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Functionalized Alginate as Immobilization Matrix in Enantioselective L (+) Lactic Acid Production by *Lactobacillus delbrucekii*

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Abstract The imperative role of functionalized natural alginate in immobilization of *Lactobacillus delbrucekii* (NCIM 2365) cells in production of optically pure L (+) lactic acid was studied. *L. delbrucekii* cells were immobilized in alginate, succinylated alginate and carrageenan to evaluate the bead stability and selectivity towards production of optically pure L (+) lactic acid. The scanning electron microscopic studies of free and immobilized cells show little morphological changes. The present study highlights the use of functionalized alginate-immobilized *L. delbrucekii* cells in production of L (+) lactic acid in higher yields (0.93 Yp/s in grams) with an improved enantioselectivity (99%). In addition, they further revealed decreased by-product formation (acetic and propionic acid) when compared to free and other immobilized cell fermentation.

Keywords Alginate · Derivatization · Immobilization · Lactic acid · *Lactobacillus delbrucekii*

Introduction

Lactic acid, 2-hydroxy propionic acid, occurs as a natural organic acid having a wide range of applications in pharmaceutical, agro-, food, and in textile industries [1]. In general, lactic acid can be obtained either by chemical synthesis or by microbial fermentation [2]. Chemical synthesis gives a racemic mixture, i.e., L (+) and D (−) lactic acid isomers, while the microbial fermentation have the advantage in producing an optically pure lactic acid [3]. L (+) lactic acid obtained from biorenewable resources is gaining importance worldwide due to increased use of this optically pure isomer in biodegradable polymers of medical importance and as green solvents [4, 5]. Henceforth, many lactic-acid-producing microbial species have been isolated, purified, and characterized for their potential to produce optically pure lactic acid. However, these methods have a limited scope in large-scale commercial production due to the

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reduced cell growth and product formation and increase in fermentation time [6]. Many technological improvements have been studied to obtain high-yielding strains and improvement in fermentation process for economic production of the L (+) lactic acid [7]. Recent advancements in immobilized-cell technology exhibited many advantages like cell-retaining capacity, reduced susceptibility to contamination, and reuse of the biocatalyst with higher product conversion capability over free-cell fermentations [8, 9]. Microbial cell immobilization using alginate was known as economic and simple compared to other matrices; however, this matrix has certain disadvantages like losing its stability under extreme pH and certain ionic concentrations [10, 11]. Hence, attention is devoted towards the search of new and novel matrices (carriers or supports) that differ in reactive groups to increase cell density and product yields by immobilize microbial cells [12]. Le-Tien et al. [13] demonstrated that functionalized immobilization matrices enhanced the mechanical stability of probiotics like viable *Lactobacillus* cells under gastric-fluid environment. Till date, there are no reports on use of functionalized alginate-immobilized beads in fermentative production of acidic compounds such as succinic acid, lactic acid, propionic acid, etc. Keeping this in view, the present study describes the impact of functionalized alginate as immobilization matrix increasing the bead towards stability, cell anchorage, and production of optically pure lactic acid with less by-product formation. The results clearly indicate that the modified alginate beads effectively retained higher *Lactobacillus delbrucekii* cell mass, when compared to natural alginate beads, with improved enantioselective lactic acid production and reduced by-product formation.

Materials and Methods

Organism and Medium

The organism, *L. delbrucekii* (NCIM 2365) was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. The culture was maintained on deMan–Rogosa–Sharpe (MRS) agar (Hi-media) stabs and subcultured twice in a month. Sodium alginate (A2158; viscosity approximately 250 cps at 25°C) was procured from Sigma-Aldrich, USA. All chemicals and solvents used in this study were of analytical grade and procured from standard firms.

Succinylation of Alginic Acid and Analysis

Alginate succinylation was performed according to modified method of Phillips et al. [11]. Sodium alginate (5 g) was solubilized in 450 ml of warm distilled water, and the solution pH was adjusted to 8.5 using 1 M NaOH solution. To this, 20 g of succinic anhydride was dissolved and incubated for 1 h at room temperature with constant stirring. The medium was then neutralized, and the derivatized alginate was obtained after precipitation in ethanol. The degree of succinylation was determined by the titration method as described by Wurzburg [14]. Further characterization of normal and succinylated alginate was done using Fourier transform infrared (FTIR) and X-ray photoelectron spectroscopic chemical analysis after drying overnight at 80°C in hot-air oven.

Cell Immobilization

L. delbrucekii cells were immobilized in κ-carrageenan, sodium alginate and succinylated-alginate beads using 2% (*v*/*v*) inoculum with an initial cell concentration of approximately

10^5 CFU/ml under sterile conditions. To these, beads having diameter in the range of 1.4 to 2.0 mm were selected by wet sieving and used for subsequent fermentation experiments. The equal amount of cell biomass was used in free- and immobilized-cell-fermentation studies. To 4% sodium alginate/succinylated alginate solution, an equal volume of 24-h grown *L. delbrucekii* cell culture was added and mixed thoroughly. The resultant cell suspension was dropped as droplets into 2% calcium chloride solution to get the calcium alginate/succinylated alginate-immobilized beads. In κ-carrageenan immobilization, 4% solution was prepared at 70°C, cooled to room temperature, and equal volumes of bacterial suspension and κ-carrageenan solution were mixed and immobilized by dropping into 2% KCl solution at 10°C. The resultant immobilized beads were washed with sterile distilled water, and the immobilized cell beads were incubated at constant shaking condition at 150 rpm at 37°C. The stability of immobilized beads was measured in terms of time taken for dissolution of five beads from each matrix in 3 M phosphate buffer (pH 5.0).

Fermentations

Lactic acid fermentations were performed using free and immobilized microbial cells. For development of inoculum, batch fermentations were first carried out for cell colonization in immobilized beads independently by incubating 2–3 days in the shaking incubator (LabTech-LSI-3016 R) using MRS medium (pH 6.4) in the 250-ml conical flask at 37°C. Lactic acid production with free and immobilized cell fermentations was performed at 37°C using the production medium (glucose, 100 g/l; corn steep liquor, 68 ml/l; trace mineral solution, 1 ml/l; and $CaCO_3$, 100 g/l adjusted to pH 6.4) and 24-h grown inoculum (5%, *v*/*v*) having optical density 1.0 at 600 nm. The cell-free samples were collected at predetermined time intervals and analyzed using high-performance liquid chromatography (HPLC). Repeated batch fermentations were carried out regularly in a fresh medium after every 144 h using the immobilized cell beads. The process was repeated till the lactic acid production continued by viable cells present in the immobilized matrix. The data presented in this study was average values of three repeated experiments.

Cell Mass Estimation

The cell mass present in immobilized beads was estimated by dissolving five numbers of beads in 5.0 ml of phosphate buffer (3.0 M, pH 5.0) for 30 min under constant shaking at room temperature. The total number of released cells was determined by standard plate-count method using agar plates after incubating at 37°C for 24 h. At the end of each batch, the cell densities in the beads were enumerated using similar method to study the cell loss upon repeated use [15]. Viable cell counts were performed in duplicate and expressed in CFU/ml of immobilized beads.

Scanning Electron Microscopy

For microscopic studies, immobilized beads containing *L. delbrucekii* cells were transferred to vials and fixed in 3.5% gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4°C. These cells were then post fixed by incubating in 2% aqueous osmium tetroxide in the same buffer for 2 h. The samples were then dehydrated by gradient alcohol series and dried to the critical point by incubating in an Electron Microscopy Science CPD unit. The dried samples were then mounted over the stubs with double-sided conductivity tape. Finally, a thin layer of platinum metal was applied over the sample using an automated sputter coater (JEOL JFC-

1600) for about 90 sec. The samples were then scanned under scanning electron microscope (model: JOEL-JSM 5600) at various magnifications using 5 kV (accelerating voltage) at RUSKA Lab, college of Veterinary Sciences, ANGRAU, Hyderabad, India [16].

HPLC Analysis

Concentrations of glucose and organic acids (lactic, formic, propionic and acetic acids) present in filtered fermentation culture broth were determined by HPLC using GROM Resin ZH column (250×8 mm) using mobile phase 5 mM H_2SO_4 and absorbance at 210 nm. The optical purity of the lactic acid was analyzed using a chiral column [chiral pak MA (+) obtained from Daicel Chemical] using 2 mM $CuSO_4$ as eluent and absorbance at 250 nm. All the experiments were carried out in three replicates, and mean values were reported.

Fourier Transform Infrared Spectroscopy Analysis

FTIR studies were conducted using Thermo Nicolet Nexus 670 Spectrometer. Demoisturized samples before and after succinylation (1–2 mg) were homogenized with 100 mg of dry KBr and made into pellets. These pellets were analyzed for transmittance in the range of 4,000 to 400 cm^{-1}.

X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) measurements were obtained on a KRATOS-AXIS 165 instrument equipped with dual aluminum–magnesium anodes using Mg Kα radiation. The X-ray power supplied was 15 kV and 5 mA. The pressure of the analysis chamber during the scan was 10^{-9} Torr. Carbon 1s photoelectron line was used for energy calibration. The peak positions were based on calibration with respect to the 'C 1s' peak at 284.6 eV. Normal and succinylated alginate samples were analyzed for electrostatic resolution at anode (Mg) 150 W with step-up voltage of 100 meV. Based on the binding energy (eV) resolution between 280 to 300 intensity, the composition was calculated by the total number of surface charge groups specially C–C/C–H, C–O, and C=O present using a nonlinear square method with the convolution of Lorentzian and Gaussian functions after the polynomial background subtraction from the raw data.

Results and Discussion

To understand the impact of functionalized alginate as matrix material for production of L (+) lactic acid by immobilized *L. delbrucekii* cells, the natural alginate was derivatized using succinic acid. The degree of succinylation was determined based on the number of carboxy charges found [13]. It was noticed that the number of carboxy charges were 5.5± 0.2 in succinic anhydride-derivatized polymer, while in natural alginate, it was 2.7±0.3. This was further confirmed by FTIR spectral studies. Alginate spectra indicated the absorption bands at 1,418 and 1,615 cm^{-1} for carboxylate anions, whereas on succinylation, an additional tiny new peak was observed at 1,735 cm^{-1} due to carbonyl-stretching vibration of alginate. XPS analysis of alginate and derivatized alginate showed (Table 1) variation in C 1s (C–C, C–N, C–O, O–C–O/O=C–N, and O=C–O) peaks confirming the

Table 1 XPS data on curve fitting of C 1s peaks of alginate and succinylated alginate.

State	C–C	C–N	C–O	O–C–O/O=C–N	O=C–O/
Binding energy (eV)	284.6	285.2	286.3	287.9	289.7
Alginate	51.6	3.9	26.2	14.5	3.9
Succinylated alginate	51.7	2.4	28.5	13.1	4.3

functionalization of alginate. The O/C ratio was observed to be 0.4 for alginate and 0.44 for succinylated alginate.

Higher lactic acid production was observed with succinylated alginate immobilized beads compared to natural alginate and other immobilized cell fermentations (Fig. 1). Lactic acid production varied (11–23%) with immobilized matrix, indicating the role of matrix material significance on *L. delbrucekii* cell metabolism and subsequent lactic acid production. Further evaluation of data indicated that the overall improvement of lactic acid with immobilized cells compared to free-cell fermentations was found in the range of 22 to 111% (Fig. 1). In comparison with literature, the present study indicated maximum lactic acid productivity (0.86 g l^{-1} h^{-1}) with succinylated alginate-immobilized cells.

Substrate (glucose) consumption data suggested that the derivatized alginate-immobilized cells showed maximum carbon consumption when compared to other immobilized cells (Fig. 1). It was observed that there was no correlation with the carbon utilization from the fermentation broth, indicating difference in metabolic activity in different immobilized *L. delbrucekii* cell fermentations. However, further evaluation of carbon source (glucose) utilization pattern under different immobilization fermentation conditions with respect to fermentation time (in days) suggest that the sugar was metabolized during 2 to 4 days of fermentation. (Fig. 2). Whereas correlation between product production and substrate utilization (Yp/s) was noticed to be varied from 0.64 (alginate beads) to 0.83 (succinylated alginate beads), only 0.45 was observed with free cells.

One of the major drawbacks in microbial lactic acid fermentations was reduction of medium pH during fermentation, which affects the immobilized bead stability. Chemical modification of this matrix material has shown to improve the stability under acidic environment in control release of embodied material under acidic environment [17]. The succinylated alginate beads showed higher mechanical stability (dissolution time 35±2 min)

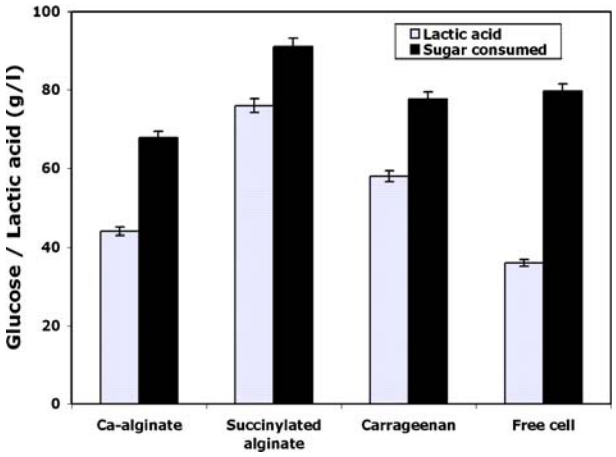

Fig. 1 Influence of immobilization methodology on lactic acid production and glucose utilization by immobilized and free *L. delbrucekii* cells during lactic acid fermentation

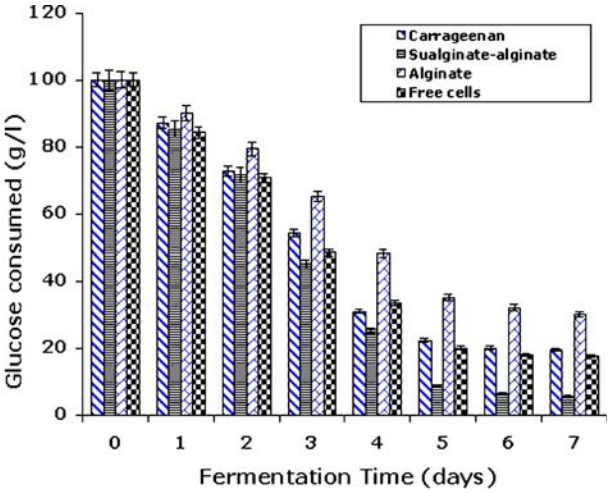

Fig. 2 Carbon source utilization pattern by different immobilized and free cells of *L. delbrucekii* during lactic acid production

compared to alginate beads (dissolution time 20±2 min) and κ-carrageenan beads (dissolution time 25±2 min) indicating durability under acidic environment with increased shelf life.

Reusability studies of these immobilized cell beads in lactic acid production revealed that the derivatized alginate beads were more stable (eight cycles) when compared to other immobilized beads (five cycles; Table 2). In addition, the effective L (+) lactic acid yield with modified alginate immobilized cells was not altered much during reusable studies and found to be 0.93 Yp/s even at eighth cycle fermentation. The optically pure lactic acid production (ee %) was improved with increase in fermentation time with succinylated alginate immobilized cell (ee 99%) fermentation compared to natural alginate (ee 90%).

The impact of succinylated alginate in production of enantioselective L (+) lactic acid by immobilized *L. delbrucekii* cells was evaluated and compared with free and other immobilized-cell fermentations. This study revealed that the L (+) lactic acid and by-product production by alginate and succinylated alginate immobilized cells. In case of alginate-immobilized cells, by-product production was noticed from the fourth day of fermentation onwards, whereas it was noticed only after the sixth day of fermentation samples (Table 3) with succinylated alginate-immobilized cells. In addition, metabolite production was shown to progressively increase from

Table 2 Reusability studies of immobilized *L. delbrucekii* cells for lactic acid production.

Sample number	Fermentation cycles	Lactic acid produced (g/l)[a]		
		Carrageenan	Succinylated alginate	Na-alginate
1	1	58.1±2.03	76.1±2.66	43.7±1.53
2	2	57.8±2.02	76.5±2.67	44.5±1.56
3	3	58.9±2.06	77.3±2.70	45.6±1.59
4	4	60.4±2.11	83.5±3.24	44.7±1.56
5	5	57.3±2.00	76.4±2.67	43.9±1.54
6	6	58.1±2.03	75.4±2.64	42.9±1.50
7	7	56.3±1.97	76.1±2.66	41.7±1.46
8	8	55.2±1.93	75.4±2.64	42.1±1.47

[a] The values are mean ± SD of three different experiments.

Table 3 Byproduct production profile during fermentation by alginate and succinylated alginate immobilized cells of *L. delbrucekii*[a].

Sample number	Name of the product (g/l)	Fourth day broth		Fifth day broth		Sixth day broth	
		Alginate	Succinylated alginate	Alginate	Succinylated alginate	Alginate	Succinylated alginate
1	Lactic acid	20.90±0.41	32.42±0.65	30.84±0.62	47.80±0.95	40.63±0.08	60.32±1.20
2	Acetic acid	01.13±0.02	ND	02.56±0.05	ND	03.63±0.07	00.25±0.01
3	Propionic acid	01.89±0.03	ND	03.58±0.07	ND	05.65±0.11	00.46±0.02

ND Not detectable

[a] The values are mean±SD of three different experiments.

5.4 to 8.9% and 9.04 to 13.9% for acetic acid and propionic acid, respectively, with increase in fermentation time from the fourth to sixth day by alginate-immobilized cells. This data depicted that the by-product formation was minimal in the present study compared to the acetic acid production observed with other lactic acid-producing microbial strain [18]. The results also revealed that these by-product production was found to be only 0.7 (acetic) and 1.4% (propionic acid) with succinylated alginate-immobilized cells, suggesting the succinylated alginate was the better immobilization matrix choice for production of optically pure lactic acid with minimal by-product formation. These advantages may offer an economic production of high-quality optically pure L (+) lactic acid production by using derivatized alginate as microbial cell immobilization matrix.

Electron microscopy revealed prominent morphological variations with immobilized *L. delbrucekii* cells (Figs. 3, 4 and 5). The surface of the alginate bead was smooth and showed a less porous nature when compared to the derivatized alginate immobilized beads. More microbial cells were observed in the modified alginate beads compared to alginate beads indicating improved cell anchorage. The microbial cell length (as measured with inbuilt software facility) varied from 1.5 to 2.02 μm and 1.21 to 1.63 μm in natural

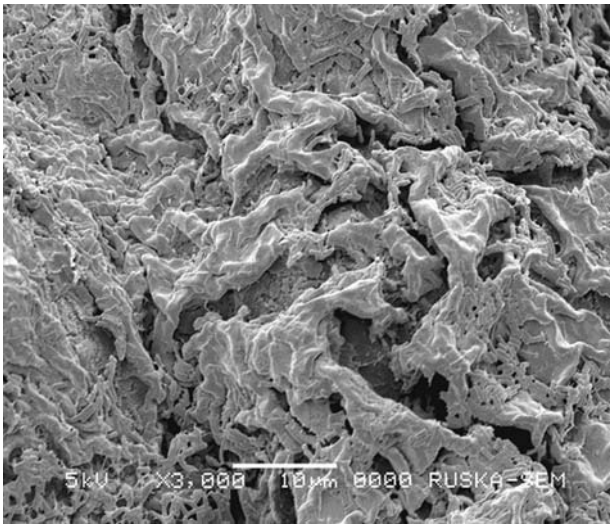

Fig. 3 Scanning electron micrographs of *L. delbrucekii* cells immobilized using ca-alginate

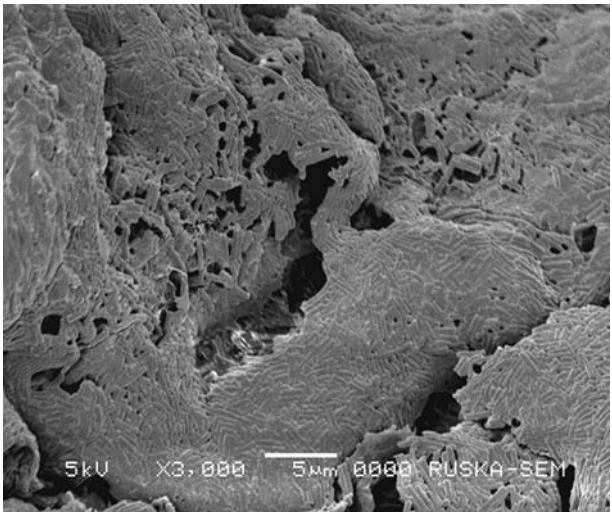

Fig. 4 Scanning electron micrographs of *L. delbrucekii* cells immobilized using succinylated-alginate

and succinylated alginate beads, respectively. Under carrageenan immobilization conditions, cell length varied in the range of 1.38 to 2.18 μm. From batch to batch, the cell length variation was observed to be approximately 3–5%.

The production of optically pure lactic acid using immobilized microbial cells may be advantageous over conventional free-cell fermentation process. Hence, metabolic variations in different immobilization conditions were studied by analyzing the production of lactic acid isomer and other by-products during fermentation. The by-product (acetic acid and propionic acid) formation was found to be significantly less in succinylated alginate immobilized cell fermentation compared to that of natural alginate beads (approximately seven to eight times higher in alginate-immobilized cell fermentation broth; Table 3). The data suggested that functionalized alginate beads show higher substrate utilization and enhanced lactic acid production with higher enantioselectivity and low by-product formation. In

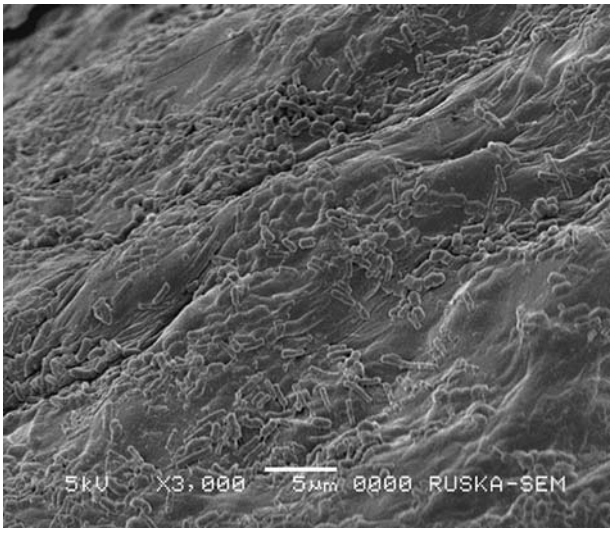

Fig. 5 Scanning electron micrographs of *L. delbrucekii* cells immobilized using carrageenan

general, microbial lactic acid fermentation can synchronously produce other byproducts such as acetic, propionic, and formic acids [19]. The amount of these metabolites can greatly influence the downstream process and quality of optically pure L (+) lactic acid produced. However, the production of selective lactic acid using immobilized microbial cells may be advantageous over conventional free-cell fermentation process. Thus the present study assumes importance in understanding the role of succinylated alginate as immobilization matrix to produce optically pure L (+) lactic acid with less by-product formation, which are important for process economy and future commercial biomedical application. Although the functionalization of alginate matrix was reported to improve the viability of the *Lactobacillus* cells in nutraceutical formulation [18], to the best of our knowledge, the present study emphasizes successful application of the derivatized alginate for *L. delbrucekii* cell immobilization and in production of optically pure (99%) L (+) lactic acid, which was higher compared to literature reports [20, 21].

Conclusion

The present study emphasizes successful application of the derivatized alginate beads in immobilization for *L. delbrucekii* cells in production of optically pure L (+) lactic acid with significantly less by-product formation compared to other immobilization matrices. Our results confirm that the *L. delbrucekii* cells immobilized in succinylated alginate beads provided better stability and durability under an acidic environment when compared to alginate beads. The resultant immobilized beads showed increased cell mass entrapment and production of L (+) lactic acid with higher yields (0.93 Yp/s) and enantioselectivity (99%).

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