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Evaluation of Ethanol Production from Tannin-Reduced Carob Pod Extracts by Zymamonas Mobilis

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A B S T R A C T

Regarding some reported antimicrobial properties of tannins; *Zymomonas mobilis* was used to obtain ethanol from tannin-reduced carob pod extract (TR-CPE). Culture of 50 ml volume containing 7.5 g sugar at pH 5.5 and 0.03 g bacterial inoculums with shaking at 80 rpm was used. Using response surface methodology (RSM), the maximum ethanol concentration of 5.34 % w/v (higher than that reported earlier, 4.01% w/v) was obtained at the optimized addition levels of yeast extract, peptone, and fermentation time; 0.13 g, 0.62 g, and 43.78 h, respectively. Carob pods extract (CPE) containing 62.23gl^{-1} sugar was treated with 3 gl^{-1} gelatin to decrease its tannin content by 57.87 %. Sugar loss was not observed during gelatin treatment. The results revealed that there was no significant difference in ethanol production, yield, and productivity between TR-CPE and non-TR-CPE. In conclusion, tannin showed no inhibiting effect to maximum ethanol production by *Zymomonas mobilis*.

Keywords: Carob pod extracts, Ethanol production, Tannin-reduced, *Zymomonas mobilis.*

1. Introduction

Carob is a leguminous evergreen tree which reaches 15 to 17 m in height. There are few diseases which the tree may be infected with. The tree does best in Mediterranean-type climate (-6.67 to 50°C). It flourishes in widely soils, but is not tolerant to acid or wet soils; it is however, extremely drought-tolerant [1]. About 92,000 ha worldwide were planted with carob trees in 2010 for a total production of 153,000 tons of carob beans. Average worldwide yield is 1.7 t/ha [2]. But, in modern orchards production potential is higher than this average, i.e., 5-7 t/ha. In producing countries, carob pods have traditionally been used as animal and human food and currently the main use is the seed for gum extraction, carob bean gum (CBG) or locust bean gum (LBG) [3]. Carob pod contains 45 to 56.10% total sugar and13.60 to19.00% reducing sugar [4, 5]. Carob composition differs between cultivars and it contains sucrose, glucose, and fructose, regardless of the variety and origin [6]. The mature fresh fruit is made up of about 90 % pod (known as kibble) and 10% seed [3].

Ethanol has attracted worldwide interest as a renewable energy source with high performance and low environmental impact motor fuel [7]. Ethanol is a renewable energy source because it is produced via the microbial fermentation by using agricultural based carbohydrates such as starch, sugar, or cellulose with microorganisms such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Zymomonas mobilis* (*Z. mobilis*). Regarding

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the high sugar content in carob pod, there have been some studies on the production of the value added product, ethanol, from it with *S. cerevisiae* and *Z. mobilis*. Roukas investigated the production of ethanol from carob pod extract by *S. cerevisiae* in static and shake flask fermentation and obtained a maximum 75 g l^{-1} concentration of ethanol when the shake flask fermentation was used with an initial sugar concentration of 200 g l^{-1} [8]. Turhan *et al*. used the carob pods extract with an initial sugar concentration of 115.3 g l^{-1} for ethanol production by *S. cerevisiae*. They obtained final ethanol concentration of 42.6 g l^{-1}, while production rate was 3.37 g l^{-1} h^{-1} [9]. Ethanol production from carob pod extract was also investigated by *Z. mobilis* fermentation in shake flask. The maximum ethanol concentration of 39.30 g l^{-1} was obtained using concentrations of (g l^{-1}): inoculums bacterial dry weight, 0.34 g; initial sugar, 115.60; peptone, 8.60; yeast extract, 8.60; and culture time of 36 h [5]. *Z. mobilis* has also been investigated by Mazaheri *et al*. to produce bio ethanol from carob pod particles mixed with wheat bran by solid-state fermentation. They obtained a maximum 0.3 g of ethanol per l g initial sugar present in carob pod particles of 1 mm size at 31°C with initial moisture content of 80% [10].

It should be noted that aqueous extract of carob pod is rich in tannins [11]. Tannins are astringent, bitter-tasting plant polymers that bind and precipitate proteins [12] and have been reported to have both bacteriostatic and bactericidal effects on *Cell vibriofulvus* [13]. Yoshida *et al*. suggested that tannins have antioxidant property by their radical-scavenging effect [14]. Carob pod extracts of different compositions were examined to produce ethanol producing yeast, *S. cerevisiae*. The maximum growth yield (Yx/s) was obtained in CPE medium contained higher nitrogen source and lower tannin [15]. Shuping Yan *et al*. reduced the tannin level of grain sorghum flour (GSF) by 20% via ozonation. Then, they compared ethanol producing activity of *S. cerevisiae* in the two ozone-treated and non-treated GSF media. It was revealed that ethanol yields from all the ozone-treated GSFs were significantly higher than that from the non-treated control GSF. Also, the fermentation efficiencies of ozone-treated GSFs were 2-5% higher than that of the control flour [16]. Ling Ying *et al*. investigated the effect of tannins present in cassava on its alcohol fermentation. However, the results obtained indicated that there was no correlation between tannin content and starch liquefaction, saccharification and liquor output rate; thus, they showed that the effects of tannin content on liquefying enzyme and saccharifying enzyme could be neglected and the effect of inhibition on yeast in cassava fermentation was not apparent [17].

Tannins and tannin-like substances are well known for their ability to form complexes by combining with substrates and other organic compounds. Three mechanisms were identified regarding tannin toxicity in microorganisms:
Enzyme inhibition and substrate deprivation, action on membranes, and metal ion deprivation. Enzyme inhibition and substrate deprivation are characteristic of tannin/protein interactions [18]. Gelatin has been used for the clarification or fining of wine since the Roman civilization and probably before that as well [19]. Cashew apple juice was clarified by adding 1% gelatin to remove tannin and suspended solids before statistical screening of medium components on ethanol production from it using *Saccharomyces diasticus* [20]. As already mentioned, the activity of *S. cerevisiae* may be influenced or not influenced by the tannins present in different media. *S. cerevisiae* and *Z. mobilis* both produce ethanol as a primary metabolite. For that reason, the amount of ethanol produced by *Z. mobilis* in low tannin level CPE may be different from that of high tannin containing CPE.
Since, there is no data available on the effect of tannins regarding the ethanol production from CPE using *Z. mobilis*, the aims of this research were:

- To find a way to reduce tannin contents of CPE. Gelatin was used for this purpose.
- To optimize the rates of nutritive materials, peptone and yeast extract, required to be added to the tannin-reduced culture as well as culture time to reach the maximum ethanol production by *Z. mobilis* from the initial sugar content of CPE. Response surface methodology (RSM) was employed to reach this goal.

- To evaluate *Z. mobilis* ethanol production in TR-CPE and non-TR-CPE under the optimized conditions and comparing the performance of *Z. mobilis* in the two different culture media used.

2. Materials and Methods

A. Microorganism and growth Culture

The strain used was *Z. mobilis* PTCC 1718, obtained from the Persian Type Culture Collection. The dried strain was reactivated and grown for 17 h at 30°C and 120 rpm in a medium containing10 g l^{-1} peptone from meat (peptone) (Merck, Darmstadt, Germany), 10 gl^{-1} yeast extract (Merck), and 20 gl^{-1} glucose (Merck). Developed inoculums were mixed with sterilized glycerol (121°C and 15 min) and kept frozen for future use at -70°C in 2ml micro tubes.

B. Preparation of CPE

Carob pods were obtained from a Cypriot carob field. CPE was prepared by a procedure similar to that described by Roukas [21]. Details of the procedure used in this research were the same as used by Vaheed *et al*. [5].

C. Gelatin treatment of CPE

Edible gelatin (Gelita AG, Eberbach, Germany) solution was prepared by dissolving 1.25 g of gelatin in 32.50 ml distilled water while heating (not above 70°C to avoid possible decomposition). This solution was added to 500 ml of preheated CPE (70°C) while being stirred slowly. The pH of the CPE had been adjusted previously to 3.6-3.7 using 1 N HCl. This mixture was left to cool to room temperature and then centrifuged to separate its tannin-reduced CPE, supernatant, from the precipitate (15650 g and 15 min).

D. Fermentation of gelatin-treated CPE and non-gelatin treated CPE

Proportional volumes of gelatin-treated and untreated CPE, containing 15 g total sugar, while their pH were adjusted previously to 5.5 (using 1 N HCl or 1 N NaOH), were transferred into two separate 100ml Erlenmeyer flasks and were enriched each with 1.12 g of yeast extract (Merck) and 1.12 g of peptone from meat (Merck). The mixtures were sterilized at 121°C for 15 min. Then, they were inoculated with 34 ml of bacterial suspension (0.06 g bacterial dry weight) and the volumes of the mixtures were adjusted to 100 ml by adding sterilized distilled water. These cultures were incubated at 30°C and 80 rpm for 80 h.

E. Statistical experimental design

Response surface methodology was used to design experiments to optimize the conditions for obtaining maximum amount of ethanol in tannin reduced CPE. The software, design expert dx7 (trial version), was used to design these experiments. The variables were time (24-48 h), yeast extract (0.00-0.75 g /50 ml culture), and peptone from meat (0.00-0.75 g /50 ml culture). Each factor was assessed at five coded levels (-1.414, -1, 0, +1, and +1.414), as shown in Table 1. Constant factors in these experiments were the following: pH, 5.5; initial sugar in 50 ml culture,7.5g; culture volume, 50 ml; and 80 rpm.

F. Fermentation treatments according to RSM-designed experiments in TR-CPE

A proportional volume of gelatin-treated CPE containing 7.5 g total sugar at a pH of 5.5 (adjusted by 1 N HCl or 1 N NaOH) was transferred to a 50-ml Erlenmeyer flask. The weighed amounts of yeast extract (Merck) and peptone (Merck), as specified in Table 1, were mixed into the CPE. This mixture was sterilized at 121°C for 15 min. Then, the sterilized mixture was inoculated with 17ml of bacterial suspension (0.03 g of bacterial dry weight) and the volume of culture was adjusted to 50 ml by adding sterilized distilled water. This culture was incubated at 30°C and 80 rpm for different periods as indicated in Table 1.

G. Fermentation of TR-CPE at optimized conditions

A proportional volume of TR-CPE containing 7.5 g total sugar at a pH of 5.5 (adjusted by 1 N HCl or 1 N NaOH) was transferred into a 50-ml Erlenmeyer flask. Yeast extract (0.13 g) and peptone (0.62 g) were added to the flask. Then, this mixture was inoculated with 17 ml of bacterial suspension (equivalent to 0.03 g of dried bacteria) and its volume was brought up to 50 ml by adding sterilized distilled water.

Finally, the culture was incubated at 30°C and 80 rpm for 43-79 h.

H. Analytical procedures

H.1. Bacterial mass determination

The dry bacterial mass concentration in the inoculum culture was determined to be g l^{-1} by measuring its optical density (OD) at 600 nm and using a calibration graph in the range 0.15-0.35 g l^{-1} bacterial dry weight ($R^2 = 0.996$).

*H.2. Determination of moisture and total sugars in carob pods powde*r

Moisture content in each sample of carob pods powder was determined by drying it at 70°C to constant weight. The weight difference between its dried and primary state was reported as moisture content of the samples. Sugar was extracted from carob pods powder by heating 0.22g of it with 20 ml of distilled water at 85°C for 3 h, while stirring. Then, total sugars as well as reducing sugars were determined according to section H.3 in this paper.

H.3. Sugar determination

Total sugars (glucose, fructose, and sucrose) as well as reducing sugars (glucose and fructose) were determined using the 3, 5-dinitrosalicylic acid (DNS) method [22, 23] as described by Vaheed *et al*. [5].

Table 1. Quantitative values of the coded parameter levels and RSM-designed experiments accordingly for TR-CPE as well as the ethanol produced at 30°C, pH 5.5, and 80 rpm.

Code	Variables	Units	Levels				
			-α (-1.414)	-1	0	+1	+α (+1.414)
A	Time	h	24	28	36	44	48
B	Yeast extract	g/50 ml	0	0.13	0.38	0.62	0.75
C	Peptone	g/50 ml	0	0.13	0.38	0.62	0.75

Run	A: Time (h)	B: Yeast extract (g)	C: Peptone (g)	Response Ethanol produced (g)
1	28.13	0.62	0.62	1.799
2	24.00	0.38	0.38	1.549
3	36.00	0.38	0.38	2.285
4	36.00	0.38	0.38	2.188
5	43.87	0.13	0.13	2.486
6	48.00	0.38	0.38	2.549
7	28.13	0.13	0.62	1.451
8	36.00	0.00	0.38	2.153
9	36.00	0.38	0.38	1.722
10	36.00	0.38	0.00	2.097
11	36.00	0.38	0.38	2.34
12	36.00	0.38	0.75	2.132
13	43.87	0.62	0.62	2.229
14	36.00	0.75	0.38	2.472
15	28.13	0.13	0.13	1.333
16	43.87	0.13	0.62	2.653
17	43.87	0.62	0.13	2.285
18	36.00	0.38	0.38	1.965
19	28.13	0.62	0.13	2.208
20	36.00	0.38	0.38	2.063

H.4. Ethanol determination

The ethanol produced was distilled out from the fermented medium as an aqueous solution, which its ethanol content was determined as w/v% by the Caputi *et al*. method [24]. Absolute ethanol (Merck) was used to prepare a standard graph in the range 0.00–4.5 g ethanol/100 ml water– ethanol solution ($R^2 = 0.998$).

H.5. Determination of tannin in CPE

This method is based on the reduction of iron (III) to iron (II) by tannin. The reduced iron (II) reacts with 1, 10-phenanthroline to form a colored complex, which its absorbance was measured at 540 nm [25]. Tannic acid (Merck) was used to prepare a calibration graph by plotting absorbance versus concentration of tannic acid in the range of 1.17-5.8 µg ml^{-1} (R^2= 0.978). Then, the tannin content was reported as the tannic acid equivalent.

H.6. Determination of total protein concentration

The total protein determination test kit (Pars Azemun, Karadj, Iran) based on the Orsoanneau *et al*. [26] method was used for determination of total protein. The reagent used was composed of pyrogallol red (60 µmol l^{-1}), sodium molybdate (40 µmol l^{-1}), and detergents. The standard solution used was bovine serum albumin (500 µg l^{-1}). A cell with 1 cm path length was used. Aliquots, 20 µl, of the sample or standard solutions were mixed with 100 µl of the reagent solution followed by incubation for 10 min at 20-25°C. Then, the absorbance of these reaction mixtures was determined within 30 min at 600 nm against a reagent blank (20 µl of distilled water and 1000 µl of reagent solution). The procedure described above was for protein determinations in the range of 20 to 1000 µg l^{-1}. Therefore, a dilution was made if required.

3. Results and discussion

A. Moisture and sugar contents of carob pods powder

The carob pods powder used contained 9.09±0.00 (n=3) moisture, 56.10±1.14 (n=3) total sugars, and 19.00±2.67 reducing sugars (all as weight %). These values are close to those obtained by Salem and Fahad, 9.30% and 51.6% for moisture and total sugar, respectively in carob pods powder they studied [27].

B. Efficiency of sugar extraction from carob pods and tannin content of the CPE

A 2.7-l CPE was obtained from 300 g of carob pods powder. Its sugar concentration was determined to be 52 g l^{-1}, which means 468 g of sugar is obtained from one kg of carob pods powder. Turhan *et al*. reported 461.6 g sugar for each kg of carob sample, at the optimum extracting conditions of 80°C, 2 h, and 1:4 fruit to water ratio [9]. Furthermore, the CPE also contained tannins as 4244.74±346.66 mg l^{-1} (n=3) tannic acid equivalent and protein as 57.91±1.04 mg l^{-1} (n=3). According to Avallone *et al*. carob pod contains carbohydrate (45%), appreciable amounts of protein (3%), and polyphenols (0.019%) as mean value [28]. Yousif and Alghazawi reported that the roasted carob powder contained 9.00, 5.82, and 3.75% moisture, protein, and tannins, respectively [4]. The amounts of the compounds transferring to the aqueous extracting phase depend upon conditions employed, for example, Manal *et al*. obtained three types of CPE with different levels of the total sugar, protein, and tannin contents using different conditions, whereas the carob powder which they used was the same [15]. The analysis conducted on a sample taken from the CPE concentrating process showed that its sugar and tannin concentrations were 62.23± 0.96 g l^{-1} (n=3) and 5083.53±419.68 mg l^{-1}, respectively leading to a ratio of tannin to sugar as 81.68 mg g^{-1} for the CPE used in this research. Using the easy procedure, DNS method, to determine total as well as reducing sugars in CPE is satisfactory. Because, sugar profiles of carob pod has already been determined by Biner *et al*. using HPLC. They indicated that carob pods contained sucrose, fructose, and glucose regardless of the variety and origin [6]. The ratio of tannin to sugar obtained in this research is between those two values reported by two other research teams; Yousif and Alghazawi, and Avallone *et al*. The 96.9 mg g^{-1} ratio of tannin to sugar has been obtained for a carob pod analysis conducted by Yousif and Alghazawi. Another ratio, namely 42.22 mg tannin/g carbohydrate, has been obtained in an analysis performed by Avallone *et al*. [4, 28]. However, the weight contribution of tannins in CPE depends on carob cultivar and extracting conditions.

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C. Tannin reduction from CPE

The isoelectric point of gelatin ranges between 4.8 and 9.4, with acid processed gelatins having higher isoelectric points than alkali processed gelatins [29].

Therefore, at a pH of 3.6-3.7, which is close to that of wine [30], one would expect most of the acidic groups in the protein (gelatin) to be uncharged. Tannin molecules containing benzene rings with adjacent hydroxyl groups as are present in gallic acid are proposed to be the major source of hydrogen bonds, which are the basis of complex formation between gelatin and tannins [31]. Gelatin was useful to eliminate phenolic compounds, which are responsible for astringency, bitterness, and color in wine [32]. For comparative purposes, colorimetric methods to determine the tannin content of plant samples frequently use catechin as a standard, and the results are quoted as "catechin equivalent" [33].

The light absorption intensity (OD) of the prepared CPE in this research was scanned for maximum absorption wavelength, which was 550 nm in the range 400-700 nm. The sugar concentration of the CPE used for tannin-reducing studies was as 62.23±0.96 g l^{-1}. Five identical 10-ml volume samples of this CPE (pH 5.4) were mixed with different quantities of gelatin to determine the optimum amount of gelatin needed to reduce the tannin in the CPE. The pH of these samples were adjusted (by 1 N HCl) previously to 3.6-3.7, as is the pH of wine which is subjected to gelatin fining operations [30].

A gelatin solution of 40 g l^{-1} was used to add gelatin to the samples in these treatments. This solution also showed its maximum absorbance at 550 nm, which was the same as observed for 100 µg l^{-1} of tannic acid solution. The quantity of gelatin added, optical density (OD), and the total protein content of the solution phase of the CPE-gelatin mixtures are presented in Table 2 and Figure 1. As shown in Figure 1, an increase in total protein level in the solution phase of the CPE-gelatin mixtures were concomitant with the increase in amounts of gelatin added to the CPE.

Table 2. Amount of gelatin used, optical absorbance, and protein concentration of the solution phase of the CPE-gelatin mixture.

Run	Gelatin used, g for 10 ml of CPE	Absorbance of solution at 550 nm (Blank, distilled water), n = 3	Protein concentration of solution part, mg l^{-1}, n = 3
1	0.00	0.344 ± 0.001	69.35 ± 0.00
2	0.02	0.096 ± 0.002	315.05 ± 12.25
3	0.03	0.074 ± 0.005	953.75 ± 16.35
4	0.04	0.0955 ± 0.005	1797.70 ± 122.60
5	0.05	0.134 ± 0.001	2687.85 ± 130.80

This phenomenon implied that some of the gelatin added in each treatment did not combine with tannin or other gelatin-combining agents presented in CPE, but rather remained as free dissolved protein in the solution phase of CPE-gelatin mixture which led to an increase in its light absorbance after the minimum OD value observed (at the point of 0.03g gelatin added to 10 ml of CPE). OD decreasing trend between points 1 and 3 on the absorbance graph in Figure 1 is because of partial removal of tannins from the solution phase.

Whereas, the gradual increase of OD between points 3 and 5 is because of changing up the gelatin level in the solution phase of the CPE-gelatin mixtures. As already mentioned, the wavelength of the maximum absorbance of the non-gelatin treated CPE and the gelatin solutions were the same (550 nm). Therefore, it was not possible to use the absorption tool to investigate the tannin levels in the solution phase of the CPE-gelatin mixtures after the minimum value of OD observed. So the gelatin addition level of minimum OD observation was selected as the optimum level for gelatin use as tannin reducing agent (3 g l^{-1}). Gelatin is suggested to be used at the rate of 30-240 mg l^{-1} for wine fining [30]. In another example, the cashew apple juice was

clarified by 1% gelatin which was added to it to remove tannin and suspended solids before being used for ethanol production [34]. The sugar concentration of the CPE was measured before and after the gelatin treatment, the values obtained were as 62.23±0.96 and 61.08±1.63 g l^{-1}, respectively (n=3). These values are not significantly different from each other (p=0.05). In other words, sugar loss was not observed after gelatin treatment. The tannin content of the gelatin-treated CPE was determined to be 2224.16 ±26.92 µg ml^{-1}, (n=3). The weight ratio of tannin to sugar content for gelatin-treated CPE was determined to be as 36.40 mg g^{-1}, while, this ratio for non-gelatin treated CPE was 81.65 mg g^{-1}, as previously mentioned. That is to say, gelatin treating of CPE reduced its tannin content to 44.58% of its original level.

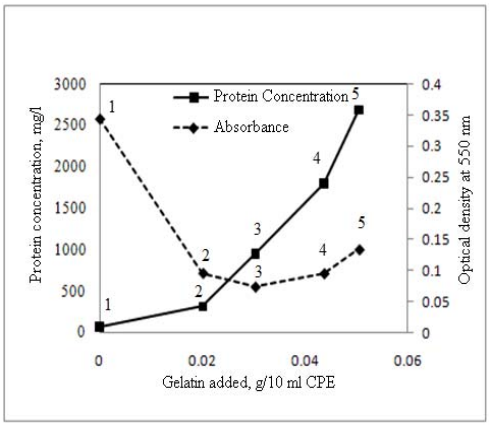

Fig. 1. Gelatin used for 10 ml of CPE as well as optical absorbance and protein concentration of the solution phase of the CPE-gelatin mixture.

D. Comparison of Z. mobilis performance in gelatin-treated and untreated CPE

The experiments were conducted at 30°C, a pH of 5.5, and 80 rpm. Relative weight amounts of the nutrients, peptone and yeast extract, to the initial sugar content were the same as used in our previous work to obtain the maximum ethanol production by *Z. mobilis* fermentation [5]. Ethanol produced from gelatin-treated and untreated CPE were 5.40 ± 0.18 g and 5.33 ± 0.15 g, respectively (n=3). Based on the statistical analysis of the results, no significant difference (p=0.01) was observed between the two amounts of ethanol produced (g g^{-1} initial sugar) in the two different cultures. On the other hand, some solids were formed and remained at the end of fermentation period in the two culture media which were determined to be different by weight; 0.12 ±0.02 g, (n=3) for tannin-reduced culture and 1.37 ±0.07 g (n=3) for culture containing untreated CPE. This difference was proposed to be because of the different amounts of tannin presented in the two cultures which combined with the protein containing nutrients, peptone and yeast extract, in the medium. Therefore, TR-CPE with initial sugar concentration of 15 w % was studied independently to optimize the addition levels of peptone, yeast extract, and fermentation time as well. A central composite design (CCD) was developed by employing the software design expert dx 7 (trial version). Twenty different experiments were designed and conducted [at temperature of 30°C; pH 5.5; 80 rpm; initial sugar (sugar), 7.5 g (15% w/v); inoculums bacterial dry weight (DW), 0.03 gr in 50-ml Erlenmeyer flask. Each factor was tested at five different levels (- α, -1, + 1, and + α) as presented in Table 1. Results were analyzed by the above mentioned software and the *p*-values were estimated (Table 4). In this case, A and AB are significant model terms (p = 0.05). The R^2 value is 0.84, which is an indication of good fit between experimental data and the regression equation. The lack of fit for response surface 2FI (two factor interaction) model is insignificant (p = 0.05).

The final formulas to predict ethanol production (g in 50 ml culture) in terms of coded factors and actual factors are shown as Equations (1) and (2) for the specified variable ranges of:

A: (Time), 28.13-43.87 h; B: (yeast extract), 0.13-0.62 g; C: (peptone), 0.13-0.62 g.

Ethanol production=2.10 +0.35A+0.086B-0.010 × C - 0.23AB+0.050AC - 0.094BC (1)

Figure 2 (a) represents the model-predicted values versus the actual values of ethanol produced. Clustering of the points around the diagonal line indicates a satisfactory correlation between the experimental and predicted values. Figure 2 (b) predicts that when the actual values of both yeast extract and peptone are 0.38 g, an increase in culture time will result in higher ethanol production, so that the maximum ethanol will be produced at the maximum incubation period

(time) of 43.87 h. According to this figure, more ethanol is produced as the fermentation time increases. For this reason, the confirmation test was repeated using different extended incubation periods (55, 67, and 79 h) to investigate the possible effect of increase in fermentation time on ethanol production.

Table 3. Comparative data obtained from *Z. Mobilis* performance in gelatin-treated and non-gelatin-treated CPE media.

Factor	Gelatin treated CPE	Untreated CPE
Initial sugar, (g*)	14.10	14.10
Residual sugar (g)	2.56±0.07, n = 3	2.55±0.10, n=3
Sugar consumed (g)	11.54 (82 %)	11.55 (82 %)
Ethanol produced (g)	5.40±0.18, n = 3	5.33±0.15, n=3
Ethanol yield, g g^{-1} sugar consumed	0.47	0.46
% of theoretical ethanol yield	85.10	84
Ethanol produced, g g^{-1} initial sugar	0.38±0.01	0.38±0.01

*Remained sugar after 6-ml volume sample removal. Therefore, practical volume of each sample became 94 ml.

Ethanol production= -1.08213+0.079075×Time+5.22546×Yeast extract-0.39404×Peptone-0.1193×Time×Yeast extract+ 0.025958×Time×Peptone– 1.54970×0.11933× Time ×Yeast extract+0.025958×Time×Peptone–1.54970× Yeast extract × Peptone (2)

The ethanol produced in these three repeating experiments was 2.64±0.14, 2.74±0.16, and 2.64±0.14, respectively.

These new quantities were not different from that obtained in 43.87 h incubation period (p=0.01). Consequently, it was concluded that the fermentation time of 43.87 h was sufficient for maximum ethanol production.

The effects of significant variables on ethanol production are shown as Figures 3 (a,b) and 4 (a,b). Figure 3(a) predicts that when the actual value of peptone is 0.38 g in 50 ml of culture medium, the maximum ethanol would be produced for 0.13 g yeast extract and 43.87 h incubation period. Furthermore, the antagonistic interaction between these two factors is presented in Figure 3(b).

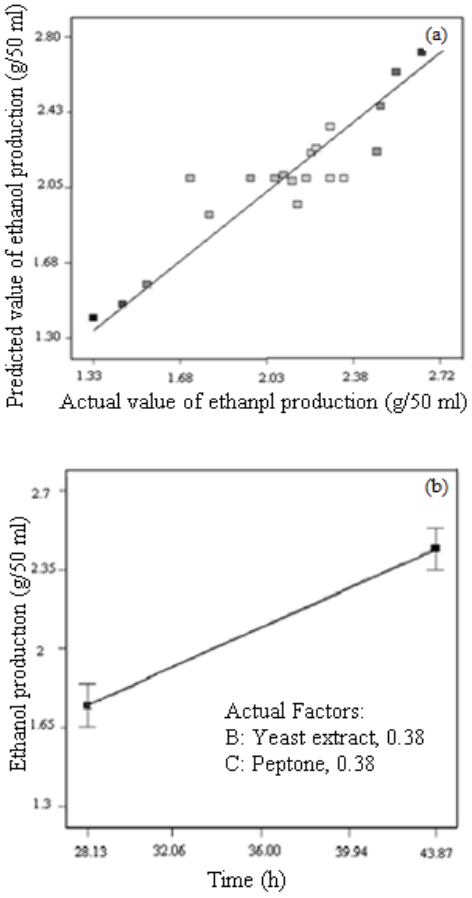

Fig. 2. Plots of predicted versus actual values of ethanol produced (a) and the effect of time on ethanol produced in TR-CPE medium (b).

Figure 4 shows that the effect of time at low level of yeast extract is greater than that at higher level of this nutrient. According to Figure 4 (a), the maximum ethanol production will occur at 0.13 g peptone and 0.62 g of yeast extract. The interaction between these two factors is shown in Figure 4 (b) in which, the effect of yeast extract is shown to be positive at low level of peptone, and close to zero at high level of this nutrient. This observation is an indication that these two nutrients can be replaced by each other. The optimal conditions for maximum ethanol production were obtained by further numerical analysis of the response levels (ethanol produced) using the software (design expert dx7 trial).

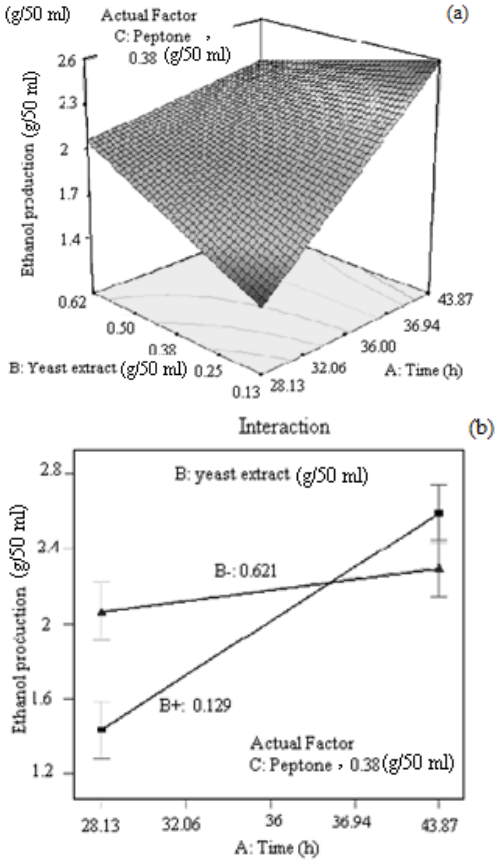

Fig. 3. Plots of the interaction between time and yeast extract, (a) and (b), on ethanol production at the actual value of peptone as 0.38 g/50 ml.

The optimum levels of factors to the maximum ethanol production were obtained as: yeast extract, 0.13 g; peptone from meat, 0.62 g; and time 43.78 h. A confirmation experiment was conducted under these optimal values in TR-CPE as well. Other conditions were the same as used in the RSM experiments (culture volume, 50 ml; initial sugar, 7.5 g; temperature, 30°C; pH, 5.5; rpm, 80; and bacterial dry weight, 0.02 g). Aeration and high shaking rate showed negative effect on *Z. mobilis* growth and also on its ethanol producing performance [5, 35].

Additionally, *Z. mobilis* produced the highest ethanol yield in "artichoke juice" at static conditions, i.e., less aeration conditions [36]. Hence, in all fermentation experiments small volume Erlenmeyer having less aeration surface and low shaking rate (80 rpm) were used to have less aeration. Results of this confirmation experiment are presented in Table 5. The amount of ethanol produced in the confirmation experiment was within the 95% prediction interval (PI) (95% PI low, 2.20 g and 95% PI high, 3.13 g).

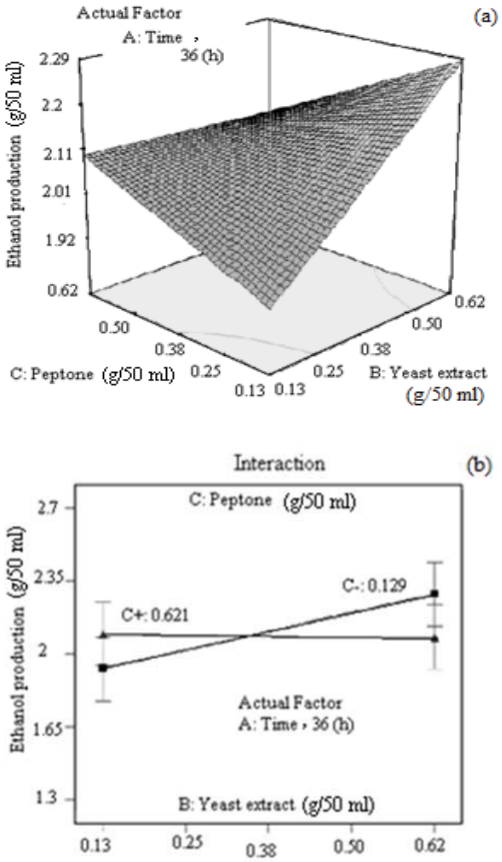

Fig. 4. Plots of the interacting effect of peptone and yeast extract on ethanol production in the actual fermentation period 36 h, (a) and (b).

Therefore, the model obtained was useful to predict the results and also to optimize the experimental conditions to maximize *Z. mobilis* ethanol fermentation in TR-CPE. A comparative experiment was also conducted using original CPE (non-gelatin-treated CPE) observing conditions the same as optimum obtained for TR-CPE. The tannin concentration in this case was so much higher than that of TR-CPE case, that is, 2.24 times more than that of confirmation tests (81.65 mg g^{-1} initial sugar/36.4 mg g^{-1} initial sugar). The results of this experiment are shown in Table 5 in comparison to those of confirmation test. The ethanol produced in untreated CPE was the same as in the confirmation experiment, with no significant difference from that of TR-CPE (p = 0.01). The

ethanol productivity and ethanol yields also were not significantly different between these two culture media (p = 0.01). Lin Ying *et al*. investigated the effect of tannin on alcoholic fermentation of cassava. The results indicated that there was no correlation between tannin content and starch liquefaction, saccharification, and liquor output rate. Thus, they showed that the effects of tannin content on liquefying enzyme and saccharifying enzyme could be neglected, and the effect of inhibition on yeast in cassava fermentation was not apparent [18]. However, Mullins and Lee showed that the mash prepared from high-tannin varieties of grain sorghum supports a significantly lower rate of ethanol fermentation than those of lower tannin varieties [37]. The numerical values of the ethanol yield and productivity in this research (Table 5) may be compared with those obtained by On soy *et al*. for *Z. mobilis* ethanol production in the jerusalem artichoke substrate (ethanol yield and productivity were 0.47g g^{-1} sugar utilized and 1.33 g l^{-1} h^{-1}, respectively) [36]. Higher ethanol concentration was obtained in TR-CPE medium at optimized conditions, i.e., 5.34 w% versus previously obtained 4.01 w%. Whereas, weight of nutrients consumed (peptone and yeast extract), 0.29 g g^{-1} ethanol produced, was 34.09% less than that of earlier reported nutrients consumption as 0.44 g g^{-1} ethanol produced [5]. According to sugar extraction efficiency and the data shown in Table 5, the ethanol yield for carob pods used is 154 g kg^{-1} carob pods powder. This criterion can be used for feasibility studies concerning the ethanol production from carob pod powder by *Z. mobilis*.

4. Conclusion

The following conclusions can be drawn from this study:

Gelatin was useful to reduce the tannins in CPE to 44.58 % of its initial value. No sugar loss was observed during the gelatin-treating process. Tracking the total protein content in the solution phase of the gelatin-CPE mixture showed that the entire gelatin added to the CPE did not form complex with tannin or other protein-binding agents but, some of it accumulated as uncombined in the solution phase. Tannin reduction practice in CPE has no significant effect on *Z. mobilis* ethanol fermentation, ethanol yield and productivity. Therefore, tannin reduction operations are not necessary to maximum ethanol fermentation by *Z. mobilis* in CPE. RSM is a good method to find the optimum levels of nutrients, peptone and yeast extract, as well as culture time for the maximum ethanol production in TR-CPE. Higher ethanol concentration and lower amounts of nutrients consumption, relative to initial sugar presented were obtained in this research compared with earlier reported concentrations and nutrients consumption.

Table 4. Estimated factor coefficients and associated p-values of the CCD model for response (Ethanol Produced).

Factor	Coefficient estimate [1]	p-value [2]
Model		0.0001 significant
Intercept	2.10	
A-Time	0.35	<0.0001
B-Yeast extract	0.086	0.1034
C-Peptone	-0.010	0.8411
AB	-0.23	0.0024
AC	0.050	0.4289
BC	-0.094	0.1516
Lack of Fit		0.9284, not significant

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

[2] p-values: 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., it is a parent term of a significant interaction).

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Table 5. Confirmation experiment results for *Z. mobilis* performance at optimized conditions for maximum ethanol production in TR-CPE and comparing the results with those of non-TR-CPE culture.

Factor	TR CPE, n=3	Non-TR CPE, n =3
Initial sugar, g	7.5	7.5
Residual sugar, g	2.08± 0.12	1.80±0.07
Sugar consumed, g	5.42± 0.12	5.71±0.07
Ethanol produced, g	2.67± 0.06	2.76±0.12
Ethanol yield, g g^{-1} sugar consumed	0.49± 0.01	0.49±0.02
Ethanol produced, g g^{-1} initial sugar	0.36± 0.01	0.37±0.02
Ethanol productivity, g $l^{-1}h^{-1}$	1.24± 0.03	1.29±0.05

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