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Continuous Ethanol Production from Nonsterilized Carob Pod Extract by Immobilized *Saccharomyces cerevisiae* on Mineral Kissiris Using a Two-Reactor System

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ABSTRACT

The continuous production of ethanol from nonsterilized carob pod extract by immobilized *Saccharomyces cerevisiae* on mineral kissiris using one- and two-reactor systems has been investigated. A maximum ethanol productivity of 9.6 g/L/h was obtained at an initial sugar concentration of 200 g/L and $D = 0.4\ h^{-1}$ with 68% of theoretical yield and 34% of sugar utilization using the one-reactor system. At $S_0 = 200$ g/L, $D = 0.05\ h^{-1}$, 83% of theoretical yield, and 64% of sugar utilization, an ethanol productivity of 2.6 g/L/h was achieved. In the two-reactor system, a maximum ethanol productivity of 11.4 g/L/h was obtained at $S_0 = 200$ g/L and $D = 0.4\ h^{-1}$ with 68.5% of theoretical yield and 41.5% of sugar utilization. The two-reactor system was operated at a constant dilution rate of $0.3\ h^{-1}$ for 60 d without loss of the original immobilized yeast activity. In this case, the average ethanol productivity, ethanol yield (% of theoretical), and sugar utilization were 10.7 g/L/h, 71.5%, and 48%, respectively.

Index Entries: Carob pod extract; *Saccharomyces cerevisiae;* continuous culture; two-reactor system; kissiris.

INTRODUCTION

The carob pod is the fruit of the carob tree (*Ceratonia siliqua*), which is mainly cultivated in the Mediterranean countries and in some semiarid regions of North America. The annual world production is about 340,000–

400,000 metric tons. Greece is a main producer with an annual harvest of 21,000 t (*1*). Carob pod consists of the kibble and the seeds, which contain a storage polysaccharide (galactomannan, also known as locust bean gum) that is highly valued in the food, textile, and cosmetic industries. The carob kibble contains the following (expressed as g 100 g^{-1} of kibble), moisture 10–15; protein 3–4; pectin 1–2; cellulose 7; hemicellulose 5; phenolic compounds 20; fat 0.5–1.0; and ash 2–3 (*1*). The carob pod is used as animal feed, in the preparation of antiarrheic and antiemetic products, baking pastries, and as a cocoa substitute (*2*). Because of the high concentration of sugars in the carob kibble, it is important to develop new and more attractive uses for these sugars. Recently, the production of ethanol from carob pod by free and immobilized *Saccharomyces cerevisiae* cells has been described (*1,3–6*).

In the last few years, research has been directed toward the production of ethanol by immobilized microbial cells using continuous culture. This immobilization technology, when compared with the other continuous processes, has several advantages, including prevention of organism washout, high biomass density, high yield, easy control, low risk of contamination, and operation with high dilution rate (*5*). Continuous ethanol production by immobilized *S. cerevisiae* and *Zymomonas mobilis* cells in different matrices, such as Ca-alginate, k-carrageenan, glass, vermiculite, porous brickets, PVC-flake, ceramic matrix, celite R-633 microcarriers, and g-alumina pellets, has been reported (*5,7–12*). Kissiris (also known as elaphropetra or Thiraiki gi) is a volcanic rock comprised of volcanic glasses with a petrification similar to that of granite. It is formed by the foam thickening of volcanic lava, and is characterized as a natural glass foam with porosity and relatively high specific surface area. It contains 70% SiO_2, 13% Al_2O_3, and other inorganic oxides, such as Na_2O, K_2O, CaO, P_2O_5, MgO, MnO, FeO, Fe_2O_3, and TiO_2 (*13*). In Greece, it occurs in the Aegean islands, Sandorini, Milos, and Nissiros in a layer 30–50 m thick. It is a cheap mineral material with a price not exceeding $40/t (*14*). Recently, the mineral kissiris was used as immobilization matrix of *S. cerevisiae* cells to produce ethanol from raisin extract and molasses using batch and continuous culture (*8,13,14*).

The aim of this investigation was to examine the potential of carob pod as a source of ethanol production by *S. cerevisiae* cells immobilized on mineral kissiris using continuous culture.

MATERIALS AND METHODS

Microorganism and Substrate

Compressed bakers' yeast *S. cerevisiae* (ZANAE Co., Thessaloniki, Greece) was used throughout this investigation. Carob pods (cultivar Tylliria) were obtained from the local market. The carob pod extract was

prepared as described previously (*1*). The solution (pH 4.7) was diluted with distilled water or concentrated at 50°C under vacuum to contain 14 and 20% initial sugars. Samples of carob pod extract prepared in this way (production medium) were used for the production of ethanol by immobilized *S. cerevisiae* cells on kissiris.

Immobilization of Cells

The Greek mineral kissiris was used for the immobilization of *S. cerevisiae* cells. The pieces of kissiris (0.5–1.0 cm in diameter) were washed with tap water and dried overnight at room temperature. A glass bioreactor and two successively connected reactors of 4.5-cm diameter and 40-cm height were used for the fermentation of carob pod extract to ethanol by immobilized *S. cerevisiae* cells on kissiris. A shallow layer of glass beads was placed at the bottom of each column. The reactors were sterilized at 121°C for 15 min. After cooling, each column was filled with 400 g of kissiris and 400 mL of sterilized culture medium (glucose 10%, yeast extract 0.5%, malt extract 0.5%, and peptone 0.5%, pH 5.0) containing 8 g of compressed bakers' yeast. The top of the reactors was fitted with a plastic lid with one opening to allow removal of fermentation broth and to prevent washout of the kissiris. The substrate was allowed to ferment without feeding. After 6 h, culture medium was continuously pumped into the columns at dilution rate of 0.1 h^{-1} via a tube passing through the lid to the base of each reactor. Another tube from the medium surface was used for channeling of the fermentation broth out of the column. The reactors were operated continuously for 20 h to absorb the cells on the surface of kissiris.

Fermentation Conditions

In the case of the one-reactor system, the carob pod extract was pumped from the bottom of the reactor, and the fermentation broth exited from the top of the column. In the case of the two-reactor system, the production medium was pumped from the bottom of the first reactor, and the liquid effluent passed through the bottom inlet of the second reactor. The final effluent overflowed from the top exit into a collection flask. The bioreactors were incubated at 28°C in a thermostatically controlled chamber. Continuous operation was initiated at a dilution rate of 0.05 h^{-1} with medium of 140 g/L initial sugar concentration, pH 4.7. The dilution rate was increased stepwise from 0.05 to 0.4 h^{-1} until steady-state operation was achieved at each rate. Then, the sugar concentration of the feed input was increased from 140 to 200 g/L, and the reactors were operated at different dilution rates (0.05, 0.1, 0.2, 0.3, and 0.4 h^{-1}) as described above.

Analytical Techniques

When steady-state conditions were reached, ethanol, residual sugars, and free cell concentration were determined as described previously (*1*). Ethanol yield and sugar utilization were expressed as g ethanol/100 g sugar utilized and g sugar utilized/100 g initial sugar, respectively. Ethanol yield (% of theoretical) was calculated by multiplying the ethanol yield by a factor of 2. The dilution rate (D) was calculated by dividing the flow rate of the medium by the liquid volume of the reactor. Ethanol productivity was calculated using the equation: $R = DP$ where D is the dilution rate (h^{-1}) and P is the ethanol concentration (g/L).

RESULTS AND DISCUSSION

Ethanol Production Using the One-Reactor System

The ethanol concentration, ethanol productivity, ethanol yield (% of theoretical), sugar utilization, and free cell concentration as a function of dilution rate using 140 and 200 g/L initial sugars and one reactor are presented in Fig. 1. In cultures grown at an initial sugar concentration (S_0) of 140 and 200 g/L, the ethanol concentration decreased drastically as the dilution rate increased from 0.05 to 0.4 h^{-1}. A maximum ethanol concentration of 53 g/L was obtained in culture grown at an initial sugar concentration of 200 g/L and $D = 0.05$ h^{-1}, whereas the lowest ethanol concentration (12 g/L) was observed in culture grown at initial sugar concentration of 140 g/L and a dilution rate of 0.4 h^{-1}. The ethanol productivity increased significantly with the increase in dilution rate from 0.05 to 0.4 h^{-1}. A maximum ethanol productivity of 9.6 g/L/h was achieved at an initial sugar concentration of 200 g/L, $D = 0.4$ h^{-1}, and 34% sugar utilization. At $S_0 = 200$ g/L, $D = 0.05$ h^{-1}, and 64% sugar utilization, an ethanol productivity of 2.6 g/L/h was obtained. Therefore, the greatest ethanol productivity was achieved at the highest dilution rate tested, but the most complete utilization of sugars occurred at the lowest dilution rate. This finding is in agreement with earlier studies (*5,10,14*). The lower ethanol concentration and, hence, lower ethanol inhibition of yeast are an adequate explanation of the increased volumetric productivity at the higher dilution rates.

The ethanol yield (% of theoretical) and the sugar utilization decreased with the increase in dilution rate from 0.05 to 0.4 h^{-1}. Maximum ethanol yield (84% of theoretical) and sugar utilization (70%) were achieved at $S_0 = 140$ g/L, $D = 0.05$ h^{-1}, whereas at the highest dilution rate tested ($D = 0.4$ h^{-1}), the above parameters decreased significantly. At $S_0 = 200$ g/L and $D = 0.05$ h^{-1}, the ethanol yield (% of theoretical) and the sugar utilization were 83 and 64%, respectively.

As shown in Fig. 1, free cells in the fermentation broth were formed during fermentation. At $D = 0.05$ h^{-1}, in cultures grown at an initial

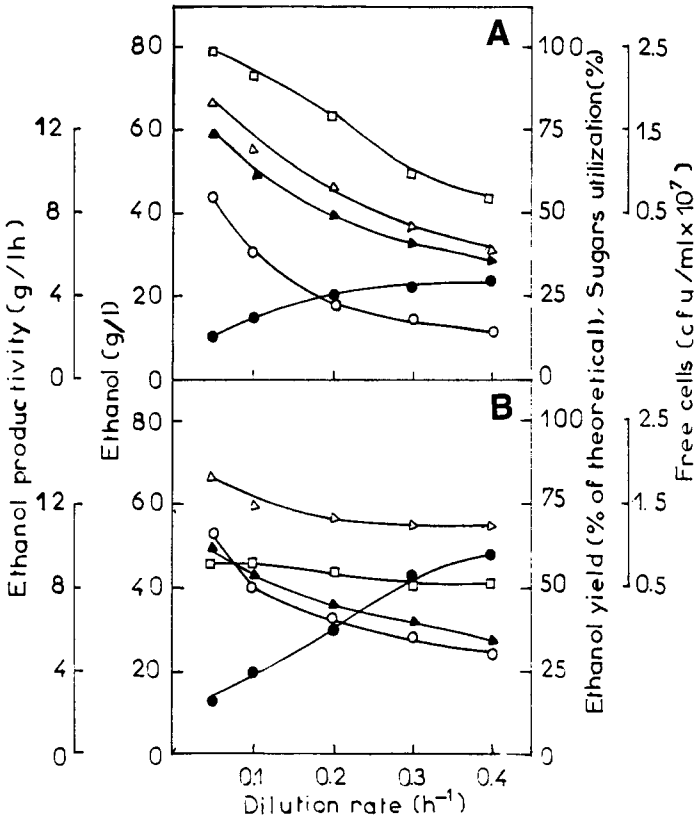

Fig. 1. Continuous ethanol production from nonsterilized carob pod extract by immobilized *S. cerevisiae* on mineral kissiris using one-reactor system (**A, B:** initial sugar concentration 140 and 200 g/L, respectively, pH 4.7, 28°C). (○) ethanol; (●) ethanol productivity; (□) free cells; (△) ethanol yield (% of theoretical); (▲) sugar utilization.

sugar concentration of 140 and 200 g/L, maximum free cell concentration was 2.5×10^7 and 8.0×10^6 CFU/mL, respectively. When the initial sugar concentration was increased from 140 to 200 g/L, a decrease in free cell number was observed. This was owing to the inhibition of growth of yeast at high sugar concentration (4). The above results showed a high concentration of free cells in the fermentation broth during ethanol production from carob pod extract by immobilized *S. cerevisiae* on kissiris in continuous culture. This may be explained by the fact that a number of immobilized cells were transported from the solid surface to the fermentation broth. This is a disadvantage for kissiris, because separation of cells from the fermentation broth before further processing is still required. Also, the free cell concentration in the waste water of the ethanol production industry causes environmental pollution. However, the ethanol productivity, the ethanol yield (% of theoretical), and the sugar utilization were at an acceptable level at low dilution rates. In addition, kissiris is a cheap mineral material compared to the other immobilization matrices. Thus,

the benefits obtained by the ethanol productivity and the low cost of material make kissiris an attractive immobilization matrix of *S. cerevisiae* cells to produce ethanol from carob pod extract.

In a previous study on the production of ethanol from nonsterilized carob pod extract by immobilized *S. cerevisiae* cells in Ca-alginate beads using continuous culture, it was found that an ethanol productivity of 6.5 g/L/h, ethanol yield of 66.5% of theoretical, and 95% sugar utilization were obtained at an initial sugar concentration of 200 g/L and $D = 0.1\ h^{-1}$ (*5*). Koutinas and coworkers (*14*) reported that an ethanol productivity of 3.6 g/L/h, ethanol yield of 80% of theoretical, and sugar utilization of 41.6% were obtained when *S. cerevisiae* immobilized on mineral kissiris in a fixed-bed reactor was grown in raisin extract containing 171.5 g/L total sugars at $D = 0.13\ h^{-1}$. Rosario and Pamatong (*7*), who studied the production of ethanol from banana extract by *S. cerevisiae* entrapped in k-carrageenan in a packed-bed reactor; found that an ethanol productivity of 9.8 g/L/h, ethanol yield of 89% of theoretical, and 88.7% sugar utilization were obtained at $D = 0.18\ h^{-1}$, whereas Dallmann and coworkers (*15*) found an ethanol productivity of 6.3 g/L/h was obtained at $D = 0.11\ h^{-1}$ when immobilized *S. cerevisiae* in Ca-alginate beads was grown in apple juice. Mehaia and Cheryan (*16*), who studied the production of ethanol from date extract containing 140 g/L total sugars by *S. cerevisiae* immobilized in a membrane reactor, found an ethanol productivity of 16.2 g/L/h with 100% sugar utilization at $D = 0.24\ h^{-1}$. Margaritis and Bajpai (*17*) investigated continuous ethanol production from Jerusalem artichoke extract using immobilized *Kluyveromyces marxianus* in Ca-alginate beads in a packed-bed reactor, and found an ethanol productivity of 22.5 g/L/h at $D = 0.5\ h^{-1}$ and 92% sugar utilization. There are several possible reasons for these differences, including the strain of organism used, the inoculum amount, chemical composition of the substrate, reactor design, the immobilization matrix, and generally, the conditions under which the fermentation takes place (pH, temperature, dilution rate, and so forth).

Ethanol Production Using a Two-Reactor System

Figure 2 shows the ethanol concentration, ethanol productivity, ethanol yield (% of theoretical), and the sugar utilization as a function of dilution rate using initial sugar concentration of 140 and 200 g/L and two reactors connected successively. The kinetic parameters (except ethanol productivity) decreased significantly with the increase in dilution rate from 0.05 to $0.4\ h^{-1}$. On the other hand, the ethanol productivity increased rapidly with the same increase in dilution rate. The maximum ethanol concentration (64 g/L) was achieved at $S_0 = 200$ g/L and $D = 0.05\ h^{-1}$, whereas the highest values of ethanol yield (84% of theoretical) and the sugar utilization (86.5%) were obtained at $S_0 = 140$ g/L and $D = 0.05\ h^{-1}$. In our previous work (*1*), it was found that the maximum ethanol concentration (75 g/L) was obtained when *S. cerevisiae* cells were grown in carob pod extract con-

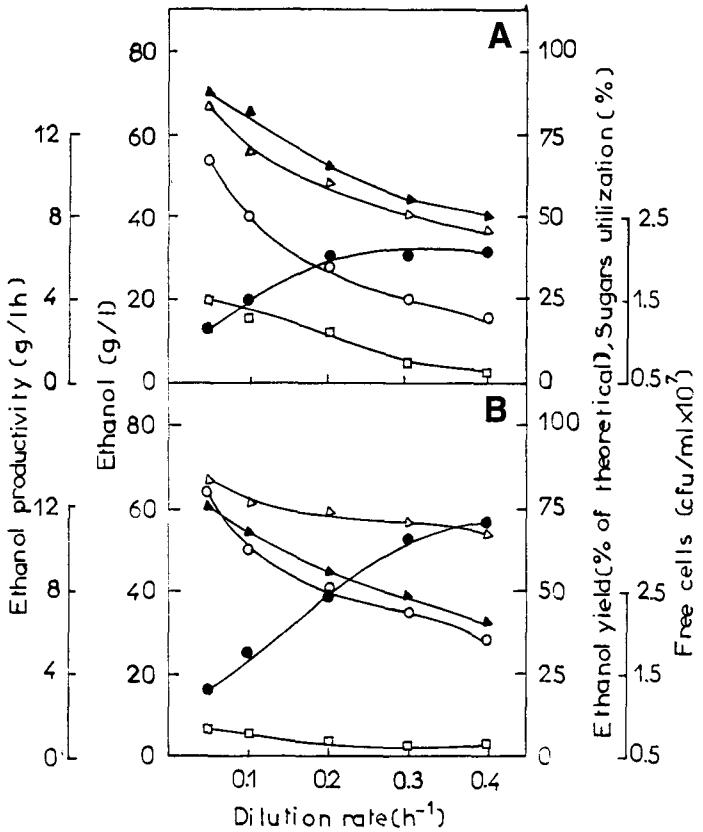

Fig. 2. Continuous ethanol production from nonsterilized carob pod extract by immobilized *S. cerevisiae* on mineral kissiris using two-reactor system (**A, B:** initial sugar concentration 140 and 200 g/L, respectively, pH 4.7, 28°C). Symbols as in Fig. 1.

taining 200 g/L initial sugars in batch culture after 48 h of incubation. As shown in Fig. 2, the culture grown at S_0 = 200 g/L gave higher ethanol productivity than those grown at S_0 = 140 g/L under the same fermentation conditions. A maximum ethanol productivity of 11.4 g/L/h was achieved at S_0 = 200 g/L and D = 0.4 h^{-1} with 68.5% ethanol yield (% of theoretical) and 41.5% sugar utilization. At S_0 = 200 g/L, D = 0.05 h^{-1}, ethanol yield of 83% (% of theoretical), and 74% of sugar utilization, an ethanol productivity of 3.2 g/L/h was obtained. Koutinas and coworkers (*14*), who studied the production of ethanol from sterilized molasses by *S. cerevisiae* immobilized on kissiris using two successively connected reactors, found an ethanol productivity of 3.0 g/L/h at D = 0.06 h^{-1} with 90% ethanol yield (% of theoretical) and 94% sugar utilization. The free cell concentration decreased with the increase in initial sugar concentration from 140 to 200 g/L. The maximum free cell concentration (1.5 × 10^7 CFU/mL) was obtained at S_0 = 140 g/L and D = 0.05 h^{-1}.

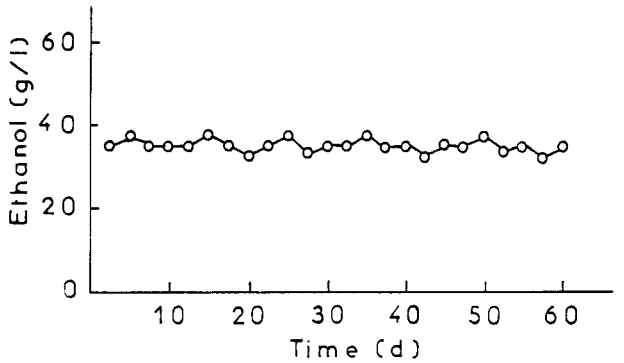

Fig. 3. Long-term continuous ethanol production from nonsterilized carob pod extract by immobilized *S. cerevisiae* on mineral kissiris using two-reactor system (200 g/L initial sugar concentration; D = 0.3 h^{-1}, pH 4.7, 28°C).

Long-Term Continuous Ethanol Production

The two-reactor system was used to study the operational stability of the immobilized bioreactor. The system was run at a constant dilution rate of 0.3 h^{-1} continuously for 60 d. Nonsterilized carob pod extract (pH 4.7) containing 200 g/L total sugars was used as production medium. Temperature was maintained at 28°C. The results of the operational stability study was presented in Fig. 3. A shown in Fig. 3, the reactor operated for a long time with a stable ethanol concentration. Moreover, other factors, such as high productivity, ease of maintenance, and absence of sterilization requirements, established the two-reactor system as a potentially useful technology for industrial production of ethanol. The ethanol concentration remained at 35–38 g/L during the 60 d of operation of the reactor. The ability of immobilized yeast to produce ethanol for a long time has not been explained yet, but may be owing to the protection of the yeast by the immobilization matrix. Rychtera and coworkers (*18*) reported that immobilized yeast can retain enzymes activities for a long time owing to the different composition of cells (proteins, lipids, RNA, DNA, and inorganic substances) compared with free yeast. Average ethanol productivity, ethanol yield (% of theoretical), and sugar utilization were 10.7 g/L/h, 71.5%, and 48%, respectively (data not shown). The high productivity shows that the carob pod extract contained the necessary nutrients to maintain the growth of yeast and the fermentation process without any supplementation. In relevant work from our laboratory, it was found that the *S. cerevisiae* cells entrapped in Ca-alginate beads retained their activity to produce ethanol for 30 d. This means that *S. cerevisiae* cells immobilized on mineral kissiris retained their ability to produce ethanol for a longer time compared to cells entrapped in Ca-alginate gel. This advantage combined with the low price of kissiris certified that kissiris is a good immobilization matrix of *S. cerevisiae* cells to produce ethanol from carob pod extract using continuous culture.

CONCLUSIONS

The results showed some important aspects of ethanol production from nonsterilized carob pod extract by immobilized *S. cerevisiae* on mineral kissiris using continuous culture. The ethanol concentration, ethanol productivity, ethanol yield (% of theoretical), and sugar utilization remained constant for a long time during the operational stability of the two-reactor system. The production of ethanol from nonsterilized carob pod extract has the advantages of saving equipment and energy cost. The mineral kissiris can be used in the ethanol-producing industry without significant modifications as an immobilization matrix of *S. cerevisiae* cells using continuous culture. A decrease in the ethanol production cost can be achieved concerning the reduction of the inoculum amount required, the high ethanol productivity, and the low price of kissiris. On the other hand, the production of ethanol from carob pod extract by immobilized *S. cerevisiae* on kissiris showed some disadvantages, such as the high free cell concentration in product stream and the low sugar utilization at high dilution rates. Generally, the carob pod extract was an attractive medium for the production of ethanol by immobilized *S. cerevisiae* on kissiris using continuous culture.

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