

## Article

# Optimization of an Industrial Medium from Molasses for Bioethanol Production Using the Taguchi Statistical Experimental-Design Method

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**Abstract:** The production of bioethanol as a clean liquid fuel in a cost-effective way is highly desired by global energetics. Sugar beet molasses is a renewable and cheap substrate for the production of biotechnological products. Therefore, the aim of the current study was the optimization of an industrial medium from molasses for bioethanol production using the Taguchi statistical experimental-design method. First, the growth rate of yeast cells and the amount of ethanol produced by the *Saccharomyces cerevisiae* strain sahand 101 were investigated in aerobic and aerobic–anaerobic conditions. The yeast strain produced 8% (*v/v*) bioethanol in a medium containing molasses with 18% Brix in aerobic–anaerobic conditions. The main factors of the medium, including molasses, ammonium sulfate, urea, and pH, were optimized for the increase of bioethanol production by the Taguchi method. Bioethanol production reached 10% (*v/v*) after optimization of the medium in flask culture. The yeast strain produced 11% (*v/v*) bioethanol in the bioreactor culture containing the optimized medium, which is an acceptable amount of bioethanol produced from molasses at the industrial scale. The results showed that the Taguchi method is an effective method for the design of experiments aiming to optimize the medium for bioethanol production by reducing the number of experiments and time.

**Keywords:** bioethanol; yeast; *Saccharomyces cerevisiae*; optimization; Taguchi method

## 1. Introduction

The exploitation of fossil fuels is responsible for 73% of global carbon dioxide emissions which are considered to be the main factor contributing to global warming and environmental changes [1,2]. The limited resources of fossil fuels, the increasing costs of these fuels, and concerns about climate change are the main reasons to trend towards biofuel production like bioethanol, biodiesel and biomethane [3]. Thus, the importance of microbial production of bioethanol has risen for many years. Efforts on the intensification of the microbial bioethanol production are continued [4]. The optimization of an industrial medium is one of the ways to increase bioethanol production. The optimized medium contains optimal amounts of all components, including carbon and nitrogen sources and growth factors, and the minimal amounts needed for growth and the production of metabolites by a microorganism [5,6]. Sugar beet molasses is available as a cheap carbon source in Europe and many countries. For example, 3.7 million tons of molasses were produced in the European Union in 2018. It is rich in sucrose, vitamins, and minerals for the growth and bioethanol production by *Saccharomyces cerevisiae* [7,8]. Molasses with high a concentration of sugar is suitable for fermentation and ethanol production. Of the world's molasses resources, 40% are used for ethanol production, especially by European countries [9,10].

Formation of a given bioproduct can be increased by modification of the culturing medium's composition. Modifications can rely on changing the medium components and their amounts. Such modifications can be driven by identified interactions between the components of the medium. Systematical optimization of a medium is done based on the design of experiments (DOE) and involves the determination of the interactions between the main factors contained in the medium. One factor at a time (OFAT) is a type of DOE which determines optimum composition of a medium by changing one factor at a time without finding the interaction between factors, and it is a time-consuming procedure. The most complete DOE is a full factorial method. In the full factorial method, all possible interactions between factors and their levels are considered. But it is an expensive and error-prone method. Fractional factorial methods are an alternative for DOE which reduce the number of experiments, time and costs of experimental run [11]. The Taguchi method is one of the simple and effective fractional factorial methods for DOE [12,13]. This method is a statistical tool for orthogonal arrays (design) with a minimum number of experimental runs. It also arranges various factors for efficient optimization in the experimental conditions and uses analysis of variance (ANOVA) for statistical analysis [14,15].

Hence, we used the Taguchi statistical experimental-design method to optimize an industrial medium, containing molasses as a cheap carbon source, ammonium sulfate and urea as nitrogen sources which are used in Iranian ethanol production plants, for increased bioethanol production by the newly isolated yeast strain *Saccharomyces cerevisiae* sahand 101. Furthermore, the efficiency of the optimized medium was evaluated in bioreactor cultivation.

## 2. Materials and Methods

### 2.1. Yeast Strain

*S. cerevisiae* sahand 101 is an industrial strain recently isolated from Iranian ethanol production plants and was used in the current study [16].

### 2.2. Media and Culture Conditions

YPD medium was used for yeast growth. An industrial medium, containing molasses with 18% Brix, 1 g/L ammonium sulfate, and 1 g/L of urea, was used as a basic medium for ethanol production and optimization. The yeast strain was cultured in the YPD agar medium for 24 h at 29 °C. Then, one colony was transferred into 20 mL of the industrial medium as seed culture in a 100 mL-shake flask and incubated for 24 h at 29 °C and 200 rpm. Seed culture of 1.5 mL was inoculated into a 100 mL of the industrial medium in a 500 mL shake-flask and incubated at 29 °C and 200 rpm for 48 h. This was aerobic cultivation. For anaerobic cultivation, the previous culture was transferred to a container and sealed by a rubber cap with a double bubble–water–air lock and incubated at 29 °C. Yeast culture was centrifuged at 10,000 rpm for 20 min, then the supernatant was used for ethanol concentration measurements [16].

### 2.3. Ethanol Assay

Ethanol concentration was determined by colorimetric assay with a sulfochromic solution. The absorbance of samples was measured by the Shimadzu UV-1800 spectrophotometer at 578 nm [17,18].

### 2.4. Measuring Yeast Cell Growth

Yeast cell growth was monitored by the counting method using a hemocytometer [19].

### 2.5. Optimization of the Ethanol Production Medium by the Taguchi Experimental-Design Method

Qualitek-4 software (version 14.5+) (Nutek Inc., Bloomfield Hills, MI, USA) was used to optimize the production medium by the Taguchi experimental-design method [20]. Four main factors of the industrial medium including molasses, ammonium sulfate, urea and pH at four different levels were

included in optimization design for optimization of the medium (Table 1). After that, the software recommended 16 different experiments for the run (Table 2).

**Table 1.** Selected four factors of the medium and their levels for the design of experiments by the Taguchi method.

Factor	Level 1	Level 2	Level 3	Level 4
Brix of molasses (%)	15	18	21	24
Ammonium sulfate (g/L)	1	2	3	4
Urea (g/L)	1	2	3	4
pH	4	5	6	7

**Table 2.** Recommended experiments by the Qualitek-4 software.

Experiment No.	Molasses (% Brix)	Ammonium Sulfate (g/L)	Urea (g/L)	pH
No. 1	15	1	1	4
No. 2	15	2	2	5
No. 3	15	3	3	6
No. 4	15	4	4	7
No. 5	18	1	2	6
No. 6	18	2	1	7
No. 7	18	3	4	4
No. 8	18	4	3	5
No. 9	21	1	3	7
No. 10	21	2	4	6
No. 11	21	3	1	5
No. 12	21	4	2	4
No. 13	24	1	4	5
No. 14	24	2	3	4
No. 15	24	3	2	7
No. 16	24	4	1	6

## 2.6. Bioreactor Cultivation

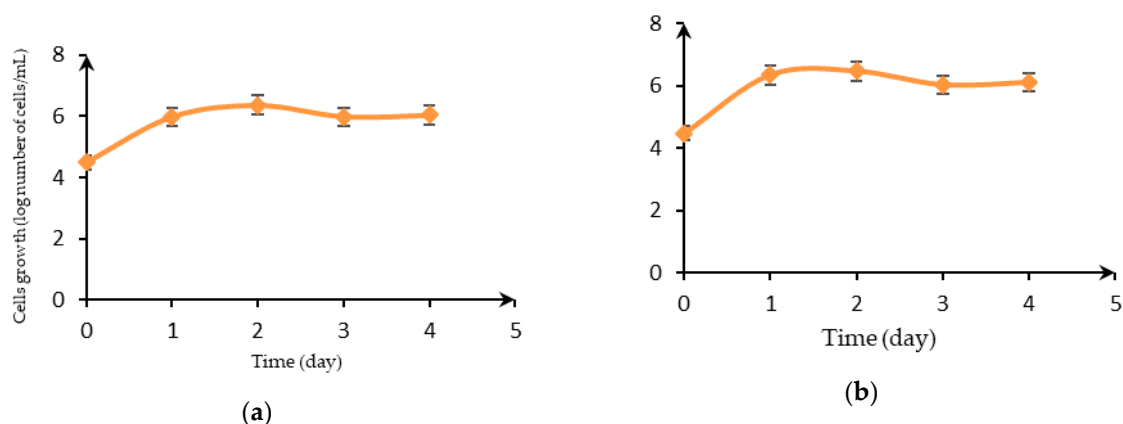
The yeast strain was cultivated in a 5 L bioreactor (Winpact, Taoyuan, Taiwan) containing a 2 L volume of the optimized medium in order to scale up the production of bioethanol. The bioreactor was equipped with two RDT6 Rushton turbines and a built-in digital controller for pH, temperature, agitation, and dissolved oxygen (DO), along with peristaltic pumps for adding acid, base, antifoam, and nutrients. The setpoint for pH and DO concentration was controlled by online monitoring using a pH sensor (Mettler–Toledo InPro3030/325, Urdorf, Switzerland) and a DO sensor (Mettler–Toledo InPro6800/12/320, Urdorf, Switzerland), respectively. The yeast strain was cultured in 200 mL YPD at 29 °C and 200 rpm for 24 h and inoculated at an initial optical density of 0.1, at 600 nm into the bioreactor. The aerobic condition towards biomass propagation was achieved at 29 °C at 150 rpm and an airflow rate of 1 vvm for 24 h. The foam level in the reactor was monitored by an antifoam probe placed 10 cm from the top of the vessel, and pH was adjusted at a value of  $7 \pm 0.1$  by addition of NaOH 3 N or H<sub>3</sub>PO<sub>4</sub> 3 N. Afterwards, and in order to produce bioethanol, the anaerobic conditions were implemented by sparging nitrogen gas into the medium [19,21].

## 3. Results

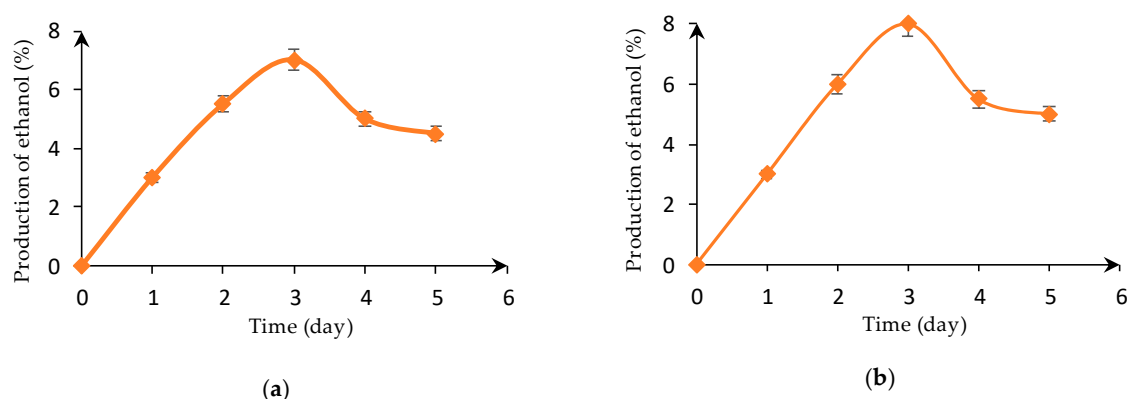
### 3.1. Yeast Cell Growth and Ethanol Production in the Industrial Medium

*S. cerevisiae* sahand 101 was cultured in the industrial medium in shake-flask batch cultures under aerobic and aerobic–anaerobic conditions in order to determine the yeast strain ability for growth and ethanol production. Biomass was propagated in aerobic conditions, but ethanol was produced in anaerobic conditions. Figure 1 shows the growth of yeast cells in both aerobic and aerobic–anaerobic

conditions. The highest amount of ethanol production after three days was 7% (*v/v*) in aerobic conditions (Figure 2a) and 8% (*v/v*) in aerobic–anaerobic conditions (Figure 2b). The results show that ethanol production in aerobic–anaerobic conditions is better than in aerobic conditions only.



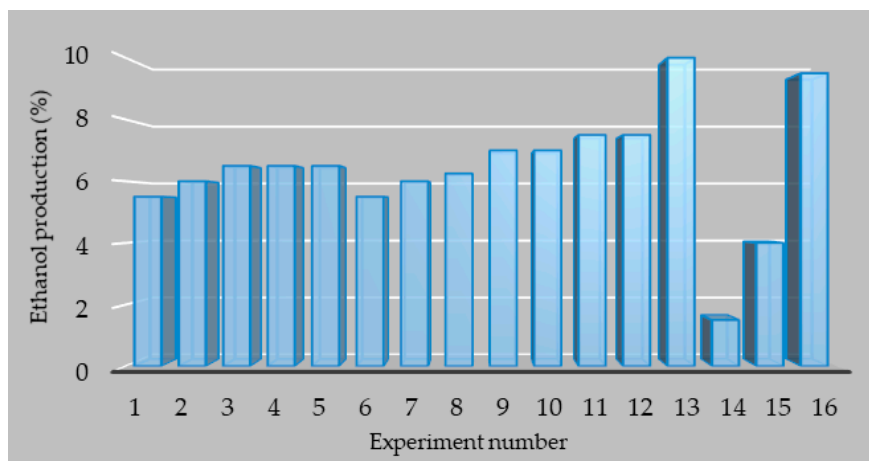
**Figure 1.** Growth curves of *S. cerevisiae* sahand 101 in the industrial medium in aerobic conditions (a) and in aerobic–anaerobic conditions (b).



**Figure 2.** The production of ethanol by *S. cerevisiae* sahand 101 in the industrial medium in aerobic conditions (a) and in aerobic–anaerobic conditions (b).

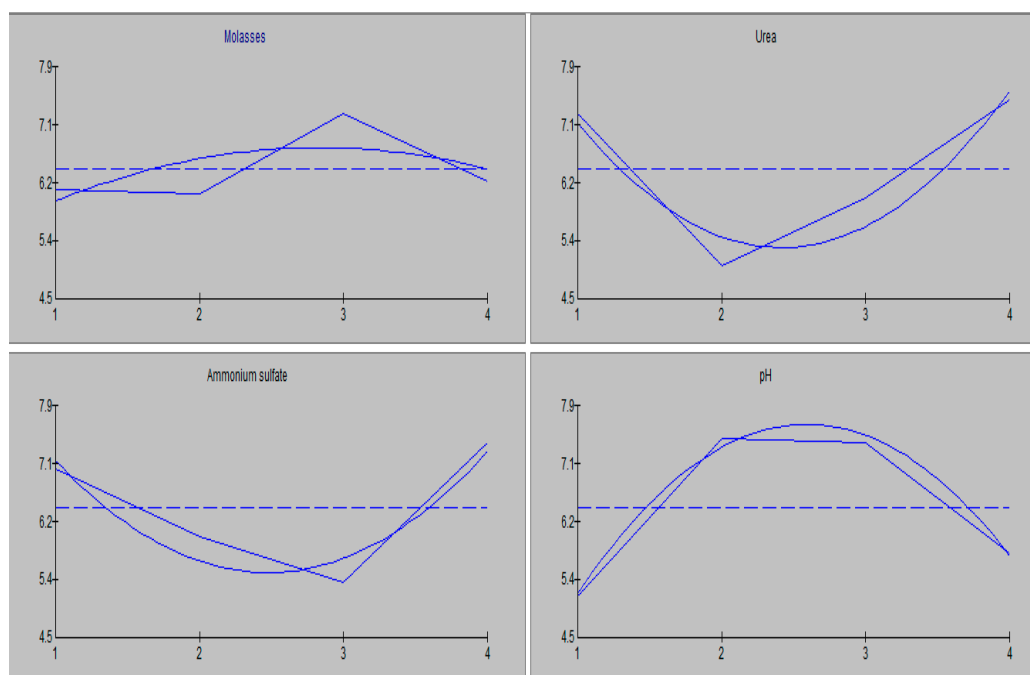
### 3.2. Optimization of the Industrial Medium for Increasing Ethanol Production

Four factors in four levels of the industrial medium including molasses (15%, 18%, 21%, and 24% Brix), ammonium sulfate (1, 2, 3 and 4 g/L), urea (1, 2, 3 and 4 g/L), and pH (4, 5, 6 and 7) were selected for optimization with the Taguchi method. Qualitek-4 software recommended 16 experiments with different combinations. Ethanol production in the 16 experimental variants after 72 h of culturing are presented in Figure 3.



**Figure 3.** The results of ethanol production by *S. cerevisiae* sahand 101 in 16 experiments designed by the Taguchi method.

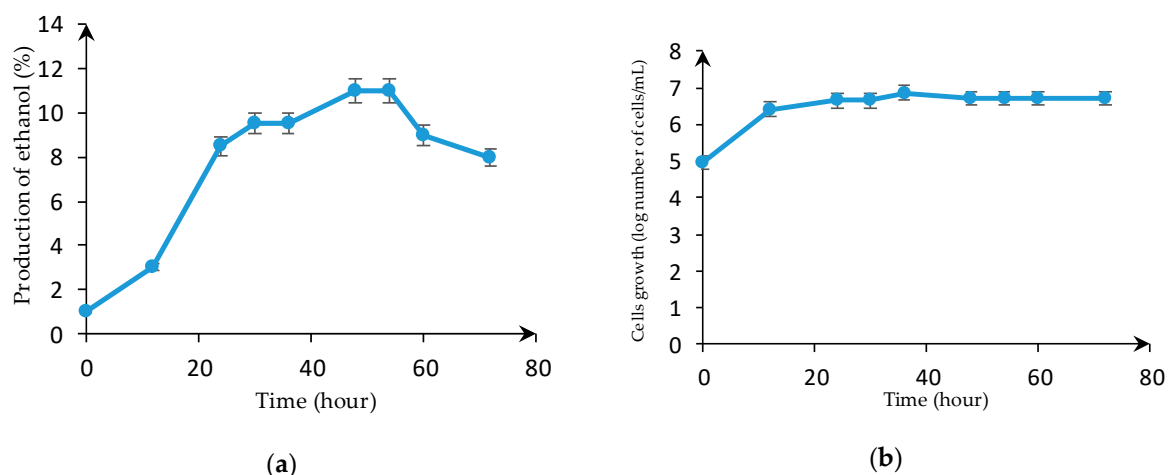
The results of the 16 experiments were analyzed by the software. According to software analysis (Figure 4), level 3 of carbon sources (molasses with 21% Brix), level 4 of ammonium sulfate and urea (4 g/L) and level 2 of pH (pH = 5) were the best and optimum conditions for the production of ethanol. The highest amount of ethanol, with 10% (v/v), was achieved in the optimized medium.



**Figure 4.** Determine the best level of four selected factors optimized by Qualitek-4 software.

### 3.3. Ethanol Production in the Bioreactor

In bioreactor culture, the ethanol concentration reached 11% (v/v) after 54 h. Therefore, the yield was increased by 10% in bioreactor cultivation compared to flask cultures, in a shorter time (Figure 5).



**Figure 5.** *S. cerevisiae* sahand 101 culture in a 5 L bioreactor containing the optimized medium. (a) Production of bioethanol; (b) yeast cell growth.

#### 4. Discussion

The production of renewable biofuels is very important in order to reduce and counteract the environmentally destructive effects of fossil fuels exploitation. Bioethanol is one of the renewable biofuels that should be produced economically for its widespread application. The optimization of industrial medium and fermentation conditions is a simple and effective way to economically produce of bioethanol [22].

Molasses is a cost-effective substrate for ethanol fermentation with higher sucrose content. *S. cerevisiae* is the main industrial microorganism used for bioethanol production. Since it can utilize sucrose very efficiently, molasses, rich in this sugar, meets its physiological requirements. Molasses is being considered for the production of the first generation of bioethanol in Europe and many countries [23]. Hence, it was used as a substrate in the current study to develop and optimize an industrial medium for bioethanol production by *S. cerevisiae* sahand 101.

Newly isolated yeast *S. cerevisiae* sahand 101 was cultured in the industrial medium containing molasses with 18% Brix in aerobic and aerobic–anaerobic conditions. The highest amount of bioethanol production was 7% (*v/v*) in aerobic conditions and 8% (*v/v*) in aerobic–anaerobic conditions after 72 h. Therefore, the aerobic–anaerobic conditions are suitable for bioethanol production, because biomass was propagated in aerobic conditions and anaerobic conditions are suitable for alcoholic fermentation and ethanol production. Thammasittirong et al. produced 8.6% (*v/v*) ethanol in a medium containing molasses with 28% Brix by *S. cerevisiae* NR1 [24].

The Taguchi method reduces the number of experiments and determines the interactions between involved factors in the process for optimization. Therefore, we selected four factors with four levels of the industrial medium, including molasses, ammonium sulfate, urea, and pH, for optimization using the Taguchi method. According to the software analysis, the best composition of the optimized medium for the production of ethanol was composed of molasses with 21% Brix, 4 g/L of ammonium sulfate and urea with pH 5. The *S. cerevisiae* sahand 101 produced 10% (*v/v*) of ethanol in the optimized medium. Therefore, ethanol production in flask culture with the optimized medium was 2% more than that in the non-optimized medium, and that is a significant increase at the industrial scale.

Shankar et al. used response surface methodology (RSM) based on the central composite design to optimize the fermentation conditions of *S. cerevisiae* MTCC 170 for ethanol production. According to the analysis of design expert software, optimum conditions were a pH of 3.5, a temperature of 35 °C and an initial sugar concentration of 4% glucose. Ethanol production was 10% ethanol by optimizing factors involved in the process [25].

The Brazilian ethanol production plans utilize molasses with 15–20% Brix at the industrial level of ethanol production. Due to osmotic pressure, ethanol production is decreased when the concentration of molasses is increased [26].

Nitrogen sources play a significant role in yeast growth and ethanol production. Nitrogen sources cause glycerol formation which is regulated by osmotic pressure of the cell during ethanol production [27]. The effect of various nitrogen sources, such as urea and ammonium sulfate, on ethanol production by *S. cerevisiae* have been evaluated. Ethanol concentration reached around 6.7% after 24 h when 0.8 g/L of urea was introduced into the sweet maize medium. A concentration of about 9.7% of ethanol was obtained when ammonium sulfate was added to the medium. As a result, the nitrogen source is necessary for yeast cell growth and tolerance to ethanol [28].

Furthermore, results of previous studies show that the nitrogen source cannot increase the carbon source uptake by *S. cerevisiae*, but a suitable amount of the nitrogen source can reduce the formation of byproducts and increase the yield of ethanol production. Carbon metabolism is different when using urea as a nitrogen source compared to ammonium sulfate. Ethanol yield increases with urea because it produces NADH and osmotic pressure does not increase. Also, it can act as a molecular filter by binding to proteins and prevent the destruction of proteins during alcohol production [29].

Another key factor affecting alcoholic fermentation is the pH value. The concentration of hydrogen ions is a major factor affecting cell growth and production of metabolites. For example, acidic pH of about 4–4.2 is suitable for ethanol production by *S. cerevisiae* [30,31].

Lin et al. carried out ethanol production under anaerobic conditions at pH 3, 4, 5, 5.5 and 6. The incubation time was longer in order to produce the maximum ethanol concentration when pH was lower than 4. The production of ethanol was reduced when pH was set above 5. Therefore, pH in the range of 4 to 5 can be considered as an optimum range for the production of ethanol under anaerobic conditions [32]. Alcoholic fermentation in acidic conditions is important because the growth of harmful bacteria is stopped by acidic conditions and yeast growth is better under acidic conditions [33]. Molasses is alkaline, and the culture medium should be acidified during alcoholic fermentation. The optimized medium was used in bioreactor cultivation for scaled-up ethanol production. The bioethanol amount reached 11% (v/v) after 54 h. Therefore, ethanol production increased by up to 1% in bioreactor cultivation compared to flask cultures, in a shorter time. Ethanol production is usually within 10–14% (v/v) in the ethanol industry, with the high theoretical yield of 90–93% for the fermentation efficiency of the conversion of glucose into ethanol [34,35]. Hence, 11% (v/v) of bioethanol production, with molasses as the renewable substrate, is acceptable for an industrial scale. In the industrial scale, a 1% increase in ethanol production is considerable. The Taguchi method is an effective method for optimization of ethanol production. Our yeast strain initially produced 8% ethanol, and after optimization, this amount reached 10% in flask culture and 11% in bioreactor culture from molasses. Our results recommend that the Taguchi method can be used to optimize the production of ethanol by other yeast strains used in the ethanol industry right now.

## 5. Conclusions

Biomass propagates in aerobic condition and ethanol is produced in anaerobic conditions in flask cultures. Furthermore, nitrogen sources, beside molasses, have a major effect on the production of bioethanol, yeast cell growth, and tolerance to ethanol. The yeast strain produces 11% (v/v) bioethanol in the bioreactor culture containing the optimized medium which is an acceptable amount of bioethanol produced from molasses at the industrial scale. The Taguchi method is an effective method in the design of experiments to optimize the medium for bioethanol production by reducing the number of experiments and time.

**Author Contributions:** N.A.M. performed the experiments, analyzed the data, and drafted this paper. F.D. developed the concept, designed the experiments, and revised the manuscript for publication.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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