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# Enhancing the production of bacterial cellulose in Kombucha through the utilization of sugarcane molasses via central composite design

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**ABSTRACT**

Kombucha is a functional drink which is obtained from the symbiotic culture of acetic acid bacteria and osmophilic yeasts in sweet tea and has a sour taste. During fermentation of this product, in addition to the many metabolites that are produced, a cellulose layer is formed by *Acetobacter xyleneum* in the fermented liquid. This drink has many benefits like probiotic and antioxidant properties, prevention and treatment of cancer and diabetes, increment the body's metabolism, and etc. Also, the cellulose layer produced is used to remove metal contaminants from the effluent, treat wounds and burns, and as poultry dietary supplement. In this study, optimization of kombucha bacterial cellulose using sugarcane molasses as a source of carbon and energy, and in different environmental conditions (temperature (25, 27.5, 30 °C), incubation time (7, 11, 15 days), initial pH (5.5, 6, 6.5)) analyzed using central composite statistical plan. The results showed that concentration of sugarcane molasses, incubation time, and initial pH have significant effect on production of kombucha bacterial cellulose ($p<0.05$).



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## 1- Introduction

Kombucha is a traditional, beneficial and refreshing fermented drink that refers to the cellulose consortium of the symbiosis of osmophilic yeasts and acetic acid bacteria that are usually cultured in sweetened black tea. Yeast cells hydrolysis sucrose to glucose and fructose and use as ethanol production substrate and acetic acid bacteria converts glucose to gluconic acid and ethanol to acetic acid [1,2]. Also, the cellulose layer of kombucha is formed by the activity of acetic acid bacteria. Kombucha cultures consist of a macroscopic colony floating on the surface of the tea solution, often in the form of a container, called a kombucha fungi (tea fungi), which is not actually a fungus. This name is erroneously given due to the ability of bacteria to synthesize a floating cellulosic network on the surface of the tea solution, which appears to be surface mold in a motionless environment [3-7]. This tea has many biological properties such as antioxidant properties, reducing inflammatory problems, protection against diabetes, and etc. [8-9]. In the analysis of Kombucha drink, there are many compounds such as various organic acids, acetic, gluconic, glucoronic, citric, malic, tartaric, malonic, oxalic, succinic, pyruvic, usnic and L-lactic acid; also sugars such as glucose and fructose; Group B vitamins include $B_1$, $B_2$, $B_6$, $B_{12}$ and C; 14 amino acids, biogenic amines, purines, pigments, lipids, proteins, some hydrolytic enzymes, ethanol, active antibiotics, carbon dioxide, phenols as well as some tea polyphenols, minerals, anions and some known metabolites have been isolated from yeasts and bacteria [3,8,10-11]. The microbial composition of kombucha and its native characteristics are determined on the one hand by the climatic and geographical conditions of cultivation and on the other hand by the native species of wild yeasts and bacteria [12]. The microbial composition of kombucha depends on the origin of cultivation and affects the characteristics of the kombucha product [13-14]. Common acetic acid bacteria isolated from kombucha are Acetobacter xylinum, Acetobacter pastorianos, Acetobacter ste and Gluconobacter oxidans. The predominant bacterium is Acetobacter xylinum, which produces bacterial cellulose floating on the surface of the fermentation liquid. A wide variety of yeasts, including species of Saccharomyces, Saccharomycoides, Schizosaccharomyces, Zygosaccharomyces, Brettanomyces, Candida, Torulospora, Piscia, Mycotorula, and Mycoderma have been isolated from kombucha. Yeasts from Saccharomyces species such as Saccharomyces cerevisiae, Saccharomyces bisporus, Saccharomyces pombe, Zygosaccharomyces roxy, Zygosaccharomyces boilii and Brettanomyces genomes were isolated by several researchers [12-13,15-16]. It is worth mentioning that the microflora in the cellulose layer and the fermentation liquid match. Since the microorganisms in Kombucha are beneficial microorganisms and have high resistance, especially in acidic conditions, they can replace harmful microorganisms in the digestive system. Therefore, this drink can be considered as one of the products with probiotic and functional properties [17]. Kombucha provides a source of probiotics that help increase the metabolism of beneficial microorganisms in the digestive tract [5]. Cellulose produced from Acetobacter xylinum has unique characteristics in terms of chemical stability, molecular structure and mechanical resistance.

This cellulose network contains a symbiotic culture of bacteria and yeast, which is consumed as a delicacy in the Philippines and is used in Brazil to treat skin burns and other skin injuries [3,16]. Bacterial cellulose has been used in the production of medical wound dressings and is known as artificial skin or blue coating. These bandages are used to heal severe skin burns [18]. In addition, this cellulose layer is used as a bio-absorbent in water purification, so the cellulose layer of fungi tea treated with iron chloride is used to remove arsenic from aqueous solutions, and its maximum capacity for arsenic is $3.9\times10^{-3}$ mmol/g at pH 6 to 8. Also, this layer has been used to remove



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chromium and copper from aqueous solutions in a non-continuous (batch) absorption system, and the optimal pH value for the absorption of these elements was approximately 2 to 4. The best absorption capacity of chromium in cellulose layer of modified kombucha with NaOH was at 20 ℃ and for copper absorption at 100 ℃. Modification of the kombucha cellulose layer with HCl increased the zinc absorption capacity by four times [19]. Furthermore, as a poultry feed supplement, 150 g/kg of kombucha cellulose layer in the diet caused a significant increase in body weight, protein efficiency factor and carcass characteristics (feather weight, net weight, etc.) in the tested chickens compared to the control sample. Therefore, pure species of industrial kombucha can be used to prepare bacterial cellulose with a high degree of purity and produce a high quality beverage [6,20]. Goh et al. in 2012 investigated the effects of sucrose concentration and fermentation time on the yield of microbial cellulose and indicated that at a sucrose concentration of 90 g/L, the highest yield of bacterial cellulose was obtained (66.7%) and, the lowest yield of bacterial cellulose was produced at a sucrose concentration of 110 g/L. The gradual decline of pH in the fermentation process in the tea liquid with a concentration of 90 g/L of sucrose increased the production of bacterial cellulose as the fermentation progressed [21]. Furthermore, Sukhtezari et al. produced bacterial cellulose film utilized in antioxidant active packaging. They used *Scrophularia striata* Boiss. extract as antioxidant substrate. It was observed that applying bacterial cellulose enhanced mechanical properties of the film [22]. Antioxidant active packaging is a novel packaging technology that several researchers have investigated about it [23-26].

Sugarcane is considered as one of the most important industrial plants of the country and its side industries are very important in terms of the national economy. Molasses is one of the by-products of sugarcane, which is used as the main raw material for the production of alcohol and related industries. The amount of final molasses is usually 2.7% of sugarcane weight and its exact composition is somewhat variable, but it contains 50-60% sucrose [27-28]. In addition to sucrose, sugarcane molasses is rich in minerals and vitamins and is used as a substrate in the production of dextran, xanthan gum, monosodium glutamate, L-listin, etc. [28]. Due to its non-ionic and stable properties in operating conditions such as heat, acid and alkali, increased viscosity, solubility in water and oil, the possibility of film formation and water retention properties, this product has wide applications in pharmaceutical, biochemical and other industries [27].

Malbaša et al. in 2008 evaluated the effect of different concentrations of sucrose in sugar beet molasses on kombucha fermentation. The prepared molasses contained 50.4% sucrose. As a result of the formation of acids, the pH value decreased during the process. The decrease in pH in all samples at the beginning of fermentation continued exponentially until the third day, and after that the decrease in pH was much slower. The decline in pH value was the result of the enhancement in the content of acetic acid in fermentation systems. In general, acidity increased in all systems, and according to Duncan's test, the acidity of each system was significantly different from other systems. Concentration reduction time was used to measure the fermentation rate. In the $MC_1$ system, after 14 days, sucrose was almost completely (97%) fermented, and for the $MC_2$ and $MC_3$ systems, this value was 35% and 25%, respectively. The amount of cellulose layer in molasses fermentation systems was as follows: in $MC_1$ 260 grams, in $MC_2$ 100 grams and in $MC_3$ 85 grams. This proves the importance of the carbon source in the fermentation process, however, other factors, especially the nitrogen source, may have affected the performance of the cellulose layer. Thus, the difference in the amount of cellulose layer can be attributed to these components [7]. Malbaša et al. in 2008 compared Kombucha fermentation products on three sugar beet molasses and sucrose samples. According to the results, pH reduction had occurred in all fermentation systems. The



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highest rate of fermentation was until the third day, and after that the decrease in pH was slower, especially in the case of systems with $M_3$ molasses. In the systems with $M_1$ molasses, the pH value first decreased and then began to increase until the seventh day of fermentation. The lowest pH value was observed in systems with $M_2$ molasses, and a similar trend was also observed in the case of sucrose fermentation. The total acidity content in the metabolites of the system with molasses was doubled compared to the systems with pure sucrose. Acidity increased in systems with molasses, the most significant was in the case of samples with $M_2$ molasses. The reduction of sucrose concentration was much faster in systems with molasses. The fastest rate of sucrose reduction was in the case of sample $M_3$, when sucrose was completely consumed. The lowest amount of cellulose layer for the system with sucrose (1.17 g/L) was obtained in samples of molasses $M_1$ (154.8 g) and molasses $M_2$ (165.6 g) and The largest mass of kombucha in molasses was $M_3$ (270.8 g). Such observations may be related to the significant content of total nitrogen in molasses [29].

Sugar cane molasses can be a suitable carbon source for kombucha production due to its low price, significant amounts of sucrose and also compounds such as minerals and vitamins that are useful for the fermentation process. The aim of the current research is to investigate the effect of sugarcane molasses percentage as a carbon source in the cultivation environment, as well as the greenhouse temperature, initial pH and fermentation time to optimize the production of kombucha bacterial cellulose.

## Materials and methods

### Materials

Kombucha fungi was prepared from the collection of microbial cultures of Aksan, Isfahan. Sugarcane molasses with Brix of 89.12, sucrose percentage of 39.62 and purity degree of 46.44 was got from Imam Khomeini Agriculture Company of Ahvaz. All materials were from Merck company and analytical grade.

## 2-Methods

### Kombucha cultivation

According to the statistical plan, molasses was added at 0, 5, 10, 15, and 20% levels in one-liter glass containers containing 0.5 L of boiled water to prepare the culture medium for the batch fermentation system. The initial pH of the culture medium was adjusted using NaOH (5N) and citric acid (5N) at the levels of 5, 5.5, 6, 6.5 and 7. Then the lid of the container was covered with a linen cloth and closed with a rubber band, and finally it was sterilized using an autoclave at a temperature of 121℃ and a pressure of 1.5 bar for 15 minutes. The samples were incubated at temperatures of 22.5, 25, 27.5, 30 and 32.5 ºC for 3, 7, 10, 11, 15 and 19 days [30].

### Measurement of bacterial cellulose

To measure the amount of bacterial cellulose, the cellulose layer floating on the fermentation liquid was separated and dried with filter paper after washing with distilled water. Also, the wet weight of the cellulose layer was measured using a sensitive laboratory scale with an accuracy of 0.001 [31].

### Acetic acid bacteria and yeast count test

The counting of acetic acid bacteria and yeasts in fermentation liquid was done by standard plate counting method in yeast peptone monitor agar culture medium containing 500 mg/L cycloheximide and Saburo dextrose agar respectively. Then acetic bacteria were incubated at 28 ℃ for five days and for yeasts at 25 ℃ for 72 hours [12].

### Ethanol measurement

The amount of ethanol was measured by distillation method and using a vacuum rotary device. For this purpose, 100 ml of the fermentation sample was transferred to a round bottom flask and then the flask was connected to the device and placed in the water bath. The





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device settings are set at 78 ℃ temperature, 55 rpm speed and 45 minutes' duration and after the end of distillation and cooling the flask containing the sample, the contents of the flask were measured with a graduated cylinder. The amount of ethanol produced during the kombucha fermentation process was obtained by reducing the sample volume [32].

**pH measurement**

pH measurement was done after homogenizing the fermentation samples using an electronic pH meter calibrated with buffers 4 and 7 at 20 ℃ [21].

**Total acidity measurement**

To determine the titratable acidity, first 20 mL of the fermentation liquid was brought to 100 mL, and then the titration was done with 20 mL of the diluted sample using 0.1 N sodium hydroxide in the presence of phenolphthalein as a reagent. Titratable acidity was expressed in terms of grams of acetic acid per liter of sample using the following formula [12]:

$$A= N\times V\times E\times 100/M$$

Where, A: total acidity in terms of acetic acid, M: sample amount, N: normality of sodium hydroxide, V: amount of sodium consumed and E: equivalent of acetic acid.

**Measurement of residual sucrose**

The amount of remaining sucrose, as well as reducing sugars before and after hydrolysis was measured by Linn-ionone method.

**Design and statistical analysis**

In this study, the effect of four independent variables of sugarcane molasses concentration, incubation temperature, initial pH and fermentation time was evaluated by applying the response surface method and in the form of a central composite design on the amount of bacterial cellulose production and other quality characteristics of Kombucha (table 1). In addition, The experimental design was composed of 30 runs (15 runs, each with two replicates). The experimental design is shown in table 2. After conducting experiments and collecting information, the analysis of variance (ANOVA) and Fisher distribution were used to test the significance of the factors and their interaction effects, and the significance level was considered as $\alpha$=0.05. Finally, the optimization was calculated using the utility function and numerically analysis. Design expert statistical software version 10 was used for data analysis, numerical optimization and graphs.

**Table 1: Independent variables of the process**

| independent variable | maximum | central | minimum |
|---|---|---|---|
| molasses (%) | 15 | 10 | 5 |
| incubation temperature (℃) | 30 | 27.5 | 25 |
| Initial pH | 6.5 | 6 | 5.5 |
| Fermentation time (days) | 15 | 11 | 7 |

**Table 2: Experimental design**





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| Run | Molasses (%) | Temperature (°C) | Fermentation time | pH |
|---|---|---|---|---|
| 1 | 15 | 25 | 7 | 6.5 |
| 2 | 10 | 22.5 | 11 | 6 |
| 3 | 0 | 27.5 | 11 | 6 |
| 4 | 15 | 25 | 15 | 5.5 |
| 5 | 15 | 25 | 7 | 5.5 |
| 6 | 5 | 30 | 7 | 6.5 |
| 7 | 10 | 27.5 | 11 | 5 |
| 8 | 10 | 27.5 | 11 | 6 |
| 9 | 15 | 30 | 15 | 5.5 |
| 10 | 5 | 25 | 15 | 5.5 |
| 11 | 10 | 27.5 | 3 | 6 |
| 12 | 5 | 30 | 7 | 5.5 |
| 13 | 15 | 30 | 7 | 6.5 |
| 14 | 10 | 27.5 | 11 | 6 |
| 15 | 5 | 30 | 15 | 6.5 |
| 16 | 5 | 30 | 15 | 5.5 |
| 17 | 15 | 25 | 15 | 6.5 |
| 18 | 10 | 32.5 | 11 | 6 |
| 19 | 10 | 27.5 | 11 | 7 |
| 20 | 5 | 25 | 15 | 6.5 |
| 21 | 10 | 27.5 | 11 | 6 |
| 22 | 5 | 25 | 7 | 6.5 |
| 23 | 10 | 27.5 | 11 | 6 |
| 24 | 15 | 30 | 15 | 6.5 |
| 25 | 10 | 27.5 | 11 | 6 |
| 26 | 5 | 25 | 7 | 5.5 |
| 27 | 10 | 27.5 | 11 | 6 |
| 28 | 20 | 27.5 | 11 | 6 |
| 29 | 15 | 30 | 7 | 5.5 |
| 30 | 10 | 27.5 | 19 | 6 |

## 3-Results and discussion

### Bacterial cellulose

The amount of produced cellulose layer was influenced by the factors of percentage of sugarcane molasses, incubation temperature, fermentation time and initial pH, as well as the interaction effect of percentage of sugarcane molasses with incubation temperature and fermentation time, and the interaction effect of fermentation time with initial pH.

Fermentation time, and the square of molasses percentage had a significant effect on the production of kombucha cellulose layer ($p<0.05$). At constant temperature, increasing the percentage of sugarcane molasses from 5% to 10%, the amount of cellulose layer of kombucha produced reached 6 grams, but at higher percentages of sugarcane molasses, the production of cellulose layer of kombucha decreased (Figure 1, a). In accordance with the findings of AL-Kalifawi et al. in 2014, who demonstrated that the maximum yield of bacterial cellulose was obtained at a concentration of 100 g/L of sucrose, and this value decreased at higher sugar ratios [33]. Temperatures above 50°C prevented the growth of bacterial cellulose and film formation. By increasing the percentage of sugarcane molasses and fermentation time passing, the amount of bacterial cellulose production increased, but after the tenth day, with the





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increase in the percentage of sugarcane molasses, the amount of produced bacterial cellulose decreased (Figure 1, b). Sreeramulu et al. in 2000 reported that with the enhancement in the percentage of carbon source and incubation time, the wet weight of the cellulose layer raised until day 11-12, but in the amount of sucrose greater than 100 g/L, the growth rate of bacterial cellulose reduced [14]. At the initial pH less than 3.6, the amount of bacterial cellulose increased with the increase of incubation time (Figure 1, c) and according to the findings of Kallel et al. in 2012, the amount of cellulose layer during fermentation increased linearly by incubation time passing [34].

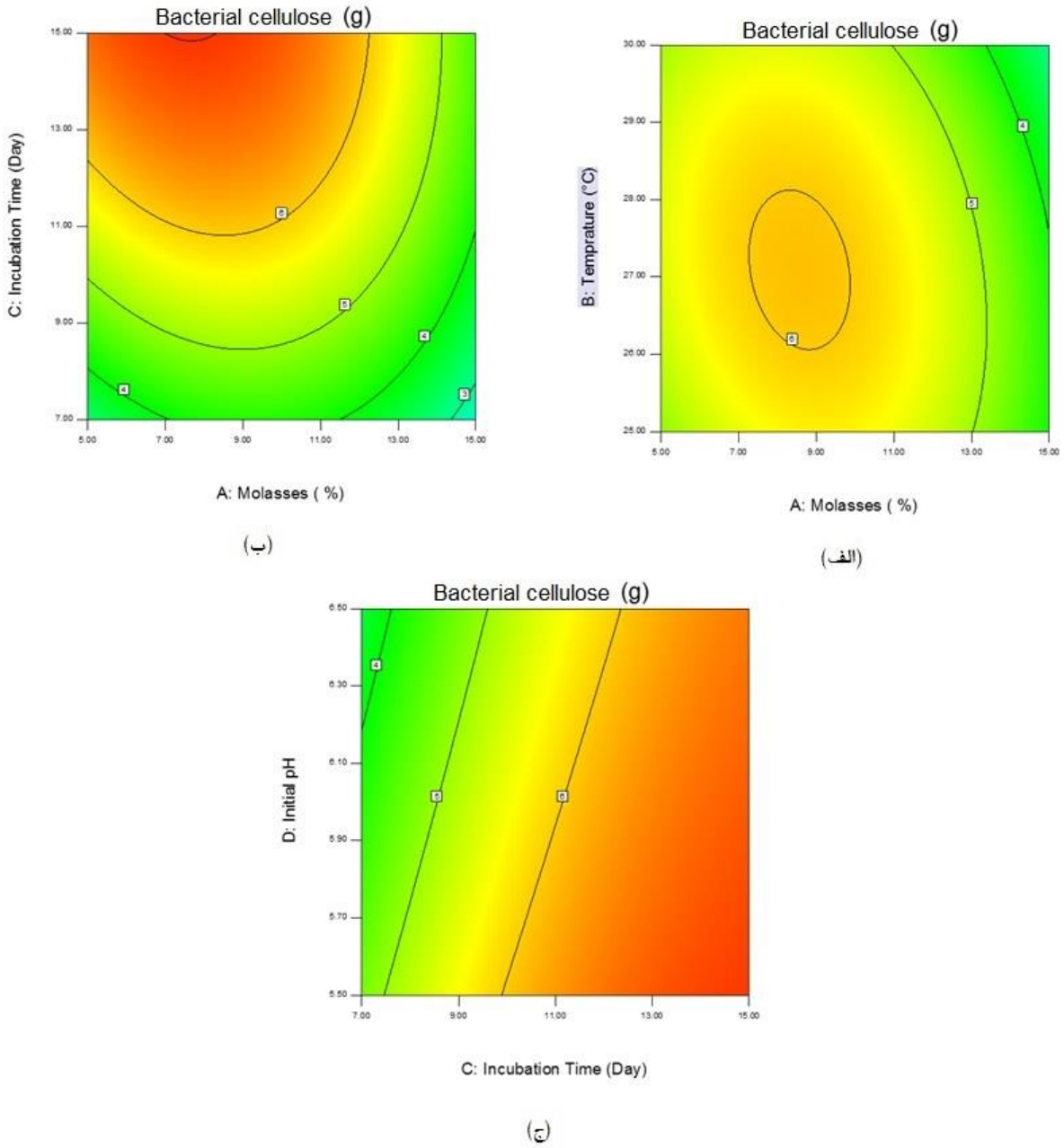


Figure 1: Bacterial cellulose production rate, a) interaction effect of sugarcane molasses percentage and incubation temperature, b) interaction effect of sugarcane molasses percentage and fermentation time, c) interaction effect of incubation time and initial pH

**Acetic acid bacteria count**

The growth rate of bacteria was affected by temperature and incubation time, as well as the interaction effect of sugarcane molasses percentage with initial pH, and incubation time with initial pH. The growth of acetic acid





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bacteria raised with the increment of incubation temperature and also with the increment of pH up to 6. After that, the number of bacteria decreased with the increase of pH (Figure 2, a). As the percentage of molasses and pH increased, the growth rate of bacteria started to increase and remained constant at pH 6.5 and the amount of sugarcane molasses 9-14%, and then it started to decrease (Figure 2, b). Goh et al. in 2012 reported that the content of acetic acid bacteria in the concentration of 90 g/L of sucrose enhanced until the eighth day, and then the growth rate lessened in higher amounts of sucrose [21].

During the fermentation process, the growth rate of bacteria increased by increasing the greenhouse time and decreasing the pH, but after the ninth day, the growth rate decreased (Figure 2, c). According to the findings of Jayabalan et al. in 2007, the growth of acetic acid bacteria raised rapidly until the ninth day and then began to diminish, which they attributed to the acid shock resulting from the decrease in pH. With the enhancement in temperature and incubation time, the growth of acetic acid bacteria raised and the growth trend continued in the temperature range of 26-28 °C between 7-9 days [3]. At a constant incubation temperature, the growth rate showed a decreasing trend with increasing incubation time (Figure 2, d).

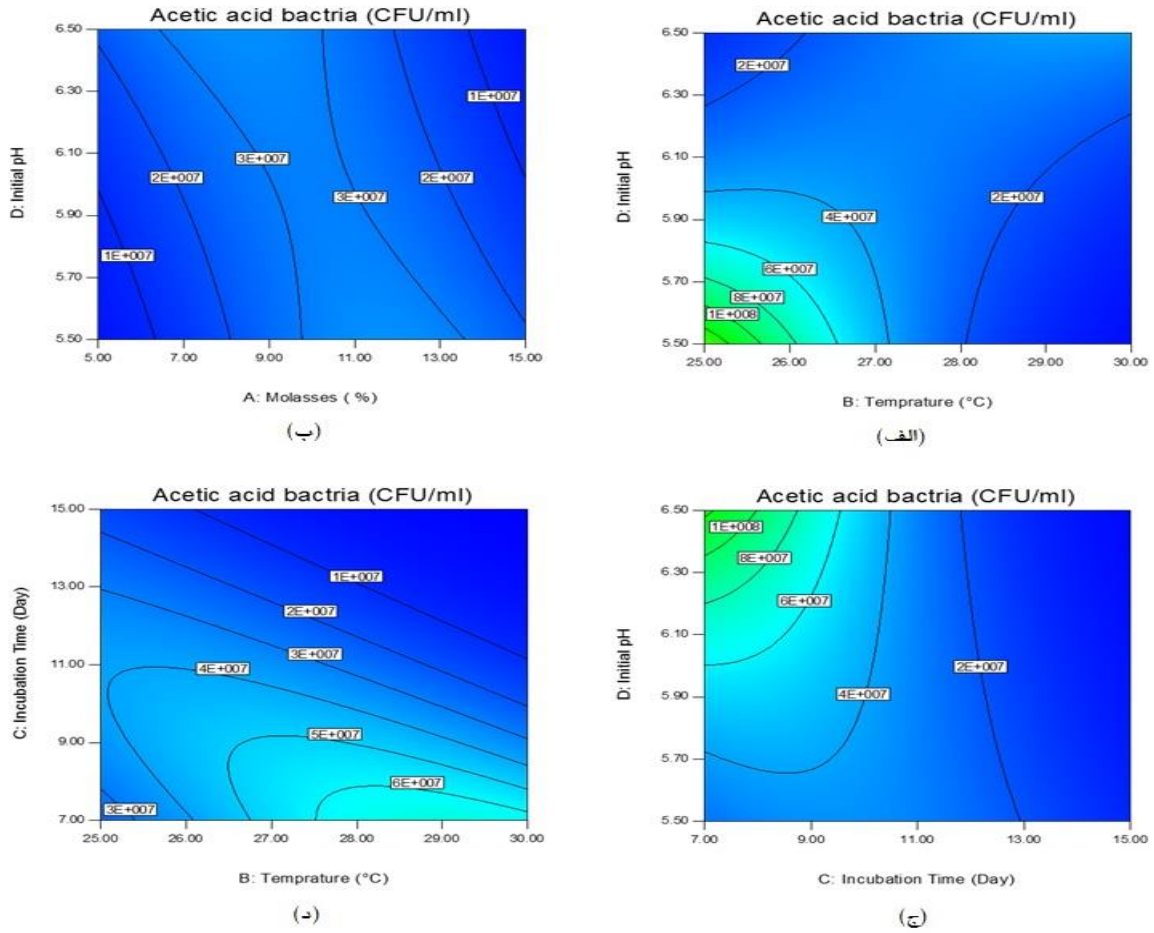


Figure 2: Counting of acetic acid bacteria, a) interaction effect of incubation temperature and initial pH, b) interaction effect of sugarcane molasses percentage and initial pH, c) interaction effect of incubation time and initial pH, d) interaction effect of incubation temperature and time



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**Yeast count**

The percentage of sugarcane molasses, temperature and incubation time, as well as the interaction of initial pH with the percentage of sugarcane molasses, temperature and incubation time had a significant effect on yeast growth ($p<0.05$). At constant pH, by increasing the percentage of sugarcane molasses up to 10%, the growth rate of yeasts increased and then decreased (Figure 3, a). With the increase of incubation temperature and initial pH, yeast growth initially raised, but then began to diminish and then raised again (Figure 3, b). At constant temperature, yeast growth increased by increasing fermentation time. In the early days, the growth rate was higher, but then it decreased (Figure 3, c). According to the findings of Velicanski et al in 2013, the growth of yeasts increased initially with the increase of incubation time, and the growth rate reduced with the fermentation time passing [35]. At first, with increasing pH to less than 6 and increasing the incubation time, the growth rate decreased, and at higher pH, the growth rate decreased in less time. At constant pH, the growth of yeast decreased in more than 11 days (Figure 3, d). It was similar to the findings of Talawat et al. in 2006, with the increase in fermentation time and pH, the growth rate enhanced and after 13 days, it reduced, and the maximum growth of yeast occurred in average amounts of 35 g/L [36].



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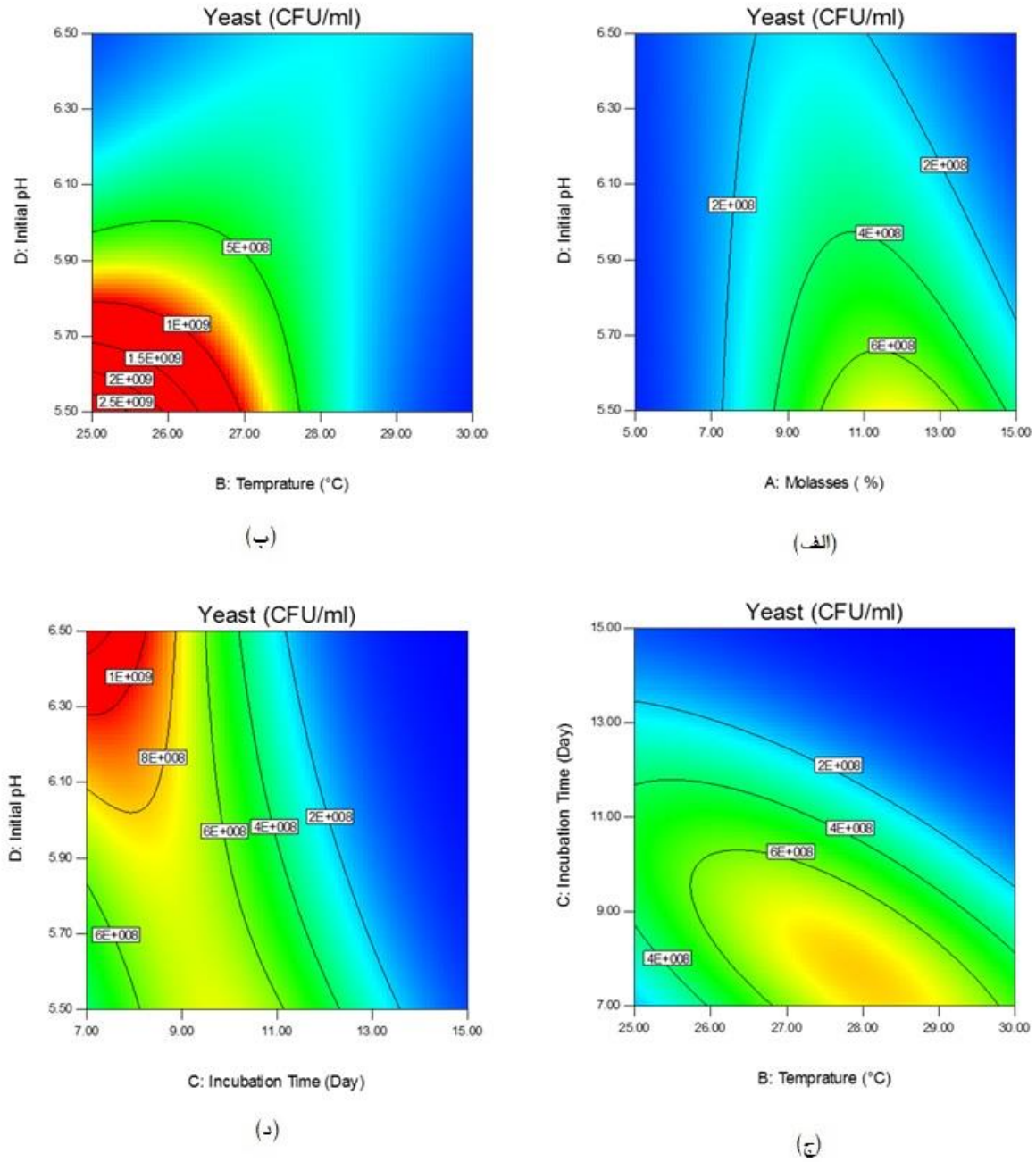


Figure 3: Enumeration of yeasts, a) interaction effect of sugarcane molasses percentage and initial pH, b) interaction effect of greenhouse temperature and initial pH, c) interaction effect of temperature and incubation time, d) interaction effect of incubation time and initial pH

**Ethanol Production**

Changes in the amount of produced ethanol under the influence of the studied parameters can be seen in (Figure 4). Ethanol production was affected by sugarcane molasses percentage, incubation temperature and interaction effect of sugarcane molasses percentage with temperature ($p<0.05$). In relation to the interaction effect of sugarcane molasses percentage and greenhouse time, at a constant time, the amount of ethanol increased with the increase of sugarcane molasses percentage. At 9% to 15% molasses, the amount of ethanol remained constant at 5 g/L and then started to raise again. Also, regarding the interaction effect of sugarcane molasses percentage and





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initial pH, at constant pH, the amount of ethanol raised by increasing molasses percentage. Regarding the interaction effect of temperature and incubation time, with the increase of both fermentation variables, ethanol increased at first, but started to decrease at a temperature above 28°C.

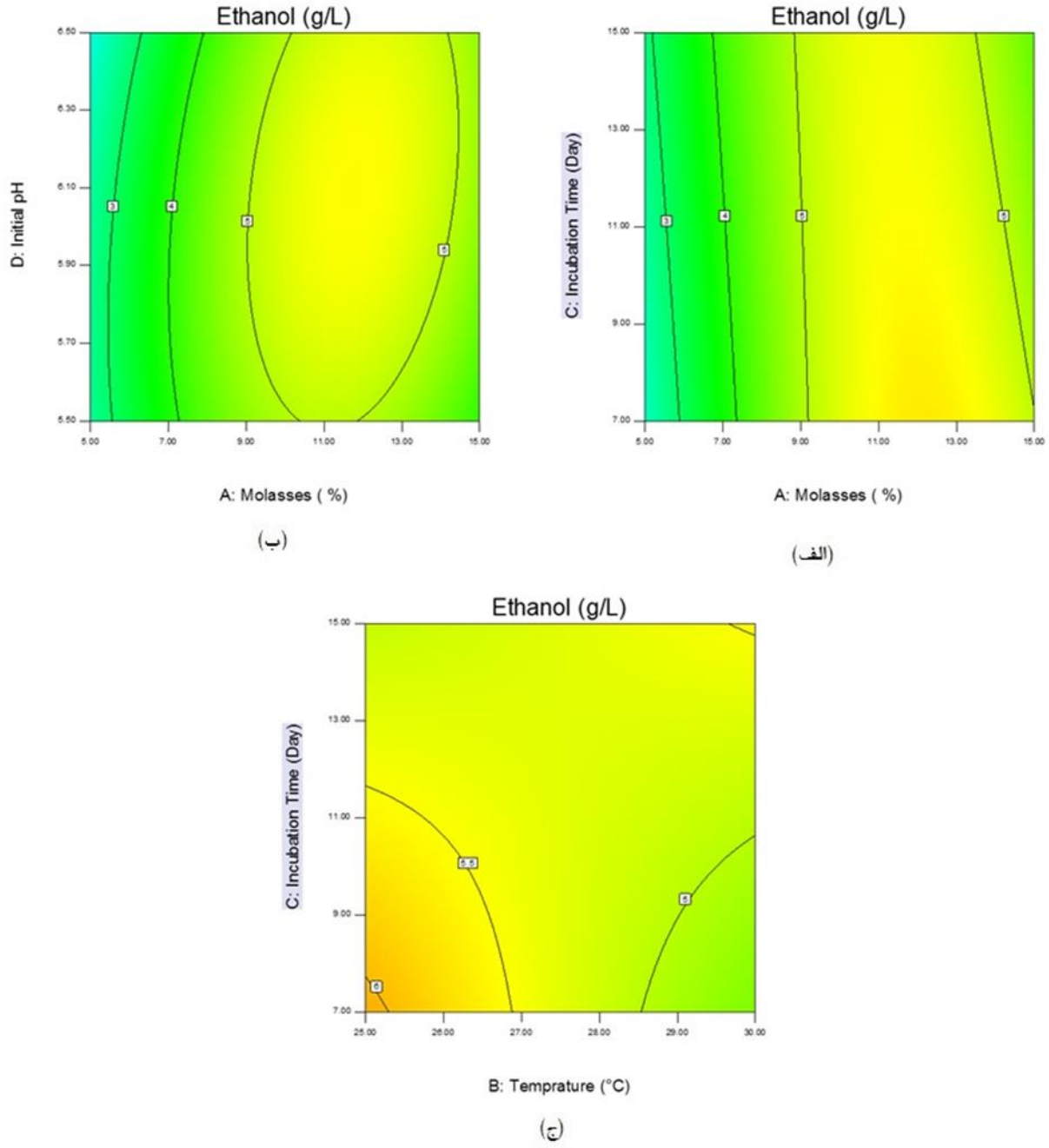


Figure 4: Changes in produced ethanol, a) the interaction effect of sugarcane molasses percentage and hothouse time, b) the interaction effect of sugarcane molasses percentage and initial pH, c) the interaction effect of temperature and hothouse time

**pH variations**

Measurement of pH variations of fermented





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samples on different days of incubation showed that the pH decreased during the fermentation period (Figure 5, a). The reason for the decrease in pH is due to the production of organic acids by kombucha microorganisms. Incubation time, percentage of sugarcane molasses, initial pH, as well as the interaction effect of time with the percentage of sugarcane molasses and the interaction effect of incubation temperature with initial pH had a significant effect on pH ($p<0.05$), but the incubation temperature did not have a significant effect on pH variations during the fermentation process ($p<0.05$). Regarding the interaction between the percentage of sugarcane molasses and the incubation time, in the fermentation samples prepared with different percentages of sugarcane molasses, the pH decreased regularly with the increase in the fermentation time, but by increasing the amount of sugarcane molasses during the fermentation period, the reduction in pH lessened. The highest pH value was observed in amounts greater than 13% of sugarcane molasses, which is in accordance with the findings of Malbasa et al. in 2008 [29]. Regarding the interaction effect of temperature and incubation time by simultaneously increasing both variables pH decreased rapidly. The highest amount of changes obtained at 30°C and between 9-13 days, which is in accordance with the findings of Lonkar et al. in 2006 [37]. In relation to the interaction between incubation time and initial pH, at constant initial pH, with the increase of incubation time, the final pH decreased steadily, and its greatest changes occurred between days 10-13. Similar results was observed by the study conducted by Heydari et al. examined the production of fermented kombucha drink using apple vinegar, white tea, and date syrup. The researchers found that the pH decreased, while the cellulosic layer and acidity increased during fermentation, which was influenced by the concentration of date syrup and the duration of fermentation ($p<0.05$) [38].



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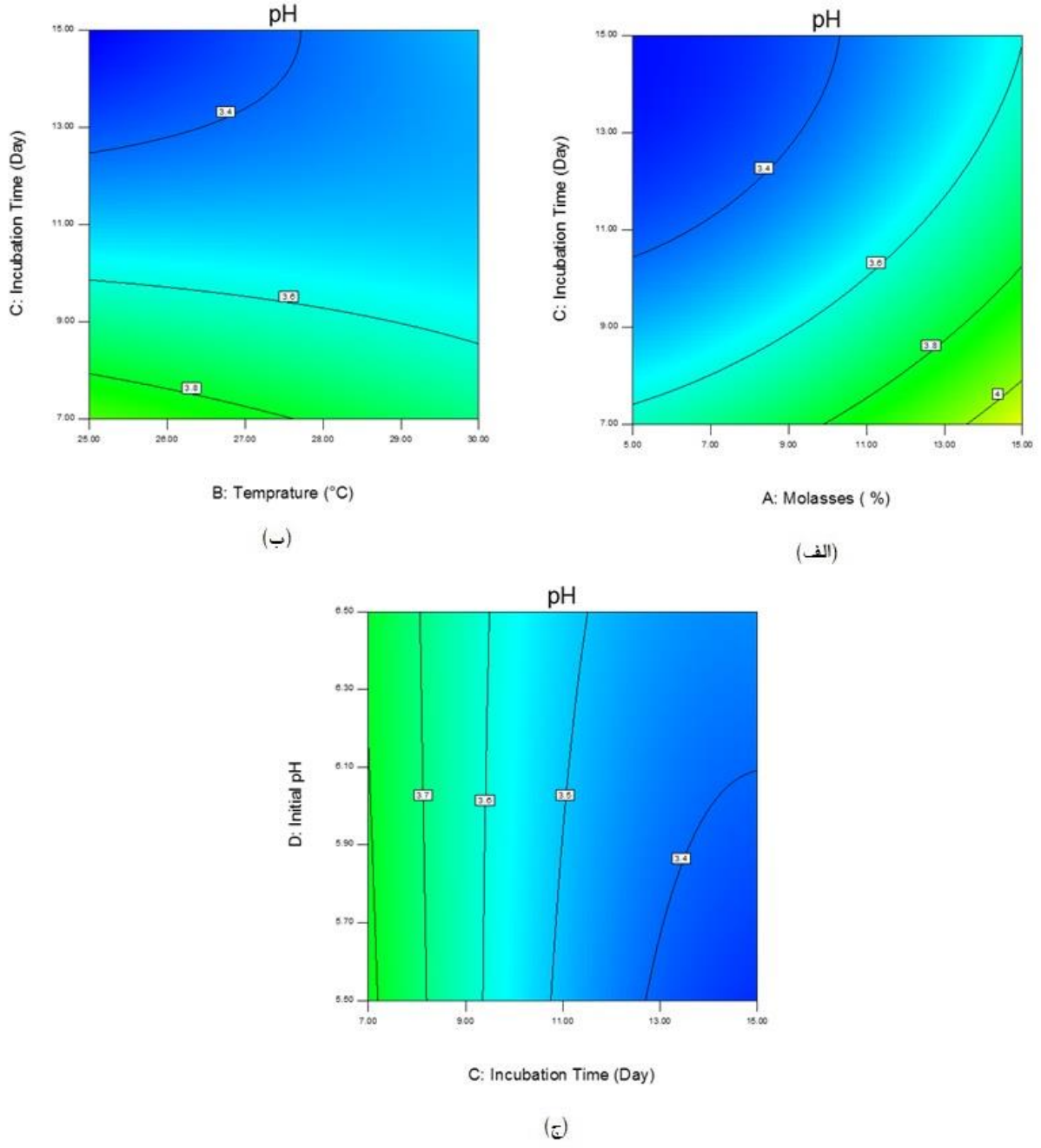


Figure 5: pH changes during the fermentation process, a) interaction effect of sugarcane molasses percentage and incubation time, b) interaction effect of incubation time and temperature, c) interaction effect of incubation time and initial pH

**Total acidity**

As seen in (Figure 6), the amount of total acidity increased during the fermentation process. The amount of total acidity is affected by sugarcane molasses percentage, incubation time and also the interaction effect of sugarcane molasses percentage with temperature and incubation time ($p<0.05$). By keeping the percentage of sugarcane molasses constant and increasing the incubation time, the amount of total acidity increased to 6 g/L, which is in agreement with the findings of Talawat et al. in 2006 [33]. With the increase in the percentage





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of sugarcane molasses and the incubation temperature, the amount of total acidity increased. Also, with the increase in incubation temperature, acidity increased due to the production of organic acids, which is in accordance with the findings of Yavari et al. in 2010 [11].

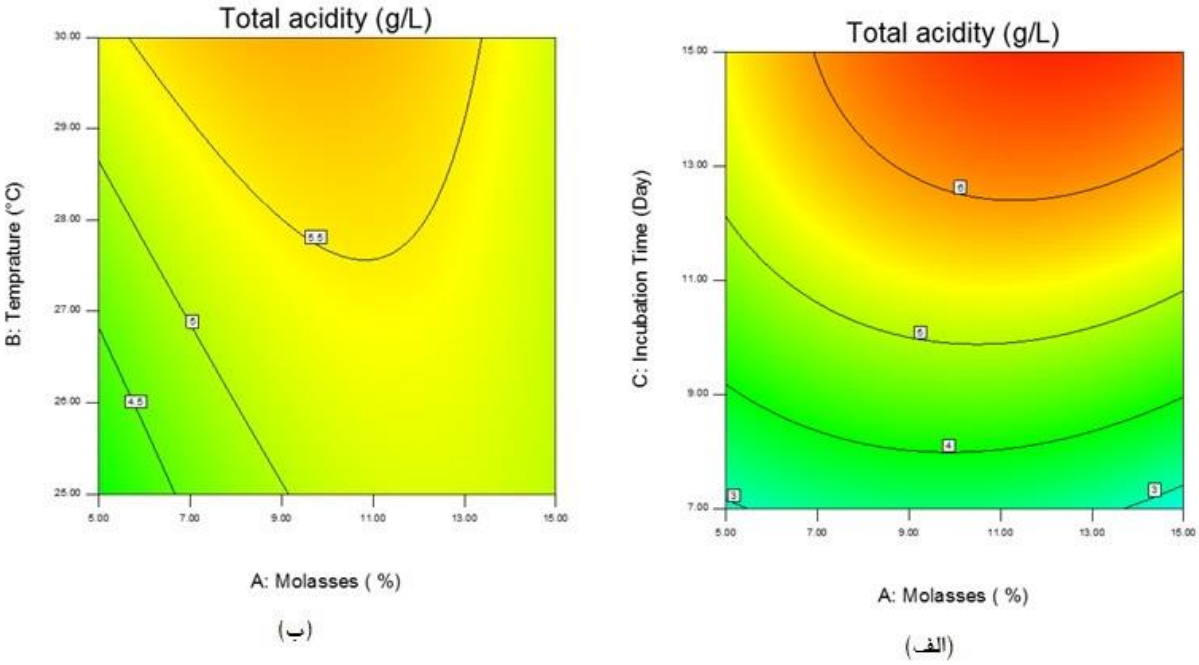


Figure 6: Total acidity changes, a) the interaction effect of sugarcane molasses percentage and incubation time and b) the interaction effect of sugarcane molasses percentage and incubation temperature

**Residual Sucrose**

Sucrose was metabolized during fermentation and its amount decreased (Figure 7). The amount of sucrose consumption during the fermentation process was affected by the percentage of sugarcane molasses, incubation time, as well as the interaction effect of sugarcane molasses percentage with initial pH, temperature and incubation time, likewise the interaction effect of incubation time with incubation temperature ($p<0.05$). The results showed that at a constant temperature, with the increase in the percentage of sugarcane molasses, the consumption of sucrose increased and its consumption was superior in higher amounts of sugarcane molasses. Also, with the increase of both variables (cane molasses percentage and incubation temperature), sucrose consumption increased continuously, and in higher percentages of sugarcane molasses, sucrose was almost completely consumed until the end of the fermentation period, the results of the study were consistent with the findings of Malbasa et al. in 2008 [28]. It is worth noting that at constant initial pH, sucrose consumption increased uniformly with increasing sugarcane molasses percentage. At constant incubation temperature, with increasing fermentation time, sucrose in the medium decreased and reached 0.8% on the 14th day of fermentation, which is consistent with the findings of Lonkar et al. in 2006 [34].



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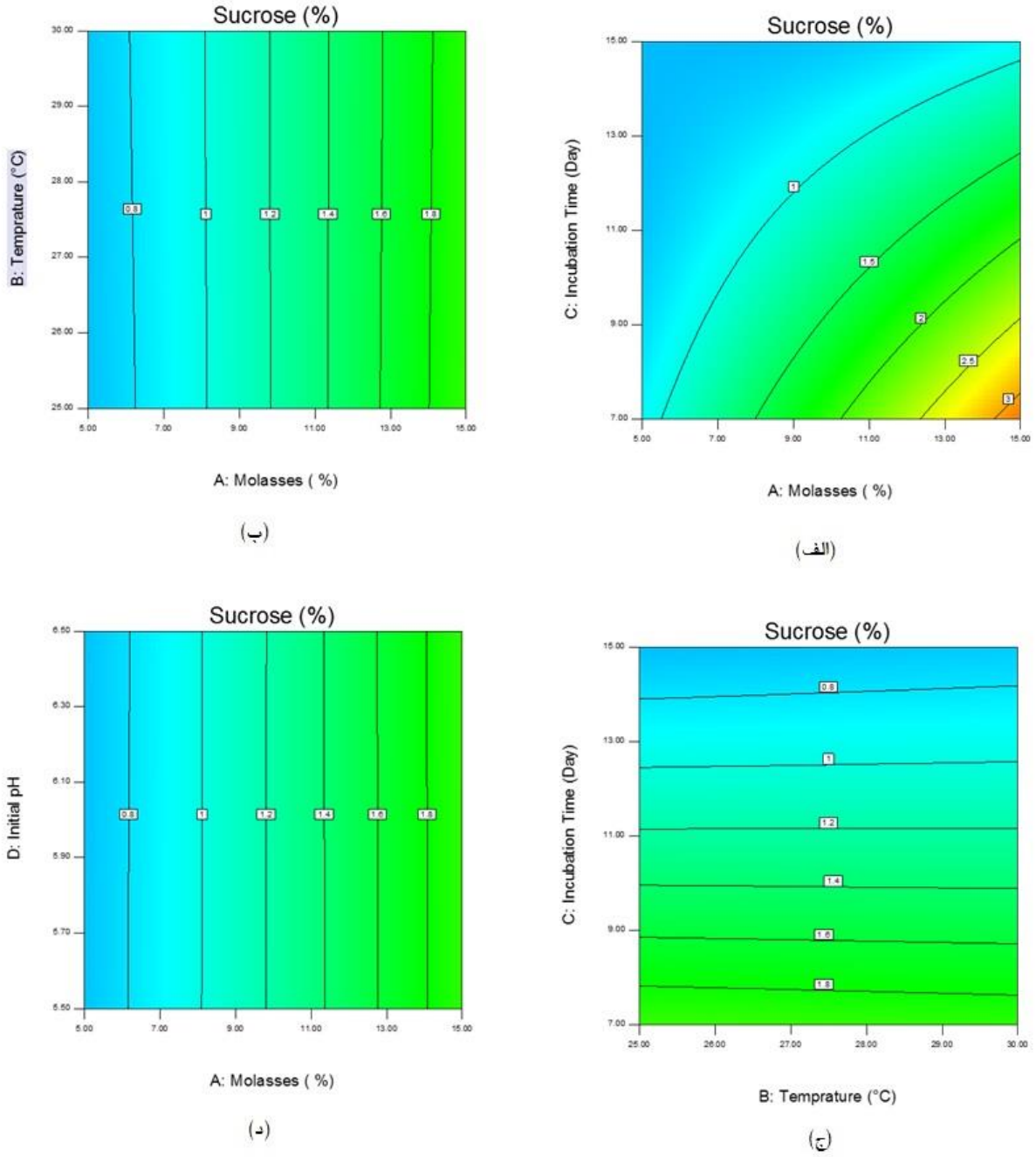


Figure 7: Changes in sucrose content during the production process of kombucha, the interaction effect of a) sugarcane molasses percentage and incubation time, b) sugarcane molasses percentage and incubation temperature, c) temperature and incubation time, d) sugarcane molasses percentage and initial pH

**Optimization**

For this purpose, using the numerical optimization of the maximum amount of bacterial cellulose produced, the minimum amount of ethanol produced and the minimum amount of remaining sucrose in the culture medium were considered as the main conditions and the optimal conditions of the process were obtained in 5% sugarcane molasses, 15 days of





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incubation storage, incubation temperature of 27 ℃ and initial pH of 6.5. In optimal conditions, the values of the studied responses will be as follows, which has a utility function of 0.71: final pH 3.33, cellulose layer 6.5 grams, total acidity 5.3, remaining sucrose 0.67%, ethanol 2.24%, the total count of acetic acid bacteria $8.1*10^5$ CFU/mL and yeasts $4\times10^5$ CFU/mL.

## 4-Conclusion

In this research, it was found that the concentration of sugar cane molasses, initial pH, incubation time and incubation temperature are effective in the production of cellulose layer in the fermentation process of kombucha. The results showed that the pH decreased due to the fermentation of hydrolyzed sucrose by yeasts and the production of organic acids, but its decrease was less in higher concentrations of sugarcane molasses. Moreover, during the fermentation process, ethanol is produced, which acetic acid bacteria consume and produce bacterial cellulose. According to the obtained results, sugarcane molasses can be a cheap carbon source and suitable alternative for kombucha fermentation.

## 5-References

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