

# Parametric Optimization of Lactic Acid Production by Immobilized *Lactobacillus casei* Using Box-Behnken Design

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## Abstract

Technological optimization of process parameters poses one of the open challenge for fermentative lactic acid (LA) production. Hence optimization of process parameters viz. sugar concentration, pH, biomass, incubation temperature and incubation time for maximizing fermentative lactic acid production from molasses sugar and corn steep liquor as a low cost carbon and nitrogen source, respectively by immobilized *Lactobacillus casei* MTCC 1423 cells has been carried out using Box Behnken Design (BBD). By applying multiple regressions on experimental data, quadratic models have been realized, explaining role of each variable and their quadratic interaction on LA production, LA productivity and yield coefficient. Analysis of variance has demonstrated that models are significant. The maximum LA production (132 g/L fermentor volume), LA productivity of 2.36 g/(L×h) and yield coefficient of 0.936 g/(g substrate) have been estimated by the quadratic regression model for optimum process parameters values of sugar concentration (194 g/L), pH (6.85), biomass (310 mg, CDW), incubation temperature (37°C) and incubation time (57 h). The optimization validated experiments had resulted in LA production of 130±2.1 g/L fermentor volume; LA productivity of 2.28±0.037 g/(L×h) and yield coefficient of 0.921±0.003 g/(g substrate) and which are substantially higher than those obtained with free cells of *Lb. casei* MTCC 1423 (2%, v/v inoculum size) at obtained optimized process parameters values. Thus resulted quadratic models provided an opportunity for scaling up the lactic acid production process and demonstrated the economic potential of using agro industrial waste molasses sugar for lactic acid production by *Lb. casei* MTCC 1423.

## Keywords

immobilization, lactic acid, *Lactobacillus casei*, molasses

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## 1 Introduction

Lactic acid (LA) is GRAS (generally recognized as safe) grade one, being declared safe by the United States Food and Drug Administration. Lactic acid, one of the functional, valuable and versatile compounds has been utilized globally for synthesizing various compounds in food, textile, pharmaceutical, cosmetics and chemical industries [1]. In recent time, its market demand has been increased manifold since naturally producing lactic acid acts as feedstock for biocompatible and bioabsorbable Poly lactic acid (PLA) which has a widespread variety of applications and is an effective alternative to petrochemical plastics hence ultimately leading to a considerable diminution in carbon dioxide net emission [2, 3].

Lactic acid can be produced on industrial scale by fermentation or chemical synthesis method. Fermentative lactic acid production, a green method has attained a remarkable place worldwide attributed to the escalating global energy and environmental issues. In recent years, microbial conversion of renewable raw materials into valuable compounds has become an important objective in industrial biotechnology. Moreover it also offers an advantage in terms of low production temperature, low energy consumption, etc. [2].

Fermentation process using immobilized cells has recently gained a considerable scientific and industrial interest. Cell immobilization is an approach to bring improvements in the fermentation performance because immobilized cells exhibit numerous advantages over free (suspended) cell, like accomplishment of high cell densities in the bioreactors, higher productivities due to cell growth within the immobilizates, feasibility of continuous processing at high dilution rate, ease in product separation, preservation of biosynthetic activity of the cells for longer duration etc [4-9]. The immobilization also gives an additive advantage of easier removal of biocatalyst from the fermentation media subsequently facilitating their reusability in repeated batch fermentation cycles [10]. The relative weakness of adsorptive binding forces poses a major disadvantage [11]. However, proper selection of immobilization techniques and supporting materials can minimize the disadvantages of immobilization [12].

The price of biological lactic acid is considerably higher in comparison to chemical lactic acid, mainly because of the high cost of the carbohydrate sources [13]. The production cost of microbial lactic acid can be significantly reduced by using the cheap raw materials like starchy, cellulosic materials, algal biomass and waste or side stream feed stocks [14, 10]. But the starch-based substrates compete with food resources as a large part of the earth's population is malnourished, due to poverty and inadequate food production [15]. A complicated pretreatment hydrolysis processing is required for cellulosic biomass-derived substrates. The significant reduction in the manufacturing cost of lactic acid must be accomplished by seeking the possible use a waste/by product such as sugarcane molasses containing "simple sugars" which is considered to be preferred potential renewable raw material for microbial lactic acid production by sucrose positive biocatalysts [16].

The primary microbial sources of lactic acid are lactic acid bacteria (LAB) and filamentous fungi, although the latter exhibits relatively lower productivity. Homofermentative LAB is an imperative aspect in developing an economical and efficient bioprocess for lactic acid production. Homofermentative LAB genera include *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Pediococcus* species [14, 17]. *Lactobacillus* is the main genus which can be employed for lactic acid production as free or immobilized cells [9]. The *Lactobacillus* related strains utilized by various researchers for lactic acid production with immobilized cells are *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus rhamnosus* etc. [9, 10, 20, 40, 41].

In the field of fermentation technology, the cell growth and metabolic products accumulation are strongly dependent on the various parameters like temperature, pH, time, carbon sources & its compositions etc. [18]. Rational experimental design and optimization of fermentation is required for finding the major factor influencing the fermentation. In comparison to single parameter optimization, the optimization by response surface methodology offers more advantages like saving time, space and raw material [19].

Keeping in view the above, the present study has been carried out to optimize the process parameters for maximizing the production, productivity and yield coefficient during the bioconversion of sugarcane molasses into lactic acid by immobilized *Lactobacillus (Lb.) casei* MTCC 1423 cells using response surface methodology (Box-Behnken Design).

## 2 Materials and methods

### 2.1 Materials

Sugarcane molasses (agro industrial waste) obtained from Bhagwanpura Sugar Mill Limited Dhuri was used as substrate, Punjab, India. Corn steep liquor (CSL), waste water as a nitrogen source was procured from Sukhjeet Industries, Phagwara, Punjab, India. Sugarcane molasses and corn steep

liquor were stored at 4°C and no pretreatment was applied. Sodium alginate (alginic acid sodium salt from brown algae with medium viscosity) was obtained from Sigma-Aldrich. All other chemicals (analytical grade or HPLC grade for HPLC analysis) have been procured from HiMedia Laboratories Pvt. Limited, Mumbai (India), Merk India Ltd., Mumbai (India), Fluka Goldie Chemika-Biochemica, Mumbai (India).

### 2.2 Microorganism

*Lb. casei* MTCC 1423 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Freeze-dried microbes were cultured for 20 h at 37°C (1%, w/v) in sterile MRS (de Mann Rogosa Sharpe) broth. The obtained culture was sub-cultured (37°C, 20 h) twice in sterile Man Rogosa Sharpe (MRS) broth with inoculum size of 1% (v/v) for activation and adaptation.

### 2.3 Fermentation media

The carbon source, molasses was diluted with deionized H<sub>2</sub>O to achieve the required sugar concentration for fermentation. Fermentation media was composed of molasses sugar concentration of (125-225 g/L), MnSO<sub>4</sub> (20 mg/L), CaCO<sub>3</sub> (25%, w/w with respect to sugar content) and CSL (2.5%, v/v). The pH was adjusted with 4.0 N NaOH and conc. H<sub>2</sub>SO<sub>4</sub>. Erlenmeyer flasks containing 50 mL fermentation medium were sterilized (121°C, 15 psi for 20 min) before subjecting to fermentation.

### 2.4 Immobilization of *Lb. casei* MTCC 1423 cells and bead coating

The cultivation of *Lb. casei* MTCC 1423 cells for immobilization was carried out in MRS broth for 24 h at 37°C temperature. Cells were harvested aseptically by centrifuging (6700 rpm x 12 min at 4°C) using temperature controlled centrifuge (Eppendorf) and washed twice with phosphate buffer (0.1 M, pH 7.0) and were used for accomplishing the immobilization in sodium alginate (2%, w/v) matrix. The cell dry weight (CDW) equivalent to cell wet weight (CWW) of *Lb. casei* MTCC 1423 utilized for immobilization in sodium alginate matrix had been determined from the calibration curve. The known amount of harvested cells were placed in an oven at 65°C for 72 h and thereafter the cell dry weights were measured to obtain the calibration curve.

The *Lb. casei* MTCC 1423 immobilization was accomplished in accordance to the procedure adopted by Idris et al. [20] and Kaleem et al. [21]. The known amount (CWW) (equivalent to 200, 300 and 400 mg, CDW) of harvested cells of *Lb. casei* MTCC 1423 were aseptically transferred into sterilized (121°C for 20 min) sodium alginate (2%, w/v) solution (7.5 mL) and mixed well. Biocatalysts entrapped in beads of 2.5±0.2 mm size were thus obtained by the drop wise addition of cells and sodium alginate solution mixture aseptically with the help of sterilized syringe, into a 0.2 [M] sterile solution of calcium chloride. After 30 min beads were sieved out and followed

by washing with 0.85% (w/v) sodium chloride solution. Wet weight of beads was determined to account for any material and cell content loss during immobilization process and no appreciable losses were noticed.

For achieving reduction in the cell leakage, alginate beads thus obtained were double layer coated with chitosan and sequentially with alginate. The coating of bead containing *Lb. casei* cells was accomplished by following the method as described by Klinkenberg et al. [22] at ambient temperature. The sodium alginate beads containing *Lb. casei* MTCC 1423 were aseptically immersed in sterilized chitosan (0.4%, w/v) solution ( $pH=5.6$ ) for 45 min. The chitosan coated beads after sieving were immersed and stirred for 15 min in sterilized solution of sodium chloride (0.2 M) and calcium chloride (0.05 M). The beads were then transferred into sterilized sodium alginate solution (0.5%, w/v) and stirred for 10 min before sieving. The beads were again put in the solution of sodium chloride (0.2 M) and calcium chloride (0.05 M) for 15 min after washing with sterilized demineralized water. The double layered coated biocatalysts were stored in peptone solution (0.75%, w/v) at 4°C for further utilization in fermentation broth (within 30 min). The known amount of beads (on the wet basis) containing equivalent amount of *Lb. casei* MTCC 1423 cells (i.e. 200, 300 and 400 mg, CDW) were transferred to the Erlenmeyer flasks containing sterilized 50 mL fermentation medium.

## 2.5 Analytical methods

The lactic acid concentration in fermentation samples was analyzed by the HPLC method [23] using Shimadzu LC 2010 CHT (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump, dual wavelength UV-Visible detector and column oven. The chromatographic data were recorded and processed using LC solution software based on the peak area of the identified lactic acid. The column temperature was maintained constant at 25°C. Phosphate buffer (10 mM, pH 3.0) and acetonitrile at 95:5 % (v/v) ratio as mobile phase were utilized for isocratic elution at the flow rate maintained at 1 mL/min and in each run, the injection volume was 50  $\mu$ L. The effluent was monitored at a wavelength of 210 nm for detection and quantification of lactic acid. The samples of the fermentation broth were prepared by centrifuging at 6700 rpm for 15 min and were further diluted appropriately using the mobile phase and vacuum filtered through 0.22  $\mu$ m filter membrane. The phenol sulfuric acid method [24] was followed for the determination of total sugar concentration. Each sample was analyzed in duplicate and the lactic acid as well as sugar concentrations were quantified from the respective calibration curve generated using standard solutions.

## 2.6 Response surface methodology

Response surface methodology has been widely and successfully applied for evaluating the effect of process

variables and optimization of various bioprocesses. The optimization of lactic acid production by immobilized *Lb. casei* MTCC 1423 cells, lactic acid productivity and yield coefficient had been carried out to investigate the positive effect of the nutrients and negative effect of the toxic substances in molasses and other process variables using Box-Behnken design (BBD).

## 2.7 Statistical analysis and optimization

The statistical software Design-Expert 7.16 (Statease Inc., Minneapolis, USA) was used for experimental design, regression analysis of the experimental data, response surface graphs preparation and carrying out the numerical optimization. A suitable approximation for the true functional relationship between independent variables and responses had been obtained in the form of mathematical model by making assumption that the independent variables are continuous and controllable by experiments with negligible errors. The process behavior can be explained by a quadratic equation of the form:

$$Y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=1}^k b_{ij} x_i x_j + \varepsilon \quad (1)$$

Where  $Y$  represents the predicted responses i.e LA production, LA productivity and yield coefficient. Whereas  $b_0$ ,  $b_i$ ,  $b_{ii}$ ,  $b_{ij}$  and  $x_i$  are offset term; linear effect; squared effect; interaction effect and  $i$ th independent variable, respectively [25] and  $\varepsilon$  represents random error or allows for discrepancies or uncertainties between predicted and measured values.

The analysis of variance (ANOVA) for the response was utilized for the estimation of significance of the terms in the model through the Fisher's test for P (probability) <5%. The fitting quality of the hence generated second-order polynomial model equation was checked with the help of the coefficient of determination ( $R^2$ ). The interactive relationship between the variables and the responses had been illustrated through the three-dimensional surface plot as an outcome of the 2<sup>nd</sup> order polynomial model equation. The maximum LA production, LA productivity as well as yield coefficient along with corresponding optimal level of each independent variable (within the experimental range) were obtained by numerical optimization method [26].

## 2.8 Experimental design

The software of Design-Expert (version 7.16, Stat-Ease, Inc.) has been employed to program a Box-Behnken design (BBD) with five factors at three coded levels (Table 1). Experiments were carried out according to the design generated by the software using the production medium (50 mL) constituted of molasses sugar (125, 175 and 225 g/L), CSL (2.5%, v/v); CaCO<sub>3</sub> (25%, w/w w. r. t. sugar content) and MnSO<sub>4</sub> (20 mg/L). A total of 46 experimental run results obtained for the responses are presented in the Table 2 as average value  $\pm$  standard deviation. Average value of LA produced, LA productivity and yield coefficient for each run were considered for further analysis.

**Table 1** Process variables range for batch lactic acid production by immobilized *Lb. casei* MTCC 1423 cells

Factors	Process Parameters	Coded values	Level		
			-1.000	0.000	+1.000
1.	Sugar concentration, $C_s$ (g/L)	125	175	225	
Un-coded values	2.	pH	5.5	6.5	7.5
	3.	Biomass, $B_w$ (mg, CDW)	200	300	400
	4.	Incubation temperature, $T_f$ (°C)	32	37	42
	5.	Incubation time, $\tau_f$ (h)	36	48	60

Experimental combinations obtained through Box-Behnken design had been performed in triplicate for further analysis, obtaining 3D graphs as well as for numerical optimization to obtain the optimized value of the five individual parameters and responses. The plan of experimental design in un-coded form of process variables along with results have been shown in Table 2.

### 3 Results and discussion

#### 3.1 Lactic acid production

##### 3.1.1 Regression model

The regression equation was developed as a result of application the multiple regressions with backward elimination regression (alpha to exit = 0.100) on experimental data. The quadratic model (coded forms) explaining the role of each variable and their quadratic interaction on the lactic acid production,  $C_{LA}$  (g/L) thus obtained is as follows:

$$\begin{aligned} \text{Lactic acid } (C_{LA}) = & 109.47 + 18.85 * C_s + 2.35 * pH \\ & + 0.25 * B_w + 6.16 * T_f + 25.92 * \tau_f - 2.39 * C_s * B_w \\ & + 3.48 * C_s * T_f + 10.41 * C_s * \tau_f - 4.34 * T_f * \tau_f \\ & - 16.49 * C_s^2 - 2.88 * pH^2 - 9.39 * B_w^2 - 9.14 * T_f^2 \\ & - 10.43 * \tau_f^2 \end{aligned} \quad (2)$$

The quadratic model (Eq. (2)) has fourteen terms comprised of five each of linear and quadratic terms alongwith four two-factorial interactions. The statistical significance of Eq. (2) was checked by analysis of variance (ANOVA). The significance of each of the coefficients was checked through probability, P ( $p > f$ ) values (Table 3). The terms having  $p > f$  values  $< 0.05$  are identified as significant terms while the terms having P-value  $> 0.1$  indicates that the model term is insignificant [27]. Smaller the values of |P|, more significant is the correlation with the corresponding coefficient [28]. It can be concluded from Table 3 that all the parameters play a significant role in lactic acid production from molasses by immobilized *Lb. casei* MTCC 1423 due to the significant first-order main and the square effect of all factors. Significant interactions between  $C_s$

&  $B_w$ ,  $C_s$  &  $T_f$ ,  $C_s$  &  $\tau_f$ ,  $T_f$  &  $\tau_f$  and highly significant model has also been demonstrated by the ANOVA (Table 3).

The satisfactory ( $> 0.98$ ) coefficient of determination values ( $R^2$ ) for the LA production ( $P \leq 0.05$ ) is an indicative of a good agreement between experimental results and predicted values and suggested that only 1.18% of the variation was not explained by the model [27]. The significant model best fit has been certified by the ‘‘Lack of Fit F-value’’ of 4.63 for lactic acid production which implies that it is not significant relative to the pure error [25]. The sufficiently close in values of adjusted  $R^2$  and predicted  $R^2$  implies that the model values are in good agreement.

##### 3.1.2 Interactive effect of variables on lactic acid production

The response surface graphs (Fig. 1-4) were obtained using the Design-Expert 7.16 software to understand the individual as well as interactive effect of variables on the lactic acid production and for obtaining their optimum levels.

The interactive effect of biomass loading and substrate concentration on the lactic acid production is illustrated in Fig. 1. Regardless of substrate concentration, an increase in the lactic acid production has been registered with the enhancement in the concentration of entrapped *Lb. casei* MTCC 1423 cells ( $\leq 300$  mg, CDW) and highest biomass loading ( $\approx 400$  mg) has caused a decrease in production. However, at the low substrate concentration, lactic acid production was comparatively lower. This may have been resulted in due to limited availability of substrate for microbial growth and hence it secretes less lactic acid. Moreover at small initial cell density, distinct and large micro colonies formed due to cell growth and their size got increased with decreasing initial cell density [29]. At the lower biomass loading, the lactic acid production has been observed to be increased with an increase in the initial sugar concentration and it tends to decrease at higher sugar concentration (225 g/L). The lactic acid production was observed to decrease at the higher cell concentrations and higher sugar concentration. This could be attributed to the depletion of substrate by the high population of microbes for maintenance and growth, repressive effect of molasses as a result of increased viscosity and sugar inhibition [19].

Lactic acid production has been noticed to be enhanced, depending on the sugar concentration and the incubation temperature (Fig. 2). An increase in the LA production with the increase in the incubation temperature and sugar concentration was observed which may be due to the influence of temperature on substrate and product diffusion through the beads [9]. A gradual decrease in lactic acid production was obtained towards higher incubation temperature while the enhanced production and significant interactive effect was found at around 37°C. As the incubation temperature was increased towards 42°C, there was a slight decrement in the lactic acid production.

**Table 2** Experimental design of process variables and values of experimental data for lactic acid production by immobilized *Lb. casei* MTCC 1423 cells

Process parameters					Responses		
Sugar conc. (g/L)	pH	Biomass (mg, CDW)	Incub. temp. (°C)	Incub. time (h)	Lactic acid production (g/(L fermentor volume))	Productivity (g/(L×h))	Yield coeff. (g/g substrate)
175	6.5	200	37	60	120.67±1.81	2.01±0.03	0.924±0.004
175	6.5	300	37	48	107.32±1.63	2.24±0.03	0.930±0.002
125	6.5	300	32	48	64.07±1.02	1.33±0.02	0.900±0.006
175	6.5	300	32	60	115.17±1.81	1.92±0.03	0.910±0.004
125	7.5	300	37	48	71.69±1.16	1.49±0.02	0.900±0.003
175	6.5	300	37	48	109.62±1.75	2.28±0.04	0.940±0.005
225	6.5	300	37	60	131.59±2.02	2.19±0.03	0.920±0.004
225	5.5	300	37	48	108.07±1.65	2.25±0.04	0.890±0.007
175	6.5	300	37	48	111.45±1.71	2.32±0.04	0.930±0.004
175	7.5	300	37	36	72.2±1.20	2.01±0.03	0.905±0.005
175	6.5	300	32	36	54.01±0.92	1.50±0.02	0.900±0.001
175	6.5	400	37	60	123.2±1.94	2.05±0.03	0.930±0.004
225	6.5	300	32	48	93.81±1.52	1.95±0.03	0.910±0.004
175	6.5	400	37	36	65.18±1.12	1.81±0.03	0.895±0.006
175	6.5	300	42	60	121.69±1.92	2.03±0.03	0.912±0.002
175	7.5	300	42	48	105.49±1.57	2.20±0.03	0.901±0.001
225	6.5	300	42	48	113.47±1.65	2.36±0.04	0.908±0.003
125	6.5	300	37	60	74.07±1.23	1.23±0.02	0.890±0.002
175	7.5	300	32	48	92.29±1.4	1.92±0.03	0.900±0.003
175	7.5	200	37	48	101.89±1.64	2.12±0.03	0.903±0.006
175	5.5	200	37	48	93.07±1.41	1.94±0.03	0.890±0.003
175	6.5	400	42	48	94.24±1.49	1.96±0.03	0.900±0.004
175	6.5	300	42	36	75.89±1.12	2.11±0.03	0.895±0.008
175	5.5	300	37	36	69.38±1.11	1.93±0.03	0.902±0.002
175	5.5	300	32	48	89.47±1.45	1.86±0.03	0.912±0.005
175	7.5	400	37	48	98.89±1.59	2.06±0.03	0.910±0.007
175	5.5	300	37	60	119.86±1.75	2.00±0.03	0.900±0.004
175	5.5	300	42	48	100.67±1.72	2.10±0.03	0.900±0.008
125	6.5	300	42	48	69.81±1.11	1.45±0.02	0.914±0.004
225	6.5	300	37	36	63.79±0.95	1.8±0.03	0.905±0.003
175	6.5	300	37	48	110.23±1.83	2.30±0.04	0.937±0.002
225	6.5	200	37	48	106.78±1.52	2.22±0.03	0.925±0.004
175	6.5	300	37	48	109.67±1.80	2.28±0.04	0.932±0.003
175	6.5	200	37	36	60.72±0.84	1.69±0.03	0.925±0.004
175	6.5	400	32	48	83.04±1.38	1.73±0.03	0.900±0.003
125	6.5	400	37	48	67.58±1.12	1.41±0.02	0.910±0.007
175	5.5	400	37	48	93.07±1.41	1.94±0.03	0.900±0.004
225	6.5	400	37	48	101.5±1.52	2.11±0.03	0.920±0.006
125	5.5	300	37	48	70.87±1.21	1.48±0.02	0.900±0.004
175	7.5	300	37	60	127.68±2.05	2.13±0.03	0.930±0.003
225	7.5	300	37	48	111.89±1.81	2.33±0.04	0.920±0.008
175	6.5	200	42	48	94.24±1.36	1.96±0.03	0.905±0.004
175	6.5	300	37	48	108.52±1.62	2.26±0.04	0.935±0.003
125	6.5	200	37	48	63.3±1.05	1.32±0.02	0.920±0.008
175	6.5	200	32	48	83.04±1.34	1.73±0.03	0.930±0.006
125	6.5	300	37	36	47.91±0.81	1.33±0.02	0.905±0.005

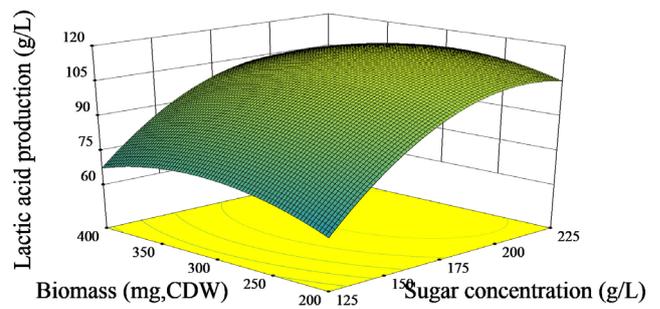
**Table 3** Regression model and ANOVA for lactic acid production by immobilized *Lb. casei* MTCC 1423

Source	Sum of squares	Degree of freedom	Mean square	f-value	p > f
Model	21309.23	14	1522.09	186.72	< 0.0001
Sugar conc. ( $C_s$ )	5685.16	1	5685.155	697.40	< 0.0001
pH	88.1721	1	88.17	10.82	0.0025
Biomass ( $B_w$ )	0.56	1	0.56	0.07	0.7952
Incubation temp. ( $T_p$ )	632.52	1	632.52	77.59	< 0.0001
Incubation time ( $\tau_p$ )	11281.1	1	11281.13	1383.86	< 0.0001
( $C_s$ ). ( $B_w$ )	22.85	1	22.85	2.802	0.1042
( $C_s$ ). ( $T_p$ )	48.44	1	48.44	5.94	0.0207
( $C_s$ ). ( $\tau_p$ )	433.47	1	433.46	53.18	< 0.0001
( $T_p$ ). ( $\tau_p$ )	58.98	1	58.98	7.24	0.0114
( $C_s$ ). ( $C_s$ )	2434.49	1	2434.46	298.64	< 0.0001
(pH). (pH)	70.65	1	70.65	8.67	0.0061
( $B_w$ ). ( $B_w$ )	736.17	1	736.17	90.31	< 0.0001
( $T_p$ ). ( $T_p$ )	736.30	1	736.33	90.32	< 0.0001
( $\tau_p$ ). ( $\tau_p$ )	838.35	1	838.34	102.84	< 0.0001
Residual	252.71	31	8.15		
Lack of Fit	242.62	26	9.33	4.63	0.0475*
Pure Error	10.086	5	2.02		
Cor Total	21561.94	45			
Standard Deviation = 2.86		$R^2 = 0.9882$			
Mean = 92.87		Adjusted $R^2 = 0.9830$			
Coefficient of variation (C.V. %)=3.074		Predicted $R^2 = 0.9669$			
Predicted residual error of sum of squares (PRESS)= 712.56		Adequate Precision = 55.69			

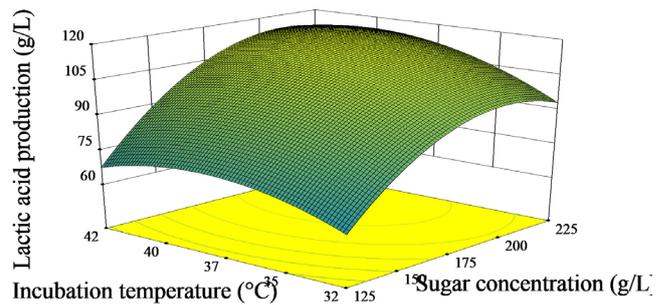
\* non-significant at 5 % level

Since *Lb. casei* being a thermophilic or mesophilic could produce lactic acid within a range of 30 and 44°C and production tends to be got reduced at higher or lower temperature than optimum due to decrement in catalytic activities of the cells [26-30, 31]. The lactic acid production may have decreased at higher sugar concentration due to the increase in the concentration of metal ions such as calcium, sodium, iron, magnesium, copper etc. and suspended colloids which might be present in molasses causing toxic or inhibitory effect on the cells [25].

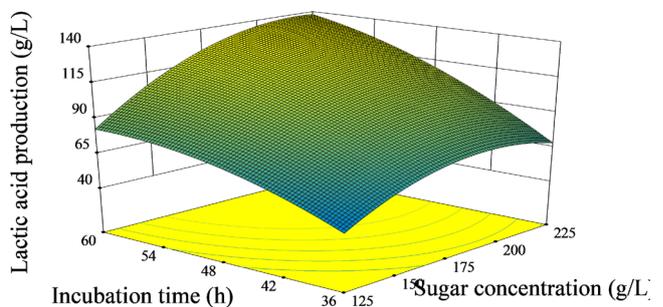
Lactic acid production has been observed to be varied extensively and simultaneously by incubation time and sugar concentration (Fig. 3). The increase in lactic acid production was more significant in the early phase of incubation time ( $\leq 54$  h) for all sugar concentration.



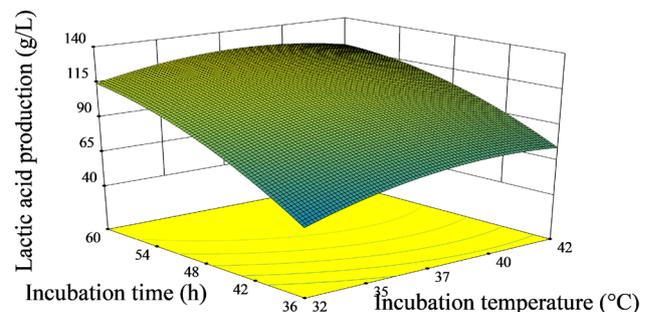
**Fig. 1** Lactic acid production by immobilized *Lb. casei* MTCC 1423 as a function of biomass and sugar concentration



**Fig. 2** Lactic acid production by immobilized *Lb. casei* MTCC 1423 as a function of incubation temperature and sugar concentration



**Fig. 3** Lactic acid production by immobilized *Lb. casei* MTCC 1423 as a function of incubation time and sugar concentration



**Fig. 4** Lactic acid production by immobilized *Lb. casei* MTCC 1423 as a function of incubation time and incubation temperature

At higher sugar concentration the lactic acid production has been noticed to be increased continuously with the incubation time and remained nonetheless significant at 60 h of incubation time because increasing the level of fermentable sugars not only increases availability of sugars to the microbes in the fermentation media but also some other nutritional substances that are suitable for production [25]. At low sugar concentration, the lactic acid production did not increase with prolonged incubation time due to early depletion of substrate.

A sharper initial increase was illustrated during the first period (48 h) of fermentation at the low incubation temperature (32-37°C) range (Fig. 4). The increase was followed by a slow reduction that might be because of decrease in the cells' catalytic activity at higher incubation temperature [9]. At higher incubation temperature, the lactic acid production tends to stabilize after a shorter incubation time. Higher production of lactic acid by immobilized *Lb. casei* MTCC 1423 could be obtained in medium with high incubation time at a moderate incubation temperature. Lower production was obtained with lower incubation time and temperature.

## 3.2 Lactic acid productivity

### 3.2.1 Regression model

The regression equation for LA productivity,  $P_{LA}$  (g/(L×h)) was obtained after backward elimination regression (alpha to exit = 0.100) on experimental data. The quadratic model (in coded forms) thus obtained is as follows:

$$\begin{aligned} \text{Lactic acid productivity } (P_{LA}) = & 2.28 + 0.4 * C_s \\ & + 0.0475 * pH - 0.015 * B_w + 0.1394 * T_f \\ & + 0.083 * \tau_f + 0.0725 * C_s * T_f + 0.125 * C_s * \tau_f \\ & - 0.125 * T_f * \tau_f - 0.356 * C_s^2 - 0.0494 * pH^2 \\ & - 0.2177 * B_w^2 - 0.187 * T_f^2 - 0.222 * \tau_f^2 \end{aligned} \quad (3)$$

The quadratic model (Eq. (3)) has thirteen terms which comprises of five each linear and quadratic terms along with three two-factorial interactions. The statistical significance of Eq. (3) was checked by analysis of variance (ANOVA).

Significant interactions between  $C_s$  &  $T_f$ ,  $C_s$  &  $\tau_f$  and  $T_f$  &  $\tau_f$  have been indicated by the analysis of variance. ANOVA (Table 4) has also demonstrated that the model is highly significant.

### 3.2.2 Interactive effect of variables on lactic acid productivity

It is evident from the Table 4 that for LA productivity, the interaction among incubation temperature and sugar concentration is significant. Fig. 5 has displayed that with the enhancement in sugar concentration (up to approx. 210 g/L) irrespective of incubation temperature, the LA productivity has risen to maximum. This indicates a consistent and promising efficiency of *Lb. casei* MTCC 1423 cells even at concentrated

**Table 4** Regression model and ANOVA for lactic acid productivity by immobilized *Lb. casei* MTCC 1423

Source	Sum of squares	Degree of freedom	Mean square	f-value	p > f
Model	4.63	13	0.356	115.186	< 0.0001
Sugar conc. ( $C_s$ )	2.56	1	2.56	828.032	< 0.0001
pH	0.036	1	0.0361	11.677	0.0017
Biomass ( $B_w$ )	0.0036	1	0.0036	1.164	0.2886
Incubation temp. ( $T_f$ )	0.311	1	0.3108	100.53	< 0.0001
Incubation time ( $\tau_f$ )	0.111	1	0.111	35.76	< 0.0001
( $C_s$ ). ( $T_f$ )	0.021	1	0.021	6.801	0.0137
( $C_s$ ). ( $\tau_f$ )	0.063	1	0.0625	20.22	< 0.0001
( $T_f$ ). ( $\tau_f$ )	0.063	1	0.0625	20.22	< 0.0001
( $C_s$ ). ( $C_s$ )	1.106	1	1.106	357.83	< 0.0001
(pH). (pH)	0.0213	1	0.0213	6.881	0.0132
( $B_w$ ). ( $B_w$ )	0.414	1	0.414	133.79	< 0.0001
( $T_f$ ). ( $T_f$ )	0.305	1	0.305	98.58	< 0.0001
( $\tau_f$ ). ( $\tau_f$ )	0.429	1	0.429	138.96	< 0.0001
Residual	0.0989	32	0.0031		
Lack of Fit	0.0949	27	0.00351	4.395	0.0527*
Pure Error	0.004	5	0.0008		
Cor Total	4.728	45			
Standard Deviation = 0.0556			$R^2 = 0.9791$		
Mean = 1.92			Adjusted $R^2 = 0.9706$		
Coefficient of variation (C.V. %) = 2.89			Predicted $R^2 = 0.9482$		
PRESS = 0.245			Adequate Precision = 35.49		

\* non-significant at 5 % level

form of substrate since the byproducts/wastes from sugar manufacturing process are rich in mixed carbohydrates which provides an additive advantage in enhancing LA productivity and process economy [32].

The LA productivity can also be seen achieving maxima on increasing the incubation temperature up to 40°C giving an impression that the *Lb. casei* MTCC 1423 cells is capable of balancing the first and last biochemical reaction up to this temperature during the conversion of molasses sugar into lactic acid [33]. The LA productivity has been found to be consistently decreased as sugar concentration as well as incubation temperature has increased beyond optimal thereafter. This implies that LA productivity has got influenced due to LA accumulation at higher sugar concentration or substrate constituents' complexity and decrement in microbial activity at high temperature.

Both incubation time and sugar concentration has shown significant interactive effect on LA productivity (Table 4). The

lactic acid productivity irrespective of the sugar concentration has gradually increased with the increase in incubation time up to 54 h (Fig. 6). Further enhancement in time has resulted in a decrement in productivity. Similarly irrespective of the incubation time, the LA productivity has been found to be increased with the increase in sugar concentration up to 200 g/L and thereafter decrement has been observed which likely has indicated the onset of the substrate inhibition influence. Hence the LA productivity tends to decrease as the incubation time and sugar concentration simultaneously on approaching towards the high level of each.

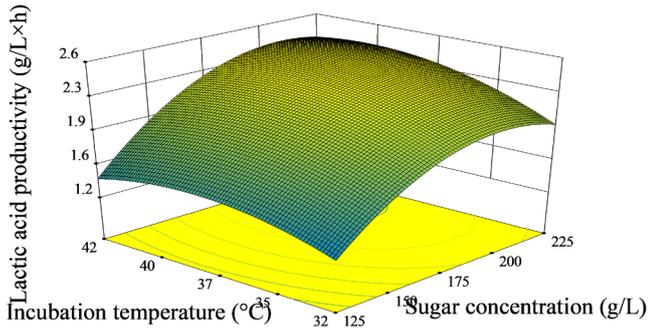


Fig. 5 Lactic acid productivity as a function of incubation temperature and sugar concentration for LA production by immobilized *Lb. casei* MTCC 1423

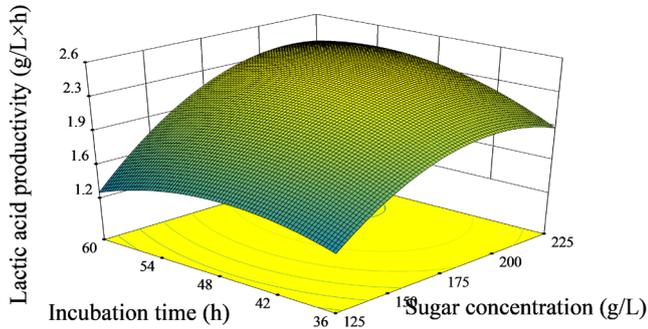


Fig. 6 Lactic acid productivity as a function of incubation time and sugar concentration for LA production by immobilized *Lb. casei* MTCC 1423

At low (-1) level of incubation time, with the increase in incubation temperature, levelling off the LA productivity has been observed as the temperature approaches its high (+1) level (Fig. 7). However at high (+1) level of incubation time the LA productivity was found to be decreased with the increase in incubation temperature just after a slight initial increase in it. At low level of incubation temperature the LA productivity has initially increased but no significant enhancement in the lactic acid productivity for incubation temperature of higher than 54 h has been noticed and it has decreased with an increase in the incubation time at high level of incubation temperature once the incubation time has increased beyond 48 h.

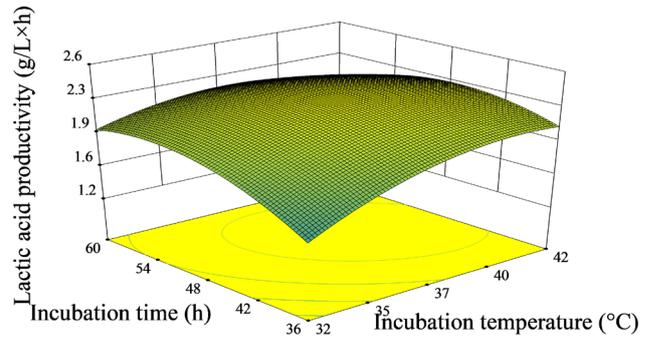


Fig. 7 Lactic acid productivity as a function of incubation time and incubation temperature for LA production by immobilized *Lb. casei* MTCC 1423

### 3.3 Yield coefficient

#### 3.3.1 Regression model

The regression equation was developed on the application of the multiple regressions with backward elimination (alpha to exit = 0.100) on experimental data for yield coefficient,  $Y_{LA/S}$  (g/(g substrate)). The quadratic model (in coded forms) hence generated is as follows:

$$\begin{aligned} \text{Yield coefficient } (Y_{LA/S}) = & 0.934 + 0.00181 * C_s \\ & + 0.00487 * pH - 0.00356 * B_w - 0.000169 * T_f \\ & + 0.00713 * \tau_f + 0.0075 * C_s * pH + 0.00675 * pH * \tau_f \\ & + 0.00625 * B_w * T_f + 0.009 * B_w * \tau_f - 0.01029 * C_s^2 \\ & - 0.01886 * pH^2 - 0.00863 * B_w^2 - 0.01613 * T_f^2 \\ & - 0.00938 * \tau_f^2 \end{aligned} \quad (4)$$

The quadratic model (Eq. (4)) has fourteen terms comprises of five each linear and quadratic terms along with four two-factorial interactions. The statistical significance of Eq. (4) was checked by analysis of variance (ANOVA). The analysis had shown that there were significant interactions between  $C_s$  &  $pH$ ;  $pH$  &  $\tau_f$ ;  $B_w$  &  $T_f$  and  $B_w$  &  $\tau_f$ . ANOVA (Table 5) of the regression model demonstrates that the model is significant.

#### 3.3.2 Interactive effect of variables on yield coefficient

The yield coefficient had displayed a parabolic behaviour with the increment in  $pH$  as well as sugar concentration with significant shifting towards higher level of both the parameters (Fig. 8). The microbial growth as well as the synthesis of metabolic enzymes which further in turn synthesis new protoplasm has been regulated and limited by  $pH$  [34]. It has been demonstrated that the yield coefficient maxima could be achieved at optimal conditions of near 6.75 of  $pH$  and sugar concentration of 175 g/L (approx.). Further enhancement in  $pH$  and sugar concentration had led to a significant decrease in yield coefficient. This indicates that higher  $pH$  as well as enhanced sugar concentration has negative effect on the yield coefficient as the shift in  $pH$  towards alkalinity or

acidulous influences the reaction pathways during the conversion of molasses sugar into lactic acid associated with the inhibiting factor of molasses at higher concentration [35].

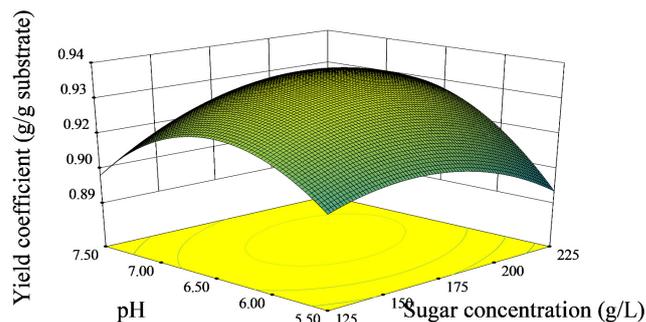
**Table 5** Regression model and ANOVA for yield coefficient by immobilized *Lb. casei* MTCC 1423

Source	Sum of squares	Degree of freedom	Mean square	f- value	p > f
Model	0.006718	14	0.00048	9.52	< 0.0001
Sugar conc. ( $C_s$ )	0.000053	1	0.000053	1.043	0.315
pH	0.000352	1	0.000352	6.976	0.0128
Biomass ( $B_w$ )	0.000203	1	0.000203	4.029	0.0535
Incubation temp. ( $T_p$ )	0.000046	1	0.000046	0.904	0.3491
Incubation time ( $\tau_p$ )	0.000812	1	0.000812	16.12	0.0004
( $C_s$ ). (pH)	0.000225	1	0.000225	4.465	0.0428
(pH). ( $\tau_p$ )	0.000182	1	0.000182	3.616	0.0665
( $B_w$ ). ( $T_p$ )	0.000156	1	0.000156	3.10	0.0881
( $B_w$ ). ( $\tau_p$ )	0.000324	1	0.000324	6.429	0.0165
( $C_s$ ). ( $C_s$ )	0.000924	1	0.000924	18.34	0.0002
(pH). (pH)	0.003137	1	0.003134	62.24	< 0.0001
( $B_w$ ). ( $B_w$ )	0.000649	1	0.00065	12.88	0.0011
( $T_p$ ). ( $T_p$ )	0.002269	1	0.0023	45.026	< 0.0001
( $\tau_p$ ). ( $\tau_p$ )	0.000767	1	0.00077	15.22	0.0005
Residual	0.001562	31	0.0000504		
Lack of Fit	0.00148	26	0.000057	3.4717	0.0847*
Pure Error	0.000082	5	0.000164		
Cor Total	0.00828	45			
Standard Deviation = 0.0071			$R^2 = 0.811$		
Mean = 0.912			Adjusted $R^2 = 0.726$		
Coefficient of variation (C.V. %)=0.779			Predicted $R^2 = 0.557$		
PRESS= 0.0037			Adequate Precision = 10.22		

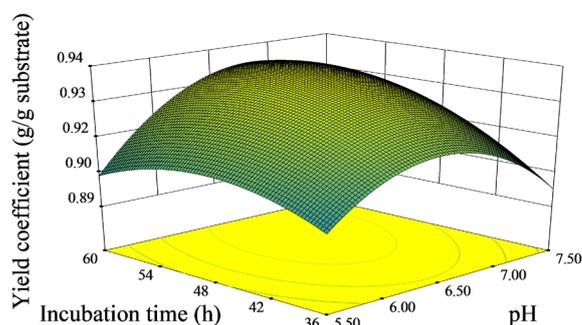
\* non-significant at 5 % level

As evident from the Fig. 9 that the yield coefficient has increased with the increase in incubation time up to approx. 50 h and 58 h of incubation time at low and high pH levels respectively and further enhancement in incubation time has resulted in decrement in yield coefficient. While the yield coefficient at low and high level of incubation time has been noticed to be increased with the increase in pH (up to 6.65) and thereafter it has shown a decrement. This suggests the negative impact of low and high pH at longer incubation time on yield coefficient as the pH influences the metabolism of the microbes. Low value of yield coefficient has been observed at low pH in comparison to that at high pH irrespective to the incubation time which might be attributed to the low acidogenic bacterial

activity at low pH [36] resulting in utilization of more molasses sugar for the maintenance of the cells.



**Fig. 8** Yield coefficient as a function of pH and sugar concentration for LA production by immobilized *Lb. casei* MTCC 1423

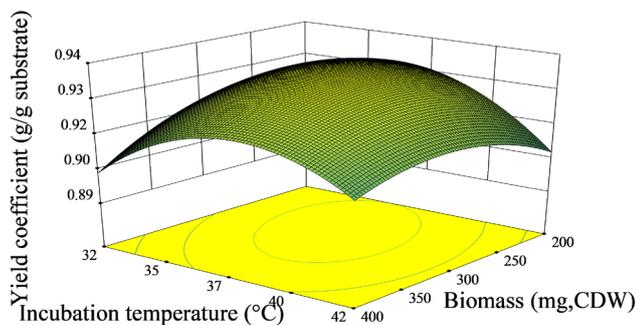


**Fig. 9** Yield coefficient as a function of incubation time and pH for LA production by immobilized *Lb. casei* MTCC 1423

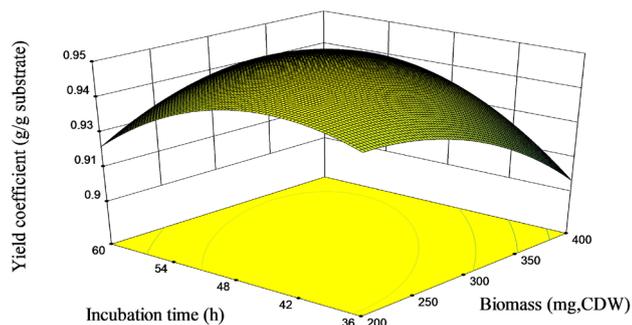
Higher incubation temperature and high biomass loading has clearly depicted a negative interactive effect on the yield coefficient (Fig. 10). At low level of incubation time the yield coefficient has consistently decreased with the increase in biomass while at high level of temperature it initially has increased but afterwards a decrement has been noticed with the increase in biomass content. Since each bead contains limited space for the growth as well as maintenance and survival of the biomass entrapped in it hence with the enhancement in biomass content the availability of the substrate transported into the bead matrix becomes limited. Moreover the cell growth inside the beads can have affect on the mass transfer and fermentation efficiency profoundly causing reduction in yield coefficient [37]. Low value of yield coefficient at low and high temperature irrespective of the biomass content may be attributed to the low metabolic rate [34].

Yield coefficient maxima could be visualized to be achieved at 55 h (approx.) and between 54 and 56 h of incubation time for low (-1) level and high (+1) level of biomass (CDW), respectively. The yield coefficient has consistently decreased at low level of incubation time with the increase in the biomass (CDW).

However at high level of incubation time, the yield coefficient has initially increased with the enhancement in biomass loaded in beads (up to 350 mg, CDW), but beyond that it has decreased.



**Fig. 10** Yield coefficient as a function of incubation temperature and biomass for LA production by immobilized *Lb. casei* MTCC 1423



**Fig. 11** Yield coefficient as a function of incubation time and biomass for LA production by immobilized *Lb. casei* MTCC 1423

### 3.4 Optimization of lactic acid production

Since a higher LA production combined with high LA productivity and high yield is highly desirable from techno-economic point of view. Hence a numerical optimization using RSM was applied to obtain the optimized conditions for maximizing LA production, LA productivity and yield coefficient. It has been estimated that highest lactic acid production of 132 g/L, lactic acid productivity of 2.36 g/(L×h) and yield coefficient of 0.936 g/(g substrate) could be obtained under the optimized conditions. The optimized conditions are sugar concentration: 194 g/L; pH: 6.85; biomass: 310 mg (CDW); incubation temperature: 37°C and incubation time: 57 h which were validated experimentally. In our previous study, it has been observed that the double layer coated beads (alginate) entrapping *Lb. casei* MTCC 1423 cells has exhibited a potential of elevated immobilization efficiency and consistent reusability performance up to ninth cycle (each of 72 h) with sugarcane molasses as substrate.

### 3.5 Validation of results

In order to validate the optimized values of the process variables for the maximum production of the lactic acid, experiments were conducted in triplicate using the optimized conditions obtained. A close correspondence between the values of model prediction and experimental data was observed. The optimization validated experiments had resulted in LA production of 130±2.1 g/(L fermentor volume); LA productivity

of 2.28±0.037 g/(L×h) and yield coefficient of 0.921±0.003 g/(g substrate) at obtained optimized process parameters values. Lactic acid production of 110±1.9 g/(L fermentor volume), LA productivity of 1.93±0.033 g/(L×h) and yield coefficient of 0.91±0.002 g/(g substrate) was obtained, on performing the experiments for LA biosynthesis by free cells of *Lb. casei* MTCC 1423 under the above optimized conditions i.e. molasses sugar concentration: 194 g/L; pH: 6.85; incubation temperature: 37°C and incubation time: 57 h with inoculums size of 2% (v/v) (observed optimal during our previous study for LA production from molasses by free cells of *Lb. casei* MTCC 1423) while keeping other fermentation conditions same as mentioned in materials and method section of this study. The high lactic acid productivity observed during the validation experiments by immobilized *Lb. casei* MTCC 1423 cells is characterized by high cell densities retained in the fermentation media and long term stability. A maximum of 26.6 g/L lactic acid was reported to be synthesised by *Lb. casei* ssp. *Lb. rhamnosus* ATCC 11979 cells immobilized in alginate/chitosan complexes with solid and liquid core at optimum temperature of 42°C in 44 h with optimum pH of 6.5 [9]. A maximum rate of 2.16 g/(L×h) and a yield of 0.81 g/(g substrate) using food waste as carbon and nitrogen source was reported by Daniel et al. for lactic acid production using *Streptococcus* sp. Strains [38].

Lactic acid concentration ranging of 120.02-129.45 g/L and productivity in the range of 2.5 to 2.69 g/(L×h) during 1-5 batches of repeated fermentation with NVLS as immobilization matrix was observed by Sailaja et al. [39]. Lactic acid production of 136 g/L from whey by *Lactobacillus casei* NRRL B-441 cells immobilized in Ca-alginate/chitosan coated beads using yeast extract as nitrogen source has been reported by Goksungur et al. [40]. The maximum lactic acid concentration of 42.19 g/L & 47.60 g/L, process productivity of 1.69 g/(L×h) & 1.41 g/(L×h) and average yield coefficient of 0.96 g/(g substrate) and 0.96 g/(g substrate) was achieved by Djukic-Vukovic during the LA fermentation of liquid stillage by *Lb. rhamnosus* ATCC 7469 cells immobilized onto zeolite and Mg modified Zeolite without mineral or nitrogen supplementation, respectively [10, 41]. Maximum LA preparation of 70 g/L & 93 g/L and average LA productivity of 2.7 g/(L×h) & 4.7 from glucose (100 g/L) by using 7.5 g of dry cells/L of *Lb. casei* and *R. oryzae* immobilized in Ca-alginate gel & PVA cryogel, respectively has been reported by Maslova et al. [42].

### 4 Conclusion

Homofermentive lactic acid production by immobilized cells of *Lactobacillus casei* MTCC 1423 was found to be promising value added utilization of agro industrial by-product molasses and industrial waste corn steep liquor as carbon and nitrogen sources, respectively. Lactic acid production of 130±2.1 g/(L fermentor volume), LA productivity of 2.28±0.037 g/(L×h) and yield coefficient of 0.921±0.003 g/(g substrate) has been

experimentally obtained under the optimized conditions of process variables as 194 g/L sugar concentration; 6.85 pH; 310 mg (CDW) biomass; 37°C incubation temperature and 57 h incubation time resulted from numerical optimization for maximizing lactic acid production, LA productivity and yield coefficient using Box-Behnken Design.

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