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# Optimization of process variables for enhanced lactic acid production utilizing *paneer* whey as substrate in SMF

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**Abstract**

In the present study lactic acid production was enhanced by optimizing the three process variables viz; inoculum size, temperature and pH using three factor five level CCRD (central composite rotatable design) by *Lactobacillus delbruckii* under SMF (submerged fermentation process). *Paneer* (dairy by-product) whey was used as sole substrate for lactic acid production. Design Expert 8.0.2.0 software depicted that an optimum concentration of 8% (v/v) size of inoculum, 5.50 pH and 36.53°C temperature gave lactic acid and biomass yield of 5.61 g/L and 4.27 g/L, respectively. Lactic acid production was scale up in 7.5 L bioreactor under optimized conditions and it gave lactic acid and biomass yield of 39.2±1.4 and 47.6±0.8 g/L, respectively. $\mu_g$, $Y_{P/S,}$ $Y_{P/X}$ and productivity were found to be 0.14 $h^{-1}$, 0.66 g/g, 0.7 g/g and 1.98 g/L· h, respectively. *Leudking Piret* equation deduced that lactic acid production was growth associated which varies from earlier reports. Lactic acid was characterized by FTIR (Fourier transform infrared spectroscopy) and HPLC (High performance liquid chromatography).

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## 1. Introduction

Presently, fossil oil reserves are decreasing, oil prices are fluctuating, and the $CO_2$ produced by oil consumption in contributing to global warming. Alternative sources are necessary for energy generation, as transport fuel or feedstock for the production of bulk chemicals. Biomass is the only valid alternative for the synthesis of bulk chemicals and microbial bioconversion and it requires the key technologies for the conversion of biomass into products of commercial interest [1]. In order to compete with petrochemical processes, microbial conversions should be executed with high productivity and yield. The dairy industry generates enormous liquid waste i.e. whey annually, whose disposal requires a huge investment. Approximately, 84% of the total milk used for manufacturing cheese and *paneer* (a sort of cheese which is an un-aged, acid-set dairy product common in India, which is similar to acid-set fresh mozzarella cheese except that it does not have added salt, is discarded, as whey [2]. Most milk plants do not have proper treatment systems for the

disposal of whey and the dumping of whey comprises a significant loss of potential food and energy, as whey retains about 55% of total milk nutrients. Among the most abundant of these nutrients are lactose, soluble proteins, lipids and mineral salts. Most of the work has been carried out on the fermentation of whey from cow milk; however, few reports are available showing lactic acid production from *paneer* whey [3, 4]. Although several possibilities of *paneer* whey utilization have been explored, a major portion of the *paneer* whey produced is discarded as effluent. Its disposal as waste poses serious pollution problems for the surrounding environment, since it affects the physical and chemical structure of soil, resulting in a decrease in crop yield and when released into water bodies, reduces aquatic life by depleting the dissolved oxygen [5]. Thus, whey poses a major threat to environmental and human health, for which an effective and permanent solution is urgently needed.

In recent times, major portion of whey is utilized in production of biodegradable packaging material precursors such as lactic acid, valeric acid etc. Lactic acid popularly known as milk acid is a chemical compound that plays significant role in various food, pharmaceutical, leather, and textile industries [6, 7]. Currently, lactic acid production is produced by variety of microorganism including Lactobacillus species, *Saccharomyces cerevisiae*, *Lactobacillus delbruckii*, *Lactobacillus coryniformis* and *Lactobacillus lactis* have drawn most researchers' focus for lactic acid production [8, 9, 10, and 11]. However, the fermentative production by these strains required expensive substrates and high downstream processing cost. Recently a new microbial system has been developed in *E.coli* which synthesizes polyester intracellularly [12]. But, this alternative approach also failed due to low productivity and low tolerance of recombinant bacterium towards lactic acid. Thus the major problem is to realize the highly efficient lactic acid producing condition utilizing cheaper substrate.

In the present work, an attempt was made to enhance lactic acid production utilizing dairy industry by- product (*paneer* whey) as a source of lactose by *Lactobacillus delbruckii* in submerged fermentation process. Optimization of cultural conditions was done by CCRD and kinetic study of lactic acid production was studied in a 7.5 L bioreactor in submerged fermentation process.

## 2. Materials and Methods

### 2.1. Microorganism and culture conditions

*Lactobacillus delbrueckii* NCIM 2025 was selected for lactic acid production from whey. Bacterial inoculum was prepared in MRS broth. From the stock culture, the inoculum was transferred to 100 mL of sterilized MRS broth taken in culture flasks. The flasks were incubated for 24 h at 37$^{o}$C and refrigerated at 4$^{o}$C until used as working culture. Working cultures were propagated on MRS broth by transferring every 2 weeks. Active cultures for inoculation (10%, v/v) were prepared from the working cultures again using MRS as culture medium.

### 2.2. Maintenance media

Maintenance media contained following components in 1 L distilled water: Trypticase 10 g, Yeast extract 5 g, Tryptose 3 g, $KH_2PO_4$ 3 g, $K_2HPO_4$ 3 g, Tween 80 1 mL, Sodium acetate 1 ml, L-Cystine HCL 200 mg.

### 2.3. Production media

Production media contained whey suspension 100 mL, yeast extract 3 g, $MnSO_4$ 0.5 g and $CaCO_3$ 0.2 g, respectively. 10% (v/v) Inoculum (10% v/v) was added in the production media and then kept in the incubator plus shaker (Sigma Si-Louis, USA) for 24 h at different temperatures as designed by design expert trial 8.0.2.0.

### 2.4. Whey pre-treatment

Paneer whey was obtained from sweetmeat shop (Kheer Sagar, Varanasi, India). Precipitation was used to remove salts from whey. pH of the whey was adjusted in the range of 7-10, by adding NaOH and then its temperature was raised to 70$^{o}$C. Mineral salts were precipitated and then removed by centrifugation. For the removal of proteins, whey was autoclaved at 121$^{o}$C (15 lb pressure for 15 min). Then, the pH of the whey was brought down to 4.6-4.7 by adding 0.1 N HCl. The denatured proteins were then removed by decantation [13].

### 2.6. Fermentations

Submerged fermentation trials were conducted in 250 mL erlenmeyer flasks containing 100 ml whey suspension on a rotary shaker (Sigma Si-Louis, USA) at different experimental conditions as designed by design expert trial 8.0.2.0. Temperatures ranging from 24 to 45$^{o}$C, pH in the range of 2 to 7 and inoculum size ranging from 3 to 10% (v/v) were used for investigating the influence of temperature, pH and inoculum size on the of lactic acid and biomass yield. The fermentation time in each trial was 36 h and each trial was performed in duplicate.

### 2.6. Scale up in 7.5 L bioreactor

Inoculum for fermentation were prepared in shake flasks containing pre-sterilized cultivation medium held at 37$^{o}$C for 24 h. Experimental runs were performed in 7.5 L bench top bioreactor (BioFlo/Celligen 115, New Brunswick, USA) with a working volume of 3.4 L. The fermentation media were sterilized in the autoclave at 121$^{o}$C for 15 min. The bioreactor was inoculated with 275 mL of inoculum (8% v/v). The fermenter was equipped with control panel for different variables viz; temperature, pH, agitation and dissolved oxygen (DO). pH level

was regulated by 0.1 N NaOH/HCl. The required NaOH was supplied by the peristaltic pump and another peristaltic pump was used to take samples to be analyzed. The fermentation temperature was kept at 37°C. pH of the culture broth was maintained at 7 by automatic addition of acid or base by pH–mV controller (Mettler Toledo, USA). Dissolved oxygen was measured by DO probe (Mettler Toledo, USA).

### 2.7. Analytical methods

Proximate analysis of paneer whey was done to estimate total solid, fat, protein, lactose and ash content of paneer whey by the method as described previously with slight modifications [14]. Lactic acid analysis was done by titrimetric method and spectrophotometric method [15]. Dry dell mass was analyzed by taking 20 ml of culture broth which was centrifuged at 10000 rpm for 10 min at 4°C and cell pellet was obtained. The cell pellet was washed with saline water (NaCl 0.8% (w/v)) and then dried in aluminum dish at 90°C for 24 h in hot air oven.

### 2.8. Statistical optimization

Statistical optimization of media was done by using software tool RSM (Response Surface Methodology). Three factors were chosen at 5 levels for the optimization process viz; inoculum size (1.3, 3.0, 5.50, 8.00 and 9.70 %), pH (2.98, 4.00, 5.50, 7.00 and 8.02) and temperature (24.89, 30.00, 37.50, 45.00 and 50.11°C) and 2 responses i.e. lactic acid and biomass concentration were observed. Experiments were designed by Design expert DX 8.0.2.0 software, USA. Analysis of data generated during the present investigation was carried out using RSM by employing CCRD. The experimental data obtained from the design were analysed by the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x^2_i + \sum \beta_{ij} x_i j_i$$

Where $Y_i$ was the predicted response, $x_i x_j$ were independent variables, $\beta_0$ was the offset term, $\beta_i$ was the linear coefficient, $\beta_{ii}$ was the quadratic coefficient and $\beta_{ij}$ was the interaction coefficient.

### 2.9. Sample preparation for FTIR measurement

The sample preparation was carried out as suggested by [16] with some modifications. After cultivation of Lactobacillus on production media, 20 ml of broth was centrifuged at 8000 rpm on cold centrifuge (Sigma, USA) and 100 µL of suspension was taken. The concentration of the suspension was adjusted so that the intensity of the amide I band (1655 $cm^{-1}$) in the IR spectrum was between 0.35 and 1.25, which is within the linear range of the DTGS detector. Of each suspension, 35 µL was transferred to an IR transparent optical crystal (ZnSe) in a multi-sample cuvette (Bruker Optics, Germany). The sample was dried under moderate vacuum (0.1 bar) using anhydrous Silica gel (Prolabo, France) in a desiccator to form films suitable for FTIR analysis. The FTIR measurement was performed with a Biomodule (Bruker Optics, Germany) specially designed for microorganisms, coupled to an Equinox 55 spectrometer (Bruker Optics, Germany). The spectra were recorded in the region between 4000 and 500 $cm^{-1}$ with a spectral resolution of 6 $cm^{-1}$ and an aperture of 5 mm.

### 2.10. Sample preparation for HPLC analysis

2 µL of sample was analysed through HPLC (Shimadzu, Japan) with UV detector at 210 nm using RP C-18 column. The operating conditions comprised: mobile phase 5 mM $H_2SO_4$, flow rate 0.4 mL/min, column temperature 60°C, and injection volume 10 µL. Quantification was based on internal standard method.

## 3. Results and Discussion

### 3.1. Proximate analysis of paneer whey

Proximate analysis results (Table 1) clearly depicts that *paneer* whey obtained from cow milk contained 4.5±0.05% lactose which was comparable to lactose content of cheese whey [17]. Similarly other contents like protein, fat, ash and total solid were also comparable to cheese whey. However, the pH of *paneer* whey was lesser than cheese whey which facilitates lactic acid production by *Lactobacillus duelbrueckii*. The high acidity of *paneer* whey could be due to the presence of citric acid added during coagulation of *paneer* [18].

**Table 1.** Compositional analysis of *paneer* whey

| Components | Composition (%) |
|---|---|
| Total solids | 7.15±0.34 |
| Fat | 0.8±0.04 |
| Protein | 0.45±0.05 |
| Lactose | 5.37±0.68 |
| Ash | 0.63±0.07 |

n=3, SD=0.5

### 3.2. Effect of inoculum size, temperature and pH on lactic acid production

*Lactobacilus delbrueckii* was selected for lactic acid production utilizing *paneer* whey as a carbon source since it is a non-spore forming, catalase negative, fastidiously acid tolerant and strictly fermentative strain [19]. RSM was employed to check the best operating parameters and select optimum fermentative conditions. The average lactic acid concentration varied from 2.8 to 5.6 g $L^{-1}$ (Table 2). The maximum and minimum scores were obtained in experiment no. 15 and 4, respectively (Table 3). In experiment no. 15, the level of size of inoculum, temperature and pH were 9.7% (v/v), 37.5°C and 5.5,

respectively while the experiment no.4 comprised 5.5% (v/v) size of inoculum, 50.11°C temperature and 5.5 pH, respectively. In order to determine the maximum lactic acid concentration corresponding to the optimum levels of different parameters, a second order polynomial model was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second order polynomial model (equation 1) explained the role of each variable and their second order interactions in producing lactic acid. The data fitted the following linear model:

$$\text{Lactic acid} = +0.49 +0.065A -7.056E -003B +0.021C -1.250E - 003AB -0.026AC -0.01BC +2.420E -003A^2 -0.068B^2 -9.955E - 003C^2 \quad (1)$$

The coefficient of determination ($R^2$) was 0.90. The adequate precision was 11.69. Hence, the model could be used to navigate the design space. The linear model was significant ($P<0.01$). The probability (p) values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of the p-value, the more significant the correlation with the corresponding coefficients effects. The coefficient estimates of lactic acid production model (Table 4) showed that the level of inoculum size, temperature and pH had positive effect on lactic acid production but only the level of inoculum size and temperature had significant effect on lactic acid production ($P<0.05$). The graphical representation of the response shown in Figure 1(a-f) helped to visualize the effect of inoculum size (A), pH (B) and temperature (C) on the lactic acid yield. The influence of inoculum size, ranging from 3% (v/v) to 10% (v/v) on lactic acid production was investigated at varying pH and temperature. As shown in Table 2 lactic acid production increased substantially from 2.8 to 5.6 g $L^{-1}$, when inoculum size increased from 3 to 8% (v/v, $P<0.05$). Further increases in inoculum size (beyond 8%) had no significant effect on lactic acid production ($P>0.05$). Similar results were previously reported which suggests that high inoculum size could result in a negative impact on lactic acid production, although it could shorten the total fermentation time by 12 h [20]. Figure 1A shows the response surface plot for lactic acid production as influenced by inoculum size and temperature which depicts the linear relationship between inoculum size and temperature rate increment on lactic acid production. It can be seen from the Figure 1B that with increasing level of inoculum size, the yield of lactic acid in production media increased up to 8% (v/v) of inoculum size and obvious increase in the lactic acid score was observed at pH 5.5, which is in correlation with previous finding [21]. An optimal pH value of 5.7 for Lactobacillus strains was previously reported which is higher in comparison to the present finding [22]. This optimal pH agrees well with Bergey's manual (1974), which gives for Lactobacilli a value of 5.5 - 5.8 or less. pH 5.5 was used for batch cultures of *Lactobacillus bulgaricus* [23] and continuous cultures [24]. In the current study, the Lactobacillus strain was found to be more acid tolerant than *Lactobacillus helveticus* which showed a pH optimum of 5.9, using corn steep liquor [25]. The hydrogen ion concentration (pH) of the media during fermentation affects microbial growth and product formation rate. pH affects at least two aspects of microbial cells, i.e. the functioning of its enzymes and transport of nutrients into the cell. It can limit the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm. It has been observed that at suitable pH, RNA and protein synthesis is enhanced which shows profound effect on lactic acid production [26].

**Table 2.** Constraints fixed for optimization of size of inoculums, pH and temperature levels in production of lactic acid from whey

| Name | Goal | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|---|---|---|---|---|---|---|
| A- Inc size | In range | 3 | 8 | 1 | 1 | 3 |
| B- Temp | In range | 30 | 45 | 1 | 1 | 3 |
| C-pH | Target | 4 | 7 | 1 | 1 | 3 |
| Lactic acid (g/l) | Maximize | 2.6 | 5.6 | 1 | 1 | 3 |
| Biomass (g/l) | Maximize | 2.9 | 4.5 | 1 | 1 | 3 |

**Table 3.** Responses obtained by varying parameters involved in lactic acid production

| Run | Size of Inoculum (% v/v) | Temperature (°C) | pH | Lactic acid (g/l) | Biomass (g/l) |
|---|---|---|---|---|---|
| 1 | 5.50 | 37.50 | 5.50 | 5.20±0.20 | 4.10±0.08 |
| 2 | 8.00 | 30.00 | 7.00 | 5.50±0.40 | 4.30±0.04 |
| 3 | 5.50 | 37.50 | 5.50 | 4.90±0.60 | 3.80±0.10 |
| 4 | 5.50 | 50.11 | 5.50 | 2.80±0.30 | 3.00±0.24 |
| 5 | 5.50 | 37.50 | 5.50 | 5.00±0.40 | 3.90±0.36 |
| 6 | 8.00 | 30.00 | 4.00 | 5.10±0.30 | 4.00±0.40 |
| 7 | 3.00 | 30.00 | 7.00 | 3.10±0.40 | 3.20±0.48 |
| 8 | 8.00 | 45.00 | 5.50 | 4.80±0.10 | 3.70±0.30 |
| 9 | 5.50 | 24.89 | 5.50 | 2.60±0.30 | 2.90±0.40 |
| 10 | 1.30 | 37.50 | 5.50 | 3.80±0.20 | 3.50±0.10 |
| 11 | 3.00 | 45.00 | 4.00 | 3.10±0.24 | 3.10±0.20 |
| 12 | 5.50 | 37.50 | 5.50 | 4.70±0.34 | 3.70±0.40 |
| 13 | 8.00 | 45.00 | 4.00 | 5.10±0.40 | 4.10±0.20 |
| 14 | 5.50 | 37.50 | 5.50 | 4.80±0.24 | 3.80±0.24 |
| 15 | 9.70 | 37.50 | 5.50 | 5.60±0.30 | 4.50±0.36 |
| 16 | 5.50 | 37.50 | 2.98 | 4.20±0.18 | 3.50±0.20 |
| 17 | 5.50 | 37.50 | 5.50 | 5.10±0.24 | 3.90±0.24 |
| 18 | 5.50 | 37.50 | 8.02 | 4.50±0.10 | 3.6±0.40 |
| 19 | 3.00 | 45.00 | 7.00 | 3.90±o.30 | 3.5±0.18 |
| 20 | 3.00 | 30.00 | 7.00 | 4.50±0.20 | 3.7±0.10 |

**Table 4.** ANOVA for response surface linear model of coded factors for yield of lactic acid production

| Factor | Sum of Squares | Df | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Model Significant | 0.23 | 3 | 0.077 | 9.05 | 0.001 |
| A-Inc size | 0.007 | 1 | 0.071 | 8.42 | 0.010 |
| B- Temp | 7.447E-003 | 1 | 7.447E-003 | 0.88 | 0.361 |
| C-pH | 0.15 | 1 | 0.15 | 17.84 | 0.006 |
| Residual | 0.14 | 16 | 8.458E-003 | NA | NA |
| Lack of fit | 0.091 | 11 | 8.290E-003 | 0.94 | 0.570 |
| Pure total | 0.044 | 5 | 8.827E-003 | NA | NA |
| Cor Total | 0.36 | 19 | NA | NA | NA |

NA- Not Applicable

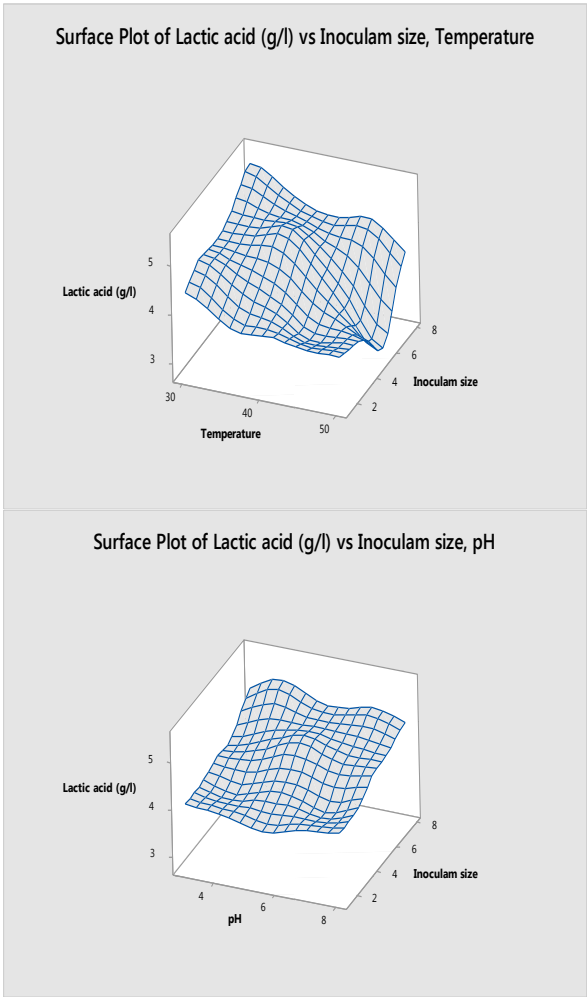



**Figure 1:** (A), Influence of temperature and inoculum size on lactic acid production (B), Influence of pH and inoculum size on lactic acid production.

Figure 1C suggests that with increasing temperature, the yield of lactic acid in production media increased up to around 37.5°C and noticeable increase in the lactic acid was observed at pH 5.5 keeping the inoculum size at fixed concentration. Table 2 indicates that lactic acid production increased from 2.6 g/L to 5.6 g/L with increase in temperature from 24 to 37.5°C due to increased lactose utilization at elevated temperature, however at temperature higher than 37.5°C lactic acid production was reduced which is in correlation with the previous finding [27]. The temperature giving the highest productivity was in some cases lower than the temperature resulting in highest lactic acid concentration and yield, whereas in others the same temperature gave the best results in all conditions. For *Lactobacillus amylophilus*, the optimal temperatures were 25 and 35°C for maximum productivity and yield, respectively [28] *Lactobacillus casei* and *Lactobacillus paracasei* showed maximum lactic acid production at 44°C [29].

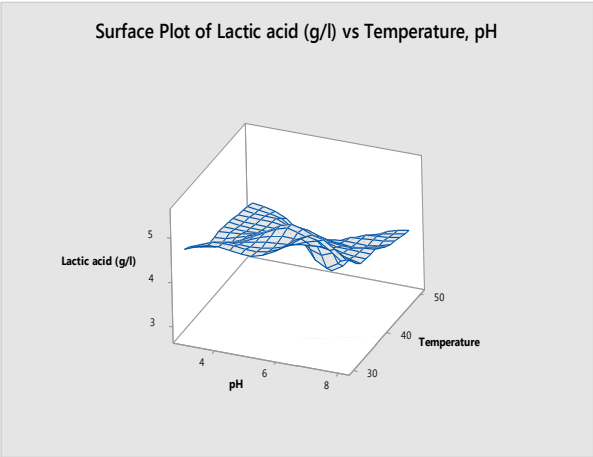



**Figure 1 (C),** Influence of pH and temperature on lactic acid production

### 3.3. Effect of inoculum size, temperature and pH on biomass yield

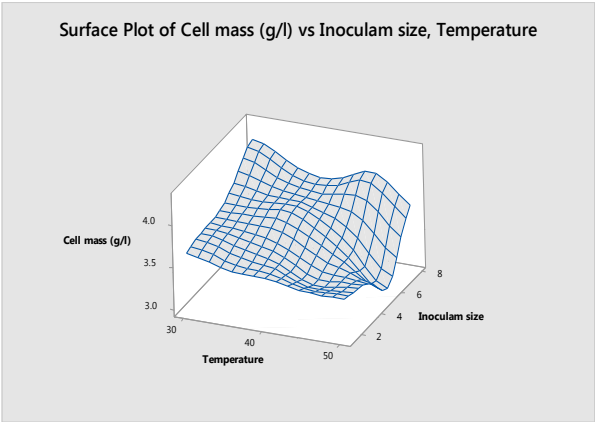
ANOVA and regression analysis revealed that linear effects, square effects, interaction effects and outputs were quite significant for production of lactic acid and biomass yield (Table 5). The cubic model in Eq. (2) with 19 terms contained 3 linear terms, 3 quadratic terms, 7 two factorial interactions, 3 cubic terms and 1 three factor interactions. The model F-value of 5 implies that the model was significant. The average biomass varied from 2.9 g $L^{-1}$ to 4.5 g $L^{-1}$ (Table 1). The minimum and maximum score were obtained for experiment no. 9 and 15, respectively. In experiment no. 15, the level of size of inoculum, temperature and pH were 9.7% (v/v), 37.5°C and 5.5, respectively, while the experiment no. 9 had the level of inoculum size 5.5% (v/v), temperature 24.89°C and pH 5.5, respectively (Table 2). The data fitted the following cubic model:

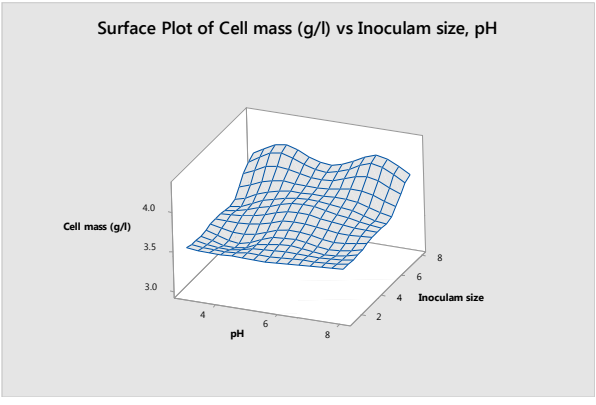
Biomass= +3.859E -003 +3.135E -004A -4.626E -005B +7.089E -005C -2.500E -005AB -1.250E -004AC -1.000E -004BC +9.404E -005$A^2$ -2.772E -004$B^2$ -6.506E -005$C^2$

The coefficeint of determination ($R^2$) was 0.89. The predicted $R^2$ of 0.8544 was in reasonable agreement with adjusted $R^2$ of 0.7322. Adequate precision ratio of 10.054 indicates adequate signal (Ratio>4 is desirable). Hence, the model could be used to navigate the design space. The coefficient estimates of biomass cubic model (Table 5) showed that the level of inoculum size and temperature had a significant effect (P<0.05) on biomass. The interactive effects of these factors were non-significant except for interactive term ABC. The quadratic terms for the effects of inoculum size and pH were positive but were insignificant, however quadratic effect of temperature was significant (P<0.05). Figure 1D shows the response surface plot for biomass production score as influenced by inoculum size and temperature. It can be seen from the Figure 1D that with increasing inoculum size, the yield of biomass in production media increased up to 8% (v/v) of inoculum size. The biomass also increased apparently up to 44°C but it sharply decreased above 44°C and was found to be maximum at 37°C which mimics the earlier findings that high temperatures were suitable for Lactobacillus cell growth [30]. Biomass was increased due to increased specific growth rate of microbe at elevated temperature [31]. Figure 1E shows the response surface plot for biomass production score as influenced by inoculum size and pH levels, which clearly indicates that with increasing level of inoculum size, the yield of biomass in production media increased up to 8% (v/v) of inoculum size and obvious increase in the biomass score was observed at pH 5.5. Maintenance of pH is important during lactic acid fermentation to provide the optimum pH for the organism to allow it to utilize maximum substrate as reported earlier [32]. Effect of pH control on lactic acid fermentation of starch was earlier studied with *Lactobacillus manihotivorans* LMG 18010T and it was found that its growth was arrested at pH 5 but it grew actively at pH 6.5 which shows that *Lactobacillus delbrueckii* is more acid tolerant and it can actively grow in acidic pH which will minimize whey processing cost [33]. Figure 1F represents the response surface plot for biomass production score as influenced by temperature and pH levels which clearly indicates that with increasing temperature, the yield of biomass in production media increased up to around 37.5°C and noticeable increase in the biomass score was observed at pH 5.5 keeping the inoculum size at fixed concentration zero level.

**Table 5.** ANOVA for Response surface linear model of coded factors for biomass yield of lactic acid production

| Factor | Sum of Squares | Df | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Model Significant | 5.348E-006 | 13 | 4.11 E-0.077 | 5.00 | 0.029 |
| A-Inc size | 5.00E-007 | 1 | 5.00E-007 | 6.07 | 0.048 |
| B- Temp | 5.00E-009 | 1 | 5.00E-009 | 0.061 | 0.813 |
| C-pH | 5.00E-009 | 1 | 5.00E-009 | 0.061 | 0.813 |
| ABC | 1.280E-006 | - | - | 15.55 | 0.007 |
| $A^2B$ | 1 | - | - | 0.081 | - |
| $A^2C$ | 1 | - | - | 0.157 | - |
| $AB^2$ | 1 | - | - | 0.316 | - |
| $C^3$ | 0 | - | - | - | - |
| Residual | 4.904E-007 | 6 | 8.458E-003 | - | - |
| Lack of fit | 6.398E-010 | 1 | 8.290E-003 | 6.458E-003 | 0.938 |
| Pure total | 4.933E-007 | 5 | 8.827E-003 | - | - |
| Cor Total | 5.842E-006 | 19 | - | - | - |



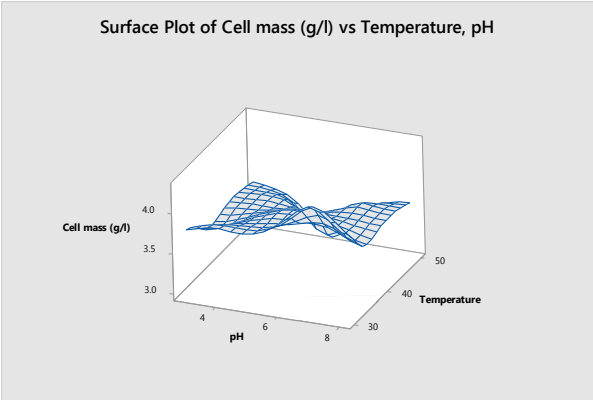



**Figure 1:** (D), Influence of inoculum size and temperature on biomass yield (E), Influence of pH and inoculum size on biomass yield (F), Influence of temperature and pH on biomass yield

### 3.4. Optimization of levels of inoculum size, pH and temperature in production of lactic acid

The 3D response surfaces are the graphical representations of the regression equation and were used to study the interaction of the variables and to locate the optimum level of each variable for maximum response. Each 3D response surface for lactic acid production represents the different combinations of two test variables at one time while keeping the other variable at its respective zero level. The convex response surfaces suggest that there are well-defined optimum variables. If the surfaces are rather symmetric and flat near the optimum, the optimized values may not vary widely from the single variable conditions. From the results obtained by analysis of data obtained through production of lactic acid and biomass growth from whey, suitable levels of inoculum size, pH and temperature were selected for further investigation. The suggested formulations with desired level of inoculum size, pH and temperature with predicted score of lactic acid and biomass, were obtained and are presented in Table 6. The obtained responses indicate that out of 8 formulations, the formulation 1 had better yield of lactic acid of 5.61 g $L^{-1}$ than all other formulations and also the desirability was 0.95, highest among all other formulations. Hence, formulation 1 with 8% (v/v) inoculum size, 36.53$^{o}$C temperature and 5.5 pH was selected, which had the highest predicted scores as 5.61g $L^{-1}$ for lactic acid and 4.3 g $L^{-1}$ for biomass. Further validation was done by performing experiment at designed condition which gave maximum lactic acid yield of 5.59 g $L^{-1}$ and 4.21 g $L^{-1}$ of biomass and it showed 98% resemblance with expected result under similar conditions designed by software tool.

**Table 6.** Predicted scores of the suggested formulations of lactic acid production by design expert 8.0.2.0

| Inoculum size (% v/v) | Temperature ($^{o}$C) | pH | Lactic acid (g/l) | Biomass (g/l) | Desirability |
|---|---|---|---|---|---|
| 8.00 | 36.54 | 5.50 | 5.4 | 4.3 | 0.950 |
| 8.00 | 36.40 | 5.50 | 5.6 | 4.1 | 0.950 |
| 8.00 | 36.34 | 5.50 | 5.1 | 3.8 | 0.950 |
| 8.00 | 36.76 | 5.50 | 4.6 | 3.6 | 0.950 |
| 8.00 | 36.90 | 5.50 | 4.2 | 3.4 | 0.950 |
| 8.00 | 37.01 | 5.50 | 3.8 | 3.0 | 0.950 |
| 8.00 | 38.22 | 5.50 | 3.4 | 2.8 | 0.946 |
| 8.00 | 36.24 | 5.47 | 3.2 | 2.6 | 0.944 |

### 3.5. Scale up in 7.5 L bioreactor

Shake flask study was then scaled up to a lab scale bioreactor. The culture was grown in a 7.5 L bench top bioreactor (BioFlo/Celligen 115, New Brunswick, USA) to study lactic acid production in batch cultivation. Figure 2 represents the lactic acid production under optimized condition by *Lactobacillus delbrueckii* NCIM 2025 utilizing 2.8 L paneer whey with initial concentration of lactose at 45 g $L^{-1}$. pH was kept at 5.5±0.1 throughout the production process and temperature was maintained at 45$^{o}$C. Agitation speed was set at 200 rpm and aeration rate during lactic acid production was kept at 0.5 L $min^{-1}$ in order to prevent the precipitation of lactic acid. Figure 2 clearly depicts that after a lag phase of 4 h biomass increased to 47.6 g $L^{-1}$ at 15 h. Maximum lactic acid concentration was production of 39.2 g $L^{-1}$ was recorded after 14.8 h of fermentative production. Previously maximum lactic acid concentration of 22.6 g $L^{-1}$ has been reported utilizing date juice as substrate [34]. Paneer whey proximate analysis reveals that it contains approximately 4.5% lactose. Lactose concentration decreased to 3.8 g $L^{-1}$ at the end of production phase in comparison to initial concentration of 45 g $L^{-1}$. Figure 2 clearly represents that approximately 50% lactose (23 g/L) was converted to lactic acid in mid exponential phase and approximately 15% conversion of lactose to lactic acid occurred in de- acceleration phase and stationary phase. It can be also clearly deduced from Figure 2 that almost complete exhaustion of lactose occurred before 15 h. Lactic acid yields (YP/S, YP/X) in terms of substrate consumed and cell biomass produced were found to be 0.66 g/g and 0.7 g/g, respectively

which is higher than the previous findings [35]. In the present study the productivity was found to be 1.98 g/L.h and µg was found to be 0.14 $h^{-1}$. Batch cultivation study was carried out to understand the kinetics of lactic acid production under controlled condition of temperature, pH, agitation and aeration.

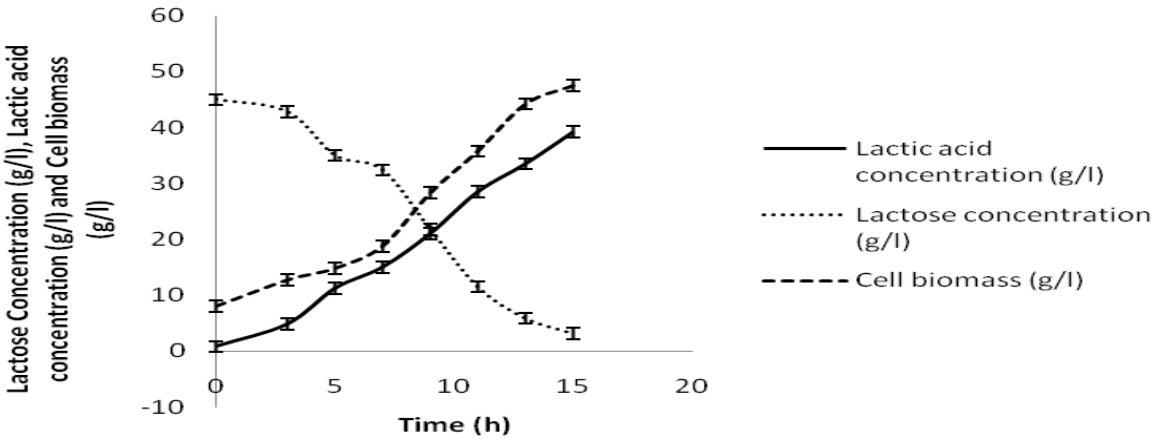



**Figure 2.** Time profile of lactic acid and biomass production under optimized condition in 7.5 L bioreactor (BioFlo/Celligen 115, New Brunswick, USA).

In order to study the kinetics of lactic acid production, *Luedking-Piret* equation was applied which reveals that lactic acid production is growth associated. Figure 2 represents that rate of product (lactic acid) formation is dependent on cell biomass concentration and it follows the following equation:

$$q_p = (Y_{P/x})\ \mu_g$$

Where, $q_p$ is product uptake rate and $\mu_g$ represents specific growth rate of microbe. $Y_{P/x}$ is product yield in terms of cell mass produced. Current study revealed that lactic acid production under optimized conditions is growth associated which varies from earlier studies which reported that lactic acid production is mixed-growth associated.

### 3.6. Characterization of lactic acid using FTIR and HPLC

FTIR analysis of lactic acid recovered showed two different types of O-H bond, the one in the acid and the simple "alcohol" type in the chain attached to the -COOH group (Figure 3). The O-H bond in the acid group gets absorbed between the range of 2500 and 3300 $cm^{-1}$ and the one in the chain between 3230 and 3550 $cm^{-1}$. When these two are taken together, give immense trough covering the whole range from 2500 to 3550 $cm^{-1}$ and lost in the trough will be absorptions due to the C-H bonds. The presence of strong C=O shows absorption at about 1730 $cm^{-1}$ (Figure 4). HPLC profile of lactic acid produced on optimized media with sharp peak at retention time of 9.86 min represented lactic acid (Figure 4).

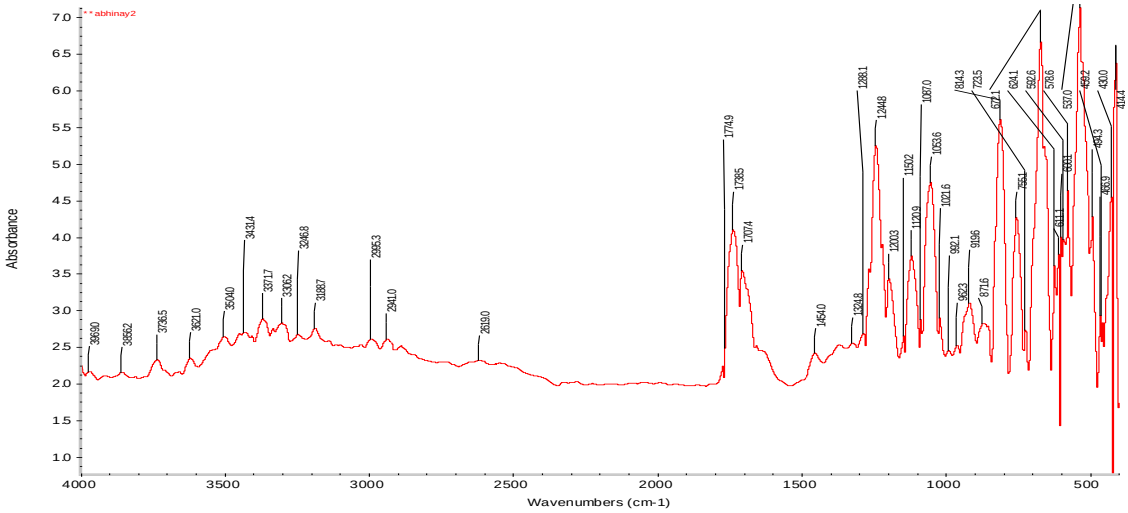



**Figure 3.** Spectrum of lactic acid produced by *Lactobacillus delbrueckii* using FTIR

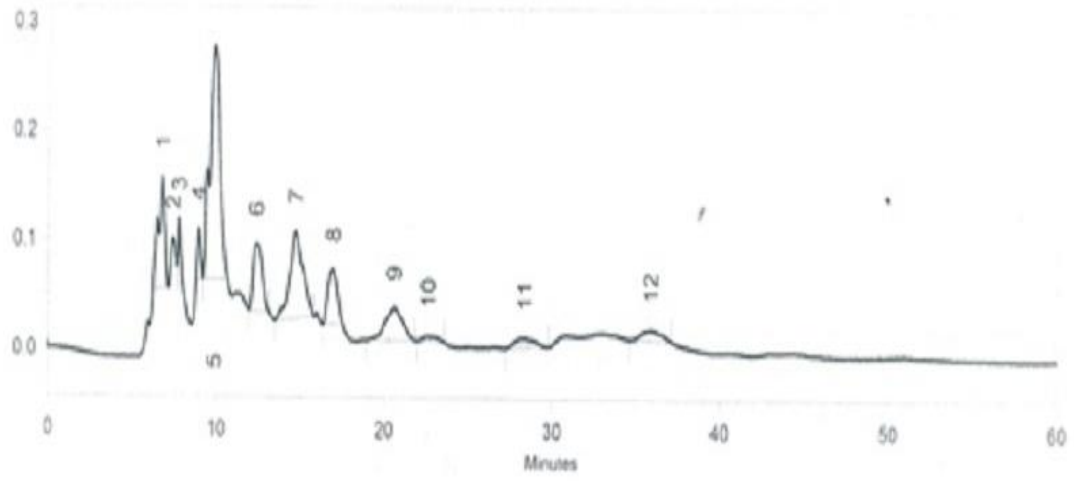



**Figure 4.** Chromatogram of lactic acid sample: citric acid (1), sucrose (3), lactic acid (5), acrylic acid (6), cis-aconitic acid (7) and linear dimer (8)

## 4. Conclusion

Fermentation carried under optimized condition on 7.5 L bioreactor comprising: 8% (v/v) size of inoculum, 5.5 pH and 36.53$^{o}$C temperature, showed maximum lactic acid yield of 70% on substrate (paneer whey) consumed with productivity of 1.98 g/L.h after 15 h of batch cultivation. Kinetic study of lactic acid production suggests that lactic acid production is growth associated, which makes the overall production process simpler and cost effective. Growth kinetic study can be utilized for development of a mathematical model in batch cultivation. Batch cultivation study can be further modified by carrying out production in fed batch/continuous process.

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## 6. Conflict of Interest

All the authors mutually agree to submit the article in the journal of Applied Food Biotechnology and there is no conflict of Interest.

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