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ORIGINAL ARTICLE

Optimization of fermentation condition for production of lactic acid from khat ("*Catha edulis*") waste by using immobilized *Lactobacillus plantarum*

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Abstract
Production of lactic acid from lignocellulosic biomass is becoming more popular as a way to solve the problem associated with first-generation biomass. In the present study, the optimum fermentation parameters for maximum lactic acid production from khat waste biomass by using immobilized *Lactobacillus plantarum* were investigated. The effect of immobilizing the cell on the yield of lactic acid has been investigated, and the yield obtained from immobilized *Lactobacillus plantarum* cell is 32.78% higher than the free cells. The effects of three process parameters (incubation temperature, pH, and incubation time) on lactic acid yield were investigated. The optimization of lactic acid yield and fermentation parameters was done via response surface methodology with a central composite design. Based on the analysis, the optimum fermentation parameters were found to be incubation temperature of 42.3 °C, incubation time of 40.0 h, and pH of 6.18. Under these conditions, the maximum lactic acid yield obtained was 23.05 g/L. The experimental yield of lactic acid (22.98 ± 0.10 g/L) is in smooth agreement with the predicted one (23.05 g/L), indicating the fitness of the quadratic model used. Generally, the findings of the study suggest that utilization of khat waste as a potential carbohydrate source and immobilization of *Lactobacillus plantarum* cells for lactic acid production is worthful.

Keywords Khat waste · Immobilized *Lactobacillus plantarum* · Fermentation · Lactic acid

1 Introduction

Nowadays, researchers have been focusing on the sustainable management of plastic wastes on earth by finding eco-friendly alternative resources for the production of plastics. As an alternative to petroleum-based plastics, the production of bioplastics is gaining popularity in front of many researchers. The findings of many studies conducted so far revealed that products with similar performance can be achieved by using biomass feedstocks instead of fossil-based resources [1, 2]. Therefore, the production of bioplastic has been attracting the idea of researchers and governments.

Recently, several bioplastic products have been researched, developed, and overgrown. The sustainability and biodegradability of these products increase their attractiveness. If disposed to the environment, they would be decomposed easily through the actions of microorganisms [3, 4]. These include several polyesters (e.g., polylactic acid and polyhydroxyalkanoates), numerous polypeptides (i.e., soy proteins, collagen, and gelatin), and some polysaccharides (i.e., starch, chitin, pectin) obtained from biological feedstocks. Among these, polylactic acid (PLA) is one of the most commercially successful bioplastic product [5]. As compared with most other biodegradable plastics, it has better mechanical strength, biocompatibility, and transparency. It is commonly produced from a lactic acid monomer via ring polymerization reaction [6].

Lactic acid (LA) is a potential building block for PLA synthesis. Furthermore, it has various applications in different sectors including cosmetic, pharmaceutical, food, polymer, and chemical industries [7]. The commercial LA production began in Japan in 1950 [8]. Nowadays, it is produced commercially either by chemical synthesis or

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microbial fermentation [9]. Chemical synthesis involves the derivation of LA from petrochemical products, which relies on fossil fuel depletion and generates the racemic LA mixture (D-L-LA) via lactonitrile hydrolysis. On the contrary, the microbial fermentation method explores the possibility of using renewable resources for the production of LA products. It generates L (+) or D (−) LA isomer via microbial fermentation based on the strain selected [9, 10]. Currently, as it averts the environmental pollution caused by the petrochemical industry and the inadequate supply of fossil-based feedstocks, the microbial fermentation method for LA production has gotten significant attention [11].

The LA production is strongly dependent on the raw material cost being utilized. Thus, raw material selections for its industrial productions need to take into account the following factors. These include availability, food conflict-tion, cost, rate of production, difficulty of pretreatment, and yield [12]. By compromising the above criteria, lignocellulose biomass is the preferred feedstock to meet the current demand for LA production [13]. Khat (*Catha edulis*) is a flowering evergreen plant. It belongs to the Celastrus family. The most favorite part of the leaves is the young shoot near the top of the plant. However, the other major component, ''khat garaba,'' is simply disposed to the environment and considered as waste. However, khat waste is lignocellulose biomass, which has enough amount of cellulose and hemicellulose [14, 15]. It is abundantly available in different cities of Ethiopia like Jimma, Aweday, Wolkite, Harar, Dire Dawa, Addis Ababa, etc. [16].

Moreover, the production of LA through the microbial fermentation method mainly depends on microbes (bacteria, fungi, and yeast) used for fermentation. As compared with bacteria, the yield and productivities of fungal and yeast strains are very low [17]. Among these bacteria, the most widely used microbes for LA productions are lactobacillus species. However, factors such as incubation temperature, incubation time, and pH are substantially affecting the metabolic process of the microbes. Therefore, optimizing the fermentation parameters is mandatory to find the significant factors affecting the metabolic process. The optimization done by response surface methodology has several advantages such as saving time, space, and raw material [18].

Hence, the current study has been carried out to optimize the fermentation process parameters (incubation temperature, incubation time, and pH) for maximizing the LA production by using immobilized *Lactobacillus plantarum* cell via response surface methodology–central composite design (RSM-CCD). Sodium alginate was used for immobilization. Khat waste was hydrolyzed and then fermented to LA. Hence, this study obsoletes the idea of khat waste and makes it a potential resource for the production of LA.

2 Methodology

Khat (*Cathus edulis*) waste was obtained from different areas of chewing places in Jimma, Oromia, Ethiopia. Sodium alginate was procured from Atomic Educational Material Supply PLC in Addis Ababa, Ethiopia. All other chemicals have been procured from Fine Chemicals Supply PLC in Addis Ababa, Ethiopia.

2.1 Sample preparation for hydrolysis process

First, 1000 ml of the conical flask was washed and dried. Then, a 100 g of ground sample was added to the flask and mixed with 1000 ml distilled water. This was the sample that was ready for the subsequent process (i.e., hydrolysis process).

2.2 Acid hydrolysis process

The prepared sample was transferred to another 1000-ml conical flask. Then, 50 ml of 2.75% (v/v) of H_2SO_4 was added to the prepared sample and soak for 24 h to mix it well. The mixed sample was placed in the autoclave reactor at 105 °C for 1 h.

2.3 Neutralization process

Since the hydrolyzation process was carried out by sulfuric acid, the pH of the hydrolyzed sample is too low (pH = 4.5). Then, 10 M of NaOH was prepared for neutralization process. The sample was neutralized by adding the prepared solution of NaOH in dropwise until it was neutralized.

2.4 Centrifuge separation

The sample taken from hydrolysis had some insoluble solid parts, which should be separated before it was taken to the fermentation process. Thus, the mixture of the sample was fed to the tube of a centrifuge. After that, the samples were separated according to their phase in centrifuge with 450 rpm for 30 min. Finally, the liquid part was sent to the fermenter.

2.5 Sugar content determination

The concentration of glucose in the hydrolyzed sample was determined by using a digital UV spectrophotometer method [19, 20]. A quantitative standard glucose solution was used

to plot the calibration curve, and the glucose concentration of hydrolyzed sample was determined from it.

2.6 Microorganism

Lactobacillus plantarum was procured from the Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia. The cell was placed in freeze (−4 °C) while waiting for the preparation of MRS medium. The prepared MRS broth was sterilized in an autoclave reactor at 120 °C for 10 min. The collected *microbe* was cultured for 36 h at 37 °C in the prepared medium.

2.7 Lactobacillus plantarum immobilization

The immobilization of *Lactobacillus plantarum* was accomplished through the technique used by [7, 18]. An inoculated *Lactobacillus plantarum* was taken to the petri-dish. Then, 3% (w/v) of sodium alginate and 1.5% (w/v) calcium chloride solution was prepared with deionized water. The prepared solution was added to the cultured cells $\left(5.72 \times 10^7 \frac{\text{CFU}}{\text{ml}}\right)$ respectively. Finally, the immobilized *Lactobacillus plantarum* was collected and transferred to the prepared MRS broth and kept in incubator with shaker at 37 °C, 150 rpm, and 36-h operating conditions.

2.8 Fermentation

The lactic acid fermentation of the hydrolyzed khat waste was carried out by the *Lactobacillus plantarum*. The fermentation medium contained peptone 10.0 g/L, meat extract 10.0 g/L, yeast extract 5.0 g/L, Tween-80 1.0 g/L, K2HPO4 2.0 g/L, MgSO4.7H2O 0.2 g/L, sodium acetate 5.0 g/L, triammonium citrate 2.0 g/L, and MnSO4.4H2O 0.05 g/L, with specific concentration of hydrolyzed khat waste (glucose) 13.84 g/L. Production media was prepared in a 500-ml conical flask. Ten percent v/v of the inoculum was added in conical flasks containing 500 mL of production medium [21]. The fermentation process was carried out at 150 rpm, with their respective incubation temperature (30, 37.5, 45) °C, incubation time (10, 29, 48) hrs, and pH (5, 6, 7) in an incubator with a shaker. Moreover, the effect of cell immobilization on the yield of lactic acid was determined at different incubation time for 48 h with time intervals of 12 h. Hence, the fermentation process with free cells was carried out under operating conditions of agitation rate of 150 rpm, incubation temperature of 37.5 °C, and pH of 6.

2.9 Design of experiment

RSM-CCD was used for optimizing the three factors with two levels. The factors with their levels were tabulated as shown in Table 1.

The RSM-CCD contains 2^K, n_o, and 2 K factorial, center, and axial runs respectively. Thus, the total experimental runs (N) could be computed as follows:

$$N = 2^k + 2k + n_o \tag{1}$$

Hence, in the current study, the required experimental run is

$$N = 2^3 + 2 * 3 + 6 = 20$$

Generally, as shown in Table 2, twenty experimental runs having six center points were observed. The RSM provides the empirical relationship between the response function and parameters. The quadratic response surface model consists of all linear, quadratic, and linear interaction terms. Mathematically it was written as Eq. (2):

$$Y = \beta_O + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_i}^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \tag{2}$$

where: Y = predicted response (i.e., LA content), β_0 = constant-coefficient, β_i = linear coefficients, β_{ii} = quadratic coefficients, β_{ij} = interaction coefficients and X_i and X_j were studied independent fermentation parameters.

2.9.1 Analytical method

Determination of the concentration of LA produced was done using the UV–visible spectrophotometry method [22, 23]. The calibration curve was plotted first from the known concentration, and the concentration of LA in the fermented sample was computed from it.

Table 1 Design factors with their lower, center, and higher value

Factor	Parameter	Unit	Code	Level		
				Low	Center	High
1	Incubation temperature	°C	A	30	37.5	45
2	Incubation time	hrs	B	10	29	48
3	pH		C	5	6	7

Table 2 The value of experimental and predicted data reports from RSM-CCD

	F-1	F-2	F-3	Response	
R. No	A: incubation temperature (°C)	B: incubation time (hrs)	C: pH	LA content (g/L)	
				Actual	Predicted
1	30	10	7	13.12	13.56
2	37.5	29	6	20.98	21.73
3	47.37	29	6	22.96	22.97
4	30	10	5	9.23	9.71
5	37.5	29	4.68	20.61	19.99
6	45	48	5	22.56	22.35
7	37.5	29	7.32	21.49	21.57
8	37.5	29	6	22.12	21.73
9	30	48	7	19.89	19.64
10	30	48	5	19.98	20.15
11	37.5	29	6	22.07	21.73
12	45	10	5	17.54	18.03
13	37.5	3.99	6	16.01	14.98
14	45	48	7	21.14	20.90
15	37.5	29	6	21.21	21.73
16	37.5	54.00	6	21.34	21.83
17	45	10	7	20.87	20.93
18	37.5	29	6	21.97	21.73
19	37.5	29	6	21.31	21.73
20	27.63	29	6	17.23	16.67

3 Results and discussion

3.1 Effect of Lactobacillus plantarum immobilization on lactic acid yield

As evident from Fig. 1, during the first 22 h, the LA yield obtained from immobilized *Lactobacillus plantarum* (9.65 g/L) is less as compared with the free cells (10.74 g/L). The reason behind this result is at the outset, and there is the limitation of mass transfer (i.e., product and substrate) through the bead in immobilized cells. The immobilized cell needs time for diffusion of substrate and product into the beads and from the bead respectively. However, as the incubation time increased to 36 h, the LA yield obtained from immobilized *Lactobacillus plantarum* (17.86 g/L) is substantially higher than the free cells (13.45 g/L), in percent about 32.78% higher. This indicates that an immobilized *Lactobacillus plantarum* has high fermenting efficiency and excellent stability over a long period of the fermentation process. Owing to its capacity to overcome the substrate (glucose) and product (LA) inhibitors, the cells could utilize the hydrolyzates of khat waste efficiently and produce a good amount of LA yield. On the contrary, as incubation

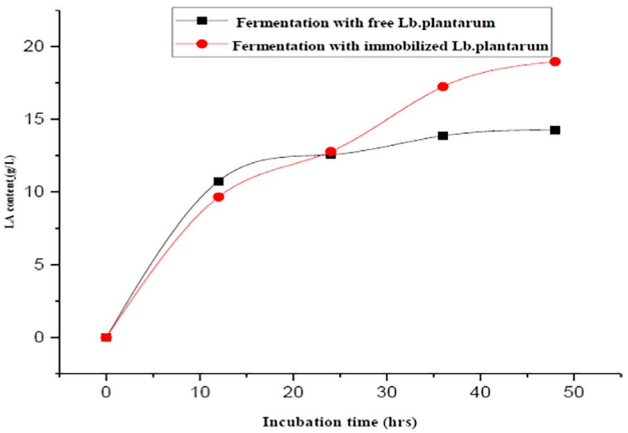

Fig. 1 Time profiles for LA fermentation by using *Lactobacillus plantarum* cell (at optimum: incubation temperature of 42.2 °C and pH of 6.18)

time increased, the free cells' fermenting efficiency was highly affected by different inhibitors, which consequently decreased the metabolic activities of cells, even up to death. Subsequently, the yield of LA obtained was low as compared to the immobilized cells.

Correspondingly, Thakur et al. [18] and Wang et al. [7] investigated a similar effect of cell immobilization on fermenting efficiency of different lactobacillus species.

They have investigated that an increment of incubation time from 20- to 42-h results in a substantial increment of the LA contents for immobilized *Lactobacilllus pentosus ATCC 8041* (60 to 105 g/L), whereas, in our study, for free cells, the LA contents were increased from 38 to 75 g/L.

3.2 Statistical analysis of the experimental (fermentation) results

The correlation between LA yield and process variable was analyzed by the RSM-CCD modeling technique. Table 2 shows the experimental data and reports obtained from design expert software.

3.2.1 Analysis of variance (ANOVA)

As shown in Table 3, ANOVA was used to confirm the statistical significance of every factor and their respective interactions in the developed quadratic response surface model as already shown in Table 3. There is only a 0.01% chance that an *F* value this large could occur due to noise. Besides, *p* values less than 0.050 indicate the model terms are statistically significant. In this case, A, B, C, AB, BC, A^2, B^2, C^2 are significant model terms. Furthermore, the lack of fit *F* value of 2.36 implies the lack of fit is not significant relative to the pure error. There is an 18.40% chance that a lack of fit

Table 3 Analysis of variance (ANOVA) for the suggested quadratic model

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*p* value	
Model	225.32	9	25.04	59.72	< 0.0001	Significant
A- incubation temperature	65.64	1	65.64	156.57	< 0.0001	
B- incubation time	77.59	1	77.59	185.08	< 0.0001	
C- Ph	4.11	1	4.11	9.82	0.0106	
AB	18.70	1	18.70	44.60	< 0.0001	
AC	0.4465	1	0.4465	1.07	0.3264	
BC	9.53	1	9.53	22.72	0.0008	
A^2	8.42	1	8.42	20.08	0.0012	
B^2	25.66	1	25.66	61.21	< 0.0001	
C^2	2.09	1	2.09	4.99	0.0496	
Residual	4.19	10	0.4192			
Lack of fit	2.94	5	0.5888	2.36	0.1840	Not significant

Table 4 Fit statistics for the suggested quadratic response surface model

Std. dev	0.6475	R^2	0.9817
Mean	19.68	Adjusted R^2	0.9653
C.V. %	3.29	Predicted R^2	0.8964
		Adequate precision	28.9645

F value this large could occur due to noise. Non-significant lack of fit is good.

3.2.2 Model adequacy analysis

The adequacy of the model can be deduced considering the regression coefficients of R^2. Hence, the relationship between the experimental and the predicted responses was determined by R^2 value. As represented in Table 4, the response of R^2 was 0.9817, which recommends that 98.17% of the response variability in LA yield can be described by the analyzed process parameters, and it could not describe nearly about 1.83% of the variation of the response. It was obtained that the value of R^2 is very close to 1, showing the closeness between the experimental and predicted value. Therefore, the model is adequate enough. Moreover, the coefficients of R^2 (0.9817) and adjusted R^2 (0.9653) shown in Table 4 indicate the close agreement of experimental and predicted values. The difference between the predicted R^2 (0.8964) and adjusted R^2 (0.9653) is less than 0.2, which shows the model is adequate.

3.2.3 Development of a model equation by using RSM-CCD

The model equation developed by using design expert software was used to determine the relationship between responses and process variables. The quadratic response surface model that relates the yield of LA with independent parameters (coded variables) was developed as shown in Eq. (3) and used for regression analysis. Since the equation in terms of coded factors can predict the response for given levels of each factor, it was selected for analysis.

$$LA\left(\frac{g}{L}\right) = 21.73 + 2.39A + 2.60B + 0.599C - 1.53AB - 1.09BC - 1.10A^2 - 1.92B^2 - 0.548C^2 \quad (3)$$

where, *A* is the incubation temperature (°C), *B* is the incubation time (hrs), and *C* is pH.

In general, the negative coefficients describe that the factors negatively affect the yield of LA, which means an increment of the factors level results in a decrement of LA yield. Whereas the positive coefficients indicate that the factors positively affect the yield of LA that means an increment of the factors level results in an increment of LA yield. As evident from Eq. 4.5, the effect of *B* on the yield of LA was dominant as compared to the effect of *A* and *C*.

3.3 Model parameters effect on the lactic acid yield

3.3.1 Incubation temperature effect

The yield of LA obtained from the fermentation process was found to be substantially affected by incubation temperature. As shown in Fig. 2, an increment of LA yield was observed with an increase in the incubation temperature. During the first 30 to 35 °C of incubation temperature, LA contents of the fermented product were very low; it was 18.86 g/L. However, as incubation temperature increased to a nearby 42 °C, the LA contents of the fermented product were significantly increased to 22.89 g/L, which is 21% higher. This result confirmed a positive effect of incubation temperature

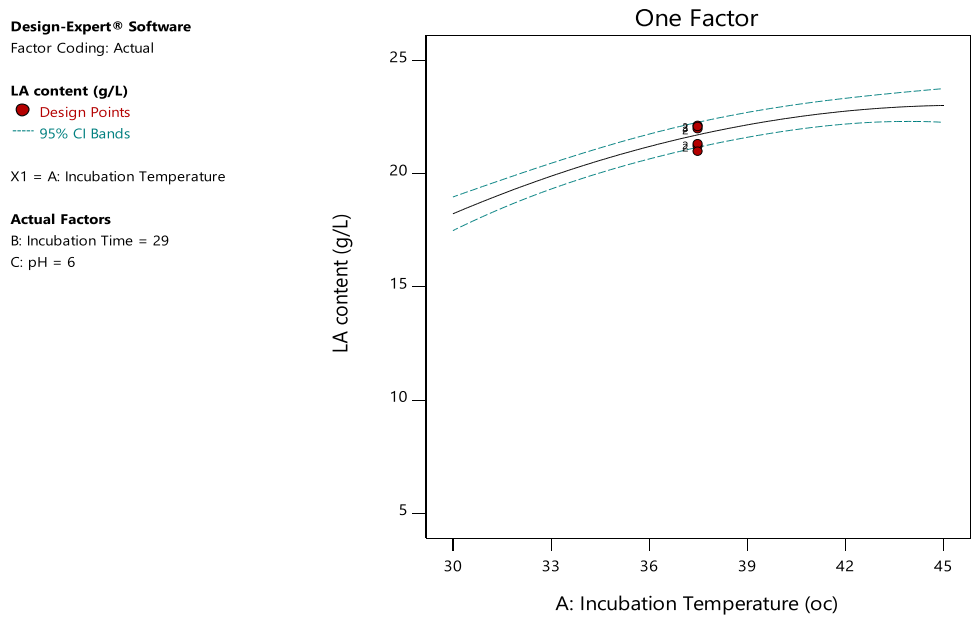

Fig. 2 Effects of incubation temperature on LA yield

on LA yield, as revealed in Eq. 3. The reason behind this result is that an increment of incubation temperature results in a substantial increment of the diffusion of substrate and product through the beads. As a result, the cells could obtain the substrate, and the metabolic process was taking place in a good manner along with the diffusion of the generated LA product from the beads. Furthermore, since *Lactobacillus plantarum* is categorized under mesophilic microorganisms (10–45 °C) with high temperature, its metabolic activity is good as the incubation temperature gets increased to a higher level [24].

In the same manner, Thakur et al. [18] observed the positive effect of incubation temperature in the fermentation process with other immobilized Lactobacillus spp. on the substrate and product diffusion through the beads. However, further increment of the incubation temperature beyond this optimum point results in the gradual decrements of the LA contents. This indicates that a higher incubation temperature beyond the optimum decreases the number of viable cells and metabolic activities of the cells. Correspondingly, Mahato et al. [23] investigated that an increment of incubation temperature from 30 to 40 °C results in a substantial increment of the LA contents from 14.89 to 31.04 g/L.

3.3.2 Incubation time effect

The yield of LA obtained from the fermentation process was also found to be dependent on incubation time. Incubation time is another important factor which substantially affects the yield of LA obtained from the fermentation process. As depicted in Fig. 3, during the first 12 h, LA content of the fermented product 16.65 g/L was obtained which is very low. Because *Lactobacillus plantarum* requires sufficient time to ferment the entire initial glucose concentration (13.84 g/L) and adapt to the new conditions; fermentation at this condition provides a low yield of LA product. Moreover, the diffusion of the substrate through the beads needs time. Accordingly, at the initial stage of fermentation, the cells could not obtain enough amount of glucose for the metabolic process and resulted in a decrement in LA yield.

However, as incubation time gets increased to 40 h, an increment of LA yield (22.67 g/L) was observed, which is 36.15% higher. This shows that in this period, the number and metabolic activity of the cells were very high since the cells adapted to the conditions and synthesized the molecules (i.e., DNA, RNA, and other molecules) which are necessary for their growth. Therefore, the cells are matured and fermented a huge amount of glucose efficiently. This performance of the cells is stable over the range of incubation time as compared with free cells. However, beyond this level of incubation time (40 h), there is a decrement of LA yield. This is due to a depletion in the substrate and the amount of available nitrogen fermentation medium for the metabolism process. The present finding is in smooth agreement with Wang et al., [7] stated the maximum LA yield was obtained at 43 h of incubation time for *Lactobacilllus pentosus ATCC 8041*.

3.3.3 Effect of pH

In addition to the above factors, pH was another major factor by which the yield of LA obtained from the fermentation process was affected. As shown in Fig. 4, an increment of LA yield was observed with the increase in the pH. During the first 5 to 5.5 values of pH, LA yields of the fermented product obtained are 20.79 g/L. However, as pH increased

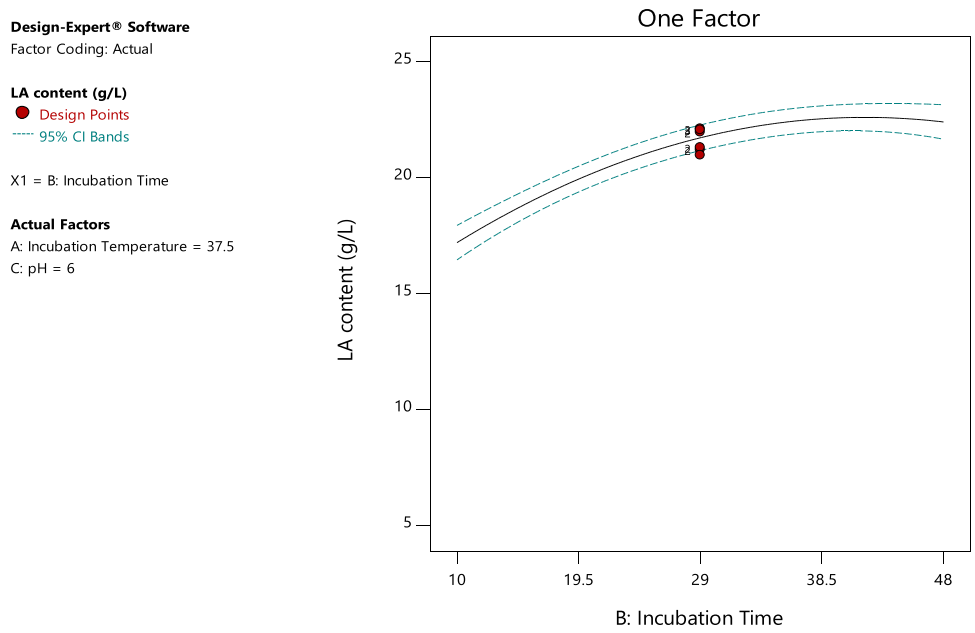

Fig. 3 Effects of incubation time on LA yield

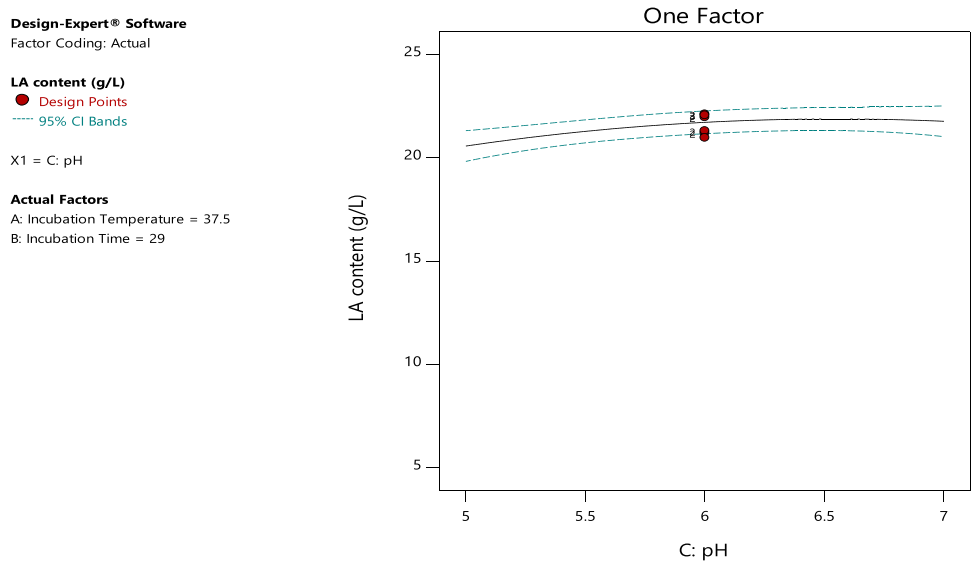

Fig. 4 Effects of pH on LA yield

to a nearby 6.18, the LA contents of the fermented product were increased to 21.83 g/L, which is 5% higher. This revealed that the lower the pH (i.e., higher H^+ concentration) of the media causes the diffusion of H^+ through the plasma membrane into the cytoplasm of the cells and leads to the decrement in the internal pH of the cells. Consequently, the drastic variation in cytoplasmic pH can harm the cells by inhibiting their activity and membrane transport of nutrients. Similarly, Panesar et al. [25] investigated the effect of pH on the cellular metabolism process and microbial growth of microbial cells. Owing to *Lactobacillus plantarum* being a prokaryotic cell, it cannot survive at lower internal pH and even die. This revealed that at lower pH of media, the metabolic activity of the *Lactobacillus plantarum* cells is less and decreased LA yield. Then, as the pH of the fermentation medium increased to 6.18, the internal pH of the cells was also kept around its normal level (i.e., around 6.5), and the plasma membrane of the cells did not affect by the pH. Accordingly, the metabolic activity of the cells is increased and their fermenting efficiency is significantly increased. Subsequently, the yield of LA obtained was increased. Moreover, Bhushan et al. [26] reported that a higher acidic pH led to changes in the intracellular ionic environment and damaged protein structure, which was harmful to cell growth, resulting in a decrement of product yield. However, the further increment of the pH of fermented

media beyond this optimal value of 6.18 leads to a less far increment of LA. As the pH of fermented broth gets larger, the growth of *Lactobacillus plantarum* cells is suppressed, and their metabolic activities are inhibited. Therefore, fermenting efficiency of the *Lactobacillus plantarum* cells was substantially decreased, and the amount of additional LA produced was significantly reduced. Accordingly, Bahry et al. [27] reported the same phenomena for the effect of pH on fermenting efficiency of an immobilized *Lactobacillus rhamnosus*, and observed 6.0 as optimum pH value.

3.4 The interaction effect of model parameters on lactic acid yield

3.4.1 Effect of incubation time and incubation temperature

The interactive effect of incubation temperature and incubation time on LA yield was depicted on the 3D surface plot in Fig. 5. As discussed in the above sections, regardless of incubation time, the increment of incubation temperature results in an increment of LA yields. The same is true for incubation time. However, upon their interaction, as evident from Fig. 5, the simultaneous increment of incubation time along with incubation temperature results in a decrement of LA yields. At a low level of incubation time, an increment of incubation temperature results in an increased LA yield. Conversely, at a high level of incubation time, an increment of incubation temperature results in a decrease in LA yield. This outcome exposed that the interaction between incubation temperature and incubation time has a negative effect on LA yield. Additionally, a negative sign coefficient of incubation temperature and incubation time in the developed model Eq. (3) indicated this effect. The reason behind this result is the fermentation carried out at high temperature (beyond optimum temperature, 42 °C) over a long period might cause the reduction in metabolic activities of the *Lactobacillus plantarum* and depletion of the substrate. Consequently, the vial cells of *Lactobacillus plantarum* substantially decreased which in turn led to the decrement of LA yield. If the cells were kept at high temperature for a long period of time, the cells would not survive to grow and possess their metabolism. Moreover, another researcher, Edris (2017), stated that the simultaneous increment of incubation temperature from 25 to 30 °C and 38 to 45 h for fermentation with using free *Lactobacillus plantarum* results in the decrement of LA contents from 16.85 to 12.48 g/L. It shows the negative effect of incubation temperature and incubation time on LA yields.

3.4.2 Effect of incubation time and pH

As revealed in Fig. 6, the interactive effect of pH and incubation time on the yield of LA was depicted on the 3D surface plot. At a low level of pH, an increment of incubation time leads to a sharper increment of LA yield. Nevertheless, at a high level of pH, an increment of incubation time results in a slight increment of LA for a period and then starts decreasing. This result showed that the interaction between incubation time and pH has a negative effect on LA yield. The negative sign coefficient of pH to incubation time in the developed model Eq. (3) showed this negative effect. The reason behind this effect is the increment of both pH and incubation might cause the reduction in metabolic activities of the *Lactobacillus plantarum*. Because, if the fermentation process was carried out a high pH of media (low H^+ concentration) for a long period, then a significant amount of H^+ would be diffused from the internal cytoplasm of the cells to the surrounding (media) which results in a decrement of the H^+ and increment of pH of the cells. Therefore,

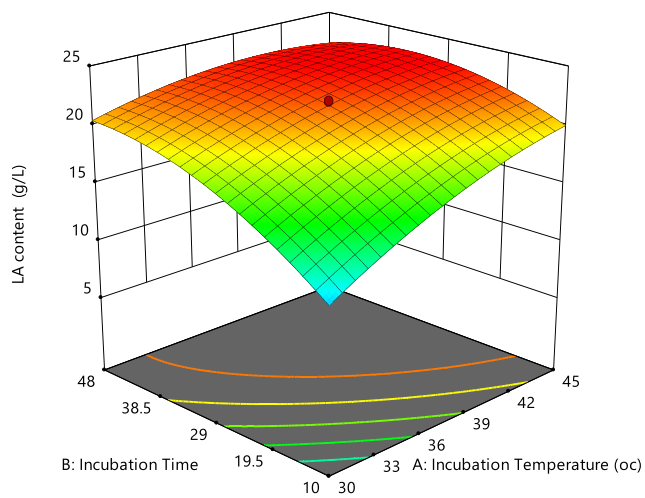

Fig. 5 The interaction effects of incubation temperature and time on LA yield

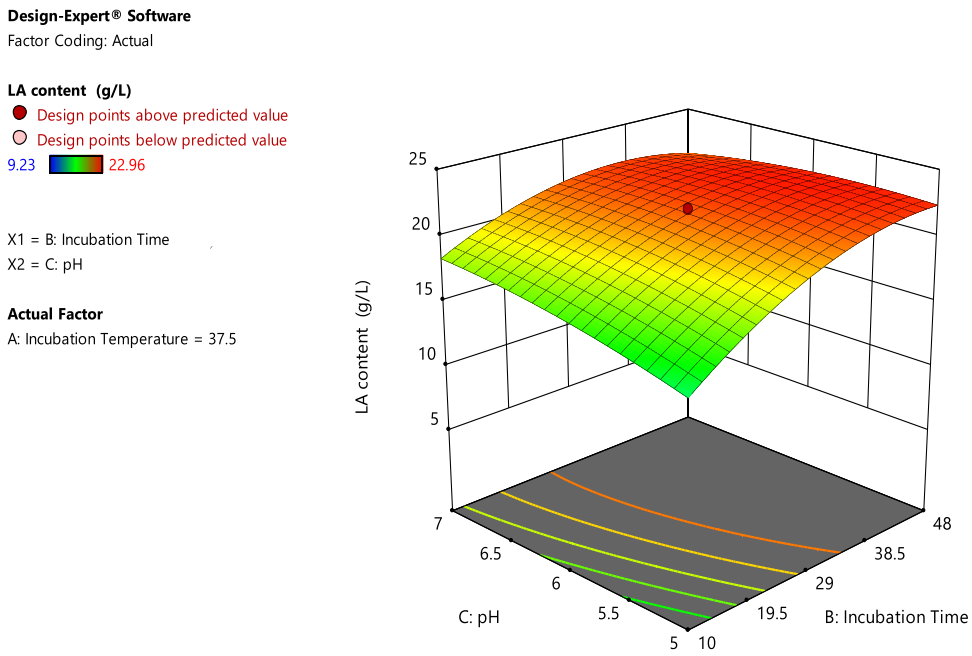

Fig. 6 The interaction effects of incubation time and pH on LA yield

if the lactobacillus species were kept at a high pH (low H^+ concentration) for a long period, the plasma membrane of the cells would be disturbed [25]. Consequently, the growth of *Lactobacillus plantarum* cells was suppressed and their metabolic activities were inhibited. In turn, the production of LA was decreased. The same investigation is reported by Bahry et al. [27]. They have reported that the simultaneous increment of incubation time from 18 to 24 h and pH from 6 to 6.5 for fermentation with *Lactobacillus rhamnosus ATCC 53,103* results in the decrement of LA contents from 21.5 to 18.75 g/L. It shows the negative effect of incubation time and pH on LA contents. It is known that when the pH approaches the pKa of lactic acid (about 3.9), the undissociated form of lactic acid plays a more inhibitory effect than the dissociated lactate form. At a pH lower than 5, the undissociated acid form cannot be neglected, whereas a pH greater than 6 leads to almost complete [27].

3.5 Numerical optimization of lactic acid yield

The aim of the present study is to optimize the yields of LA produced from khat waste biomass. Therefore, the optimum condition for the selected major fermentation parameters was determined by using design-expert software (i.e., numerical optimization feature). Totally, around 100 possible optimal solutions had been generated. However, as shown in Table 5, the most appropriate solution was selected. These include incubation temperature (42.3 °C), incubation time (40.01 h), pH (6.18), and LA content (23.05 g/L). The highest composite desirability of 1.0 at optimum conditions was obtained, which indicates the degree of satisfaction of the optimum conditions for the ultimate goal of response was successfully attained.

3.5.1 Model validation

To validate the developed model, three replications of the experiment were done. The result obtained from the experiment (22.98 ± 0.10 g/L) is very close to the result obtained from the model (23.05 g/L) used by design-expert software. Therefore, the high yield of LA obtained from this experiment revealed that the immobilized *Lactobacillus plantarum* cells performed high fermenting efficiency and outstanding stability during the fermentation process under optimum conditions.

3.6 Residual sugar determination

To determine the fermenting efficiency (i.e., glucose conversion to LA) of immobilized *Lactobacillus plantarum* in the fermentation process, the residual sugar contents of the fermented sample at optimum conditions (incubation

Table 5 The selected optimum condition by RSM

Number	Incubation temperature (°C)	Incubation time (hrs)	pH	LA content (g/L)	Desirability	
1	42.30	40.01	6.18	23.05	1.00	**Selected**

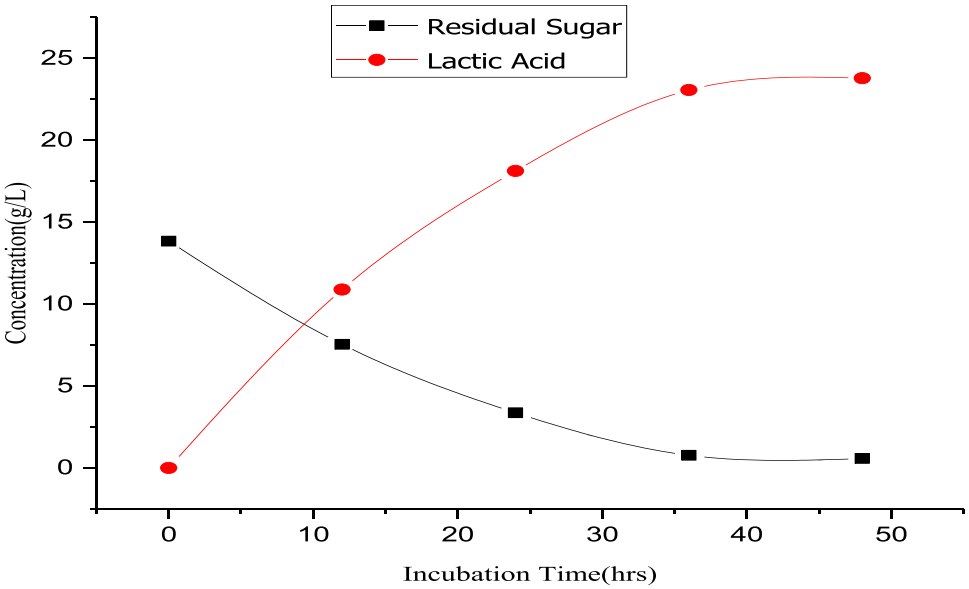

Fig. 7 Time profiles of LA and residual sugar content in the fermentation process at incubation time = 42.3 °C and pH = 6.18

temperature of 42.2 °C and pH of 6.18) were determined with the time interval of 12 h. As it was depicted in Fig. 7, during the first 12 h, about 43.13% of glucose had been converted by *Lactobacillus plantarum* and yields 10.89 g/L of LA. This indicated that, because of factors such as lack of adaption to the new condition and diffusion limitation at the starting, the cells did not convert the entire available substrate into LA. Consequently, a huge amount of glucose was left unconverted. However, as incubation time increased to 36 h (i.e., almost near optimum incubation time), 89.40% of glucose was converted and yielded 22.96 g/L of LA. Because these conditions are very suitable for the metabolism process of *Lactobacillus plantarum*, as well as the substrate and product formed were diffused through the bead properly. Thus, this outcome mentioned the utilization of immobilized *Lactobacillus plantarum* for the LA fermentation process at optimum conditions could efficiently convert the given glucose into LA product. Accordingly, Wang et al. [7] reported that fermentation with immobilized Lactobacillus species increases the fermenting efficiency and the stability of the microbes, which results in the high conversion of glucose to LA.

4 Conclusions

In the current study, optimization of fermentation conditions for the production of lactic acid from khat waste by using immobilized *Lactobacillus plantarum* was successfully attained. The effect of immobilization on the yield of LA has been investigated, and the yield obtained from immobilized *Lactobacillus plantarum* cells is 32.78% higher than the free cells. The effects of the major fermentation parameters (incubation temperature, incubation time, and pH) on the yield of LA were investigated. Response surface methodology with a central composite design was used for the optimization of LA yield and process parameters. Based on the analysis, the optimum fermentation conditions acquired by the quadratic model for maximum LA yield were 42.3 °C, 40.0 h, and 6.18 for incubation temperature, incubation time, and pH respectively. Under these optimum conditions, the LA concentration obtained from fermentation was 23.05 g/L. The result from the model also confirmed that all the independent fermentation parameters significantly affected the yield of LA and, especially, the incubation time was the dominant one. The outcome from residual sugar content determination at optimum conditions is about 1.47 g/L, which shows the utilization of immobilized L. plantarum for fermentation at optimum conditions can efficiently convert the given glucose into an LA product. Generally, this study demonstrated that the utilization of khat waste as a potential carbohydrate source and immobilization of *Lactobacillus plantarum* cells for LA production is worthful.

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Data Availability All data analyzed during this study are included in this research article.

Declarations

Conflict of interests • I confirm that I have read, understand, and agreed to the submission guidelines, policies, and submission declaration of the journal.
• I confirm that all authors of the manuscript have no conflict of interests to declare.
• I confirm that the manuscript is the authors' original work, and the manuscript has not received prior publication and is not under consideration for publication elsewhere.
• On behalf of all co-authors, I shall bear full responsibility for the submission.

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