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T. Roukas

Solid-state fermentation of carob pods for ethanol production

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Abstract The production of ethanol from carob pods by *Saccharomyces cerevisiae* in solid-state fermentation was investigated. The maximal ethanol concentration (160 ± 3 g/kg dry pods), ethanol productivity (6.7 ± 0.2 g/kg per hour), ethanol yield ($40 \pm 1.8\%$), biomass concentration ($7.5 \pm 0.4 \times 10^8$ cells/g carob pulp) and fermentation efficiency ($80 \pm 2\%$) were obtained at an inoculum amount of 3%, a particle size of 0.5 mm, a moisture level of 70%, a pH of 4.5 and a temperature of 30°C. Under the same fermentation conditions both sterilized and non-sterilized carob pods pulp gave the same maximum ethanol concentration.

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 340–400 thousand metric tons (Mulet et al. 1988). Greece is a primary producer with an annual harvest of 21 thousand tons (Roukas 1993a). From the utilization view point, 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 (Davies et al. 1971). The carob kibble contains the following (expressed as g/100 g 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; ash, 2–3 (Binder et al. 1959; Calixto and Canellas 1982; Canellas et al. 1989).

As demand for the limited global supply of non-renewable 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 worldwide interest. Recently, considerable interest has been shown in using agricultural crops and their products such as sweet sorghum, corn, apple, grape, sugar cane, sugar beets, fodder beets and Jerusalem artichoke tubers for fuel ethanol production using solid-state fermentation (Kargi et al. 1985; Sato et al. 1988; Ngadi and Correia 1992; Hang et al. 1986; Rolz and Cabrera 1980; Amin 1992; Gibbons and Westby 1988; Gibbons 1989). Carob trees have many distinct advantages over traditional crops, such as high carbohydrate yield, good growth in poor soil under favourable dry farming conditions and high tolerance to various plant diseases (Binder et al. 1959). The value of carob pods is $135/ton (Office national de statistique de Greece 1990). Although the kernels represent approximately only 10% of the weight of the pod, they contribute more than 60% of the pod market price (Canellas et al. 1989). Thus, the carob kibble can be used as a cheap carbohydrate source for ethanol production. Recently, the production of ethanol from carob pods extract by free and immobilized *Saccharomyces cerevisiae* cells in static and shake-flask fermentation has been described (Roukas 1993a, b). The production of ethanol from carob pods by solid-state fermentation has not been investigated.

The aim of this investigation was to examine the potential of carob pods as a source for ethanol production by *S. cerevisiae* cells via solid-state fermentation, as well as to study the effect of various fermentation parameters such as inoculum amount, particle size, moisture, pH and temperature on kinetic parameters of carob pod fermentation.

T. Roukas (✉)
Department of Food Science and Technology,
Aristotelian University of Thessaloniki,
Box 250, 54006 Thessaloniki, Greece

Materials and methods

Micro-organism and substrate

Compressed bakers' yeast, *S. cerevisiae* (Zanae, Thessaloniki, Greece) was used throughout this investigation. Carob pods (cv 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 and pulverized in a Waring Blender at high speed. The pulverized particles were dried overnight in an oven at 70°C and passed through sieves with a pore size of 0.5, 1.2, 2.5, and 5.0 mm.

Thirty grams of carob kibble (particle size 0.5 mm) containing 15 g initial sugars was placed in 500-ml conical flasks and moistened with the appropriate amount of distilled water in order to contain 70% moisture. The pH of the substrate was adjusted to 4.5 with 1 M HCl. The medium was sterilized at 121°C for 30 min and inoculated with 3 g compressed bakers' yeast (5.0×10^8 cells/g carob pulp). The flasks were incubated at 30°C in an incubator under stationary conditions. In the case of ethanol production from non-sterilized carob pods the fermentation conditions were the same as described above.

Study of fermentation parameters

Inoculum amount

The substrate prepared as above was inoculated with 1.5, 3, 6 and 12 g compressed bakers' yeast (2.5×10^8, 5.0×10^8, 1.0×10^9 and 2.0×10^9 cells/g carob pulp, respectively) and incubated at 30°C for 24 h.

Particle size

A set of conical-flask experiments were performed at different particle sizes (0.5, 1.2, 2.5 and 5.0 mm) with 70% moisture and pH 4.5. The flasks were inoculated with 3 g compressed bakers' yeast and incubated at 30°C for 24 h.

Moisture content

A series of conical flasks containing 30 g carob kibble (particle size 0.5 mm, pH 4.5) were moistened with an appropriate amount of distilled water in order to contain 55, 60, 65 and 70% moisture. The flasks were inoculated and incubated as above.

Initial pH

The substrate consisting of 30 g carob kibble (particle size 0.5 mm) with 70% moisture and at pH 3.5, 4.5, 5.5 or 6.5 was inoculated with 3 g compressed bakers' yeast and incubated at 30°C for 24 h.

Temperature

The medium (30 g carob kibble, particle size 0.5 mm, moisture 70% and pH 4.5) was inoculated with 3 g compressed bakers' yeast and incubated at different temperatures (25, 30, 35 and 40°C) for 24 h.

Analytical techniques

At appropriate time intervals fermentation flasks were removed and the contents analysed. The number of living cells was determined by plate counting. *S. cerevisiae* was cultivated on MYGP medium (glucose, 2%; malt extract, 0.5%; yeast extract, 0.5%; peptone, 0.5%; agar, 2%) at 30°C for 48 h. The fermented mash was mixed with 100 ml distilled water and the mixture was shaken on a rotary shaker/incubator (Lab-Line Orbit-Environ Shaker, Lab-Line Instruments) at 250 rpm for 30 min at 30°C in order to extract the ethanol and the residual sugars from the mash. The extract was then centrifuged at 4,000 *g* for 15 min and the sediment was treated again as described above for the complete extraction of the fermented materials. The supernatants of the two extraction treatments were mixed together and the mixture was distilled at atmospheric pressure until 170 ml distillate was collected. Ethanol content was determined with an alcoholometer (Dujardin- Salleron, France) at 15°C. Residual sugars (glucose, fructose sucrose and maltose) were determined as glucose by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959), after hydrolysis of sugars in 1 M HCl for 30 min at 90°C and neutralization with 1 M NaOH. Ethanol yield was expressed as g ethanol/100 g sugars consumed. Fermentation efficiency was calculated by dividing the sugars consumed during fermentation by the initial sugars and multiplying the result by 100.

Each experiment was repeated three times and the results were reported as averages ± SD of three repetitions.

Results and discussion

Ethanol production via solid-state fermentation

The production of ethanol from carob kibble (particle size 0.5 mm) by *S. cerevisiae* in solid-state fermentation is shown in Fig. 1. The concentration of ethanol increased with the increase of fermentation time. The maximum ethanol concentration (160 ± 3 g/kg dry pods) was obtained after 24 h of incubation. In a previous study maximum ethanol concentrations of 75 and 65 g/l were obtained when free and immobilized compressed bakers' yeast cells were grown in carob-pod

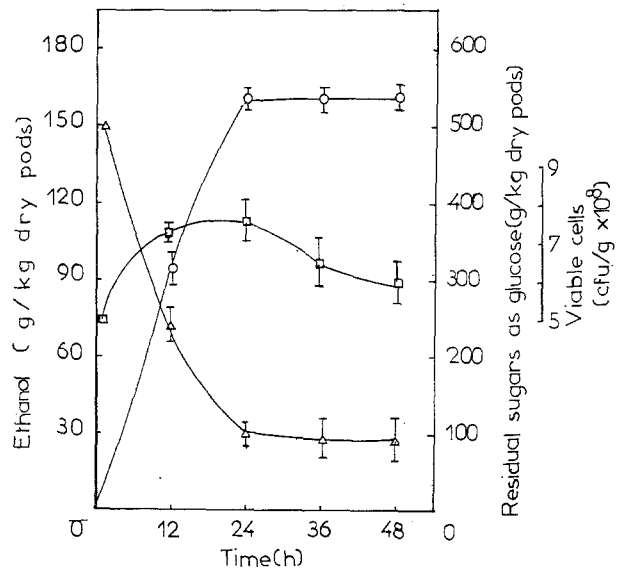

Fig. 1. Fermentation kinetics of *Saccharomyces cerevisiae* during ethanol production from carob pods in solid-state fermentation: ○, ethanol; □, viable cells; △, residual sugars as glucose. Each point is the mean ± SD of three repetitions; cfu, colony-forming units

extract after 48 and 12 h, respectively, in shake-flask fermentation (Roukas 1993a, b). Hang et al. (1981, 1986) reported maximum ethanol concentrations of 43 g/kg apple pomace and 53.5 g/kg grape pomace for various yeast strains grown in solid-state fermentation, whereas Gibbons (1989) found that a high concentration of ethanol (7.3%, v/v) was obtained when *Kluyveromyces marxianus* was grown in Jerusalem artichoke tuber pulp after 72 h of fermentation. There are some possible reasons for these differences, including the strain of organism used, the chemical composition of the substrate, the fermentation system, and the conditions under which the fermentation took place.

The viable cells number increased during the first 24 h of fermentation, after which it decreased slowly (Fig. 1). The decline in the biomass concentration could be due to the reduced substrate availability and the inhibitory effect of ethanol on yeast cells (Rosa et al. 1986; Kamini and Gunasekaran 1989). The maximum concentration of viable cells ($7.5 \pm 0.4 \times 10^8$ cells/g carob pulp) was obtained at 24 h of incubation.

As expected, the concentration of residual sugars decreased during the fermentation coinciding with an increase in biomass and ethanol production (Fig. 1). The concentration of residual sugars fell rapidly during the first 24 h of fermentation, after which it decreased slowly. This was due to a rapid increase of biomass and ethanol concentration, observed at the same time. At the time when the maximum concentration of ethanol was achieved, $40 \pm 1.8\%$ of sugars consumed was converted to ethanol. This finding is in agreement with an early study of carob-pod-extract fermentation by *S. cerevisiae* (Roukas 1993a).

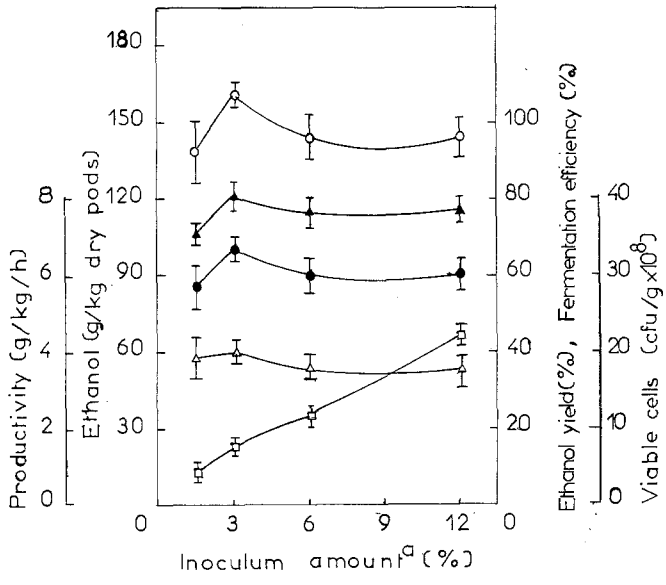

Fig. 2. Kinetic parameters of carob pods fermentation with different inoculum amounts: ○, ethanol; ●, productivity; □, viable cells; △, ethanol yield; ▲, fermentation efficiency ([a]g compressed bakers' yeast/100 g carob pulp). Each point is the mean ± SD of three repetitions

Effect of inoculum amount

The kinetic parameters (except viable cells) increased with the increase of inoculum amount from 1.5 to 3% and decreased above 3% (Fig. 2). The maximal ethanol concentration (160 ± 3 g/kg dry pods), ethanol productivity (6.7 ± 0.2 g/kg per hour), ethanol yield ($40 \pm 1.8\%$) and fermentation efficiency ($80 \pm 2\%$) were obtained with an initial inoculum of 3% (5.0×10^8 cell/g carob pulp). On the other hand, the maximum biomass concentration ($2.2 \pm 0.5 \times 10^9$ cells/g carob pulp) was achieved at an initial inoculum of 12%. These results showed that a lower ethanol concentration was obtained with a higher inoculum amount. This may result from greater use of sugars for growth and maintenance at a high biomass concentration, resulting in a lower ethanol concentration and lower ethanol yield. Kargi et al. (1985), Gibbons and Westby (1986a) and Amin (1992) studied the effect of inoculum size on ethanol production from sweet sorghum, fodder beet and sugar beet in solid-state fermentation and found that the maximum ethanol concentration was obtained at an initial inoculum of 1.0×10^7 cells/g raw sorghum, 1.0×10^8 cells/g fodder beet pulp and 5.0×10^8 cells/g sugar beet, respectively.

Effect of particle size

The effect of particle size on kinetic parameters of carob-pod fermentation is shown in Fig. 3. As shown in Fig. 3, the kinetic parameters (except ethanol yield) decreased significantly with the increase in particle size from 0.5 to 5 mm. The ethanol yield remained almost constant with the same increase in particle size. The

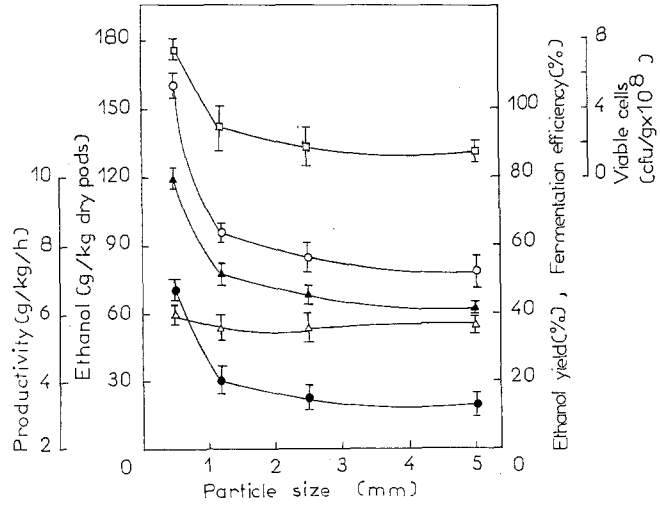

Fig. 3. Kinetic parameters of carob pods fermentation by *S. cerevisiae* at different particle size: for symbols, see Fig. 2. Each point is the mean ± SD of three repetitions

maximal ethanol concentration (160 ± 3 g/kg dry pods), ethanol productivity (6.7 ± 0.2 g/kg per hour), ethanol yield (40 ± 1.8%), biomass concentration (7.5 ± 0.4 × 10^8 cells/g carob pulp) and fermentation efficiency (80 ± 2%) were obtained with the finest particle size (0.5 mm), whereas the above parameters were decreased significantly with the coarsest particle size (5.0 mm). This indicates that in fermentation with small particles sufficient surface area was available for adequate sugar diffusion and hence yeast growth and ethanol production. On the other hand, in the case of larger particles the reduced surface area/volume ratio provided a smaller surface for yeast growth and might have inhibited penetration of yeast cells into the carob kibble particles (Gibbons and Westby 1987). The above results agree with those of Gibbons and Westby (1987) and Amin (1992), who studied the effect of particle size on ethanol production from fodder beet and sugar beet, respectively. The results showed that the maximum ethanol concentration was obtained with the finest particle size. However, the energy consumption for grinding to fine particles is high (Gibbons and Westby 1986b). So, the amount of ethanol produced should be correlated with the grinding cost.

Effect of moisture content

One important factor that affects the performance of solid-state fermentation is the moisture content of solids. The purpose of this experiment was to determine the optimum moisture level of carob kibble that would result in the highest ethanol concentration. As shown in Fig. 4, the ethanol concentration, ethanol productivity, ethanol yield, viable cell number and fermentation efficiency were increased significantly with the increase of moisture content. The highest values of fermentation parameters were achieved at a moisture level of 70%. Kargi et al. (1985) and Ngadi and Correia (1992) reported that a maximum ethanol concentration was obtained from sweet sorghum and apple pomace at a moisture level of 70 and 85%, respectively, when different strains of *S. cerevisiae* were grown in solid-state fermentation. Decreasing the moisture level from 70 to 55% resulted in a decrease in the kinetic parameters. The decrease in moisture level is advantageous since the chance of contamination of fermentation medium is reduced. However, there is a lower limit of moisture content below which yeast cells may not function to produce ethanol. This may be due to the higher osmotic pressure levels at lower moisture contents (Kargi et al. 1985). Ngadi and Correia (1992) reported that low substrate moistures in SSF resulted in suboptimal product formation due to reduced mass transfer processes such as diffusion of solutes and gas to cell during fermentation.

Effect of initial pH

The effect of initial pH on kinetic parameters of carob-pod fermentation is shown in Fig. 5. The fermentation parameters (except ethanol yield) increased drastically with the increase in pH up to 4.5 and decreased beyond this value. On the other hand, the ethanol yield remained constant over the pH range 3.5–4.5 and decreased slightly above pH 4.5. The maximum ethanol

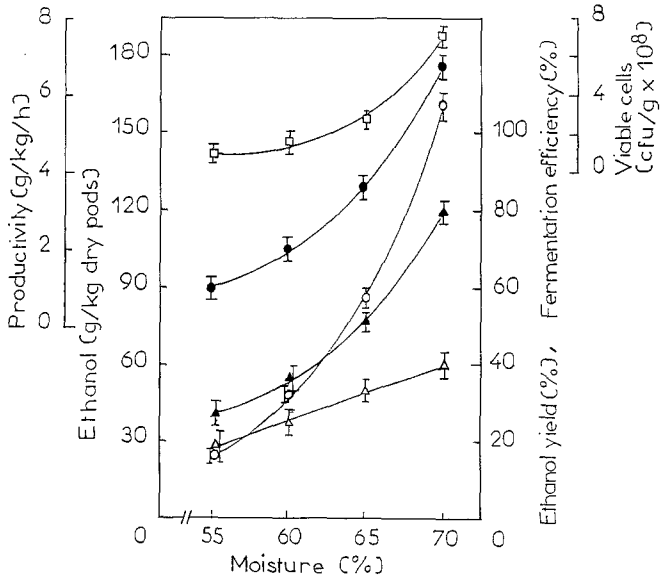

Fig. 4. Kinetic parameters of carob pods fermentation by *S. cerevisiae* at different moisture levels: for symbols, see Fig. 2. Each point is the mean ± SD of three repetitions

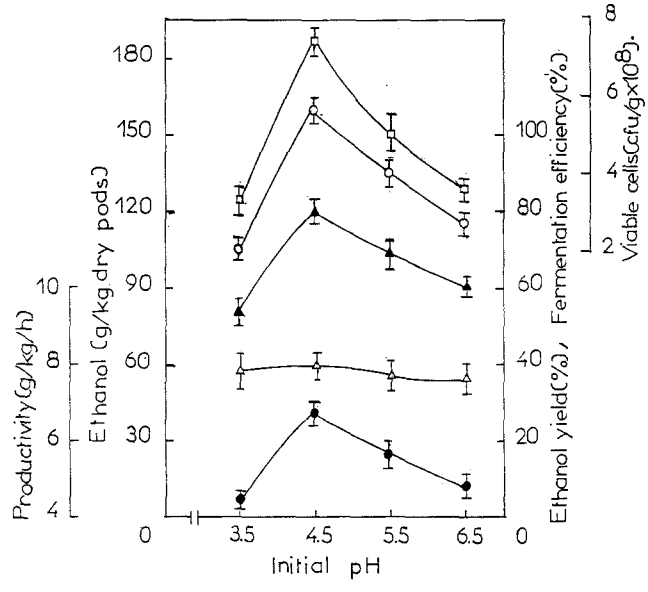

Fig. 5. Kinetic parameters of carob pods fermentation by *S. cerevisiae* at different pH values: for symbols, see Fig. 2. Each point is the mean ± SD of three repetitions

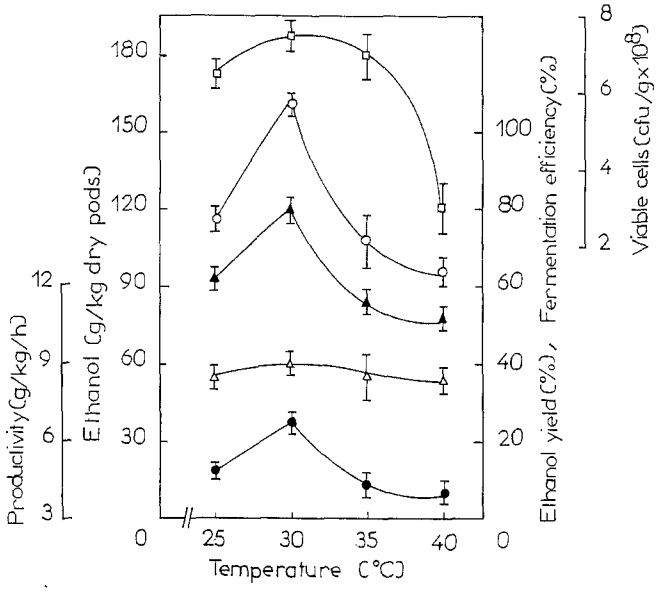

Fig. 6. Kinetic parameters of carob pods fermentation by *S. cerevisiae* at different temperatures: for symbols, see Fig. 2. Each point is the mean ± SD of three repetitions

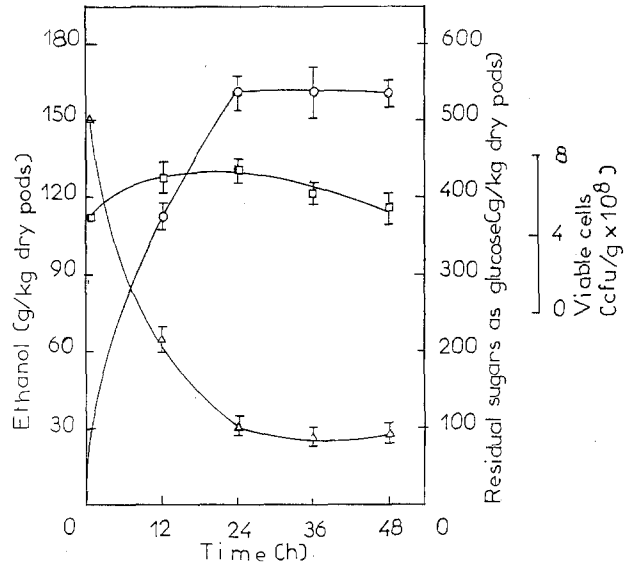

Fig. 7. Fermentation kinetics of *S. cerevisiae* during ethanol production from non-sterilized carob pods pulp in solid-state fermentation: ○, ethanol; □, viable cells; △, residual sugars as glucose. Each point is the mean ± SD of three repetitions

concentration (160 ± 3 g/kg dry pods), ethanol productivity (6.7 ± 0.2 g/kg per hour), ethanol yield (40 ± 1.8%), viable cell number (7.5 ± 0.4 × 10^8 cells/g carob pulp) and fermentation efficiency (80 ± 2%) were obtained in cultures grown at pH 4.5. Gibbons and Westby (1986c) and Gibbons (1989) studied the effect of pH on ethanol production from fodder beets and Jerusalem artichoke tuber pulp by *S. cerevisiae* NRRL Y-2034 and *K. marxianus* and found that the maximal ethanol concentration, ethanol yield and fermentation efficiency were obtained at pH 3.0–3.5. These differences were due to the strain applied and the nature of the substrate.

Effect of temperature

As shown in Fig. 6, increasing the fermentation temperature from 25 to 40°C significantly affected the ethanol concentration, ethanol productivity, viable cell number and fermentation efficiency. The ethanol yield decreased slightly at temperature values lower or higher than 30°C. The ethanol concentration, ethanol productivity and the fermentation efficiency increased significantly with the increase in fermentation temperature from 25 to 30°C and decreased drastically above 30°C. This was due to the decrease in viable cell number of temperature values above 30°C (Fig. 6). Rosa et al. (1987) reported that yeast death in the presence of ethanol at high temperatures is caused by the enhancement by ethanol of the thermosensitivity of membranes associated with the thermal death sites, whereas Bajpai and Margaritis (1987) suggested that high temperatures cause denaturation of the enzyme system of *K. marxianus*. Hang et al. (1982) studied the effect of temperature on ethanol production from apple pomace by *S. cerevisiae* and found that the maximum ethanol concentration was obtained at 30°C.

Ethanol production from non-sterilized carob pods

The ethanol production from non-sterilized carob pods is shown in Fig. 7. The concentration of ethanol increased with the increase in fermentation time. The highest ethanol concentration (160 ± 5 g/kg dry pods) was obtained after 24 h. The viable cell number followed a pattern similar to those of ethanol concentration with maximal concentration (7.3 ± 0.3 × 10^8 cells/g carob pulp) observed at the same time as the maximal ethanol concentration was achieved. No contamination of the substrate by other micro-organisms occurred. This could be due to the large number of the micro-organisms destroyed during the drying of carob kibble overnight at 70°C, the ethanol produced inhibiting the growth of contaminating micro-organisms and the high amount of the inoculum dominating the existing micro-flora. The concentration of residual sugars decreased during fermentation coinciding with an increase in biomass and ethanol production. The lowest concentration of residual sugars (95.7 ± 5 g/kg dry pods) was observed after 48 h. When the maximum concentration of ethanol was achieved, 40 ± 1% of sugars consumed was converted to ethanol, while the total amount of sugars utilized was 79 ± 1%. The above results showed that the culture grown in non-sterilized medium gave the

same ethanol concentration, ethanol productivity, ethanol yield, biomass concentration and fermentation efficiency as those grown in sterilized medium under the same fermentation conditions. Thus, the production of ethanol from non-sterilized carob pods has the advantages of saving in equipment and energy costs. In conclusion, our results showed that carob pods are an attractive medium for the production of ethanol by *S. cerevisiae* in solid-state fermentation.

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