Egypt. J. Chem. **Vol. 64,** No. 12 pp. 7017 - 7028 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Enhancement of bioethanol production by *Saccharomyces cerevisiae* under high gravity fermentation using sequential optimization strategy

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Abstract

Using high gravity fermentation for bioethanol production by *Saccharomyces cerevisiae* is faced with many challenges such as increasing osmotic stress and viscosity. Unfortunately, increasing specific gravity was accompanied by a decrease in fermentation efficiency. A two-stage sequential optimization strategy was carried out to solve this problem. The first stage was carried out using the Plackett-Burman Design in which eight factors were investigated at three molasses specific gravities (1.130, 1.145, 1.160). Urea, wheat bran, soy flour, and inoculum size were significant model terms and had positive effect on bioethanol production. When a validation test was performed using the predicted conditions, about a more than two-fold increase in fermentation efficiency (FE) was achieved (83%) at specific gravity 1.130, compared to the basal condition. The second stage (Taguchi Design) was conducted using eight factors. Urea, wheat bran, temperature, and agitation speed showed a significant effect on fermentation efficiency. A validation test was carried out and up to 83.82 % fermentation efficiency was recorded, which was very close to that achieved under normal gravity. In addition to the high value of FE, this strategy is cost-effective and time-saving.

Keywords: Bioethanol; Molasses; S. Cerevisiae; High gravity fermentation; Statistical design

1. Introduction

Bioethanol is a renewable and cleaner fuel used to mitigate the negative environmental impacts caused by the worldwide extensive consumption of fossil fuels [1, 2]. Moreover, the need for ethanol has skyrocketed recently after the outbreak of the coronavirus pandemic, especially that disinfectants and sanitizers containing ethanol are highly recommended and widely used for inactivating the COVID-19 virus [3].

Ethanol can be produced either chemically from petrochemical compounds, or biologically from carbohydrates found in natural carbon sources [4], via microbial fermentation. Petrochemicals are non-sustainable and highly demanded in a plethora of competing industrial processes. Hence, there is a global demand for producing ethanol by biological processes.

Bioethanol fermentation process depends on several factors, mainly the fermenter strain, fermentation substrate, and applied process [5]. Industrial production relies heavily on employing low costs of raw materials [6].

Fermentation process of food crops or sugarcane molasses is a widely used method of ethanol production [7]. One of the most beneficial features of using molasses and sugarcane juice is that they do not require pretreatment, milling, nor hydrolysis processes. Hence, molasses is considered one of the cheapest and most promising feedstocks for bioethanol production [8]. In addition, about 400,000 tons of molasses are produced annually in Egypt by distillery factories [9]. Several experiments have been conducted in order to increase bioethanol production some of them directed toward the use of lignocellulosic feedstocks, a process that required several pretreatment steps and produce a little amount of bioethanol. According to the results reported by El-Tayeb et al. [10], 0.52 % (v/v) of

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Receive Date: 07 June 2021, Revise Date: 22 June 2021, Accept Date: 22 June 2021
DOI: 10.21608/EJCHEM.2021.78360.3904

bioethanol concentration was the highest concentration obtained upon using three different steps of pretreatment of lignocellulosic feedstocks. Similar results (0.9 $g.L^{-1}h^{-1}$) were obtained by Darwesh *et al*. [11] after biological treatment of cellulosic materials. Another study [12], stated that 1.6 g/L of bioethanol was achieved using treated (alkali and acidic treatment) used paper. On the other hand, 18.57 g/L of bioethanol concentration was achieved by El-Mekkawi *et al*. [13] when algal biomass was used as a substrate for bioethanol production. In another approach, utilization of a high gravity source of carbon showed a significant impact on bioethanol production [14] since 20.3 % and 84.5 % of ethanol yield and fermentation efficiency, respectively, were achieved after 72 hr of fermentation.

Distillation process represents a big challenge as it is the most energy-consuming step in bioethanol production. In order to minimize the energy required for distillation, high gravity (HG) and very high gravity (VHG) fermentation can be implemented [15]. However, the high osmotic pressure caused by the high sugar content of molasses may suppress bioethanol production by causing yeast cells to shrink [16], and increasing the fermentation time [17]. Furthermore, long exposure of yeast cells to a hypertonic solution, ethanol, and other toxic metabolites decreases the fluidity of the cell membrane which may lead to the damage of its transport and metabolism systems [18, 19]. Zhang *et al*. [20] concluded that accumulated endogenous ethanol and a high concentration of substrate may inhibit the fermentation process by affecting yeast activity.

It was reported that the success of HG and VHG fermentation depended mainly on the ability of yeast cells to tolerate the osmotic stress and high ethanol concentration [21]. When *S. cerevisiae* is supplemented with adequate amounts of all required nutrients [22], in addition to supplementary materials [23], it can withstand high sugar concentrations in fermentation media. Indeed, many factors were found to affect bioethanol production including the substrate, sugar concentration, producer strain, inoculum size, agitation rate, temperature, pH, and fermentation time [5].

Broadly speaking, using the conventional approach (one factor at a time) for optimizing many fermentation factors could be inaccurate, laborious, and time-consuming. In addition, the interaction between the various factors might not be accounted for. On the other hand, statistical experimental design can allow accurate determination of optimal fermentation conditions, and it takes into account the interaction between the different factors [24].

This study aimed to utilize the agricultural and industrial wastes as a renewable and inexpensive feedstock for improving the fermentation process of bioethanol production under HG fermentation process using a sequential optimization strategy.

2. Experimental

2.1. Yeast, substrate, and media

Saccharomyces cerevisiae and blackstrap molasses used in this study were obtained from the Egyptian Sugar and Integrated Industries Company (ESIIC), Giza, Egypt. Blackstrap molasses is a byproduct produced during sugar production from sugar cane.

Yeast extract peptone dextrose medium (YEPD) was used for yeast maintaining and propagation [25]. YEPD medium composed of ($g.L^{-1}$): yeast extract, 3.0; peptone, 10.0; dextrose, 20.0. The pH was adjusted to 4.5.

Total reducing sugar and Brix of fermentation medium as well as fermentation parameters (ethanol concentration, EC; fermentation efficiency, FE and volumetric ethanol productivity, VEP) were determined. This medium comprised of ($g.L^{-1}$ of the prepared blackstrap molasses): urea, 2.0; diammonium phosphate, 2.0; magnesium sulfate heptahydrate, 0.5. The initial pH was adjusted to 4.7 by sulfuric acid. The medium was autoclaved at 121°C for 10 min. The blackstrap molasses was treated and prepared as in item (2.3). The ingredients of media were of analytical grade and obtained from different chemical suppliers.

2.2. Inoculum preparation

The inoculum was prepared using the YEPD medium. *S. cerevisiae* maintained on YEPD agar slants (1.5% agar) was used to inoculate 10 mL of YEPD broth and incubated overnight in a shaking incubator at 150 rpm and 30 °C. The overnight culture was streaked on YEPD agar plates and incubated at 30 °C for 24-48 hr. A single colony from an agar plate was used to inoculate freshly prepared YEPD broth and incubated overnight in a shaking incubator as previously described [25]. Adjustment of yeast inoculum (viable cells/mL) was carried out with methylene blue staining technique using a hemocytometer.

2.3. Pretreatment and analysis of blackstrap molasses

The raw molasses was diluted with water (1:1) w/w and treated with concentrated sulfuric acid to adjust the pH at 4.5. The treated molasses was heated at 90 °C, with continuous mixing, for one hour. The hot mixture of molasses was left to settle for 2 hr to precipitate the sludge. The supernatant was centrifuged at 3500 rpm for 10 min. The cleared supernatants were further diluted to give different specific gravities. Specific gravities of the prepared diluted molasses were

adjusted using a specific gravity hydrometer [26]. On the other hand, the specific gravity of raw molasses was determined according to A.O.A.C [27]
A Brix hydrometer was used to measure the total soluble solids (°Brix) of raw and prepared diluted molasses at the different specific gravities [28]. The total reducing sugars (TRS) and non-fermentable sugar (NFS) were determined by the volumetric method (Fehling's test) [29, 30].

2.4. Bioethanol production

Unless otherwise stated, flasks containing 200 mL fermentation medium, adjusted at different specific gravities (1.090, 1.100, 1.130, 1.145, and 1.160), were inoculated with a known number of yeast cells (5, 10, or 15 x 10^7 cells/mL) and incubated at 33 °C. Agitation was adjusted at 150 rpm for 2 hr to enhance the propagation of yeast inoculum followed by decreasing agitation speed to 60 rpm during the rest of the incubation period. After 24 hours of incubation, fermentation parameters; EC % (v/v), FE % and VEP ($g.L^{-1}.h^{-1}$) were determined.

2.5. Ethanol measurement
The fermented samples were analyzed for the estimation of EC % (v/v) by an Ebulliometer used in distillation factories [31]. Briefly, the Ebulliometry method depends on the difference in the boiling point between water and water mixture solutions; the lower the boiling point of the ethanol-water mixture the higher the concentration of ethanol [32].

2.6. Calculation of fermentation efficiency
Fermentation efficiency (FE) is defined as the ratio of the produced amount of ethanol to the theoretically calculated amount [33, 34]. FE was calculated by the following equation (No.1):
FE (%) = (quantity of produced ethanol/quantity of theoretically expected ethanol) x 100 …… (1)
For theoretical ethanol calculations, it was estimated that 100 g of glucose should yield 51.1 g or 64.75 mL ethanol [35]. Therefore, the theoretical expected ethanol could be calculated according to the equation No. 2
Theoretical expected ethanol = total fermentable sugar × 0.6475 ……... (2)

2.7. Calculation of volumetric ethanol productivity [36]
The volumetric ethanol productivity (VEP) was calculated based on the final ethanol concentration as grams of ethanol per liter per hour ($g.L^{-1}.h^{-1}$), according to the following equations (No. 3 & 4):

VEP = (EC) in fermented broth ($g.L^{-1}$)/fermentation time (hr) ……... (3)

Where

EC ($g.L^{-1}$) = EC % (v/v) x 0.789 (density of anhydrous ethanol at 20 °C) x 10 …… (4)

2.8. Optimization of HG fermentation for bioethanol production using bio-statistical factorial design

2.8.1. Plackett-Burman design
The Plackett-Burman experimental design is a fractional factorial design [37] used to reflect the relative importance of various nutritional factors on bioethanol production at different specific gravities (1.130, 1.145, and 1.160). The studied factors were urea, yeast extract, peptone, glycine, $MgSO_4.7H_2O$, wheat bran, soy flour, and inoculum size. In this design, 500-mL Erlenmeyer conical flasks containing 200 mL fermentation medium were used, and each factor was examined at two levels: (-1) for the low level and (+1) for the high level (Table 1). Fifteen experiments were generated for the 8 selected factors and carried out in duplicates.

Table 1: Plackett-Burman factors and their assigned levels.

Factor	Lower level (-1)	Higher level (+1)
Urea ($g.L^{-1}$)	1.00	2.00
Yeast extract ($g.L^{-1}$)	0.00	3.00
Peptone ($g.L^{-1}$)	0.00	2.00
Glycine ($g.L^{-1}$)	0.00	0.50
Wheat bran ($g.L^{-1}$)	0.00	10.00
Soy flour ($g.L^{-1}$)	0.00	10.00
$MgSO_4.7H_2O$ ($g.L^{-1}$)	0.25	1.00
Inoculum size (cells/mL)	5 x 10^7	15 x 10^7

The Plackett-Burman experimental design was based on the following first-order model (equation No. 5):
$Y = \beta_0 + \Sigma\beta_i x_i$ ……. (5)
Where Y is the predicted response variable, β_0 is the model intercept, β_i represents a linear coefficient and x_i is the coded variable or the level of the independent variable. Statistical evaluation of the design was conducted using Design-Expert software v 8.0.7.1. Analysis of variance (ANOVA) was performed to evaluate the design and the variables by determination of the P-value and correlation coefficient (R^2).

2.8.2. Taguchi Orthogonal Array (OAs) design
Taguchi design is usually used to determine the interactions between the factors of the optimization process [38]. And facilitate the studying of several combinations for the most significant factors and the

interaction between factors [39]. For *S. cerevisiae*, a standard orthogonal array (OAs) L-27 (3^8) was selected to examine eight-factors at three levels. The three levels of the eight factors were coded as 1, 2, and The runs involved various combinations of the levels to which the factors were set, and the diversity of factors was studied by crossing them (Table 2).

The whole experiment was performed in triplicates using 500-mL Erlenmeyer conical flasks containing 200 mL medium each. Eight different factors (urea, wheat bran, soy flour, inoculum size, temperature, agitation speed, agitation time, and fermentation time) were studied. Four of these were selected according to the results of the Plackett-Burman design previously performed. Statistical analysis and graph plotting were performed using Design-Expert software v 8.0.7.1. ANOVA was used to evaluate the effect of the independent variable on the response (FE %) and the significant results were identified by P-value of < 0.05. Multiple (R^2) and the adjusted R^2 were used as indicators to evaluate the fitness of the equation. Three-dimensional (3D) surface plots were employed to demonstrate the relationship and interaction between the variables and responses.

Table 2: Selected culture factors and their assigned levels.

Factors		Level 1	Level 2	Level 3
Medium composition	Urea ($g.L^{-1}$)	1.5	2	2.5
	Wheat bran ($g.L^{-1}$)	5	10	15
	Soy flour ($g.L^{-1}$)	5	10	15
	Inoculum size (cells/mL)	10×10^7	15×10^7	20×10^7
Fermentation conditions	Temperature °C	28	33	37
	Agitation speed (rpm)	100	150	200
	Agitation time (hr)	1	2	4
	Fermentation time (Days)	1	2	3

3. Results and discussion

The analysis of raw and diluted molasses at different specific gravities is presented in (Table 3). Molasses can be classified according to the sugar content into three different gravities; normal, high, and very high gravities. Accordingly, the total reducing sugars (TRS) of specific gravities 1.090 and 1.100 (normal gravities) were 16.2 and 17.37%, respectively (Table 3). However, TRS of specific gravities 1.130, 1.145, and 1.160 (high gravities) were 21.22, 23.02, and 24.9 %, respectively. Bai et al. and Deesuth *et al*. [15, 40] reported that normal gravity (NG) is < 180 g.L-1 total sugars, while 180-240 $g.L^{-1}$ total sugars is HG and ≥ 250 $g.L^{-1}$ total sugar is VHG. However, Thomas *et al*. [41] defined VHG for fuel alcohol production as the preparation and fermentation to completion of mashes containing 27 or more grams of dissolved solids per 100 g. The analysis of raw molasses presented in (Table 3) indicated that TRS represented 54.48% while NFS was 5.37%. Consequently, fermentable sugars represented 49.11%. This result was in agreement with previous reports which concluded that the total sugars and NFS in Egyptian cane molasses ranged from 50 to 55% and 4-5%, respectively [42, 43]. The chemical composition of molasses is not constant in all cases and is influenced by several factors such as climatic conditions, the composition of the soil, variety, and maturity of the cane, and the method of production [44].

It was shown that there is a direct relationship between molasses specific gravity and each of TRS, NFS, fermentable sugars and brix (Table 3).

Table 3: Analysis of raw and diluted molasses at different specific gravities.

Analysis	Raw molasses	Diluted molasses at different specific gravities				
Specific gravity	1.42	1.160	1.145	1.130	1.100	1.090
Total reducing sugars (%)	54.48	24.90	23.02	21.22	17.37	16.2
Non-fermentable sugars (%)	5.37	2.42	2.24	2.05	1.71	1.58
Fermentable sugars (%)	49.11	22.48	20.78	19.17	15.66	14.62
Brix°	84.8	36.5	33.43	30.34	24.39	22.32

3.1. Effect of different molasses specific gravities on ethanol production

Figure 1 illustrates that fermentation parameters including EC, FE, and VEP, were decreased by increasing molasses specific gravity. Maximum FE (85.04 % and 84.81%) was observed at 1.090 and 1.100, respectively. However, maximum EC (8.6 %) and maximum VEP (2.83 $g.L^{-1}.h^{-1}$) were obtained at specific gravity 1.100. On the other hand, the lowest fermentation parameters were recorded at the high specific gravities (1.130, 1.145, and 1.160).

The decrease in fermentation efficiency with the increase in sugar concentration (at high gravity), in the present study, is likely due to the accumulation of high amount of residual sugars and the exposure of yeast cells to high levels of oxidative stress [45], osmotic stress [46], ethanol toxicity [21], and nutrients limitation [47].

3.2. Optimization of ethanol production using Plackett-Burman design

The data presented in (Table 4 and Fig. 2) revealed that at the three models specific gravities 1.130, 1.145, and 1.160, wheat bran, soy flour, and inoculum size, were significant model terms and had a positive effect on EC % (v/v)., as P-value was less than 0.05. However, urea and glycine were significant and had a positive effect on EC % (v/v) only at specific gravity 1.130. On the other hand, the effects of yeast extract, peptone, and $MgSO_4.7H_2O$, were not determined or insignificant.

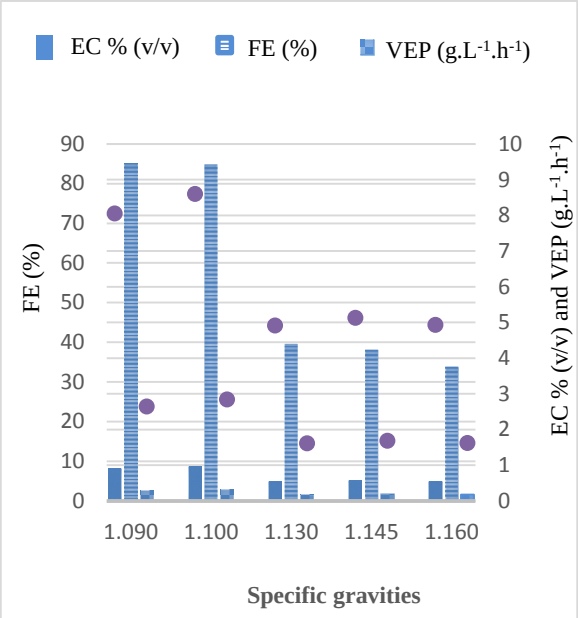

Fig. 1. Effect of different specific gravities on EC % (v/v), FE % and VEP ($g.L^{-1}.h^{-1}$). Low standard deviation (purple circles); high standard deviation (purple circles with lines).

Table 4: ANOVA analysis for Plackett-Burman designs at different specific gravities.

Source	P-value			Confidence (%)		
	1.130	1.145	1.160	1.130	1.145	1.160
Model	< 0.0001	0.0031	0.0003	> 99.99	99.69	99.97
A: Urea	0.0023	0.0881	0.0629	99.77	91.19	93.71
B: Yeast extract	ND	ND	0.2648	ND	ND	73.52
C: Peptone	ND	0.4483	0.0731	ND	55.17	92.69
D: Glycine	0.0223	ND	ND	97.77	ND	ND
E: Wheat bran	0.0006	0.0110	0.0093	99.94	98.9	99.07
F: Soy flour	0.0032	0.0026	0.0008	99.68	99.74	99.92
G: $MgSO_4.7H_2O$	ND	0.5189	ND	ND	48.11	ND
H: Inoculum size	< 0.0001	0.0019	< 0.0001	> 99.99	99.81	> 99.99

ND: not determined

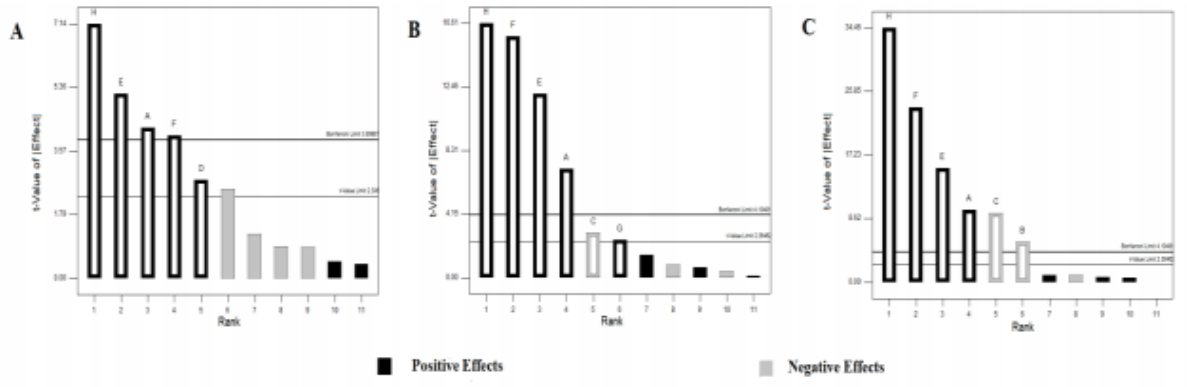

Fig. 2. Pareto chart showing positive and negative effects at different specific gravities: (A) 1.130, (B) 1.145, and (C) 1.160

Nguyen and LV [48] concluded that inoculum size is one of the most crucial factors that affect ethanol production. They reported that in presence of higher inoculum size sugar uptake was increased and led to reducing fermentation time and enhancing ethanol productivity. Consequently, larger inoculum sizes can decrease energy, labor, and capital costs [49]. Nevertheless, if the inoculum size was too large, cells would compete for nutrients and consume sugars for growth at the expense of bioethanol production [50]. Therefore, the inoculum size must be optimized.

Balakumar and Arasaratnam [51] reported that soy flour supplementation increased each of glucose fermentation, fermentation efficiency and ethanol production rate at high sugar levels and temperatures. The positive effect of wheat bran and soy flour on bioethanol production may be attributed to the presence of a high amount of lipoprotein, which is easily assimilated into cellular material [52]. The same authors stated that wheat bran may play a role in increasing the inner surface of the fermentation medium, acting as natural immobilizing material for yeast cells to adsorb on.

The current study proved also that urea plays a vital role in ethanol production only at specific gravity 1.130. This role could be due to enhancing the specific growth rate and ethanol tolerance, decreasing the byproduct formation, and increasing the ethanol yield [53]. **Theerarattananoon *et al***. [54] added that urea has a role in the elongation of the logarithmic phase during VHG fermentation.

It is well known that glycine may act as an osmoprotectant and hence improves yeast growth and fermentation which may explain the significant effect of glycine on ethanol production at 1.130 in the present investigation. Similar results were described by **Bafrncová *et al.* [23]** who reported that adding 3 $g.L^{-1}$ glycine to the production medium increased ethanol production by 50%.

3.3. Regression analysis

To establish the significance of the selected model, a regression test should be conducted and R^2 should be estimated; however, a large R^2 value may not always be a sign of a good model [68]. The close correlation of the adjusted R^2 values (0.89, 0.77, and 0.87) to the actual R^2 values (0.92, 0.87, and 0.93) for specific gravities 1.130, 1.145, and 1.160, respectively, implied the adequacy of the model. Also, the adequate precision values of the three models (16.51, 9.6, and 12.93) were higher than 4, indicating that the models are suitable to navigate the optimum production conditions [55]. In addition, the coefficient of variation (CV %) values for the three models were 5.93, 8.55, and 7.49, respectively. The lower CV values similarly indicated the reliability of the experiments [56].

After conducting validation experiments for the predicted media, it was obvious that the best FE (83%) was achieved with 1.130 specific gravity, as shown in (Table 5).

Table 5: Validation of predicted media for different molasses specific gravities.

Factor	Molasses specific gravities		
	1.130	1.145	1.160
A: Urea ($g.L^{-1}$)	1.93	1.75	1.71
B: Yeast extract ($g.L^{-1}$)	1.5	1.5	1.09
C: Peptone ($g.L^{-1}$)	1.00	0.09	0.043
D: Glycine ($g.L^{-1}$)	0.24	0.25	0.25
E: Wheat bran ($g.L^{-1}$)	9.97	6.15	5.13
F: Soy flour ($g.L^{-1}$)	7.05	9.12	9.24
G: $MgSO_4.7H_2O$ ($g.L^{-1}$)	0.63	0.3	0.63
Inoculum size (cells/mL)	14.78×10^7	14.08×10^7	14.38×10^7
Predicted EC (%)	11.2	10.11	10.54
Experimented EC (%)	10.3	10.8	7.3
Predicted EC ($g.L^{-1}$)	88.37	79.77	83.16
Experimented EC ($g.L^{-1}$)	81.27	85.21	57.60
Predicted FE (%)	90.25	75.11	72.39
Experimented FE (%)	83.00	80.24	50.15

3.4. Taguchi Design

Taguchi design was used to investigate the effect of different physical conditions (temperature, fermentation time, agitation speed, and agitation time) in addition to the significant factors previously determined by the Plackett-Burman design. The applied Taguchi design and obtained fermentation results are shown in (Table 6).

Table 6: Taguchi design variables and bioethanol fermentation efficiency at specific gravity 1.130.

Run	Factor 1 A: Urea ($g.L^{-1}$)	Factor 2 B: Wheat bran ($g.L^{-1}$)	Factor 3 C: Soy flour ($g.L^{-1}$)	Factor 4 D: Inoculum (cells/mL)	Factor 5 E: Temp (°C)	Factor 6 F: Agitation speed (rpm)	Factor 7 G: Agitation time (hr)	Factor 8 H: Fermentation time (Days)	FE %
1	2.5	5	10	15×10^7	28	200	2	1	82.04
2	1.5	5	5	10×10^7	28	100	2	1	76.94
3	2.5	15	5	20×10^7	28	200	2	3	83.74
4	2	5	15	10×10^7	33	200	1	2	83.32
5	2	15	10	20×10^7	37	100	2	2	79.07
6	1.5	10	10	10×10^7	33	150	2	3	84.17
7	2.5	5	10	20×10^7	33	100	4	2	82.47
8	1.5	10	10	20×10^7	28	100	1	2	80.77
9	2	5	15	20×10^7	28	150	4	1	82.47
10	1.5	5	5	15×10^7	33	150	2	2	84.17
11	2.5	10	15	15×10^7	33	100	4	3	82.04
12	2.5	15	5	10×10^7	33	100	4	1	84.17
13	2	10	5	15×10^7	28	150	4	2	83.74
14	2.5	10	15	20×10^7	37	150	1	1	84.17
15	1.5	10	10	15×10^7	37	200	4	1	81.62
16	2.5	15	5	15×10^7	37	150	1	2	83.32
17	2	5	15	15×10^7	37	100	2	3	79.07
18	2	10	5	20×10^7	33	200	1	3	84.17
19	2.5	10	15	10×10^7	28	200	2	2	84.17
20	1.5	15	15	20×10^7	33	150	2	1	84.17
21	2	10	5	10×10^7	37	100	2	1	80.34
22	1.5	15	15	10×10^7	37	200	4	2	81.19
23	2	15	10	15×10^7	33	200	1	1	84.17
24	2.5	5	10	10×10^7	37	150	2	3	81.19
25	1.5	15	15	15×10^7	28	100	1	3	80.34
26	1.5	5	5	20×10^7	37	200	4	3	79.92
27	2	15	10	10×10^7	28	150	4	3	82.89

ANOVA was conducted to determine the factors which have a significant effect on FE. As shown in (Table 7), the model is highly significant (P-value < 0.0001). Factors: A (Urea), B (Wheat bran), E (Temperature), and F (Agitation speed) were significant model terms.

Table 7:ANOVA analysis for the Taguchi design at specific gravity 1.130.

Source	P-value	Confidence %
Model	< 0.0001	> 99.99
A: Urea ($g.L^{-1}$)	0.0020	> 99.8
B: Wheat bran ($g.L^{-1}$)	0.0014	> 99.86
E: Temperature (°C)	< 0.0001	> 99.99
F: Agitation speed (rpm)	< 0.0001	> 99.99

HG and VHG media are more viscous than normal gravity media and carbon dioxide may not escape from viscous media as readily as it does from regular fermentation media [57]. This is probably why agitation speed was considered a significant factor as inferred by the Taguchi method. Furthermore, agitation improved the mass transfer and nutrients consumption, enhanced cell growth, and tolerance to adverse conditions [58]. **Rodmui *et al.*** [59] reported that agitation helps in accelerating the transfer of sugars and other nutrients into the cell and removing gases and other byproducts of catabolism outside the cell. The results of the current study showed that increasing the incubation temperature to 37 °C reduced ethanol concentration. This could be attributed to the denaturation of ribosomes and enzymes [60]. Furthermore, as temperature increased, the ethanol toxicity probably increased due to the combined deleterious effects of heat and ethanol on membranes [61]. **Pornpukdeewattana *et al.*** [62] suggested that 35 °C was optimum for the fermentation process.

Moreover, statistical analysis of the design showed R^2 value of 0.8925 which denotes a good correlation. The predicted R^2 of 0.7581 is in reasonable agreement with the adjusted R^2 of 0.8447. The CV is 0.95, an indicator of moderate reliability. The signal to noise ratio is 13.683 (greater than 4 is

desirable), implying that the model precision is adequate.

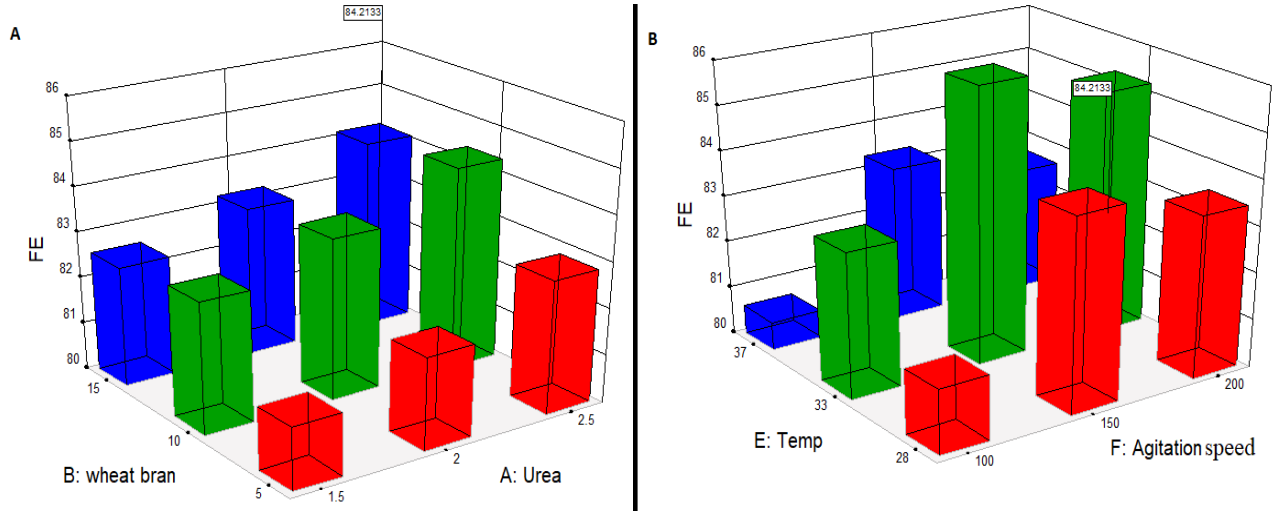

Fig. 3. (A) The interaction between urea and wheat bran and their effect on FE %, (B) The interaction between temperature and agitation speed and their effect on FE %.

To study the effect of interactions between the significant variables on bioethanol fermentation efficiency, a 3D surface plot was generated. As illustrated in Fig. 3, FE % will reach the predicted value (84.21 %) if the wheat bran and urea concentrations were adjusted to 15 and 2.5 $g.L^{-1}$, respectively. Likewise, agitation speed and temperature should be adjusted at 150 rpm and 33 °C, respectively.

A validation test was conducted using the condition predicted by the design (urea, 2.5 $g.L^{-1}$; wheat bran, 15 $g.L^{-1}$; soy flour, 5 $g.L^{-1}$; yeast extract, 1.5 $g.L^{-1}$; peptone, 1 $g.L^{-1}$; $MgSO_4.7H_2O$, 0.62 $g.L^{-1}$; glycine, 0.24 $g.L^{-1}$; inoculum size, 10 x 10^7 cells/mL; agitation speed, 150 rpm for one hour followed by 60 rpm for the rest of the incubation period; temperature, 33 °C). The calculated results of the validated test showed an obvious increase in FE %, EC % (v/v), and EC ($g.L^{-1}$) by 83.82 %, 10.4 % (v/v), and 82.07 ($g.L^{-1}$), respectively. The achieved results are similar to the results reported by **Li *et al.*** [14]**;** however, the corn starch-based medium designed by **Li *et al.*** [14] is more expensive (400 US dollars per ton of corn starch) than the validated medium of the present study which comprises mainly agricultural wastes (200 US dollars per ton of molasses). In the Egyptian Sugar and Integrated Company (El-Hawamdia, Giza, Egypt) where the present work was conducted, molasses is a waste byproduct, and hence, there is virtually no additional cost for the procurement of molasses. Instead, utilizing molasses for bioethanol production is an integrated process that valorizes a low-cost byproduct into a value-added biofuel, in line with the global trend towards adopting more sustainable approaches. Moreover, the results of the validated medium of our study showed a significant increase compared to the results stated by **Fadel *et al.*** [33]**,** **Fadel *et al.*** [9] **and Rasmey *et al.*** [63] who showed varying amounts of bioethanol concentration (9.8, 8.89, and 9.55% (v/v), respectively). Also, the experimental value of FE (83.82%) was very close to the predicted one (84.21%), proving that our findings from the Taguchi design could successfully be applied to optimize the fermentation process for bioethanol production.

4. Conclusions

It was found that the fermentation efficiency was decreased by increasing specific gravity. Under high specific gravity, FE was enhanced after optimization of inoculum size and supplementary materials, using Plackett-Burman design. Wheat bran, soy flour, and inoculum size were significant model terms and had a positive effect on the efficiency of the fermentation process at the specific gravities 1.130, 1.145, and 1.160. However, urea and glycine were effective only at specific gravity 1.130. By using the Taguchi method, FE at specific gravity 1.130 was increased up to 83.82% which was very close to the predicted one (84.21%). Noteworthy, this increase in FE is very promising at the industrial scale. Overall, our findings prove that the Taguchi design could be successfully applied to optimize the fermentation conditions for

bioethanol production as well as reducing the number and time of experiments.

5. Conflicts of interest

The authors declare that there is no conflict of interest

6. Acknowledgment

This work was supported by funds from the Distillation Factories, Egyptian Sugar and Integrated Company (El-Hawamdia, Giza, Egypt).

7. Data availability

All data generated during this investigation is included in the manuscript.

8. References

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