure 2. Effect of the presence of DC material on lactic acid fermentation kinetics using *L. brevis*.

free cells required ~4-fold higher fermentation time for complete substrate fermentation.

Comparison with Results from the Literature. Table 4 summarizes data for productivities and conversion reported in the literature for batch systems using both immobilized and free cells compared to results presented here. Supports such as alginate capsules and poraver beads and methods for lactic acid production using *Pinus pinaster* or *Eycalyptus globulus* wood proposed by Yoo et al. (*5*), Senthuran et al. (*10*), and by Parajó et al. (*2*), respectively, reported higher lactic acid productivities than immobilized *L. brevis* cells on DC material. Unfortunately, conversion is not reported in the above studies, and therefore these methods cannot be evaluated for industrial application, as conversion is probably the most important factor affecting industrial scale-up. In addition, organic supports are not cost-effective for industrial application and are mechanically unstable, whereas inorganic supports are not of food-grade purity. The method proposed by Parajó et al. (*2*), although offering interesting results as far as lactic acid productivity is concerned, employed chemical processing of samples. Therefore, their method is considered to be unsuitable for food additive lactic acid production and is not cost-effective either. On the contrary, DC material is cheap, abundant, nondestructive, and of food-grade purity, and the immobilization method is easy and simple.

Technological Consideration. A key point for the industrialization of a process is the production of a byproduct that will increase the economic efficiency of a raw material and lead to a reduction of the production cost. As a consequence, ethanol formation in the range of 0.5−1.0% can be of technological importance. Due to the low ethanol concentration, distillation to produce potable alcohol is not cost-effective. However, coproduction of potable alcohol and lactic acid would be of technological importance. To obtain this possibility, it has been proposed that the liquid waste remaining after the removal of lactic acid be diluted with molasses and then used for alcoholic fermentation. This would decrease the production cost of ethanol up to 8%. The fermentation temperature could be reduced to 15 °C in the case of glucose. At 15 °C, fermentation time is ~6 days, which is accepted by the industry producing products with lower purchase price. The increased glucose concentration led to a ~50% increase of productivity at 15 °C and to a relatively high increase of ethanol concentration. Therefore, higher sugar concentrations are considered to be more cost-effective, although a decrease in yield was reported. The sharp decrease of fermentation time from 48 h when free cells were

used to 13 h in the presence of DC material could be explained by the catalytic effect of DC material in lactic acid production. A similar catalytic effect was observed when DC material was used in the alcoholic fermentation (*17*). As a consequence, the high increase of productivity will sharply decrease production cost. The stability of productivity and yield for 10 repeated batch fermentations in whey fermentation shows an important tendency for high operational stability of *L. brevis* cells immobilized on DC material, which is an important factor for the industrialization of the process. Improved yield and productivities could be also partly attributed to continuous adjustment of the pH at 5.0−6.0. It is also stressed that the improvement of yield and productivity compared to results that have already been published will encourage further research using this biocatalyst.

Conclusion. Immobilization of *L. brevis* cells on DC material is a promising method for the industrialization of immobilized cells in lactic acid production and encourages further research.

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