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Evaluation and optimization of ethanol production from carob pod extract by *Zymomonas mobilis* using response surface methodology

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Abstract In this research, ethanol production from carob pod extract (extract) using *Zymomonas mobilis* with medium optimized by Plackett–Burman (P–B) and response surface methodologies (RSM) was studied. *Z. mobilis* was recognized as useful for ethanol production from carob pod extract. The effects of initial concentrations of sugar, peptone, and yeast extract as well as agitation rate (rpm), pH, and culture time in nonhydrolyzed carob pod extract were investigated. Significantly affecting variables ($P = 0.05$) in the model obtained from RSM studies were: weights of bacterial inoculum, initial sugar, peptone, and yeast extract. Acid hydrolysis was useful to complete conversion of sugars to glucose and fructose. Nonhydrolyzed extract showed higher ethanol yield and residual sugar compared with hydrolyzed extract. Ethanol produced (g g^{-1} initial sugar, as the response) was not significantly different ($P = 0.05$) when *Z. mobilis* performance was compared in hydrolyzed and nonhydrolyzed extract. The maximum ethanol of 0.34 ± 0.02 g g^{-1} initial sugar was obtained at 30°C, initial pH 5.2, and 80 rpm, using concentrations (g per 50 mL culture media) of: inoculum bacterial dry weight, 0.017; initial sugar, 5.78; peptone, 0.43; yeast extract, 0.43; and culture time of 36 h.

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Introduction

Limited fossil-fuel reserves, instability in the price of oil, and more recently, increasing environmental and political pressure, have provided additional motivation for researchers working on alternative fuel sources. Ethanol, as both renewable and environmentally friendly, is believed to be one of the best alternatives to gasoline, which has led to a dramatic increase in its production capacity. In practice, however, research focused on ethanol fermentation has been ongoing since the shock of the crude oil crisis in the 1970s [1].

Ethanol is most commonly produced by fermentation of agricultural starch and sugar, e.g., corn, sugar cane, and sugar beet. Another interesting and high-carbohydrate-containing agricultural crop that is a potential source of bioethanol is *Ceratonia siliqua*, more commonly known as carob. Carob is a perennial leguminous tree that is native to the Mediterranean Basin and southwest Asia. Carob is drought resistant and requires little maintenance, and a range of products are produced from its seeds and pods. Currently, world carob production is approximately 315,000 t per annum [5]. Analysis of some Turkish carob pods, both cultivated and wild, demonstrated their main sugar profile in the following ranges (g/kg): fructose 102–115, glucose 33.0–36.8, sucrose 299–384 [2]. Given the high concentration of sugars in carob kibbles and the hardy growing characteristics of the carob plant, development of methods to convert these sugars to bioethanol will serve an important role in satisfying the increasing demand for viable alternative fuel sources. However, previous

published work on the subject of ethanol production from carob pods is limited [12, 16–22, 25]. Traditionally, ethanol has been produced by batch fermentation with yeast strains that cannot tolerate high concentration of ethanol. *Zymomonas mobilis*, a Gram-negative bacterium, is considered as an alternative organism for large-scale fuel ethanol production, because of its higher sugar uptake, higher ethanol yield, and lower biomass production. This bacterium is one of the few facultative anaerobic bacteria that can metabolize glucose and fructose via the Entner–Doudoroff (E–D) pathway, which is usually present in aerobic microorganisms. However, the utilizable sugars for *Z. mobilis* are restricted to glucose, fructose, and sucrose [6]. The ethanol yield of *Z. mobilis* varies between these substrates. This is because of different by-products which are produced during *Z. mobilis* fermentation in the mentioned substrates [8, 9, 26].

As mentioned above, the sugars in the profile of carob pods match those that can be utilized by *Z. mobilis*. Considering the easy growing conditions of carob tree and specific characteristics of *Z. mobilis*, investigation of production of ethanol as a renewable source of energy from carob pod extract using this microorganism should be of high importance. Unfortunately, to the best of our knowledge, there have been no published studies regarding ethanol production from carob pod extract by *Z. mobilis*. Therefore, the aims of this research are, first, to study the ethanol-producing capability of *Z. mobilis* in carob pod extract (extract), and secondly, by applying suitable treatments and optimizing conditions, to achieve the maximum ethanol production (g g^{-1} initial sugar) in the culture (response). Then, the feasibility of *Z. mobilis* ethanol fermentation in carob pod extract medium as well as effects of important independent factors on ethanol production were studied. Plackett–Burman (P–B) and response surface methodologies (RSM) were employed to study and optimize conditions to reach the maximum ethanol production from initial sugars. Acid hydrolysis was used to convert total sugars to reducing sugars (glucose and fructose). Finally, *Z. mobilis* ethanol production practices were conducted and compared in both hydrolyzed and nonhydrolyzed media.

Materials and methods

Microorganism

The strain used was *Zymomonas mobilis* PTCC 1718, obtained from the Persian Type Culture Collection. The dried strain was activated and grown as inoculum for 17 h at 30°C in a conical flask shaken at 120 rpm in medium containing 10 g L^{-1} peptone from meat (Merck, Darmstadt, Germany), 10 g L^{-1} yeast extract (Merck), and 20 g L^{-1} glucose (Merck).

Preparation of carob pod extract

This procedure is similar to that described by Roukas [22]. Carob pods were obtained from a Cypriot local market. After removing the seeds, kibbles were ground into fine particles and were passed through a sieve of 1-mm opening. Three hundred grams of particles was mixed with 1,500 mL distilled water, and the mixture was heated in a water bath for 3 h at 85°C while stirring slowly. The mixture was filtered through cheesecloth, and the solid particles were washed with more distilled water twice. Total volume of extract and wash filtrates was 2.7 L. This liquid was then centrifuged at 4,000*g* for 15 min, and the supernatant (pH 5.4) was evaporated at 64–67°C to a concentrate of about 50%w sugar (glucose equivalent). The concentrate was stored at 4°C to make solutions of different concentrations. The concentrate is more resistant to spoiling than when diluted.

Preparation of acid-hydrolyzed carob extract and adjustment of volume and pH

In a 100-mL calibrated flask, 38.95 g carob extract [49.45%w sugar content as glucose equivalent, determined by 3,5-dinitrosalicylic acid (DNS) method as per instructions] was mixed with 30 mL distilled water, and pH of this mixture was adjusted to 1 by adding 5 N HCl. Then the solution was heated at 80–85°C for 30 min in a water bath. After cooling to room temperature, pH was adjusted to 5.2 and volume to 100 mL by simultaneous addition of 1 N NaOH and distilled water.

Feasibility study of *Z. mobilis* ethanol production in hydrolyzed carob pod extract

As a preliminary step, *Z. mobilis* ethanol-producing ability in carob pod extract medium was examined. A 30-mL volume of hydrolyzed carob pod extract with pH 5.2 and sugar content of 2.71 g (original liquid) was pipetted into a 100-mL Erlenmeyer flask. The amounts of 0.29 g yeast extract, 0.29 g peptone from meat, and 0.53 g glucose (Merck) were added to the extract. The mixture was sterilized at 121°C for 15 min, then it was inoculated with 20 mL *Z. mobilis* bacterial suspension (containing 0.018 g *Z. mobilis* based on dry weight) to become a 50-mL volume culture. This culture was incubated at 30°C and 80 rpm for 49 h.

Fermentation treatments according to Plackett–Burman (P–B) design

Amounts of additives (yeast extract, peptone from meat, and bacterial dry weight), pH, rpm, and time were specified according to Table 1 for each treatment. A 30-mL volume of nonhydrolyzed carob pod extract, with pH and total sugar content as specified in Table 1, was pipetted into a 250-mL Erlenmeyer flask (original liquid). The specified amount of yeast extract and peptone from meat were added to it. The mixture was sterilized at 121°C for 15 min, then it was inoculated with suitable volume of *Z. mobilis* bacterial suspension to provide 0.018 g bacterial dry weight. Then the volume of inoculated mixture was adjusted to 50 mL by addition of sterilized distilled water. The culture was incubated at 30°C and specified rpm for the period specified in Table 1.

Treatments according to response surface methodology (RSM) design

RSM-designed treatments were conducted in 100-mL Erlenmeyer flasks at 30°C and 80 rpm with 30-mL volume original liquid (pH 5.2), with other conditions as specified in Table 2. The procedure is the same as described for P–B treatments, with the addition that varying volumes of bacterial suspension were also used in the inoculation to provide for specified weight of *Z. mobilis* in each treatment.

Analytical techniques

Bacterial mass determination

Dry bacterial mass concentration in the inoculum culture was determined as g L^{-1} by measuring optical density (OD) at 600 nm and using a standard curve in the range 0.15–0.35 g L^{-1} bacterial dry weight ($R^2 = 0.996$).

Determination of moisture in carob pod powder

Moisture content in samples of carob pod powder was determined by drying at 70°C to constant weight. The weight difference between its dried and primary state was reported as moisture content of the samples.

Determination of sugars

Total sugars (glucose, fructose, and sucrose) were determined using the 3,5-dinitrosalicylic acid (DNS) method [13, 19] after hydrolysis of sugars at pH 1 (adjusted with 1 N HCl) and 80–85°C for 30 min and neutralization with

Table 1 P–B design experiments to investigate the effects of six selected variables (A, B, C, D, E, and F) on ethanol production by *Z. mobilis* PTCC 1718 as ethanol produced (g g^{-1} initial sugar, as response)

Run	A	B	C	D	E	F	Response, ethanol produced (g g^{-1} initial sugar), $n = 2$
1	7.00	5.00	200.00	24.0	0.00	0.00	0.0524 ± 0.0
2	7.00	5.00	200.00	48.00	0.00	0.30	0.0566 ± 0.0083
3	7.00	7.00	0.00	48.00	0.00	0.00	0.1892 ± 0.0056
4	7.00	5.00	0.00	24.00	0.30	0.30	0.2286 ± 0.0222
5	4.00	5.00	0.00	48.00	0.30	0.30	0.303 ± 0.0062
6	4.00	7.00	200.00	48.00	0.00	0.30	0.0825 ± 0.0296
7	4.00	5.00	0.00	24.00	0.00	0.00	0.2611 ± 0.0037
8	7.00	7.00	0.00	48.00	0.30	0.00	0.288 ± 0.0037
9	4.00	7.00	200.00	24.00	0.30	0.00	0.0772 ± 0.0153
10	7.00	7.00	200.00	24.00	0.30	0.30	0.0487 ± 0.000
11	4.00	5.00	200.00	48.00	0.30	0.00	0.0801 ± 0.0049
12	4.00	7.00	0.00	24.00	0.00	0.30	0.315 ± 0.0033

Indications	Factors	Low levels	High levels
A	Initial sugar, g	4	7
B	pH	5	7
C	Shaking rate per minute (rpm)	0	200
D	Time, h	24	48
E	Yeast extract, g	0	0.3
F	Peptone, g	0	0.3

Table 2 RSM-designed experiments and response obtained as ethanol produced (g g^{-1} initial sugar)

Run no.	A	B	C	D	E	Response, ethanol produced (g g^{-1} initial sugar)
1	0.013	4.87	0.17	0.17	30.95	0.316
2	0.015	5.50	0.30	0.30	36.00	0.347
3	0.015	5.50	0.30	0.30	36.00	0.342
4	0.010	5.50	0.30	0.30	36.00	0.329
5	0.015	5.50	0.30	0.30	36.00	0.295
6	0.017	6.13	0.17	0.17	30.95	0.268
7	0.015	5.50	0.60	0.30	36.00	0.324
8	0.015	5.50	0.30	0.00	36.00	0.31
9	0.017	4.87	0.43	0.43	30.95	0.335
10	0.013	6.13	0.17	0.17	41.05	0.256
11	0.013	6.13	0.43	0.17	41.05	0.313
12	0.017	4.87	0.17	0.43	41.05	0.347
13	0.013	4.87	0.17	0.17	41.05	0.326
14	0.017	6.13	0.43	0.43	41.05	0.315
15	0.017	6.13	0.43	0.17	41.05	0.329
16	0.013	6.13	0.17	0.43	30.95	0.272
17	0.013	6.13	0.17	0.17	30.95	0.214
18	0.013	6.13	0.17	0.43	41.05	0.258
19	0.015	5.50	0.30	0.30	24.00	0.325
20	0.017	6.13	0.43	0.43	30.95	0.338
21	0.015	7.00	0.30	0.30	36.00	0.277
22	0.013	4.87	0.43	0.17	41.05	0.329
23	0.017	6.13	0.17	0.43	30.95	0.328
24	0.017	4.87	0.17	0.17	41.05	0.316
25	0.015	5.50	0.30	0.30	36.00	0.335
26	0.017	6.13	0.43	0.17	30.95	0.337
27	0.017	4.87	0.43	0.17	30.95	0.337
28	0.013	6.13	0.43	0.17	30.95	0.313
29	0.013	4.87	0.43	0.43	41.05	0.328
30	0.015	5.50	0.00	0.30	36.00	0.261
31	0.013	6.13	0.43	0.43	30.95	0.314
32	0.013	6.13	0.43	0.43	41.05	0.35
33	0.015	5.50	0.30	0.60	36.00	0.342
34	0.017	4.87	0.43	0.43	41.05	0.335
35	0.013	4.87	0.43	0.17	30.95	0.321
36	0.013	4.87	0.17	0.43	41.05	0.301
37	0.015	5.50	0.30	0.30	36.00	0.329
38	0.015	5.50	0.30	0.30	48.00	0.318
39	0.017	6.13	0.17	0.43	41.05	0.333
40	0.013	4.87	0.43	0.43	30.95	0.335
41	0.017	4.87	0.17	0.43	30.95	0.301
42	0.015	5.50	0.30	0.30	36.00	0.319
43	0.017	6.13	0.17	0.17	41.05	0.304
44	0.017	4.87	0.17	0.17	30.95	0.342
45	0.017	4.87	0.43	0.17	41.05	0.33
46	0.013	4.87	0.17	0.43	30.95	0.343
47	0.015	5.50	0.30	0.30	36.00	0.338

Table 2 continued

Run no.	A	B	C	D	E	Response, ethanol produced (g g^{-1} initial sugar)
48	0.015	5.50	0.30	0.30	36.00	0.34
49	0.020	5.50	0.30	0.30	36.00	0.326
50	0.015	4.00	0.30	0.30	36.00	0.301

Indications	Factors	Low level	High level
A	Bacteria, g	0.010	0.020
B	Sugar, g	4.00	7.00
C	Peptone, g	0.00	0.43
D	Yeast extract, g	0.00	0.43
E	Time, h	24.0	48.0

1 N NaOH. Reducing sugars (glucose and fructose) were also determined by the same method but without the hydrolysis treatment. Residual sugar in culture sample was also determined by the same method used for total sugar but after exclusion of produced ethanol from fermentation medium by evaporating the sample to dry at 55°C at the end of the fermentation period. Weight of sugar was expressed as glucose equivalent. Sucrose content in each sample was calculated and expressed as glucose equivalent by subtracting the weight of reducing sugar from that of total sugar. The standard curve used in this determinations was for the range 0.1–1 g L^{-1} glucose in solution ($R^2 = 0.987$).

Ethanol determination

Produced ethanol was distilled from fermentation medium and determined as w/v% by the Arthur Caputi Jr. method [3], using absolute ethanol (Merck) to prepare a standard curve in the range 0–4.5 g ethanol per 100 mL water–ethanol solution ($R^2 = 0.998$).

Statistical experimental designs

The main effect of independent variables on ethanol production by *Z. mobilis* was studied using Plackett–Burman (P–B)-designed treatments as presented in Table 1. The six selected variables and their low (−) and high (+) values are presented in Table 1. The dependent variable (response) was ethanol produced (g g^{-1} initial sugar) in culture media. The main effects for each of these factors were defined and calculated by Eq. 1.

$$\mathrm{Ef}_i = (\bar{y}_+)_i - (\bar{y}_-)_i, \quad (1)$$

where Ef_i is the effect of the *i*th factor on the response factor, and $(\bar{y}_+)_i$ and $(\bar{y}_-)_i$ are the average response values at the high (+) and low (−) levels of the factor [4].

Response surface methodology was used to design experiments to optimize conditions for reaching the maximum response. The variables included: A = inoculum bacterial dry weight (bacteria), g; B = initial sugar content of culture (initial sugar), g; C = initial weight of peptone from meat in culture (peptone), g; D = initial weight of yeast extract in culture (yeast extract), g; E = culture time (time), h. Fifty designed experiments as well as the low and high levels of each variable are presented in Table 2.

Results and discussion

Carob powder analysis

Sugar profiles of carob pod varieties were determined by high-performance liquid chromatography (HPLC), indicating that carob pods contained sucrose, glucose, and fructose, regardless of variety and origin. The DNS method can be used to determine these kinds of sugar, using the same procedure applied by Roukas [19]. Thus, we followed Roukas for determination of sugar. Data obtained from carob pod powder analysis are reported in the second column of Table 3. Comparing these current data with the two other previously analyzed samples (Table 3, columns 3 and 4) [2, 27] showed that the total sugar content (glucose, fructose, and sucrose) of the carob used in this research as well as the weight of its reducing sugar contribution (glucose and fructose) as a fraction of total sugar content were higher.

Efficiency of sugar extraction process

Sugar concentration in 2.7 L extract obtained from 300 g carob pod powder was 52 g L^{-1}. Based on data in Table 3, extraction efficiency was calculated as 83.81%.

Table 3 Analysis of carob pod powder used in this research, in comparison with two other previously analyzed samples

Characteristic factor	Sample used in this research	Sample 1 [2]	Sample 2 [27]
Moisture, weight%	9.09 ± 0, $n = 2$	–	11.7
Total sugars[a], weight%	56.10 ± 1.14, $n = 3$	53	45.0
Reducing sugars[b], weight%	19.00 ± 2.67, $n = 3$	14.8	13.6
% of reducing	33.87	27.9	30.22

[a] Sucrose, glucose, and fructose

[b] Glucose and fructose

Fermentation and optimization results

There was no published previous experience regarding feasibility of *Z. mobilis* fermentation on carob pod extract. So, a feasibility test was carried out initially using a relatively soft medium in which: (1) all sugars were converted to simple sugars (glucose and fructose) by acid hydrolysis, and (2) 0.53 g pure glucose was added to the extract in addition to its inherent sugar content, giving a total of 3.24 g sugar content.

The results of the feasibility test (Table 4) showed that *Z. mobilis* was able to produce ethanol in carob pod extract medium. Therefore, studies were extended by performing Plackett–Burman design experiments in two repetitions, as shown in Table 1. Data from the feasibility test and following literature review aided in selecting temperature of 30°C and the low and high values of the five selected variables, also presented in Table 1. Molasses fermentation was studied by Cazetta et al. in the range of 24–48 h with 0.2 g L^{-1} *Z. mobilis*. Shaking rate was zero at low value and 180 rpm at high value. Temperature of 30°C and pH in the range 5.0–7.0 were reported as optimum in their study [4]. Media components were optimized at pH 5.5 in *Z. mobilis* ethanol fermentation by Sreekumar et al., and concentration of 120.4 g L^{-1} was reported as optimum for glucose [24]. Initial sugar selection was 4.0 g (close to the value of 3.24 g used in our feasibility test) and 7.0 g per 50 mL (close to 120.4 g L^{-1}) at its low and high level, respectively.

According to the P–B responses presented in Table 1, the variables time (+0.0029), yeast extract (+0.0116), peptone (+0.0145), and pH (+0.0031) had positive effects, whereas the variables initial sugar content (−0.0427) and rpm (−0.1980) showed negative effects. So, agitation (200 rpm) exhibited a negative effect. However, slow shaking is useful for culture homogenization. To study the probable negative effect of slow shaking, treatment 12, which showed higher response, was repeated ($n = 2$) and compared with the same treatment at 80 rpm ($n = 2$), but in 100-mL Erlenmeyer flasks instead of 250-mL (practical volume/actual volume was increased, i.e., aeration surface was decreased). Inoculation was conducted with a newly prepared bacterial suspension, and the responses obtained (0.32 ± 0.01 and 0.33 ± 0.01 ethanol, g g^{-1} initial sugar, respectively) were not different from each other ($P = 0.05$).

The highest ethanol yield by *Z. mobilis* in "artichoke juice" fermentation was observed at pH 5 and static condition [15]. *Z. mobilis* grows best within the temperature range of 30–35°C and is able to grow at pH values of 4.0–7.0 [14]. The optimal condition for *Z. mobilis* ethanol production from kitchen garbage was temperature of 30°C and pH 5 [11]. As pointed out by various researchers, conditions of temperature of 30°C and pH 5 were recognized as suitable for *Z. mobilis* ethanol fermentation. Similarly, Plackett–Burman (P–B) experiments in this study showed a slight positive effect in moving from pH 5.0 to 7.0, slow shaking (80 rpm) showed no negative effect on response, but a negative effect was observed in moving from 4.0 to 7.0 g initial sugar (in 50 mL culture medium). Therefore, normally interesting variables, namely bacteria, initial sugar, peptone, yeast extract, and time, were selected for further study at temperature of 30°C, pH 5.2, and 80 rpm. A central composite design (CCD) response surface methodology (RSM) was developed by making use of Design expert dx7-trial software. Fifty different treatment experiments were designed as

Table 4 Mean results of the feasibility fermentation test conducted in hydrolyzed carob pod extract

Initial sugar, g	Residual sugar at end of fermentation, g	Used sugar, g	Ethanol produced, g	Ethanol yield, g g^{-1} sugar used	Sugar used, %[a]	Theoretical ethanol yield, %[b]
3.24	0.30 ± 0.01, $n = 3$	2.94	1.10 ± 0.08, $n = 3$	0.37	90.74	73.36

[a] Percentage sugar used = (2.94/3.24) × 100

[b] Theoretical ethanol yield (%) = (1.10 × 100)/(2.94 × 0.51) (Theoretically, 1 g glucose is converted to 0.51 g ethanol and 0.4889 g CO_2) [16]

presented in Table 2. Low and high values of each variable as well as the observed responses are also presented in Table 2. Responses were analyzed by the software. The coefficient estimates of coded factors and significance level of factors are presented in Table 5a. The R^2 value was 0.7354. Data indicated that A (bacteria), B (initial sugar), C (peptone), D (yeast extract), B^2, and C^2 were significant factors in the proposed model ($P = 0.05$). The model was simplified by omitting insignificant terms, and data were reevaluated. In this simplified model, the coefficients were changed only slightly (Table 5b). The R^2 value was changed to 0.6545, and lack of fit was insignificant ($P = 0.05$). The final equation giving the response as a function of actual factors is given as Eq. 2 in the below-specified ranges:

$$\begin{aligned}&\text{Response}\left(\text{ethanol produced }\left(\text{g g}^{-1}\text{initial sugar}\right)\right)\\&= +0.38767 - 24.55663 \times \text{bacteria} + 0.061107\\&\quad\times \text{initial sugar} - 0.34933 \times \text{peptone} + 0.046695\\&\quad\times \text{yeast extract} + 5.06760 \times \text{bacteria} \times \text{initial sugar}\\&\quad+ 0.12610 \times \text{initial sugar} \times \text{peptone} - 0.017416\\&\quad\times (\text{initial sugar})^2 - 0.39651 \times (\text{peptone})^2. \qquad (2)\end{aligned}$$

The specified factor ranges for Eq. 2 were as follows: bacteria, 0.013–0.0170 g; initial sugar, 4.87–6.13 g; peptone, 0.17–0.43 g; yeast extract, 0.17–0.43 g; time, 24–48 h; ethanol produced, 0.21–0.35 g g^{-1} initial sugar.

The effects of significantly affecting variables on the response are shown in Figs. 1, 2, and 3. Figure 1 predicts that, when the levels (in g) of bacteria, initial sugar, and peptone are 0.015, 5.50, and 0.30, respectively, and culture time is 36 h, increase of yeast extract in the studied range would result in increase in response, with no interactions for the other four factors.

Figure 2 shows the interaction effects of initial sugar and bacteria. When peptone and yeast extract are both 0.30 g and the culture period is 36 h, predicted conditions for maximum response are the factor combination of about 0.017 g bacteria and 5.31 g initial sugar. The interaction effect of initial sugar and peptone is shown in Fig. 3. This figure predicts that, under conditions of 0.015 g bacteria, 0.30 g yeast extract, and culture period of 36 h, ethanol produced (g g^{-1} initial sugar) would be at its maximum for approximately 5.47 g initial sugar and 0.43 g peptone. The optimal conditions for response were obtained by further numerical analysis of the response surface using the software. The solution to the maximal response was: bacteria, 0.017 g; initial sugar, 5.78 g; peptone, 0.43 g; yeast extract, 0.43 g; time, 36 h.

A confirmation experiment, under the above-mentioned conditions, was conducted to confirm the optimal conditions obtained from the statistically based experimental design. The result was proven to be 0.34 ± 0.02 ethanol (g g^{-1} initial sugar) ($n = 3$), which was within the 95% confidence interval of the prediction (0.29–0.37 ethanol, g g^{-1} initial sugar). Therefore, the model was useful to predict the response and for optimization of experimental

Table 5 Estimated factor coefficients and associated significance levels of the (**a**) CCD model for response (ethanol produced, g g^{-1} initial sugar), (**b**) simplified CCD model for response (ethanol produced, g g^{-1} initial sugar)

Factor	Coefficient estimate[a]	*P*-value[b]
a		
Model		0.0003
Intercept	0.33	
A-Inoculum	6.969E-003	0.0213
B-Sugar	−0.010	0.0010
C-Peptone	0.013	<0.0001
D-Yeast extract	5.890E-003	0.0488
E-Time	8.393E-004	0.7715
AB	6.719E-003	0.0530
AC	−6.156E-003	0.0748
AD	−1.281E-003	0.7033
AE	−2.188E-004	0.9481
BC	0.010	0.0053
BD	5.281E-003	0.1237
BE	2.969E-003	0.3801
CD	−3.219E-003	0.3419
CE	−1.906E-003	0.5715
DE	−1.781E-003	0.5969
A^2	−3.775E-004	0.8823
B^2	−7.183E-003	0.0081
C^2	−6.565E-003	0.0146
D^2	−6.427E-004	0.8011
E^2	−1.438E-003	0.5738
b		
Model		<0.0001
Intercept	0.33	
A-Inoculum	6.969E-003	0.0152
B-Sugar	−0.010	0.0005
C-Peptone	0.013	<0.0001
D-Yeast extract	5.890E-003	0.0383
AB	6.719E-003	0.0420
BC	0.010	0.0032
B^2	−6.927E-003	0.0058
C^2	−6.308E-003	0.0113

[a] Term coefficient: the model coefficient or parameter for this particular term; since this value is expressed in coded units, its relative magnitude can be compared with other term coefficients to estimate relative effect

[b] *P*-value: to confirm that each term has a *P*-value less than 0.05 or at least less than 0.10. If a term is not significant, it should be removed from the model unless it is needed to satisfy hierarchy (i.e., is a parent term of a significant interaction)

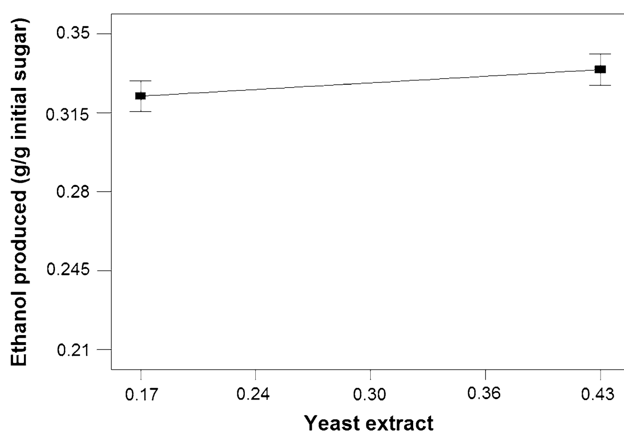

Fig. 1 The increasing effect of yeast extract on response (ethanol produced g g^{-1} initial sugar). Actual factors: bacteria, 0.015 g; initial sugar, 5.50 g; peptone, 0.30 g; time, 36.0 h

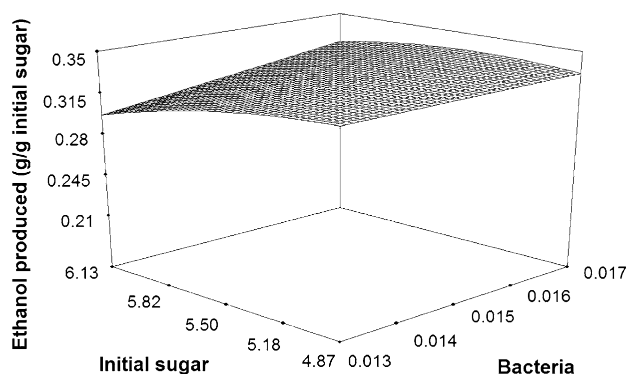

Fig. 2 Interaction effect of initial sugar and bacteria on response (ethanol produced, g g^{-1} initial sugar). Actual factors: peptone, 0.30 g; yeast extract, 0.30 g; time, 36.0 h

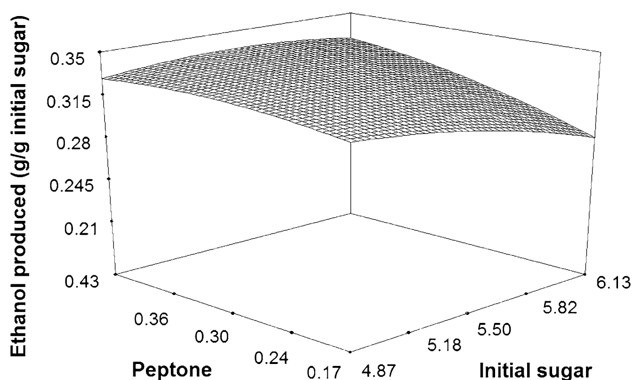

Fig. 3 Interaction effect of initial sugar and peptone on response (ethanol produced, g g^{-1} initial sugar). Actual factors: bacteria, 0.015 g; yeast extract, 0.30 g; time, 36.0 h

conditions. The volume of the different sources in this experiment were: sugar-containing extract, 29.5 mL (pH 5.2); bacterial suspension, 15 mL (pH 4.7, OD obtained at 600 nm for sevenfold-diluted suspension was 0.2742); distilled water, 5.5 mL (pH 6.8). Final pH at the end of the fermentation period was 4.8, which is still in the good working range for *Z. mobilis*. Sugar content of sterilized original liquid in this experiment (5.77 ± 0.18 g, $n = 3$) was not significantly different ($P = 0.05$) from its value before sterilization (5.78 g). Therefore, sterilization did not have a negative effect on sugar content of culture.

Hydrolysis of carob pod extract

Reducing sugar determination was performed after hydrolysis treatment. No significant difference ($P = 0.05$) was observed between its value and the total sugar content determined for untreated solution (19.26 versus 19.23 ± 0.74 g), indicating complete conversion of sugars to glucose and fructose after hydrolysis treatment.

Comparison of *Z. mobilis* fermentation performance in nonhydrolyzed and hydrolyzed carob pod extract, and ethanol production

The optimized conditions were used to compare *Z. mobilis* fermentation performance in both nonhydrolyzed and hydrolyzed carob pod extract. The results are presented and compared in Table 6. Despite the lack of significant difference ($P = 0.05$) between the responses obtained (ethanol produced g g^{-1} initial sugar), residual sugar in hydrolyzed media was lower than in nonhydrolyzed medium ($P = 0.05$). Therefore, ethanol yield in hydrolyzed medium was lower than in nonhydrolyzed medium. This different ethanol yield between hydrolyzed and nonhydrolyzed media is because of different by-products which are produced from sucrose and fructose during fermentation practice. To explain this, *Z. mobilis* can effectively ferment only glucose alone, which then accounts for as much as 97% of the theoretical ethanol yield [23]. According to Lee and Huang, in fermentation of mixtures composed of glucose and fructose, ethanol yield was lower due to formation of sorbitol as a by-product [10]. The growth of *Z. mobilis* on sucrose is accompanied by extracellular formation of fructose oligomers levan and sorbitol, which results in significant decrease in ethanol yield [23]. The results presented in Table 6 can be compared with the performance of *Saccharomyces cerevisiae* in carob pod extract (sugar content 115.3 g L^{-1}) studied by Turhan et al. [25]. Sugar utilization was 87.95% (94.97 g L^{-1}), theoretical ethanol yield was 87.95%, and ethanol produced was calculated to be 0.3695 g g^{-1} initial sugar. In the *Z. mobilis* fermentation under the various conditions, different levels of ethanol yield and sugar utilization were demonstrated. Cane molasses was fermented to 0.2790 g ethanol per g initial sugar with productivity of 1.1625 g L^{-1} h^{-1} [4]. Fermentation of glucose resulted in 0.5 g ethanol per g initial glucose [24]. In fermentation of

Table 6 Performance of *Z. mobilis* fermentation in nonhydrolyzed and hydrolyzed carob pod extract media: comparative data table

Factor	Nonhydrolyzed	Hydrolyzed
Initial sugar in 30 mL original liquid as glucose, g	5.78 (115.6 g L^{-1})	5.78 (115.6 g L^{-1})
Ratio of reducing sugar to total sugar	33.87	100
Remaining sugar in 50 mL culture media after sterilization and removal of 0.5 mL test sample from original liquid	5.67 ± 0.17[a] (113.45 g L^{-1})	5.69 ± 0.10[b] (113.82 g L^{-1})
Residual sugars after 36-h fermentation period, g	1.58 ± 0.06[a]	1.25 ± 0.09[a]
Sugar utilized, g	4.09 (81.82 g L^{-1})	4.45 (88.902 g L^{-1})
Ethanol produced, g	1.95 ± 0.09[a] (38.93 g L^{-1})	2.00 ± 0.03[a] (40.07 g L^{-1})
Ethanol produced, g g^{-1} initial sugar	0.34	0.35
Percentage sugar utilization	72.13	78.21
Theoretical ethanol yield (%)	93.48	88.13
Productivity, g h^{-1} L^{-1}	1.09	1.12

[a] $n = 3$

[b] $n = 2$

cane juice, theoretical ethanol yield and sugar utilized were, respectively, 89.4% and 95% [7]. Theoretical ethanol yield of 82.35% and productivity of 0.65 g L^{-1} h^{-1} were observed in *Z. mobilis* fermentation of acid-hydrolyzed Jerusalem artichoke juice. However, different productivity as well as pH decrease were observed when implementing different conditions [15]. In this study, *Z. mobilis* fermentation showed similar ($P = 0.05$) ethanol production (g g^{-1} initial sugar) of 0.34 ± 0.02 and 0.35 ± 0.00, respectively, in nonhydrolyzed and hydrolyzed carob pod extract. Based on the sugar extraction efficiency and carob pod powder analysis, calculations show ethanol production of 175.84 ± 10.34 g kg^{-1} carob pod powder on a dry basis. This value can be compared with 160 ± 3 g ethanol produced in *Saccharomyces cerevisiae* fermentation of carob pod slurry studied by Roukas. Theoretical ethanol yield and sugar utilization in this slurry fermentation practice were 80 ± 2% and 40 ± 1.8%, respectively [20].

Ethanol productivity

Ethanol productivities calculated as ethanol produced g L^{-1} h^{-1} were also analyzed by the software, and Eq. 3 was obtained for productivity prediction as a function of actual factors ($P = 0.05$).

$$\begin{aligned}\text{Productivity}(\text{g}\,\text{L}^{-1}\,\text{h}^{-1}) = &+2.38730 - 95.32591 \\ &\times \text{bacteria} + 0.34175 \times \text{initial sugar} - 1.46755 \\ &\times \text{peptone} + 0.16043 \times \text{yeast extract} - 0.078587 \\ &\times \text{time} + 19.35116 \times \text{bacteria} \times \text{initial sugar} + 0.46708 \\ &\times \text{initial sugar} \times \text{peptone} - 0.059256 \times (\text{initial sugar})^2 \\ &- 1.24251 \times (\text{peptone})^2 + 7.13015\text{E}-004 \times (\text{time})^2 \end{aligned} \quad (3)$$

The R^2 value of this equation was 0.8931.

The productivity obtained at optimized conditions was calculated as 1.09 ± 0.06 g L^{-1} h^{-1}. This value falls within the 95% confidence interval of the prediction (1.13–0.88 g L^{-1} h^{-1}). It is predicted that productivity can be improved by increasing the amount of yeast extract (Fig. 4). This trend is the same as was observed for ethanol production (g g^{-1} initial sugar) in Fig. 1, with productivity showing an upward trend with increasing bacteria and initial sugar (Fig. 5). The increasing trend of productivity with increasing initial sugar and peptone is presented in Fig. 6, for which maximum productivity is predicted at around 0.43 g peptone and 6.13 g initial sugar.

Conclusions

The results of this work show that *Z. mobilis* is able to utilize sugars present in carob pod extract for ethanol production. Acidic hydrolysis of carob pod extract is useful to convert its sugar content to reducing sugars (glucose and fructose). Amounts of ethanol produced (g g^{-1} initial sugar) in nonhydrolyzed and hydrolyzed carob pod extract media were not significantly different ($P = 0.05$). Therefore, there was no need to apply hydrolysis treatment for ethanol production by *Z. mobilis*. The data obtained in this research are comparable to those of *Z. mobilis* performance in cultures from various other agricultural sources. Carob growing practice is applicable to supply a new raw material for ethanol production by *Z. mobilis*. Response surface methodology was a useful tool to optimize fermentation conditions for maximizing ethanol production from carob pod extract. RSM was also useful for productivity prediction. As demonstrated, there was no need to aerate carob pod extract medium for *Z. mobilis* ethanol production. Increasing the amount of yeast extract, peptone, and

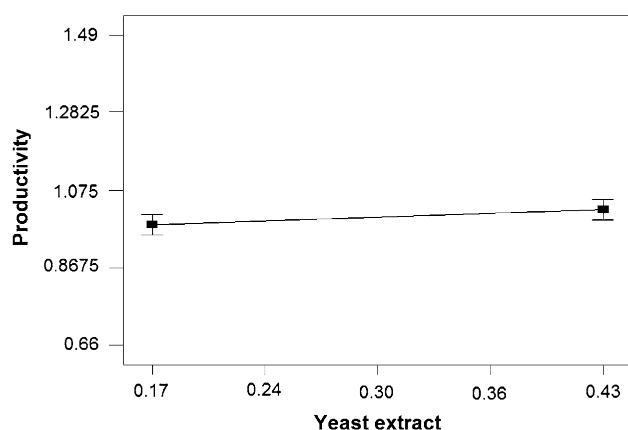

Fig. 4 Effect of yeast extract on productivity. Actual factors: bacteria, 0.015 g; initial sugar, 5.50 g; peptone, 0.30; time, 36.0 h

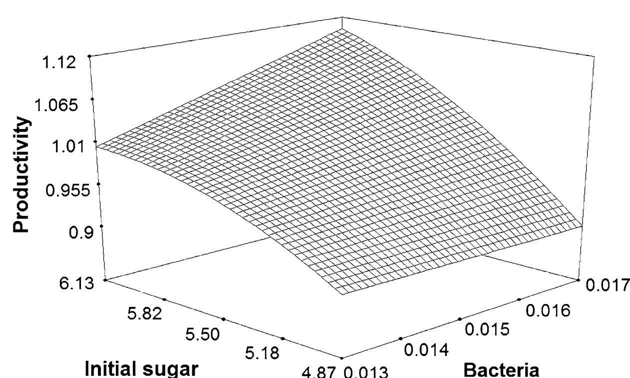

Fig. 5 Interaction effects of the amounts of bacterial dry weight and initial sugar on productivity. Actual factors: peptone, 0.30 g; yeast extract, 0.30 g; time, 36.0 h

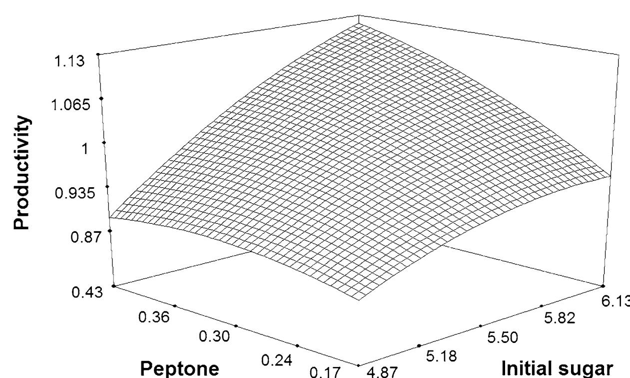

Fig. 6 Interaction effects of the amounts of initial sugar and peptone on productivity. Actual factors: bacteria, 0.015 g; yeast extract 0.30 g; time, 36.0 h

bacteria had a positive effect on ethanol production. However, dependent on conditions implemented, above a certain initial sugar concentration, ethanol production showed a downward trend. Ethanol productivity (g L^{-1} h^{-1}) was increased with increasing amounts of yeast extract, bacteria, peptone, and initial sugar in fermentation medium.

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