# Optimization of Ethanol Production from Cheese Whey Fermentation in a Batch-Airlift Bioreactor

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## Abstract

In this work, *Kluyveromyces fragilis* yeast was used for bio-ethanol production from cheese-whey in batch fermentation. The present study consisted of two steps: The first was a central composite design (CCD) for evaluating of important factors including: pH, initial lactose concentration (L), yeast cells concentrations (Y) and temperature (T). In order to optimize the fermentation process, response surface methodology (RSM) was used in this stage. The best operating conditions were found to be pH = 5.3, L = 41.8 g/l, Y = 0.57 g/l and T= 30.8°C. The second step was to determine the effect of aeration rate on the fermentation process in an airlift bioreactor. The best conditions were the aeration rate of 0.4 vvm with 89.28% of ethanol production yield. In this research, the concentrated cheese whey was also used for obtaining a bio-ethanol fermentation product.

**Keywords:** Fermentation; Bio-ethanol; Cheese whey; Response surface methodology; Airlift bioreactor

## Introduction

Cheese whey is a yellowish liquid remaining after milk coagulates during cheese production. It is a by-product of the manufacture of cheese and has several commercial uses. Cheese whey is produced in huge amounts and is a significant environmental problem due to the high levels of organic matter content [1]. Cheese whey represents a biochemical oxygen demand (BOD) of 30-50 g/l and a chemical oxygen demand (COD) of 60-80 g/l lactose is largely responsible for the high BOD and COD, since protein recovery reduces only about 12% of the whey COD [2-4]. On the other hand, whey retains much of the milk nutrients, including functional proteins and peptides, lipids, lactose, minerals and vitamins and therefore has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource [5,6]. In Iran, about 1.8 million tons of whey which is the by-product of cheese producing factories is produced each year. The changing of whey into alcohol due to the low price of whey (compare to the price of other raw materials) has become the focus of considerable attention in the world. Use of whey in the preparation of ethanol was studied since 1940. Moulin et al. [7] have achieved to the 86 to 90 percent of efficiency in the medium of cheese whey permeate by using the two species of yeasts *Kluyveromyces fragilis* and *Candida pseudotropicalis*. In a study by Gawel et al. [8] with *K.fragilis* strain obtained to 10 percent of ethanol fermentation from whey in 15 days. Janssens et al. [9] reported ethanol productivity of 7.1 gL$^{-1}$h$^{-1}$ for *K. fragilis* operating with cell recycling at D = 0.15 h$^{-1}$ and for cheese whey permeate (CCWP) with 100 g/l lactose. Terrell et al. [10] reported ethanol productivity of 13.6 gL$^{-1}$h$^{-1}$ for CCWP with 150 g/l lactose concentration operating at continuous operation. Ryu et al. [11] reached to the rate of 2.1 percent of ethanol in a batch system in airlift bioreactor by using *K.fragilis* (20 L). Ferrarie et al. [12] were obtained 64 g/l of ethanol in a fed-batch system. In this research the laboratory production of ethanol by means of whey has been accomplished in an airlift bioreactor. The purposes of the present experimental study were mainly to investigate how the main operating parameters affect the process so as to determine which of them were certainly important. The goals were satisfied by means of response surface methodology through accurately designed central composite design.

## Culture Media and Batch Cultures

### Microorganism

Yeast strain used in this study was *K. fragilis* PTCC 5193, obtained from the Iranian Research Organization for Science and Technology (IROST). *K. fragilis* was maintained in agar (65g/l). The culture was sterilized in autoclave at 121°C for 20 min; the yeast inoculum was spread on the surface and incubated at 30°C for 48 hrs. At completed growth, the slants were preserved at 4°C.

### Preculture medium

The preculture medium was 13 g/l nutrient broth, 10 g/l peptone and 10 g/l yeast extract, sterilized at 121°C for 15 min and prepared with a single colony withdrawn from the slants and maintained for 48 hrs at an incubator shaker with a temperature of 35°C and velocity of 150 rpm. In all the experiments 100 ml of sterile Erlenmeyer flask were charged with a 50 ml of preculture.

### Fermentation medium

Cheese whey was the fermentation medium; containing lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5%w/v) and mineral salts (8-10% of dried extract). Whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins [3,4]. For batch experiments five hundred milliliters of erlenmeyer flasks were charged with 300 ml of cheese sterilized and deprotenized whey. It consisted of yeast extract (5 g/l), peptone (5 g/l), $NH_4Cl$ (2 g/l), $KH_2PO_4$ (1 g/l), $MgSO_4.7H_2O$ (0.3 g/l) [13,14]. Variables were pH, initial lactose concentration (L), yeast cells concentrations (Y) and temperature (T).

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## Airlift bioreactor

Figure 1 shows an airlift bioreactor contains external loop which is made of Pyrex glass. The bioreactor was fed with sweet cheese sterilized and deprotenized whey. The cell suspension was aseptically transferred to the bioreactor. Airlift bioreactor was operated at working volume of 7 liters that included 10% preculture. The regulation system allows for: temperature control at 30 ± 1°C; foam-level and pH controlled by addition of antifoam and ammonia, respectively. The set-point fixed at pH 5.0 ± 0.1. The system was aerated with filtered air at a different flow rate of 0.1, 0.4 and 0.8 vvm that was controlled using an aeration pump controller. Each run was achieved in duplicates; the average values of lactose, ethanol and biomass concentrations were calculated and monitored with respect to time.

## Analytical methods

In the Erlenmeyer flask after 48 hrs all of the samples were removed and centrifuged and in airlift bioreactor the samples were removed from the sampling ports at different heights of the column every day and centrifuged at 8000 rpm for 20 min to remove solids from the liquid media. Total reducing sugar concentrations were measured by using the phenol-acid method [2]. The samples were analyzed in triplicates 3%. Ethanol concentrations were measured using a Varian CP-3800 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and star integrator. A 2 m×1.4"×4 mm column packed with propack Q 89-100 Mesh. The column temperature was set for 75°C for 1 min and raised to 150°C with a rate of 10°C/min yielding a total hold time of 4.75 min. Temperatures of injector and detector were 150 and 200°C, respectively. Nitrogen was used as the carrier gas



**Figure 1:** Schematic of the airlift bioreactor.

with a linear velocity of 30 ml/min. Dry cell mass concentration was estimated by measuring the optical density of the sample at 600 nm in a spectrophotometer, and by its correlation with the dry cell weight (DCW) obtained gravimetrically. pH was measured using a pH meter (JEYWAY 3510). The yeast cells concentration was estimated by the dry weight method. The dry weight cell concentration was determined by filtering the sample through 0.2 μm filter paper and then dried at 105°C for 48 hrs [15].

## Experimental design

The present experimental study consisted of two steps: (1) The central composite design (CCD) aimed at determining the effects of 4 factors on the fermentation process.

(2) Determining the effect of aeration rate on the fermentation process and measuring the variation of ethanol, lactose and biomass concentration with time in airlift bioreactor.

Four factors were considered to perform for response surface methodology of CCD: pH, initial lactose concentration (L), yeast cells concentration (Y) and temperature (T), with five different levels for each of the factors. The values of the chosen factors were 4 and 6 for pH, 40 and 80 for initial lactose concentration, 0.4 and 0.8 g/l for yeast cells concentration and 30 and 38 for temperature. The range of these values was considered since it characterized the optimum range for the yeast activity and the expected range in which the process could be operated. In this study, the experimental design consisted of 25 runs and the independent variables were studied at five different levels. Table 1 shows the experimental design used for this study. All the experiments were done in duplicates and the average of ethanol production obtained was taken as the response function (RF). The Second degree polynomials, Equation (1), which contains all interaction terms, were used to calculate the predicted response:

$$RF = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j \qquad (1)$$

Where RF represents response variable, $\beta_0$ is the interruption coefficient, $\beta_i$ the coefficient of the linear effect, $\beta_{ii}$ the coefficient of quadratic effect and $\beta_{ij}$ the $ij^{th}$ coefficient of interaction effect, xi xj are input variables which influence the response variable RF; $\beta_i$ is the $i^{th}$ linear coefficient. Numerical analysis of the model was performed to evaluate the analysis of variance (ANOVA). For each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated.

## Results and Discussion

Table 1 are shown the experimental design and results of CCD of response surface methodology. The factors levels are 4 and 6 for pH, 40 and 80 g/l for L, 0.4 and 0.8 g/l for Y and 30 and 38°C for T. In the last column the obtained response function values are shown. The experimental data were statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Table 2. The ANOVA of the quadratic regression model indicated that the model was highly significant, as the F-value for the model was 11.52. This fit of the model was checked by the coefficient of determination $R^2$, which was calculated to be 0.92, indicating that 92.05 % of the variability in the response could be explained by the model. The effects of the parameters on the response function (RF) were calculated and the parameters which showed P-values less than 0.05 were taken into account in the model; the other parameters were actually undistinguishable from noise. The significant terms, as shown in Table 2, are pH, L, T, pH² and T². Then, by eliminating the other
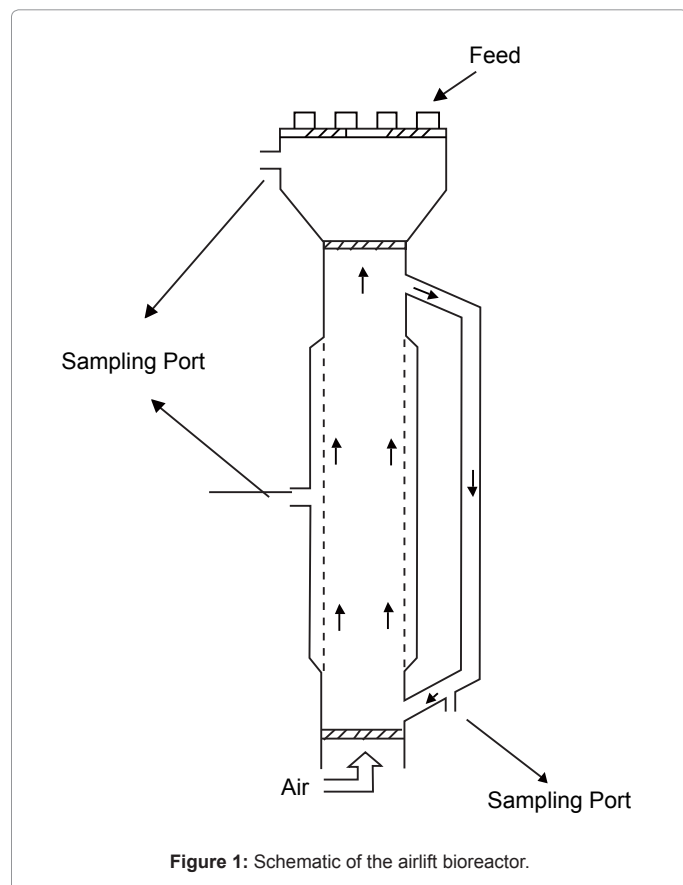
| Run | pH | Lactose (g/l) | Yeast cells concentration (Y) (g/l) | Temperature (°C) | Response Function (RF) |
|---|---|---|---|---|---|
| 1 | 5 | 100 | 0.6 | 34 | 1.35 |
| 2 | 5 | 20 | 0.6 | 34 | 1.55 |
| 3 | 4 | 60 | 0.6 | 26 | 1.47 |
| 4 | 4 | 40 | 0.4 | 30 | 1.36 |
| 5 | 5 | 80 | 0.4 | 30 | 1.05 |
| 6 | 6 | 60 | 0.6 | 42 | 0.85 |
| 7 | 6 | 40 | 0.8 | 38 | 1.17 |
| 8 | 3 | 80 | 0.8 | 38 | 1.11 |
| 9 | 7 | 60 | 0.6 | 34 | 0.90 |
| 10 | 4 | 60 | 0.6 | 34 | 0.98 |
| 11 | 4 | 40 | 0.4 | 38 | 1.05 |
| 12 | 6 | 80 | 0.4 | 38 | 0.75 |
| 13 | 6 | 40 | 0.8 | 30 | 1.48 |
| 14 | 5 | 80 | 0.8 | 30 | 1.42 |
| 15 | 5 | 60 | 0.2 | 34 | 0.75 |
| 16 | 6 | 60 | 1.0 | 34 | 0.85 |
| 17 | 6 | 40 | 0.4 | 30 | 1.50 |
| 18 | 6 | 40 | 0.4 | 38 | 1.20 |
| 19 | 6 | 80 | 0.4 | 30 | 1.45 |
| 20 | 4 | 80 | 0.4 | 38 | 1.05 |
| 21 | 4 | 40 | 0.8 | 30 | 1.08 |
| 22 | 4 | 40 | 0.8 | 38 | 0.75 |
| 23 | 4 | 80 | 0.8 | 30 | 1.03 |
| 24 | 5 | 80 | 0.8 | 38 | 0.73 |
| 25 | 5 | 60 | 0.6 | 34 | 1.59 |

**Table 1:** Experimental design and CCD results of response surface methodology.

| Model term | Coefficient | F-value | p-value |
|---|---|---|---|
| Constant | -0.85776 | | |
| PH | 1.52437 | 10.67 | 0.008 |
| L | -0.00453 | 0.06 | 0.818 |
| Y | 3.38646 | 3.88 | 0.077 |
| T | 0.37703 | 5.36 | 0.043 |
| PH$^2$ | -0.15354 | 17.77 | 0.002 |
| L$^2$ | -0.00006 | 0.42 | 0.533 |
| Y$^2$ | -4.65104 | 26.09 | 0.000 |
| T$^2$ | -0.00600 | 6.95 | 0.025 |
| PH-L | 0.00119 | 0.60 | 0.456 |
| PH-T | -0.00156 | 0.04 | 0.842 |
| PH-Y | 0.18125 | 1.40 | 0.262 |
| L-T | -0.00008 | 0.04 | 0.842 |
| L-Y | 0.00906 | 1.40 | 0.264 |
| Y-T | 0.00781 | 0.04 | 0.842 |

$R^2$ = 92.05% and adjusted $R^2$ = 80.91%

**Table 2:** Coefficient estimates and analysis of variance (ANOVA) for the response surface quadratic model.

terms from the model (except L to support hierarchy as requested from the methodology [16]), Eq. (2) was obtained as a function of the significant factors.

RF= -0.8577+ 1.5243 PH- 0.0045 L+ 0.3770 T- 0.1535 PH$^2$- 0.0060 T$^2$ (2)

Figure 2-4 show the effects of the factors on the RF. Figure 2a presenting the response surface performance as a function of both pH and L. a relatively weak effect of L and a stronger effect of pH can be noted. The best conditions were achieved at pH 5.3 and L 41.8 g/l. With increasing lactose level of 40 to 60 g/l, RF decreased because of cellular osmotic pressure limiting. Figure 2b shows the effect of pH and T on ethanol production while the third variable is fixed at its middle level.
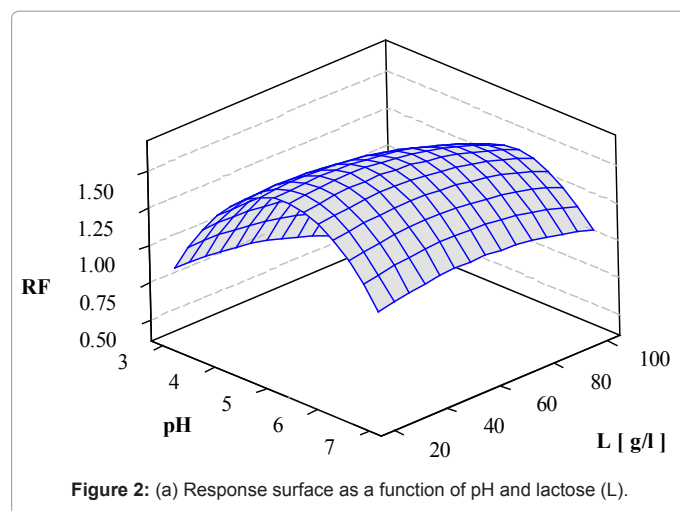


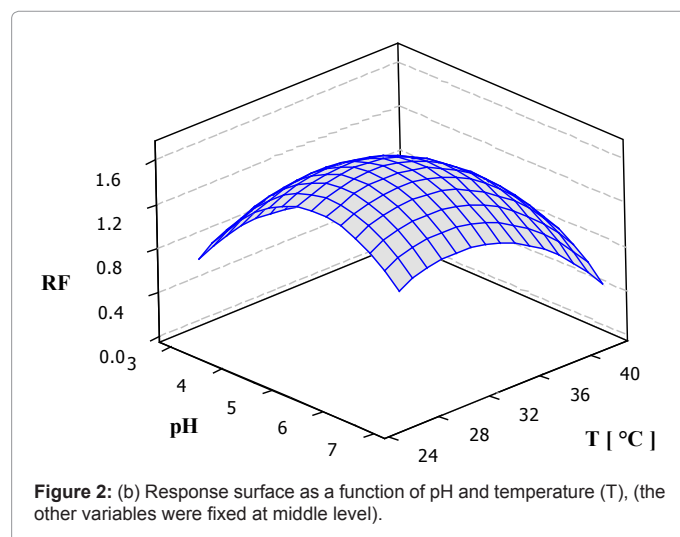**Figure 2:** (a) Response surface as a function of pH and lactose (L).



**Figure 2:** (b) Response surface as a function of pH and temperature (T), (the other variables were fixed at middle level).
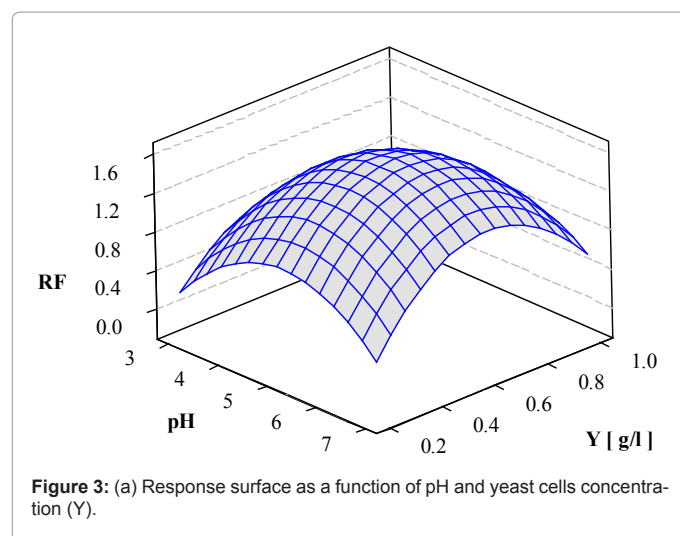


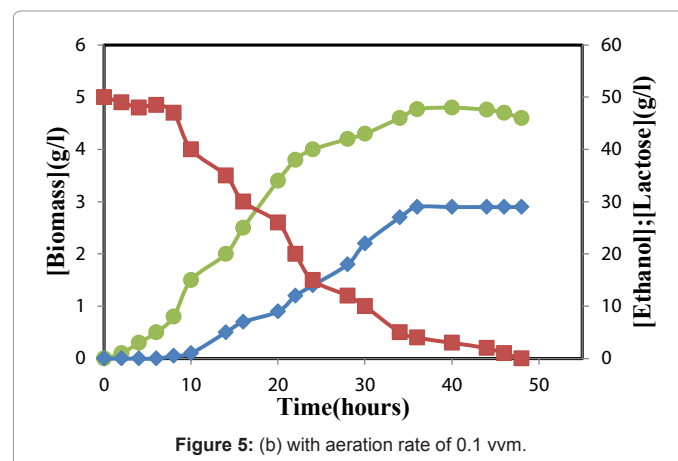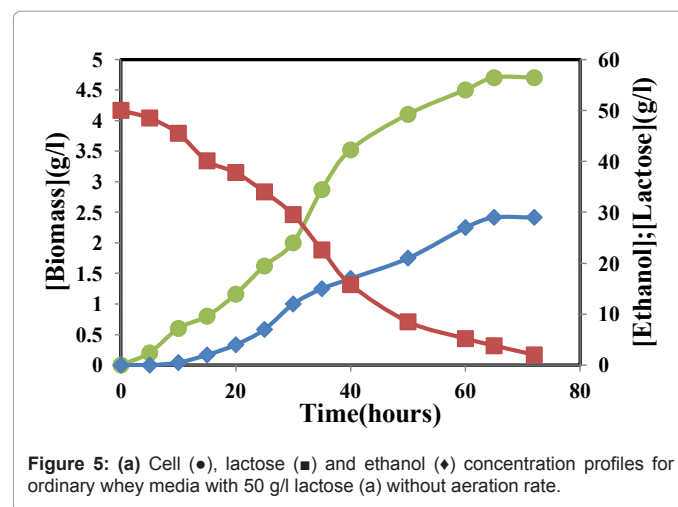**Figure 3:** (a) Response surface as a function of pH and yeast cells concentration (Y).
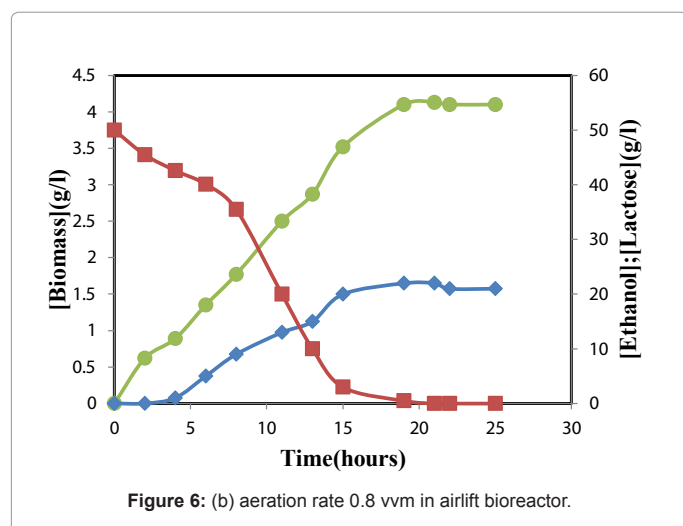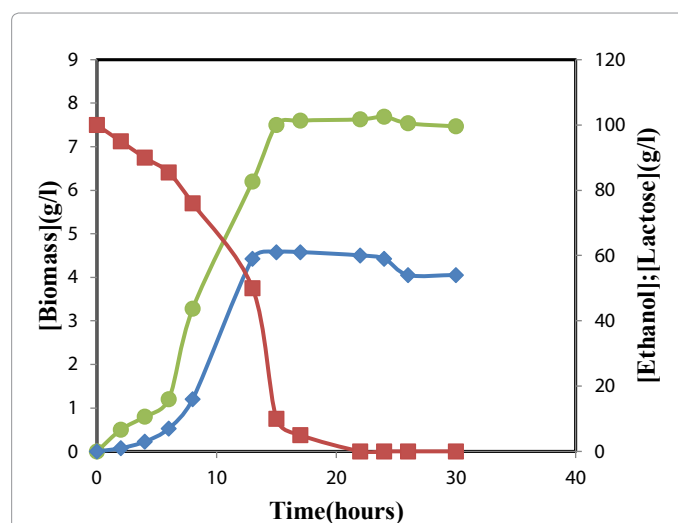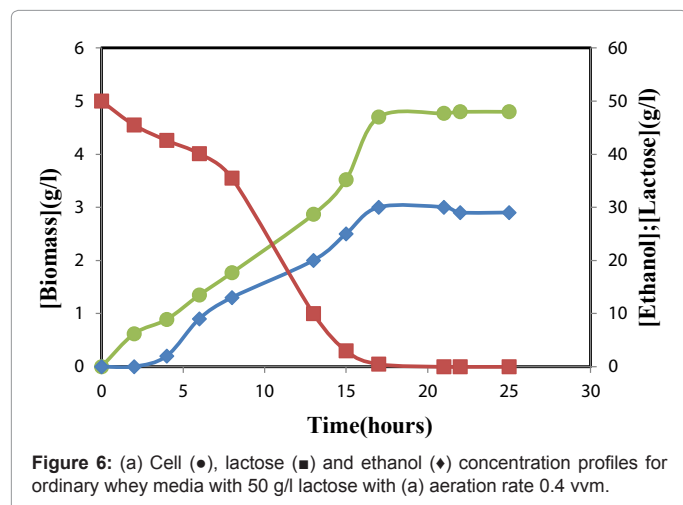
Maximum ethanol production was recorded in the middle levels of both the factors while further increase in the levels resulted in a gradual decrease in yield. It is evident that a pH value around 5.3 and T around

**Figure 3:** (b) Response surface as a function of lactose (L) and yeast cells concentration (Y), (the other variables were fixed at middle level).



**Figure 4:** (a) Response surface as a function of lactose (L) and temperature (T).



**Figure 4:** (b) Response surface as a function of yeast cells concentration(Y) and temperature (T), (the other variables were fixed at middle level).
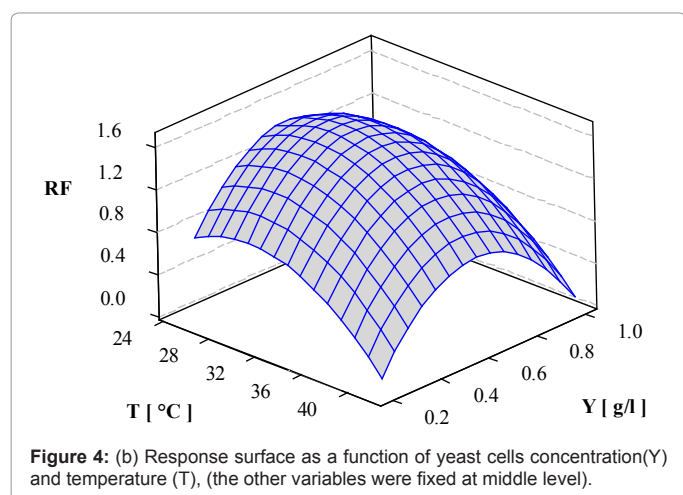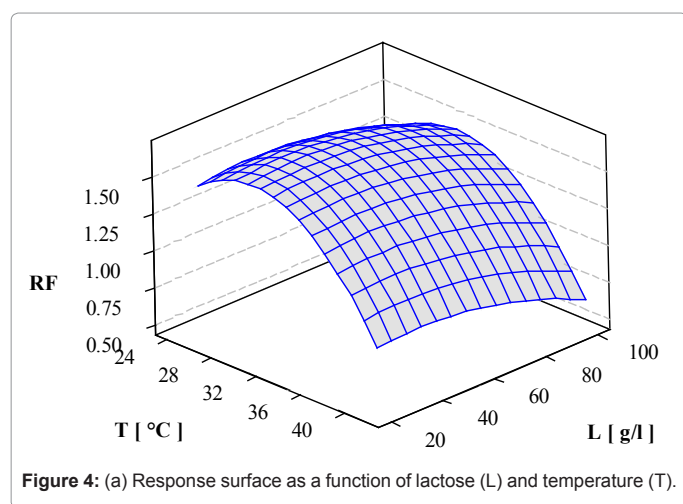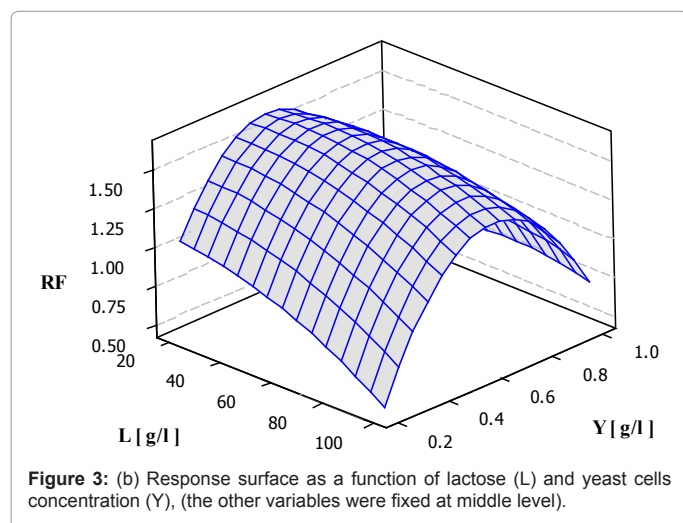
30.8°C improves the fermentation process. Results presented in Figure 3a shows the interaction between pH and Y; the best conditions were obtained at pH 5.3 and Y 0.57 g/l. The worst conditions were achieved at pH 3 and at a Y of both 0.2 and 1 g/l. Figure 3b showing the RF dependence on both L and Y, confirms that it should be advisable to use intermediate Y values and low L values. Figure 4a presents the response surface versus L and T; it strengthens the conviction that CW fermentation process is enhanced by relatively low T values. Factor L has a weak effect on the RF, even though better results were achieved with the lowest value of L, about 41.8 g/l. Figure 4b exhibits a strong response surface dependence on both Y and T; Moreover, a good system behavior corresponding to a RF of 1.4, is obtained at Y= 0.57 g/l and T=30.8°C. The RSM showed that the best set of operating conditions as following: pH= 5.3, L= 41.8 g/l, Y= 0.57 g/l and T= 30.8°C, with a predicted value of the RF of 1.69 (the optimization was strictly performed in the considered range of the factors). Figures 5 and figure 6 show the effect of aeration rate on the ethanol production and the time course of cell, lactose and ethanol concentrations, using ordinary whey media as substrate, in Erlenmeyer and airlift bioreactor cultures. Figure 5a Shows the maximum amount of alcohol produced in non-aerated conditions in Erlenmeyer, after 65 hours from when fermentation started, is 2.9 (w/v) percent. Figure 5b shows that ethanol production is 2.9 (w/v) percent after 36 hours for aeration rate of 0.1 vvm in airlift bioreactor. Comparing these two figures shows that amount of ethanol productions are as the same clearly but the duration time of figure 5b to reach the maximum amount of alcohol is shorter, because the aeration operation makes the yeast growth faster. Figure 6a shows the ordinary whey media fermentation with 0.4 vvm aeration rate in the bioreactor. The maximum amount of alcohol production after 17 hours was obtained 3 (w/v) percent. Figure 6b shows that the



**Figure 5: (a)** Cell (●), lactose (■) and ethanol (♦) concentration profiles for ordinary whey media with 50 g/l lactose (a) without aeration rate.



**Figure 5:** (b) with aeration rate of 0.1 vvm.

**Figure 6:** (a) Cell (●), lactose (■) and ethanol (♦) concentration profiles for ordinary whey media with 50 g/l lactose with (a) aeration rate 0.4 vvm.



**Figure 6:** (b) aeration rate 0.8 vvm in airlift bioreactor.



**Figure 7:** Cell (●), lactose (■) and ethanol (♦) concentration profiles for concentrated whey media with aeration rate 0.4 vvm and lactose 100 g/l.

lactose concentration (L) yeast cells concentration (Y) and temperature (T) were estimated. After having accomplished the ANOVA test on the complete quadratic model, all the negligible effects were removed in order to improve the model predictive performance. A response surface quadratic model was obtained as a function of the only significant effects, pH, L, T, $pH^2$ and $T^2$. On the other hand, an adjusted $R^2$ value of 0.92, testified a good model correlation performance. The optimization showed that the best set of operating parameters to operate the fermenter was 5.3 for pH, 41.8 g/l for initial lactose concentration, 0.57 g/l for yeast cells concentration and 30.8°C for temperature. Also in this research the laboratory production of ethanol by means of whey has been accomplished in an airlift bioreactor. The major aim of this stage is determining the effect of aeration rate on the fermentation process in the airlift bioreactor (7L) and measuring the variation of ethanol, lactose and cell (biomass) concentration with time. Experiments at this stage in four cases, the fermentation in normal whey media with 50 g/l lactose without aeration rate in the Erlenmeyer flask and with aeration rate of 0.1, 0.4 and 0.8 vvm in airlift bioreactor were conducted and compared together. The best conditions were the aeration rate of 0.4 vvm with 89.28% of ethanol production yield (Alcohol production was 3 (w/v) percent). It was 90.7% at the optimal conditions in concentrated whey media with 100 g/l lactose.

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highest value of alcohol production is 2.2 (w/v) percent with 0.8 vvm aeration rate in the nineteenth hours of the fermentation time. As can be seen with the increase of aeration rate of 0.1 to 0.4 vvm the amount of alcohol remained approximately constant, but the duration time to reach the maximum amount of alcohol was lower and with further increase of aeration rate of 0.4 to 0.8 vvm the alcohol production rate was decreased, because the fermentation process was anaerobic in nature but the yeast to grow needed a small amounts of oxygen and the excess of the required was reduced the rate of production. The optimum aeration rate for alcohol production is 0.4 vvm. Figure 7 illustrates that the amount of alcohol produced is 6.1 (w/v) percent in concentrated whey media of 100 g/l lactose and 0.4vvm aeration rate. The use of yeast extract (5 g/l), peptone (5 g/l), $NH_4Cl$ (2 g/l), $KH_2PO_4$ (1 g/l), $MgSO_4.7H_2O$ (0.3 g/l) and lactose (50 g/l) increased amount of alcohol considerably (Approximately 3 percent).

### Conclusions

Central composite design and response surface methodology can be used for the purpose of finding the maximum production of ethanol by *Kluyveromyces fragilis*. This design based on the analysis of 25 experiments, involving the anaerobic fermentation of lactose contained in cheese whey, was performed. The effects of four factors, pH and initial

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