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Full Length Research Paper

Lactic acid fermentation from refectory waste: Factorial design analysis

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A factorial experimental design method was used to optimize the lactic acid production using *Lactobacillus bulgaricus* from refectory waste obtained from Istanbul Technical University mess hall, Turkey. Fermentation experiments were carried out in a batch type reactor system which contains refectory waste with *Lactobacillus bulgaricus* bacteria during an exposition time of 30 h. Factorial design of experiments was employed to study the effect of three factors namely temperature (30 and 45°C), substrate concentration (10 and 60 g/L) and pH (4.0 and 6.0) at two markedly different levels. The main effects and interaction effects of the three factors were analysed using statistical techniques. A regression model was recommended and it was found to fit the experimental data very well. The results were analysed statistically using Student's t-test, and analysis of variance was used to define the most important process variables affecting the production of lactic acid by fermentation. In the present study, the most significant factor affecting lactic acid fermentation was found to be the initial substrate concentration.

Key words: Refectory waste, lactic acid, factorial design, *Lactobacillus bulgaricus*.

INTRODUCTION

Lactic acid is an organic acid (α-hydroxy-propionic acid) used for a wide variety of industrial applications. In food industry, it is used as an acidulant, a preservative and an antimicrobial agent. For pharmaceutical applications, lactic acid can be used as electrolytes and mineral sources. For technical applications lactic acid can be used as neutralizers, solvents, cleaning agents, slow acid release agents and metal complexing agents. It has also been used in cosmetic industry as pH buffer, antimicrobial, skin rejuvenating and skin lightening. A large number of carbohydrate materials have been used, tested or proposed for the manufacture of lactic acid by fermentation (Vick Roy, 1985). There are two isomers of lactic acid, these are D(-) and L(+) forms, which differ only in their optical properties, but are identical in their physical and chemical characteristics. L(+)-Lactic acid is biodegradable and can be metabolized by the human body and this property has resulted in the application of lactic acid in biomaterial and biomedical field (Hunger, 1984).

Lactic acid is produced by chemical synthesis and by microbial fermentation. By chemical synthesis method, racemic mixture of lactic acid is produced, while by microbial fermentation method L(+) and D(-) lactic acids can be produced according to the type of microorganism which may be homofermentative or heterofermentative. This is an important advantage of the microbial fermentation method compared to the chemical synthesis method. At the end of the fermentation process, lactic acid exists in the complex medium of fermentation broth that contains whey proteins, biomass, salts and other impurities. Lactic acid is then recovered from this complex medium. Since the high cost of lactic acid purification process limits the utilization of this chemical, in a large scale application, a system with less raw material and fewer unit operations are needed (Narayanan et al., 2004).

Sucrose from cane and beet sugar, whey containing

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lactose, and maltose and dextrose from hydrolysed starch are presently used commercially for lactic acid production. Over the years, authors have studied a large number of carbohydrates and nitrogenous materials for production of lactic acid. These have been investigated on the basis of high lactic acid yields, optimum biomass production, negligible by-product formation, fast fermentation rate, less pre-treatment, easy downstream processing, low cost, ease of availability etc. The choice of the raw material to be used depends on the microorganisms studied and also on the product desired. Sucrose, lactose, maltose, glucose, mannitol etc. have been commercially used (Narayanan et al., 2004).

Batch fermentations are widely used method for the production of lactic acid. Fermentation conditions are different for each industrial producer but are typically in the range of 45 to 60°C with a pH of 5.0 to 6.5 for *Lactobacillus delbrueckii*; 43°C and a pH of 6.0 to 7.0 for *Lactobacillus bulgaricus*. The acid formed is neutralized by calcium hydroxide or calcium carbonate. The fermentation time is 1 to 2 days for 5% sugar sucrose such as whey and 2 to 6 days for a 15% sugar source such as glucose or sucrose. Under optimal laboratory conditions the fermentation takes 1 to 2 days. The yield of lactic acid after the fermentation stage is 90 to 95 wt% based on the initial sugar or starch concentration (John et al., 2006). The fermentation rate depends primarily on the temperature, pH, concentration of nitrogenous nutrients, and initial substrate concentration. The undissociated, electroneutral form of lactic acid rather than lactate appears to be the components which inhibits the fermentation (Vick Roy, 1985).

In the present study, lactic acid was produced from refectory waste, which was collected from Istanbul Technical University mess hall, through fermentation process and convenient conditions were optimized for the production of the highest lactic acid yield via experimental design analysis. The most important factors for the production of lactic acid using fermentation process are the substrate concentration, pH and temperature. Factorial design of experiments was employed to study the effect of these three factors on the lactic acid production. Also, this work represents the first in the literature to follow the factorial experimental design of lactic acid production using *Lactobacillus bulgaricus* from refectory waste.

MATERIALS AND METHODS

Microorganism and culture conditions

L. bulgaricus (DSMZ 20081) was used through the study. The microorganism was maintained on De Man, Rogosa and Sharpe (MRS) agar plates at 4°C and sub-cultured every 15 days. Cells for inoculation of the production medium at a level of 10% (v/v) were obtained from cultures grown on MRS broth (pH 7.0) at 37°C for 24 h in the incubator and kept at 4°C in the refrigerator. Twenty-four hour (24 h) old fresh cultures were used as the inoculum for the fermentations.

Composition of refectory waste

Reducing sugar and total carbohydrates of refectory waste was estimated by dinitrosalicylic acid and phenol sulphuric acid methods, respectively. Physiochemical properties of refectory waste were determined using standard protocols. The pH of the sample was determined using a digital pH meter.

Lactic acid fermentation

Batch experiments were performed in a temperature-controlled incubator shaker operated at 160 rpm, at 37°C. The shake flasks were 250 ml Erlenmeyer flasks containing 100 ml of refectory waste as fermentation medium (initial pH 6). Unless otherwise indicated, refectory waste was dissolved to attain 58 g/L of initial sucrose concentration and supplemented with (g/L) yeast extract (10), K_2HPO_4 (0.5), KH_2PO_4 (0.5), $MgSO_4$ (0.2) and $MnSO_4.H_2O$ (0.05). Refectory waste medium (pH 6.0) and all salt solutions were sterilized separately at 121°C for 15 min. Sterile $CaCO_3$ (10% (w/v) of the initial sucrose concentration) was added to the medium to neutralize the acid. The shake flasks were inoculated aseptically with 30-h-old fresh culture propagated in medium at 30 and 45°C (Mel et al., 2008).

Lactic acid determination

Lactic acid concentrations were analysed by high performance liquid chromatography (HPLC). The HPLC system was composed of Agilent 1100 Series. The mobile phase was 5 mM H_2SO_4 for Aminex HPX-87H column. Mobile phase was filtered through 45 µM cellulose acetate filter papers after solution preparation (Okano et al., 2009).

Experimental design and statistical analysis

In the optimization studies using factorial design analysis, initial substrate concentration, pH and temperature were varied as parameters, while the levels of other medium components were kept constant. The statistical analysis of the data was performed using Minitab Statistical Software (Release 14). In this design, there were two experimental levels (-1, +1) where –1 and +1 corresponded to low level and high level of each variable, respectively (Kotzamanidis et al., 2002).

RESULTS

Composition of refectory waste

Samples of waste collected from Istanbul Technical University mess hall were analysed for moisture, ash, protein, total reducing sugar and total carbohydrates, nitrogen and protein contents (Table 1). The refectory waste contained mainly carbohydrate components. Total carbohydrate (total reducing sugar + sucrose) and total reducing sugar content of the refectory waste were confirmed as 61.38 and 0.47% respectively. During the inversion of 1 mole of sucrose, it reacts with 1 mole of water and 95 g of sucrose to produce 100 g of reducing

Table 1. Composition of refectory waste.

Composition of medium	Percentage (%)
Moisture content	7.50
Total Ash	5.68
Total reducing sugar	0.47
Total carbohydrate	61.38
Nitrogen	2.85
Protein	10.41

Table 2. Levels of factors.

Factor	Low (-1)	High (+1)
pH	4.0	6.0
Temperature, T (°C)	30	45
Initial substrate concentration, S_0 (g/L)	10	60

Table 3. Experimental factorial design results for lactic acid.

Factor			Produced lactic acid (g/L)		
T	S_0	pH	S_F1	S_F2	S_F3
-1	-1	-1	8	6	6
+1	-1	-1	13	10	17
-1	+1	-1	20	18	22
+1	+1	-1	29	32	30
-1	-1	+1	15	12	13
+1	-1	+1	24	18	23
-1	+1	+1	27	25	29
+1	+1	+1	43	47	40

T, Temperature; S_0, ınitial substrate concentration.

sugar. From this principle, sucrose content of the refectory waste was calculated as difference between total carbohydrates and reducing sugar multiplied by 0.95 and sucrose content and was estimated at 58 g/L.

The moisture content of refectory waste was found in the range of 5 to 10%, the nitrogen content in waste was 2.85% and ash content at range 5 to 6%. The lactic acid bacteria require substrates with high nitrogen content during fermentation. The nutrients were added in the form of malt sprout, corn steep liquor, and yeast extract. Lactic acid production increased with the concentration of the supplement especially yeast extract. The highest production rate was found with addition of 5 to 15 g/L yeast extract (Lund et al., 1992). Although the refectory waste contained very little nitrogen, this concentration was adequate for lactic acid bacteria growth in the present study.

Experimental analysis

The results were analysed using Minitab 14 for windows. The main effects and interaction between factors were determined. The effect of a factor was in the change in response and production of lactic acid by a change in the level of a factor, pH, temperature and initial substrate concentration from lower to higher level (Table 2). Eight experiments were carried out and each of them was replicated three times. All possible combinations of factors were used and a matrix was established according to the high and low levels represented by +1 and -1 respectively (Table 3).

The main effects represented deviations of the average between high and low levels for each one of them. When the effect of a factor was positive, production of lactic acid increased as the factor was changed from low to high levels. The results were analysed using the software for a 95% confidence level ($\alpha = 0.05$) and main effects and interactions between factors were examined. The effects, regression coefficients, standard errors and p were shown in Table 4. The mathematical model employed for the 2^3 factorial design was:

$$S_F = A_0 + A_1*T + A_2*S_0 + A_3*pH + A_4*T*S_0 + A_5*T*pH + A_6*S_0*pH + A_7*T*S_0*pH \quad (1)$$

Table 4. Statistical parameters for 2^3 design.

Term	Effect	Coefficient	Standart error	t-statistic	p
Constant	21.9583	21.9583	0.5052	43.47	0.000
T	10.4167	5.2083	0.5052	10.31	0.000
S_0	16.4167	8.2083	0.5052	16.25	0.000
pH	8.7500	4.3750	0.5052	8.66	0.000
T*S_0	2.9167	1.4583	0.5052	2.89	0.011
T*pH	1.9167	0.9583	0.5052	1.90	0.076
S_0*pH	1.2500	0.6250	0.5052	1.24	0.234
T*S_0*pH	1.0833	0.5417	0.5052	1.07	0.300

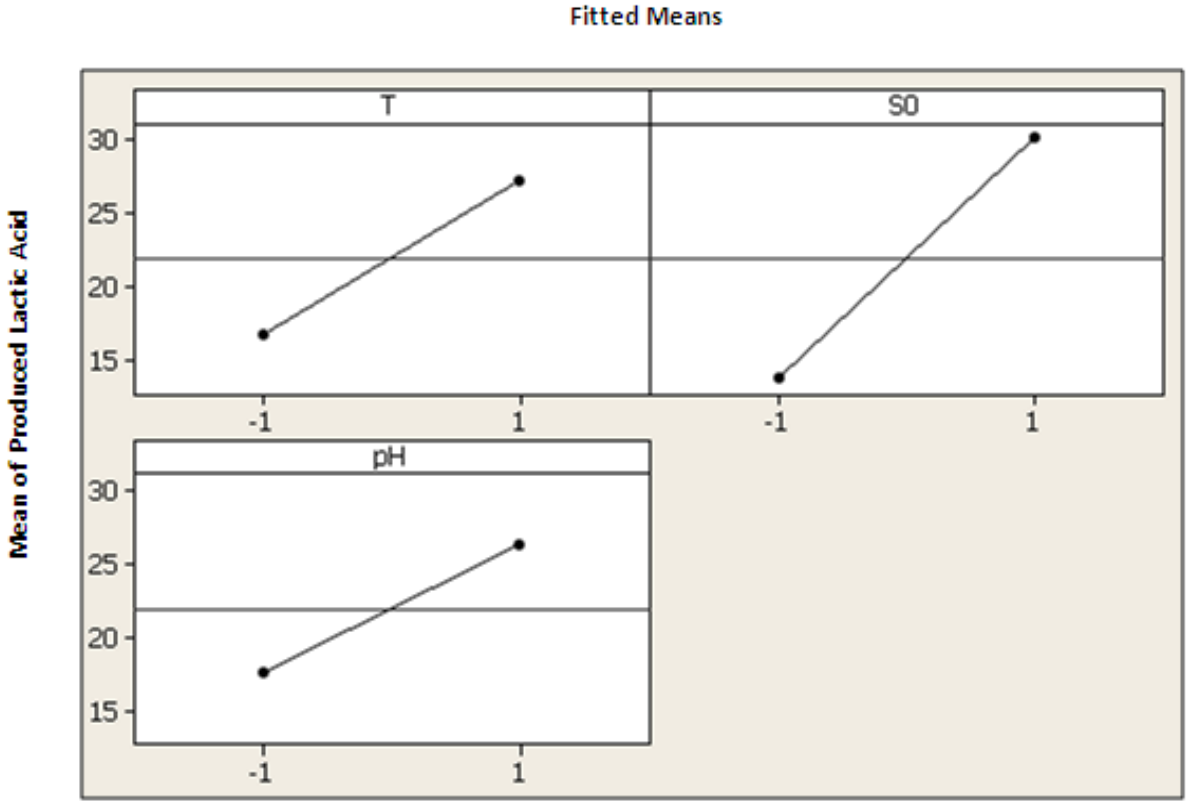

Figure 1. Main effects plot for produced lactic acid. T, Temperature; S_0, ınitial substrate concentration.

Where A_0 represents the global mean and A_i represents the other regression coefficients. Substituting the coefficient A_i in Equation (1) by their values from Table 4 we got model equation:

$$S_F = 21.9583 + 5.2083T + 8.2083S_0 + 4.3750pH + 1.4583T*S_0 + 0.9583T*pH + 0.6250S_0*pH + 0.5417T*S_0*pH \quad (2)$$

Equation (2) presented that effects of all factors were positive and results in an increase in the value of the produced lactic acid. The main effects of the control factors were presented in Figure 1. A horizontal line (parallel to the *x*-axis) indicated that no main effect was present (the control factor does not influence the objective function). If the line was not horizontal, there could be a main effect present and in this case the control factors influence the objective function. The greater the slope of the line, the stronger the effect produced.

Student's t-test

The Pareto chart (Figure 2) gave the relative importance of the individual and interactions effect. Student's t-test

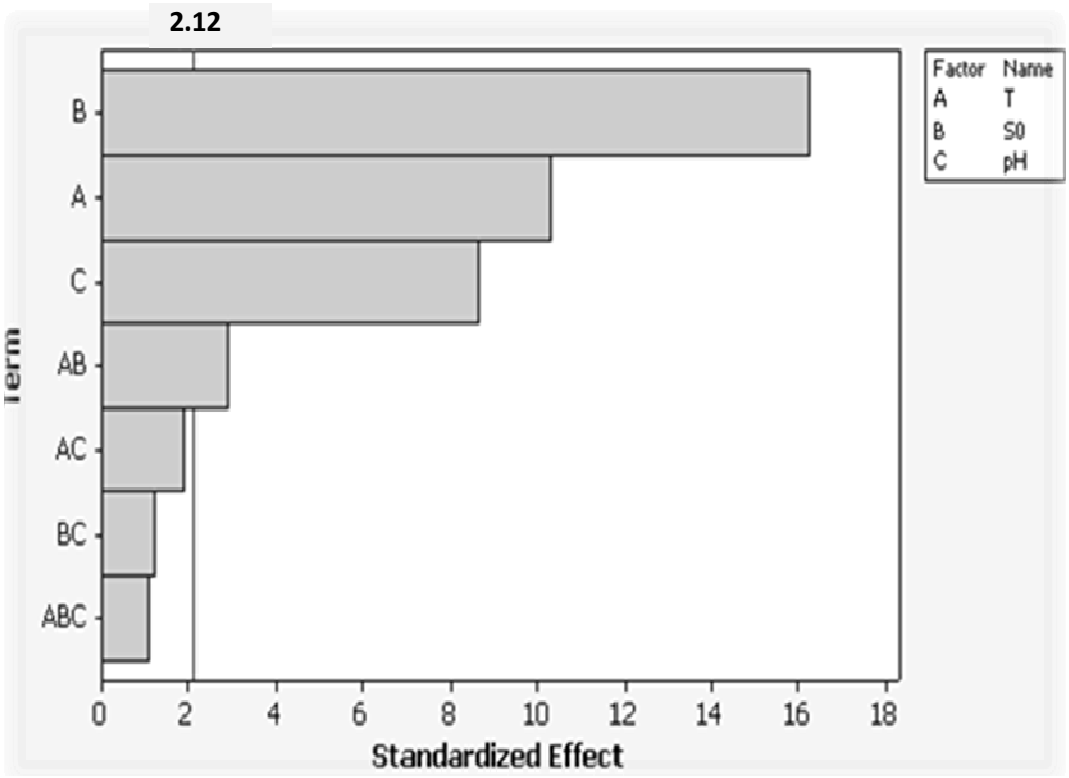

Figure 2. Pareto chart of standardized effects on the produced lactic acid concentration. T, Temperature; S_0, ınitial substrate concentration.

Table 5. Analysis of variance.

Source	Degrees of freedom	Sum of square	Mean square	F	p
Main effects	3	2727.46	909.153	148.43	0.000
2-way interactions	3	82.46	27.486	4.49	0.018
3-way interactions	1	7.04	7.042	1.15	0.300
Residual error	16	98.00	6.125		
Pure error	16	98.00	6.125		
Total	23	2914.96			

$R^2 = SS_{MODEL}/SS_{TOTAL}$; $R^2_{adj} = 1 - [(SS_{ERROR}/DF_{ERROR})/(SS_{TOTAL}/DF_{TOTAL})]$; R^2 = % 96.64 $R^2_{(adj)}$ =% 95.17.

was performed in order to determine whether the calculated effects were significantly different from zero and these values for each effect were shown in Pareto chart by horizontal columns. In our case, all control factors with a significant influence were located over the line marked at 2.12 (p = 0.05). The vertical line in the chart indicated the minimum statistically significant effect magnitude for 95% confidence level.

Analysis of variance (ANOVA)

In Table 5, the sum of squares used to estimate the factors' effects and F ratios are shown. It can be said that R^2 and $R^2_{(adj)}$ values which were important due to test obtained mathematical model were close to each other and approximately 1.0 (R^2 = 96.64%, $R^2_{(adj)}$ =95.17%) that of expected result statistically. Another important aspect was the interaction among the control factors. The interaction between the control factors can be estimated from experimental design and the results were presented in Figure 3. If the lines were parallel to each other, there was no interaction present. With the increase of the deviation degree of line from being parallel, the interactions among control factors increase. The residues were also examined for normal distribution. Figure 4 shows the normal probability plot of residual values. It could be seen that the experimental points were

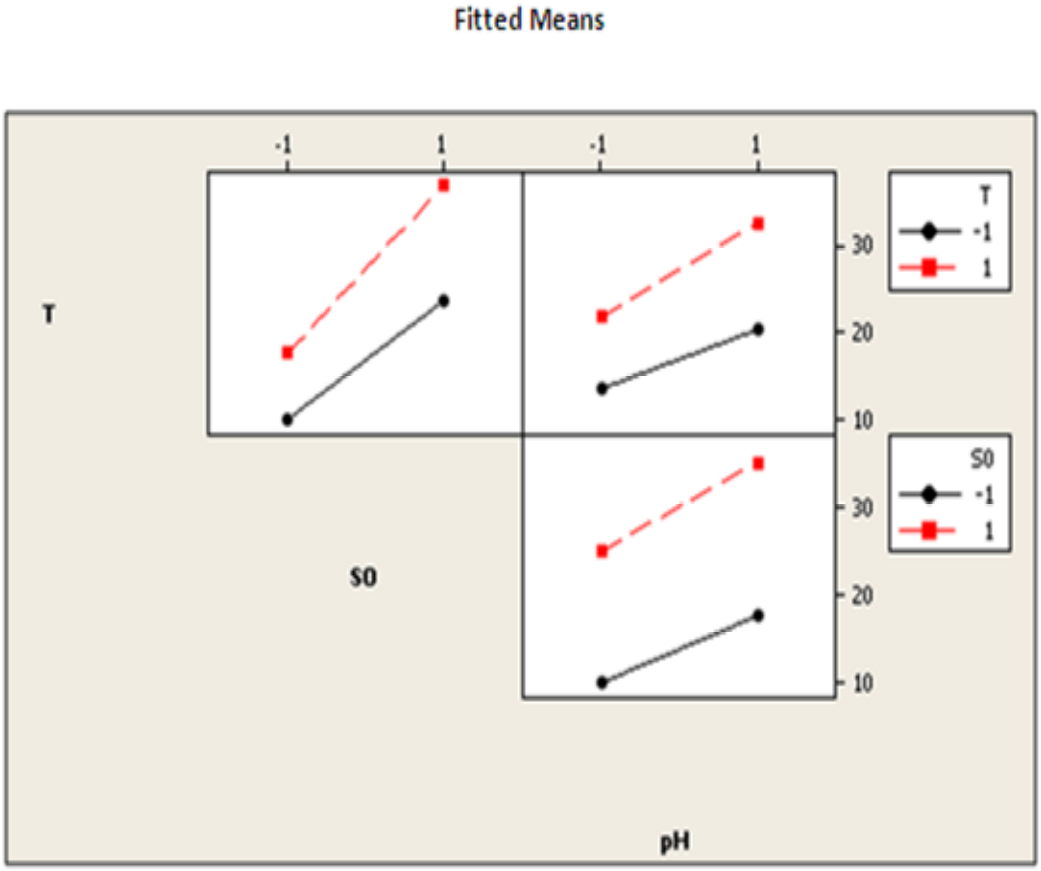

Figure 3. Interaction effects for produced lactic acid. T, Temperature; S_0, ınitial substrate concentration.

reasonably aligned, thus suggesting normal distribution.

DISCUSSION

Lactic acid starters are currently produced using pH controlled pure cultures (Beal et al., 1989), during which pH is generally regulated at an optimal value by continuously adding sodium hydroxide or ammonia in the bioreactor (Savoie et al., 2007). Various growth characteristics such as maximal biomass concentration, specific growth rate, fermentation time, substrate consumption and product yields are influenced by the pH value (Adamberg et al., 2003). Optimal pH ranges were therefore determined for several lactic acid bacteria, such as *Streptococcus thermophilus* (pH 6.5), *Lactococcus lactis* subsp. *cremoris* (pH 6.3 to 6.9) and *L. bulgaricus* (pH 5.8 to 6) (Beal et al., 1989). The effect of initial pH on the cell growth of *L. bulgaricus* during the fermentation of refectory waste was investigated and optimized in the present study.

According to the experimental results, at the initial pH of 4.0, the bacteria exhibited a prolonged lag phase and bacteria did not grow as well as at higher initial pH value. Moreover, as the initial pH increased above 4.0, the cell growth increased, however, until up to a certain limit. Beyond initial pH 6.5, its growth rate decreased again. Therefore, the optimal initial pH for the refectory waste fermentation of *L. bulgaricus* was 6.0, which is similar to those reported by Goksungur and Guvenc (1997) by using beet molasses as a substrate. Various researchers have studied the effect of pH on lactic acid production and found that the optimum pH for lactic acid production is in the range of 5.0 to 7.0 (Hofvendahl and Hagerdal, 2000; Goksungur and Guvenc, 1997). Goksungur and Guvenc (1997) showed that the effect of pH on lactic acid production was important and the optimal pH was 6.0 with the yield value 79%. That is why when the statistical analysis of the data was performed using Minitab, the levels of pH factors used in the experimental design were chosen as pH 4.0 and 6.0 (Table 2). Also, temperature is one of the most important environment factors that effect the lactic acid production. Various studies on the effect of temperatureon the lactic acid production have reported an optimal temperature between 40 to 45°C (Hofvendahl and Hagerdal, 2000). Goksungur and Guvenc (1997) reported that the optimal temperature was 45°C and this might be due to the different substrates used in the lactic acid fermentation. Maximum yield obtained with 53.61 g/L of lactic acid when the temperature was 45°C, and the

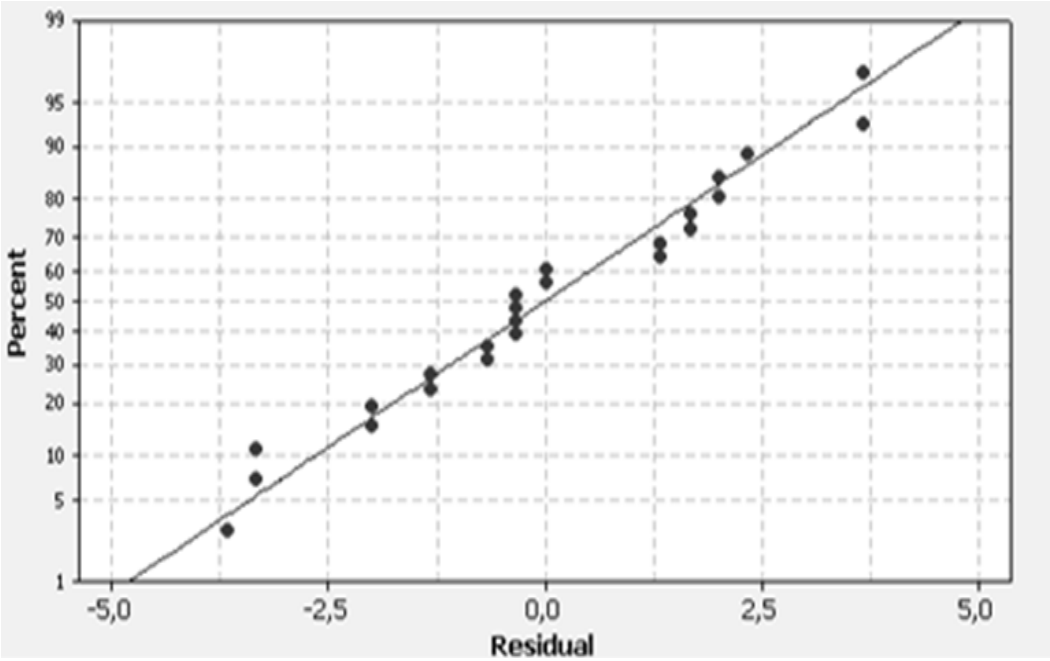

Figure 4. Normal probability plot of residual values for concentration of lactic acid vs. their expected values when the distribution is normal.

lactic acid production decreased rapidly to 25.14 g/L *Lactobacillus helveticus* used in a temperature range of 35°C. For similar reason the causes of choosing the pH levels, temperature levels were chosen as 30 and 45°C (Rakın et al., 2004).

In the present study, the Pareto chart showed that substrate concentration has a highly significant effect on the produced lactic acid concentration (Figure 2). Additionally, for initial substrate concentration at 60 g/L, concentration of lactic acid was estimated as 20 g/L. This value was considerably higher than the obtained value using initial substrate concentration as 10 g/L (Table 3); this situation was considered ideal in batch type fermentations. It was observed that as the initial substrate concentration increased to a very high point, production of lactic acid decreased clearly (results were not given), a phenomenon that can occur by substrate inhibition, product inhibition or exhaustion of one restricting nutrient or their combined effect. It was reported that in batch type, lactic fermentations have varying substrate concentrations from 20 to 100 g/L; the results for lactic acid concentrations and sucrose conversion obtained were similar to those reported in this study. It was also reported inhibition by substrate in fermentations occur using *Lactobacillus casei* NRRL B-441, and varying the glucose concentration between 80 and 160 g/L (Hujanen et al., 2001). According to these results, the effects of the high and low levels for the initial substrate concentration were chosen as 10 and 60 g/L in paralleling to the other variables.

It was observed that initial substrate concentration (S_0) whose value was 16.4167, was the most significant effect on the production of lactic acid. After that respectively T and pH main effects and $T*S_0$ binary interaction presented the statistical significance. Other binary and trio interactions were not statistically significant: T*pH, S_0*pH and $T*S_0$*pH. Similar results were obtained when p values were evaluated. From the p value which was defined as the smallest level of significance leading to rejection of the null hypothesis, it appears that the main effect of each factor and the interaction effects were statistically significant when $p<0.05$. So, it can be said that the main effects of T, S_0, pH, and interaction effect $T*S_0$ were statistically significant. On the other hand, p values of T*pH, S_0*pH and $T*S_0$*pH interaction effects were higher than 0.05, so these interaction effects were not statistically significant (Table 4).

The effects of the all variables (pH, temperature and initial substrate concentration) and their interactions on the formation of the lactic acid production were illustrated with analysis of variance (Table 5). The goodness of fit of the analysis of variance model was checked by the determination coefficient (R^2). In this case, the value of the R^2 (0.9664) for ANOVA indicates that the sample variation of nearly 97% for lactic acid was attributed to the independent variables and only 3% of the total variation could not be explained by the model. The value of the adjusted determination co efficient (R^2_{adj} = 0.9517) was also high, which stressed the significance of the model. The high value of R (0.9664) demonstrated a high degree of agreement between the experimental obser-vations and predicted values.

Conclusion

In this study, important process variable factors which affect fermentative lactic acid production were determined using a factorial experimental design technique. The results of statistical study clearly showed that initial substrate concentration was the most important parameter. Moreover the main effect of temperature, pH and interaction between temperature-initial substrate concentrations had a considerable effect on the amount of lactic acid produced. Other two-way and three-way interactions did not exhibit any statistical significance.

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