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Kinetics of Ethanol Production from Carob Pods Extract by Immobilized *Saccharomyces cerevisiae* Cells

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ABSTRACT

Kinetics of ethanol production from carob pods extract by immobilized *S. cerevisiae* cells in static and shake flask fermentation have been investigated. Shake flask fermentation proved to be a better fermentation system for the production of ethanol than static fermentation. The optimum values of ethanol concentration, ethanol productivity, ethanol yield, and fermentation efficiency were obtained at pH range 3.5–6.5 and temperature between 30–35°C. A maximum ethanol concentration (65 g/L), ethanol productivity (8.3 g/Lh), ethanol yield (0.44 g/g), and fermentation efficiency (95%) was achieved at an initial sugar concentration of 200, 150, 100, and 200 g/L, respectively. The highest values of specific ethanol production rate and specific sugar uptake rate were obtained at pH 6.5, temperature 40°C, and initial sugar concentration of 100 g/L. Other kinetic parameters, biomass concentration, biomass yield, and specific biomass production rate were maximum at pH 5.5, temperature 30°C, and initial sugar concentration 150 g/L. Under the same fermentation conditions nonsterilized carob pod extract gave higher ethanol concentration than sterilized medium. In repeated batch fermentations, the immobilized *S. cerevisiae* cells in Ca-alginate beads retained their ability to produce ethanol for 5 d.

Index Entries: Kinetics of ethanol production; batch fermentation; carob pod extract; *Saccharomyces cerevisiae*; Ca-alginate beads.

INTRODUCTION

The carob pod is the fruit of the carob tree (*Ceratonia siliqua*), which is mainly cultivated in the Mediterranean countries and in many areas of North America. The annual production is about 340,000–400,000 metric tons (*1*). Greece is a main producer, with an annual harvest of 21,000 tons in 1985 (*2*). From the utilization viewpoint, two parts can be distinguished in the pod: the kibble or "locust bean" and the seeds or "locust kernel gum," a galactomannan highly valued in the food, textile, and cosmetic industries (*3*). The carob kibble contains the following (expressed as g 100 g^{-1} of kibble), moisture 10–15; total sugars (glucose, fructose, sucrose, and maltose) 40–50; protein 3–4; pectin 1–2; cellulose 7; hemicellulose 5; phenolic compounds 20; fat 0.5–1.0, and ash 2–3 (*4–6*). Before the twentieth century, the carob pods were exclusively used as animal fodder and also for human consumption. In more recent years, most of the carob pods are still being used in animal feeds and several applications of the kibble are in use. It is used in the preparation of antiarrheic and antiemetic products, pastry baking, and as cocoa substitution (*5*). Because of the high concentration of sugars in the carob kibble it is important to develop new and more attractive uses of these sugars. The simplest way would be the manufacture of a carob sugar syrup. Previous work in this area is very scarce (*1*).

As demand for the limited global supply of nonrenewable energy resources increases, the prices of oil and natural gas keep increasing. As a result, production of ethanol from renewable carbohydrate materials for use as an alternative liquid fuel has been attracting a worldwide interest. Recently, a considerable interest has been shown in using agricultural crops and their products such as apples, oranges, peaches, watermelons, dates, raisins, cassava and sago starch, potatoes, sugar beets, sugar cane, and Jerusalem artichoke tubers for fuel ethanol production by free and immobilized cells of *S. cerevisiae, Z. mobilis, K. marxianus,* and *K. fragilis* (*7–18*). Carob trees have many distinct advantages over traditional crops such as high carbohydrate yield, good growth in poor soil under favorable dry farming conditions, and high tolerance to various plant diseases (*4*). The value of carob pods is $135/ton (*19*). Although the kernels represent approx only 10% of the weight of the pod, they contribute more than 60% of the pod market price (*6*). For this reason, the carob kibble can be used as a cheap carbohydrate source for ethanol production. The price of kibble is about $50/ton, whereas the cost of fermentable sugars from this source is $100/ton. However, very little published information is available on the utilization of carob kibble as a carbohydrate raw material to produce fuel ethanol (*5*). The production of ethanol from carob pods extract by immobilized *S. cerevisiae* cells has not been investigated.

The aim of this investigation was to examine the potential of carob pods as a source of ethanol production by *S. cerevisiae* cells entrapped in calcium alginate gel, as well as to study the effect of various fermentation

parameters such as pH, temperature, and initial sugar concentration on kinetic parameters of carob pods extract fermentation.

MATERIALS AND METHODS

Microorganism and Substrate

Compressed bakers' yeast *Saccharomyces cerevisiae* (ZANAE Co., Thessaloniki, Greece) was used throughout this investigation. Carob pods (cultivar Tylliria) were obtained from the local market. After removing the seeds, kibble was chopped into small particles ranging from 0.3 to 0.6 cm in diameter. Forty-five grams of particles were mixed with 180 mL of distilled water (solid/liquid ratio 1:4) and the mixture was shaken in a rotary shaker/incubator (Lab-Line Orbit-Environ shaker, Lab-Line Instr., Inc., USA) at 250 rpm for 2 h at 70°C in order to extract the sugars from the kibble. The extract was then centrifuged at 4,000g for 15 min and the pH of the supernatant was adjusted to 4.5 with 1*N* HCl. The solution containing 15% sugars was sterilized at 121°C for 15 min. Samples of carob pods extract so prepared (production medium) were used for production of ethanol by immobilized *S. cerevisiae* cells.

Immobilization of Cells

Five grams of compressed bakers' yeast were suspended in 20 mL sterile distilled water and the cell suspension was mixed with 20 mL of a 5% sterile alginic acid sodium salt solution (Sigma Chemical Co., St. Louis, MO, A-2033). The mixture was extruded drop by drop with a peristaltic pump into a sterile 2% $CaCl_2$ solution at room temperature while stirring it continuously. The beads (2–3 mm diameter) were hardened in $CaCl_2$ solution for 2 h. The particles were washed with sterile physiological saline to remove excess calcium ions and unentrapped cells.

Fermentation Conditions

The fermentation was carried out in 500-mL conical flasks containing 100 mL production medium and 25 g of Ca-alginate beads with entrapped cells of the microorganism (1.8×10^9 cells/g beads). The flasks were incubated at 30°C in a rotary shaker/incubator at 200 rpm or static in an incubator.

Analytical Techniques

At appropriate time intervals, fermentation flasks were removed and the contents analyzed. The concentration of living cells entrapped in Ca-alginate beads was determined by dissolving 6 beads in 10 mL of 0.3*M*

sodium citrate solution (adjusted to pH 5.0 with 1*M* citric acid) for 20 min with continuous stirring. The number of living cells liberated from the gels was determined by plate counting. *Saccharomyces cerevisiae* was cultivated on MYGP medium (glucose 2%, malt extract 0.5%, yeast extract 0.5%, peptone 0.5%, and agar 2%) at 30°C for 48 h. The fermentation broth was used for the determination of ethanol and residual sugars. Ninety milliliters of the liquor were mixed with 150 mL of distilled water and the mixture was distilled at atmospheric pressure until 170 mL distillate were collected. Ethanol content was measured with an alcohol meter at 15°C. Residual sugars (glucose, fructose, sucrose, and maltose) were determined as glucose by the 3,5-dinitrosalicylic acid (DNS) method (*20*) after hydrolysis of sugars in 1*N* HCl for 30 min at 90°C and neutralization with 1*N* NaOH.

Each experiment was repeated three times and the results were reported as averages of three repetitions. Statistical evaluation of the data was carried out through analysis of variance using the randomized block design. Comparison of the means was assessed using the LSD-test.

RESULTS AND DISCUSSION

Static vs Shake Flask Fermentation

The production of ethanol from carob pods extract by immobilized *S. cerevisiae* cells in static and shake flask fermentation is shown in Fig. 1. As shown in Fig. 1, the highest concentration of ethanol (50 g/L) was obtained in the culture grown in shake flask after 6 h of fermentation, whereas in culture grown in static fermentation the maximum concentration of ethanol (47.5 g/L) was obtained after 12 h of fermentation. Roukas and Lazarides (*21*), who studied the production of ethanol from acid hydrolyzed whey by *S. cerevisiae* cells entrapped in Ca-alginate gel found that shake flask gave higher ethanol concentrations compared to static fermentation. (16.7 vs 12.7 g/L). The higher ethanol concentration in the case of shake flask fermentation is probably owing to better growth of the yeast. As it appears in Fig. 1, within the first 6 h of shake flask fermentation, biomass concentration increased by a factor of 2, whereas it increased slightly during the first 12 h of static fermentation. D'Amore and coworkers (*22*) reported that increased yeast biomass can result in increased ethanol production. Shaking could be beneficial to the growth and performance of entrapped yeast cells by improving the mass transfer characteristics with respect to substrate, products/byproducts, and oxygen. Shaking results in mixing of the production medium outside the beads, thus helping maintain a concentration gradient between the interior and the exterior of the beads. This concentration gradient works in both directions. Through better dif-

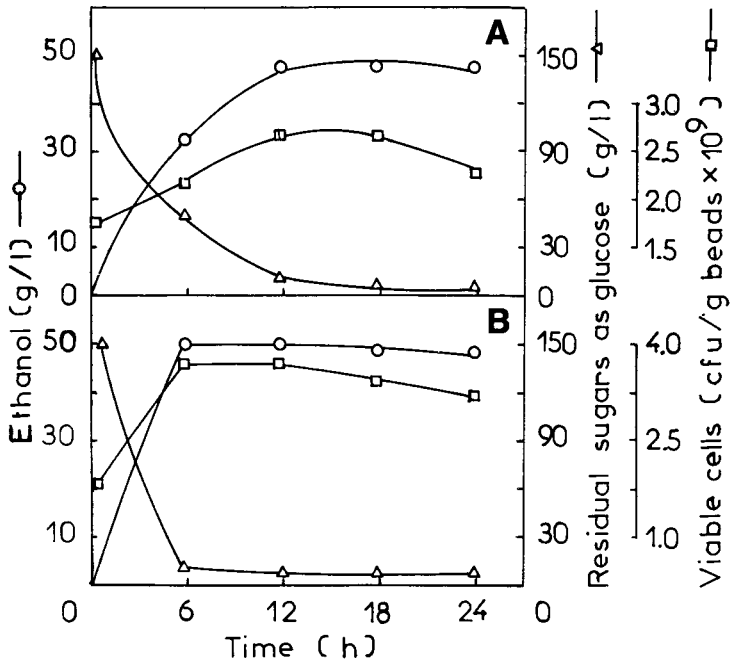

Fig. 1. Fermentation kinetics of immobilized *S. cerevisiae* during ethanol production from carob pods extract in static (**A**) and shake flask (**B**) fermentation. (Each point is the mean of three repetitions).

fusion it helps maintain a satisfactory supply of sugars and other nutrients to the entrapped cells, while it facilitates the removal of ethanol, CO_2, and other byproducts of catabolism from the microenvironment of the cells. Besides, shaking keeps the beads floating around. As a result, the entire surface of the bead is available for mass transfer. Finally, moderate shaking favors oxygen supply to the yeast and this is especially important for high biomass concentrations (*21*). Kana and coworkers (*9,10*) found that maximal ethanol concentrations of 53 and 35 g/L were obtained when immobilized *S. cerevisiae* cells on mineral kissiris and γ-alumina pellets were grown in raisin extract without any nutrients addition in static culture. Kolios and coworkers (*7*) found that maximal ethanol concentrations of 66, 39, 27, 41, and 44 g/L were obtained when free cells of various yeast strains were grown in apple, orange, peach, watermelon, and sugar beet extract, respectively, in static culture. Mehaia and Cheryan (*8*) reported that a maximum ethanol concentration (65 g/L) was obtained when free cells of *S. cerevisiae* was grown in date extract in submerged culture. There are some possible reasons for these differences, including the strain of organism used, the chemical composition of the substrate, the fermentation system, and, generally, the conditions under which the fermentation takes place.

As shown in Fig. 1, the maximum viable cell number observed at the same time as the maximum concentration of ethanol was observed. In the case of shake flask and static culture, the viable cell number decreased slightly after 12 and 18 h of fermentation, respectively. The decline in the biomass concentration could be caused by the reduced substrate availability and the inhibitory effect of ethanol on yeast cells (*14,23*). In culture grown in static fermentation, maximum concentration of viable cells was obtained on the twelfth hour, whereas in culture grown in shake flask, the maximum concentration of viable cells was found to be 6 h earlier. This means that, the better the condition of aeration was, the earlier the maximal biomass concentration occurred.

As expected, the concentration of residual sugars decreased during the fermentation, coinciding with an increase in biomass and ethanol production (Fig. 1). In the case of shake flask and static fermentation, the concentration of residual sugars fell rapidly during the first 6 and 12 h of fermentation, respectively, after which it remained practically constant. This was because of the rapid increase of biomass and ethanol concentration observed at the same time. When the maximum concentration of ethanol was achieved in cultures grown in static and shake flask fermentation, 33 and 35% of sugars consumed were converted to ethanol, respectively.

From the results presented in Fig. 1, various kinetic parameters describing the performance of the two fermentation systems are presented in Table 1. As shown in Table 1,, the kinetic parameters (except fermentation efficiency) were higher in the case of shake flask culture compared to static culture. Thus, shake flask fermentation was shown to be a better fermentation system for the production of ethanol from carob pods extract than static fermentation.

Effect of Initial pH

A set of shake flask experiments were performed at different initial pH in order to investigate the influence of initial pH on kinetic parameters of carob pod extract fermentation. The flasks containing 100 mL production medium at different initial pH (3.5, 4.5, 5.5, and 6.5) and 25 g of Ca-alginate beads were incubated at 30°C in a rotary shaker/incubator at 200 rpm. The results are presented in Table 2. The optimum pH range for ethanol concentration, ethanol productivity, ethanol yield, and fermentation efficiency was found to be 3.5–6.5, whereas the maximum biomass concentration was obtained in culture grown at pH 5.5. These results agree with those of Bajpai and Margaritis (*16*), who studied the production of ethanol from Jerusalem artichoke extract by *K. marxianus* cells entrapped in Ca-alginate beads. As shown in Table 2, the optimum pH for maximum specific ethanol production rate, biomass yield, specific biomass production rate, and specific sugar uptake rate were 6.5, 5.5, 4.5–5.5, and 6.5, respectively.

Table 1
Comparison of Static and Shake Flask Fermentation for Ethanol Production from Carob Pods Extract by Immobilized *S. cerevisiae* Cells[a]

Kinetic parameters	Static fermentation	Shake flask fermentation
Ethanol concentration P (g L^{-1})	47.5[a]	50.0[b]
Ethanol productivity, P (g L^{-1} h^{-1})	3.9[a]	8.3[b]
Ethanol yield, $Y_{p/s}$ (g ethanol g^{-1} sugar utilized)	0.33[a]	0.35[b]
Specific ethanol production rate, qp (g ethanol cfu^{-1} h^{-1})	5.8×10^{-11a}	8.7×10^{-11b}
Biomass concentration, X (cfu g^{-1} beads)	2.7×10^{9a}	3.8×10^{9b}
Biomass Yield, Yx/s (cfu g^{-1} sugar utilized)	4.8×10^{8a}	6.7×10^{8b}
Specific biomass production rate, qx (cfu g^{-1} sugar utilized h^{-1})	4.0×10^{7a}	1.1×10^{8b}
Specific sugar uptake rate, qs (g sugar cfu^{-1} h^{-1})	1.7×10^{-10a}	2.4×10^{-10b}
Fermentation efficiency (g sugar utilized) 100 g^{-1} initial sugar)	93.7[a]	93.6[a]

[a]Values are reported at the maximum ethanol concentration. Means with different letters in the same line are significantly different at the 5% level by the *t*-test.

Effect of Temperature

A series of conical flasks containing 100 mL production medium and 25 g of Ca-alginate beads were incubated at different temperatures (25, 30, 35, and 40°C) in a rotary shaker/incubator at 200 rpm in order to study the effect of temperature on kinetic parameters of carob pods extract fermentation. The results are presented in Table 3. The optimum temperature range for maximum ethanol concentration, ethanol productivity, ethanol yield, and fermentation efficiency was 30–35°C. Bajpai and Margaritis (*16*) found that the above parameters remained constant over the temperature range 25–45°C when immobilized *K. marxianus* cells where grown in Jerusalem artichoke extract in static culture. The viable cells number increased with the increase of fermentation temperature from 25 to 30°C and decreased slightly as the temperature was increased beyond 30°C. Rosa and coworkers (*15*) reported that yeast death in the presence of ethanol at high temperatures is caused by the enhancement by ethanol of the thermosensitivity of membrane associated with the thermal death sites,

Table 2
Kinetic Parameters of Carob Pods Extract Fermentation
by Immobilized *S. cerevisiae* Cells at Different pH Values[a]

Kinetic parameters	pH 3.5	pH 4.5	pH 5.5	pH 6.5
Ethanol concentration P (g L^{-1})	48.0^{ab}	50.0^{b}	48.5^{ab}	47.5^{a}
Ethanol productivity, P (g L^{-1} h^{-1})	8.0^{ab}	8.3^{b}	8.0^{ab}	7.9^{a}
Ethanol yield, $Y_{p/s}$ (g ethanol g^{-1} sugar utilized)	0.35^{a}	0.35^{a}	0.34^{a}	0.35^{a}
Specific ethanol production rate, qp (g ethanol cfu $^{-1}$ h^{-1})	9.1×10^{-11a}	8.7×10^{-11a}	8.0×10^{-11b}	9.8×10^{-11c}
Biomass concentration, X (cfu g^{-1} beads)	3.5×10^{9a}	3.8×10^{9b}	4.0×10^{9c}	3.2×10^{9d}
Biomass Yield, Yx/s (cfu g^{-1} sugar utilized)	6.4×10^{8a}	6.7×10^{8ab}	7.0×10^{8b}	5.9×10^{8c}
Specific biomass production rate, qx (cfu g^{-1} sugar utilized h^{-1})	1.0×10^{8a}	1.1×10^{8b}	1.1×10^{8b}	9.9×10^{7c}
Specific sugar uptake rate, qs (g sugar cfu $^{-1}$ h^{-1})	2.5×10^{-10a}	2.4×10^{-10ab}	2.3×10^{-10b}	2.8×10^{-10c}
Fermentation efficiency (g sugar utilized) 100 g^{-1} initial sugar)	90.3^{a}	93.6^{a}	94.0^{a}	89.6^{a}

[a]Values are reported at the 6th h of fermentation. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

Table 3
Kinetic Parameters of Carob Pods Extract Fermentation
by Immobilized *S. cerevisiae* Cells at Different Temperatures[a]

Kinetic parameters	Temperature (°C) 25	30	35	40
Ethanol concentration P (g L^{-1})	47.5^{a}	50.0^{b}	50.0^{b}	42.5^{c}
Ethanol productivity, P (g L^{-1} h^{-1})	7.9^{a}	8.3^{b}	8.3^{b}	7.0^{c}
Ethanol yield, $Y_{p/s}$ (g ethanol g^{-1} sugar utilized)	0.34^{a}	0.35^{a}	0.35^{a}	0.34^{a}
Specific ethanol production rate, qp (g ethanol cfu $^{-1}$ h^{-1})	1.2×10^{-10a}	8.7×10^{-11b}	9.5×10^{-11c}	1.4×10^{-10d}
Biomass concentration, X (cfu g^{-1} beads)	2.5×10^{9a}	3.8×10^{9b}	3.5×10^{9c}	2.0×10^{9d}
Biomass Yield, Yx/s (cfu g^{-1} sugar utilized)	4.5×10^{8a}	6.7×10^{8b}	6.2×10^{8c}	3.9×10^{8d}
Specific biomass production rate, qx (cfu g^{-1} sugar utilized h^{-1})	7.6×10^{7a}	1.1×10^{8b}	1.0×10^{8c}	6.6×10^{7d}
Specific sugar uptake rate, qs (g sugar cfu $^{-1}$ h^{-1})	3.6×10^{-10a}	2.4×10^{-10b}	2.6×10^{-10c}	4.2×10^{-10d}
Fermentation efficiency (g sugar utilized) 100 g^{-1} initial sugar)	91.0^{a}	93.6^{a}	93.3^{a}	83.6^{b}

[a]Values are reported at the 6th h of fermentation. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

whereas Bajpai and Margaritis (*16*) suggested that high temperatures caused denaturation of the enzyme system of *K. marxianus* cells. As shown in Table 3, the biomass yield and the specific biomass production rate were maximum at 30°C, whereas the specific ethanol production rate and the specific sugar uptake rate were maximum at 40°C. These results are in agreement with those obtained by Bajpai and Margaritis (*16*).

Effect of Initial Sugars

The carob pods extract was diluted with distilled water or concentrated at 50°C under vacuum in order to contain 10, 15, 20, and 25% initial sugars. The pH of the extract was adjusted to 4.5 with 1*N* HCl. Samples of carob pods extract so prepared (production medium) were used to investigate the effect of initial sugar concentration on kinetic parameters of carob pods extract fermentation by immobilized *S. cerevisiae* cells. A set of conical flasks containing 100 mL production medium and 25 g of Ca-alginate beads were incubated at 30°C in a rotary shaker/incubator at 200 rpm. As shown in Fig. 2, the ethanol concentration increased with the increase of initial sugar concentration up to 200 g/L but decreased beyond this value. The highest concentration of ethanol (65 g/L) was obtained in culture grown at an initial sugar concentration of 200 g/L after 12 h of incubation, whereas in culture grown at initial sugar concentration of 100, 150, and 250 g/L the maximum ethanol concentration was lower by 36, 23, and 7.7%, respectively. Bajpai and Margaritis (*17*), who studied the effect of initial sugar concentration on ethanol production from Jerusalem artichoke extract by *K. marxianus* cells immobilized in Ca-alginate beads, found that a maximum ethanol concentration of 112 g/L was achieved when *K. marxianus* was grown at an initial sugar concentration of 250 g/L in 20 h in static culture. Bajpai and coworkers (*24*) found that an osmotolerant *S. cerevisiae* SC 20-2 strain entrapped in Ca-alginate beads produced 132 g/L ethanol when it was grown in a chemically defined medium containing 350 g/L sucrose in static culture. Roukas and coworkers (*25*) reported that a maximum ethanol concentration (61 g/L) was obtained when immobilized *S. cerevisiae* cells in Ca-alginate beads were grown in prehydrolyzed whey containing 150 g/L lactose in shake flask culture. As reported, there are some hypotheses for these differences, including the strain used, the composition of the substrate, the fermentation system, and the conditions under which the fermentation takes place.

The viable cells number followed a pattern similar to ethanol concentration with maximum concentration observed at the same initial sugar concentration as the maximum concentration of ethanol was achieved (Fig. 2). The maximum biomass concentration (4.0×10^9 cells/g beads) was obtained in culture grown at an initial sugar concentration of 200 g/L after 12 h of incubation. When the initial sugar concentration was increased from 200 to 250 g/L, a decrease in viable cells number was observed. This

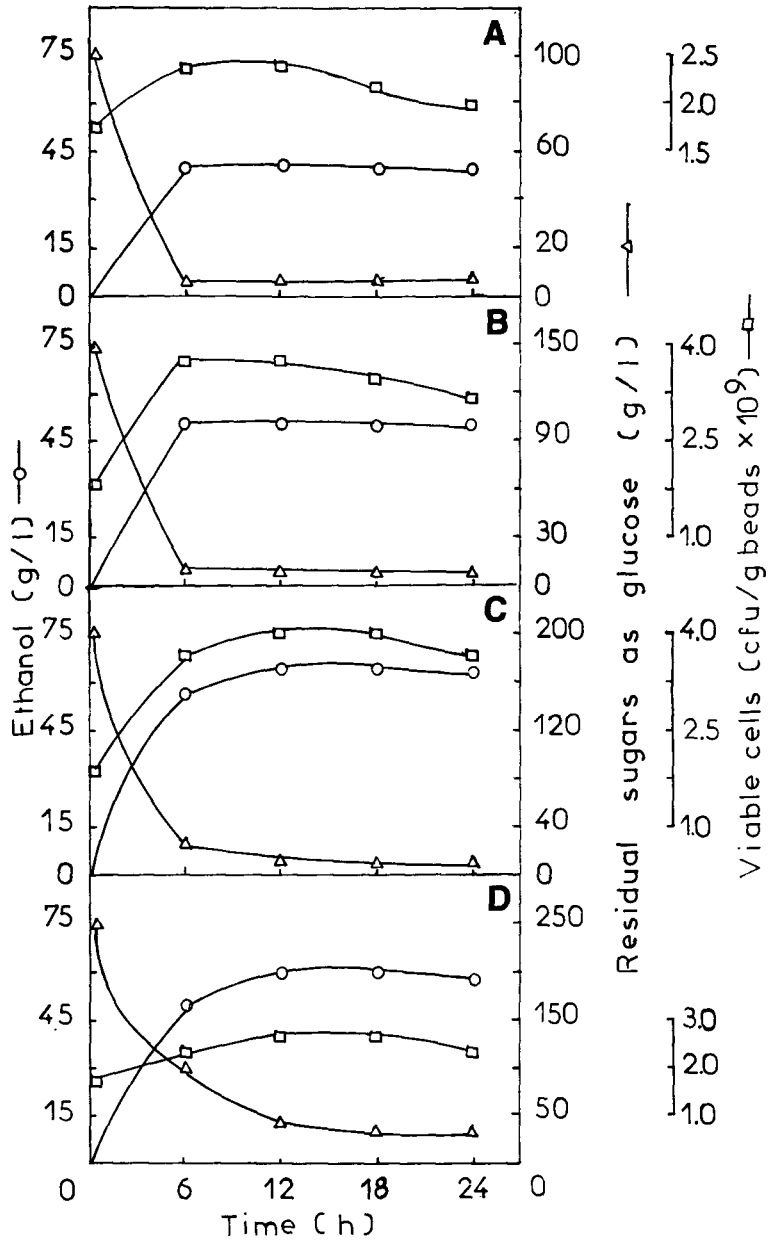

Fig. 2. Fermentation kinetics of immobilized *S. cerevisiae* during ethanol production from carob pods extract in shake flask culture at different sugar concentrations (**A, B, C, D:** Initial sugar concentration 100, 150, 200, and 250 g/L, respectively. Each point is the mean of three repetitions).

was owing to the inhibition of growth of microorganism at high sugars concentration (*23*). Roukas and coworkers (*25*), who studied the effect of initial lactose concentration on kinetic parameters of immobilized *S. cerevisiae* grown in prehydrolyzed whey, found that the maximum cell concentration increased as the initial lactose concentration increased from 50 to 150 g/L, but decreased as the initial lactose concentration increased further from 150 to 200 g/L.

As expected, the concentration of residual sugars decreased during the fermentation, coinciding with an increase in biomass and ethanol production. The residual sugar concentration was found to be very low at an initial sugar concentration equal to or below 200 g/L, but above an initial sugar concentration of 200 g/L resulted in a significant increase in the residual sugar concentration in the fermentation broth. This was expected also since there was a decrease in biomass and ethanol concentration. The increase in residual sugars was a result of the inability of microorganism to metabolize high sugar concentration (*23*). When the maximum concentration of ethanol was achieved in cultures grown at an initial sugar concentration of 100, 150, 200, and 250 g/L, 44, 35, 34, and 29% of sugars consumed were converted to ethanol, respectively.

From the results presented in Fig. 2, various kinetic parameters have been calculated and presented in Table 4. There were significant differences (at the 5% level) in ethanol concentration and ethanol productivity between cultures grown at initial sugar concentrations of 100, 150, 200, and 250 g/L. The maximum ethanol productivity (8.3 g/Lh) was obtained in culture grown at an initial sugar concentration of 150 g/L. Bajpai and Margaritis (*17*) and Bajpai and coworkers (*24*) found that a maximum ethanol productivity (5.6 and 8.8 g/Lh) was achieved when *K. marxianus* and *S. cerevisiae* cells entrapped in Ca-alginate beads were grown in Jerusalem artichoke extract and a chemically defined medium, respectively, containing 250 g/L initial sugars. There were no significant differences (at the 5% level) between cultures grown at an initial sugar concentration of 150 and 200 g/L in terms of ethanol yield and biomass concentration. The ethanol yield decreased with the increase of initial sugar concentration from 100 to 250 g/L. In contrast, Bajpai and Margaritis (*17*) found that the ethanol yield remained almost unaffected by initial sugar concentration up to 250 g/L and declined beyond that, whereas Roukas and coworkers (*25*) reported that the ethanol yield increased with the increase of initial lactose concentration from 50 to 150 g/L and decreased above 150 g/L. As shown in Table 4, increasing the initial sugar concentration from 100 to 250 g/L significantly affected (at the 5% level) the specific ethanol production rate and specific biomass production rate. The highest values of specific ethanol production rate and specific biomass production rate were obtained with an initial sugar concentration of 100 and 150 g/L, respectively. The cultures grown at an initial sugar concentration of 100 and

Table 4
Kinetic Parameters of Carob Pods Extract Fermentation by Immobilized *S. cerevisiae* Cells at Different Initial Sugar Concentration[a]

Kinetic parameters	Initial sugars concentration (g/L)			
	100	150	200	250
Ethanol concentration P (g L^{-1})	41.5[a]	50.0[b]	65.0[c]	60.0[d]
Ethanol productivity, P (g L^{-1} h^{-1})	6.9[a]	8.3[b]	5.4[c]	5.0[d]
Ethanol yield, $Y_{p/s}$ (g ethanol g^{-1} sugar utilized)	0.44[a]	0.35[b]	0.34[b]	0.29[c]
Specific ethanol production rate, qp (g ethanol cfu^{-1} h^{-1})	1.1×10^{-10a}	8.7×10^{-11b}	5.4×10^{-11c}	7.4×10^{-11d}
Biomass concentration, X (cfu g^{-1} beads)	2.4×10^{9a}	3.8×10^{9b}	4.0×10^{9b}	2.7×10^{9c}
Biomass Yield, Yx/s (cfu g^{-1} sugar utilized)	6.4×10^{8a}	6.7×10^{8a}	5.2×10^{8b}	3.3×10^{8c}
Specific biomass production rate, qx (cfu g^{-1} sugar utilized h^{-1})	1.0×10^{8a}	1.1×10^{8b}	4.3×10^{7c}	5.5×10^{7d}
Specific sugar uptake rate, qs (g sugar cfu^{-1} h^{-1})	2.6×10^{-10a}	2.4×10^{-10a}	1.6×10^{-10b}	2.5×10^{-10a}
Fermentation efficiency (g sugar utilized) 100 g^{-1} initial sugar)	93.6[a]	93.6[a]	95.0[a]	81.0[b]

[a]Values are reported at the maximum ethanol concentration. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

150 g/L resulted in a better specific sugar uptake rate and biomass yield, respectively, compared to the other sugar concentration. The fermentation efficiency remained almost unaffected by initial sugar concentration up to 200 g/L, but decreased significantly as the initial sugar concentration was increased from 200 to 250 g/L. The decreased efficiency encountered with the highest concentration treatment is probably a result of osmotic effects. It has been reported that above a critical substrate concentration, the decreased water activity and the onset of plasmolysis combine to cause a decrease in the rates of fermentation and ethanol production (25). The culture grown at an initial sugar concentration of 100, 150, 200, and 250 g/L utilized 93.6, 93.6, 95, and 81% of the sugars, respectively.

Ethanol Production from Nonsterilized Carob Pod Extract

A series of conical flasks containing 100 mL of nonsterilized carob pod extract (initial sugars 200 g/L, pH 4.5) and 25 g of Ca-alginate beads were incubated at 30°C in a rotary shaker/incubator at 200 rpm. The results are presented in Fig. 3. The concentration of ethanol increased rapidly during the first 6 h of fermentation and kept increasing at a slower rate to reach a

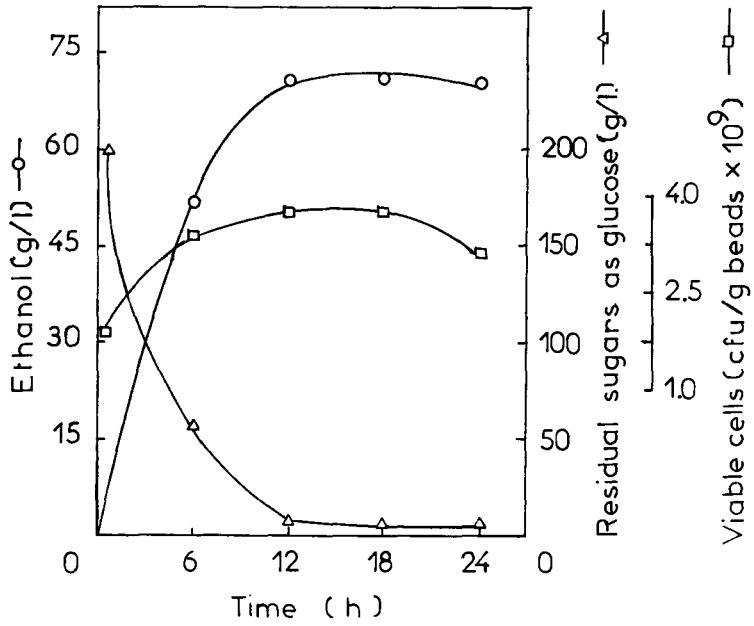

Fig. 3. Fermentation kinetics of immobilized *S. cerevisiae* during ethanol production from nonsterilized carob pods extract in shake flask culture. (Each point is the mean of three repetitions).

maximum value (71 g/L) after 12 h of incubation. The viable cell number followed a pattern similar to those of ethanol concentration with maximum concentration (3.8×10^9 cells/g beads) observed at the same time as the maximum ethanol concentration was achieved. Result of the plate counting showed no contamination of the substrate by other microorganisms. This could be caused by the large number of the microorganisms destroyed during the extraction of sugars from carob pods at 70°C for 2 h, the ethanol produced inhibited the growth of contaminating microorganisms, and the high amount of the inoculum dominated the existing microflora. The concentration of residual sugars decreased rapidly during the first 12 h of fermentation and then remained constant. This was caused by the rapid increase of biomass and ethanol concentration during the first 12 h of incubation. When the maximum concentration of ethanol was achieved, 37% of sugars consumed was converted to ethanol, whereas the total amount of sugars utilized was 95%. The above results showed that the culture grown in nonsterilized medium gave higher ethanol concentration, ethanol productivity, ethanol yield, and the same fermentation efficiency with those grown in sterilized medium under the same fermentation conditions. Moreover, the production of ethanol from nonsterilized carob pod extract has the advantages of saving in equipment and energy cost.

Table 5
Effect of Cell Recycling on Ethanol Production, Viable Cells Number, and Residual Sugars During Nonsterilized Carob Pods Extract Fermentation by Immobilized *S. cerevisiae* Cells in Ca-alginate Beads[a]

Number of recycles	Ethanol, g/L	Viable cells number, cfu/g beads	Residual sugars as glucose, g/L
1	71.0	3.8×10^9	9.5
2	72.0	3.7×10^9	8.0
3	71.5	3.8×10^9	9.0
4	69.5	3.6×10^9	11.5
5	70.0	4.0×10^9	11.0
6	70.5	3.7×10^9	11.5
7	71.0	3.6×10^9	12.5
8	70.5	3.8×10^9	12.0
9	70.5	3.7×10^9	13.0
10	70.0	3.7×10^9	13.5
11	67.5	3.0×10^9	19.5
12	64.5	2.0×10^9	26.5

[a]Recycling took place every 12 h. Each point is the mean of three repetitions.

Repeated Batch Fermentations

A set of conical flasks containing 100 mL of nonsterilized carob pods extract (initial sugars 200 g/L, pH 4.5) and 25 g of Ca-alginate beads were incubated at 30°C in a rotary shaker/incubator at 200 rpm. When maximum ethanol concentration was achieved (after 12 h of incubation) the fermentation broth was removed, the gel particles were washed twice with distilled water and fresh nonsterilized carob pod extract was added into the flasks. The above batch fermentations were repeated 12 times. As shown in Table 5, the ethanol concentration and the viable cells number remained almost constant during repeated batch fermentations up to the tenth batch and then decreased slightly. This means that immobilized *S. cerevisiae* cells in Ca-alginate gel retained their activity to produce ethanol for 5 d. This ability of immobilized cells to produce ethanol for a long time has not been explained yet. Probably, this may be due to the protection of cells by the immobilization matrix. Rychtera and coworkers (*26*) reported that immobilized cells can retain enzyme activities for a long time owing to the different composition of cells (proteins, lipids, RNA, DNA, and inorganic substances) compared to free cells. After 5 d (11th and 12th recycle) the cells begun to show signs of aging, as the slight decrease in biomass and ethanol concentration and the respective increase in residual sugars indicate.

CONCLUSIONS

The results showed some important aspects of ethanol production from carob pods extract by immobilized *S. cerevisiae* cells. Shake flask culture was a better fermentation system for the production of ethanol than static culture. The optimum conditions for carob pods extract fermentation were pH 3.5–6.5, temperature 30–35°C and initial sugar concentration of 200 g/L. In the case of Jerusalem artichoke extract fermentation where the main sugar was fructose, the optimum fermentation conditions were pH 4–6, temperature 25–45°C and initial sugar concentration of 250 g/L. Immobilized *S. cerevisiae* cells in Ca-alginate beads retained their activity to produce ethanol for a long time during repeated batch fermentations. The carob pods extract was an attractive medium for the production of ethanol by immobilized *S. cerevisiae* cells.

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