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Abstract: The continuous production of ethanol from beet molasses by Ca-alginate immobilized *Saccharomyces cerevisiae* in a packed-bed bioreactor was investigated. The temperature was maintained at 30°C and the dilution rate was 0.22 h^{-1}. Maximum ethanol (4.62%), theoretical yield (82.9%) and volumetric productivity (10.16 $gl^{-1}h^{-1}$) were obtained from the beet molasses medium containing 10.90% total sugar with 2.0-2.4 mm diameter beads prepared from 2% (w/v) sodium alginate solution. At higher substrate concentrations, substrate was recirculated through the packed-bed bioreactor to increase yields and to decrease residual sugar content. The bioreactor system was operated at a constant dilution rate of 0.22 h^{-1} for 25 days without loss of original fermentation capacity. Compared to packed-bed bioreactor, lower ethanol concentration (3.94%), theoretical yield (70.7%) and productivity (8.67 $gl^{-1}h^{-1}$) were obtained in the continuous stirred bioreactor.

Key Words: ethanol, beet molasses, calcium alginate, immobilization, packed-bed bioreactor

Dolgulu Yatak Biyoreaktörde Ca-Aljinatta Tutuklanmış Maya Hücreleri ile Pancar Melasından Etil Alkol Üretimi

Özet: Bu çalışmada dolgulu yatak biyoreaktör kullanılarak Ca-aljinatta tutuklanmış *Saccharomyces cerevisiae* hücreleri ile pancar melasından sürekli yöntemde etil alkol üretimi araştırılmıştır. Çalışma sıcaklığının 30°C, kullanılan seyreltme hızının 0.22 h^{-1} olduğu çalışmada en yüksek etil alkol (4.62%), teorik verim (82.9%) ve hacimsel verimlilik (10.16 $gl^{-1}h^{-1}$) değerleri %10.90 başlangıç şeker konsantrasyonu içeren ortamda, %2 Na-aljinattan üretilen, 2.0-2.4 mm çapındaki boncuklarla elde edilmiştir. Daha yüksek substrat konsantrasyonlarında verim değerlerini artırmak ve ortamda kullanılmadan kalan şeker miktarını azaltmak için substrat biyoreaktörden tekrar sirküle edilmiştir. Biyoreaktör 0.22 h^{-1} seyreltme hızında fermentasyon kapasitesinde önemli değişiklik olmadan sürekli sistemde 25 gün boyunca çalıştırılmıştır. Sürekli karıştırmalı tank tipi biyoreaktörde yapılan denemede ise dolgulu yatak biyoreaktöre göre daha düşük etil alkol konsantrasyonu (3.94%), teorik verim (70.7%) ve verimlilik (8.67 $gl^{-1}h^{-1}$) değerleri elde edilmiştir.

Anahtar Sözcükler: etil alkol, pancar melası, kalsiyum aljinat, immobilizasyon, dolgulu yatak biyoreaktör

Introduction

Ethanol is a natural component of alcoholic beverages and its use has seen continued growth since the late 1970s, when it was used as a product extender due to gasoline shortages caused by the OPEC oil embargoes. As a result, production of ethanol from renewable carbohydrate materials has been attracting worldwide interest and research has been directed to the production of ethanol by immobilized microorganisms using continuous culture.

Immobilized cells exhibit many advantages over free cells, such as relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control and reduced susceptibility of cells to contamination (1). Among the different cell immobilization techniques, entrapment in calcium alginate gel has been one of the most used matrices for whole cell entrapment due to its simplicity and non-toxic character. This simple and mild immobilization technique involves the drop-wise addition of cells suspended in sodium alginate onto a solution of calcium chloride whereon the cells are immobilized in precipitated calcium alginate gel in the form of beads (2). Entrapment in calcium alginate gel beads has been applied for the immobilization of a large number of different cells such as bacteria, cyanobacteria, algae, fungi, yeast, plant protoplasts, and plant and animal cells (3).

Several studies have described continuous ethanol production using Ca-alginate immobilized yeasts with different bioreactor configurations (4-9). In these studies, the most commonly used bioreactors are the continuous flow stirred tank bioreactor, fluidized-bed bioreactor and packed-bed bioreactor. Packed-bed bioreactors have become very popular in recent years due to their low manufacturing and operating costs and also due to the ease of process automation in these reactors (10).

The aim of the present study was to investigate continuous ethanol production from sugar beet molasses in a packed-bed bioreactor. Molasses, which is an abundant by-product of the sugar industry, is at present one of the least expensive sources of sugar and, in contrast to grain, it does not require hydrolysis of starch. The process was carried out in a vertical packed-bed bioreactor with beads of Ca-alginate in which *Saccharomyces cerevisiae* cells were immobilized. The effects of pH, bead diameter, alginate concentration, substrate concentration and recirculation of the substrate on the process variables were determined and the results were compared with those for the same reaction in a stirred bioreactor.

Materials and Methods

Microoganism and substrate

Compressed bakers' yeast, *Saccharomyces cerevisiae* (Pakmaya Yeast Co., İzmir), was used throughout this investigation. The organisms were maintained on Potato Dextrose Agar (Oxoid) and transferred to fresh medium every month.

Beet molasses used throughout the study was supplied by Pakmaya Yeast Co., İzmir. The molasses was diluted to 35° Brix, acidified to pH 4.0 with 4 N H_2SO_4, boiled for 5 min, centrifuged and filtered for pretreatment and clarification. In the clarification step, a part of the colored material and unknown toxic substances frequently included in the molasses were separated or inactivated and the molasses was pasteurized.

Production medium

The production medium suggested by Bravo and Gonzales (4) was used throughout the continuous fermentation experiments. The composition of the medium was (in grams per liter): total sugar from pretreated and diluted molasses, 109.0-227.0; $(NH_4)_2SO_4$, 5.19; KH_2PO_4, 1.53; $MgSO_4$, 0.55. Unless otherwise stated, pH was adjusted to 3.9 with 1 N HCl.

Cell immobilization

Prior to the immobilization step, *S. cerevisiae* cells were grown at 30°C for 36 hours in a temperature controlled shaker (B Braun Certomat). The composition of the growth medium was (grams per liter): glucose, 15; $(NH_4)_2SO_4$, 18; $(NH_4)_2HPO_4$, 10; KH_2PO_4, 5; $MgSO_4$, 5; yeast extract, 1. Fifty milliliters of this growth medium was mixed with an equal volume (1:1,v/v) of 4% (w/v) Na-alginate (Sigma, A-2033) solution. A 100 ml aliquot of alginate-cell suspension containing 2% Na-alginate (unless otherwise stated) was added dropwise to 1000 ml of 2% $CaCl_2$ with a peristaltic pump. Alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapping bacterial cells. The beads were allowed to harden for 30 min and then were washed with sterile saline solution (0.85% NaCl) to remove excess calcium ions and cells. Immediately after entrapment, the number of living bacterial cells was 1.42 x 10^7 cfu g^{-1} bead. To increase the entrapped cell population, the beads were incubated overnight in the production medium at 30°C with continuous shaking and the number of entrapped bacterial cells increased to 9.31 x 10^7 cfu g^{-1} bead. The beads were stored at 4°C in 0.2% yeast extract solution until use.

Equipment and experimental procedures

Continuous ethanol fermentation experiments were carried out using a jacketed Pyrex column reactor packed with 2.0-2.4 mm diameter (unless otherwise stated) Ca-alginate beads with entrapped yeast. The characteristics of the packed-bed bioreactor are given in Table 1. Prior to use, the bioreactor was sterilized with ethyl alcohol and then filled with Ca-alginate beads. The sterile molasses solution was fed to the bottom of the fermentor continuously by means of a peristaltic pump (Chemap AG) through sterile silicon tubing. Effluent liquid overflowed from an outlet port at the top of the bioreactor, maintaining a constant level inside the column. The beads were trapped inside the bioreactor with a metal mesh filter covered by a plastic barrier. The temperature of the bioreactor system was maintained at 30°C by circulating water at a constant temperature from a circulator bath through the jacket of the bioreactor. The dilution rate was 0.22 h^{-1} throughout the packed-bed bioreactor experiments

and the system was considered to be at steady state after at least five residence times. At higher substrate concentrations, medium was recirculated through the bioreactor for complete utilization of substrate. After steady state was achieved, samples of 10 ml were taken from the effluent stream for analysis of substrate and product concentrations.

Table 1. Packed-Bed Bioreactor Characteristics.

Packing	:	Ca-alginate beads
Inner diameter	:	1.57 cm
Column height	:	49 cm
Total reactor volume	:	95 ml
Bed volume	:	85 ml
Void volume	:	22 ml
Packing weight	:	67 g

Continuous stirred tank experiments were performed using a 500 ml aspirator bottle. Temperature was controlled at 30°C±1 on a magnetic hot plate and the working volume was 250 ml. The sterile medium was fed at a dilution rate of 0.22h^{-1} with a peristaltic pump and the effluent was received by another pump at the same speed. The sugar concentration of the medium was 10.90%. Fermentation was started by inoculating 25 g of Ca-alginate beads. Before switching to continuous fermentation, the process was carried out in batch mode during the first 18 hours. The system was considered to be at steady state after at least five residence times.

Analytical methods

Liquefaction of Ca-alginate beads was performed by dissolving 1 g of beads in 20 ml, 1% (w/v) sodium citrate solution (pH=6.0) with continuous stirring for 30 min at room temperature. For determining the concentration of viable cells entrapped in Ca-alginate beads, yeast counts were done by plating appropriate dilutions (0.1% peptone) of liquefied beads on Potato Dextrose Agar (Oxoid) and incubating them at 30°C for 48 h. Total sugar was determined according to the phenol sulfuric acid method using sucrose as the standard (11). Ethanol was assayed in a Pye-Unicam 204 GLC gas chromatograph with a flame ionization detector. The column was 4x6 mm SS 180 cm; Propak Q column. The oven, detector and injection temperatures were 160°C, 250°C and 200°C respectively. All experiments were done in triplicate samples and mean values were calculated.

The dilution rate (D,h^{-1}) was calculated by dividing the flow rate of the medium by the bed volume of the bioreactor. Ethanol productivity was calculated using the equation R=DxP where D is the dilution rate (h^{-1}) and P is the ethanol concentration (gl^{-1}). Theoretical yield was calculated as the actual ethanol produced x 100 divided by the theoretical maximum.

Results and Discussion

Effect of pH

Pretreated beet molasses containing 10.90% total sugars at different pH values (3.5, 3.9, 4.2, 4.5) were prepared and fed into the packed-bed bioreactor continuously. The dilution rate was 0.22 h^{-1} and the temperature was controlled at 30°C. Figure 1 shows the ethanol concentration and theoretical yield as a function of initial pH. As seen in Figure 1 maximum ethanol concentration (4.62%) and theoretical yield (82.9%) were obtained at pH 3.9. At pH values of 4.2 and 4.5, similar ethanol concentrations (4.46% and 4.28%, respectively) and theoretical yield values (80.1% and 76.8%, respectively) were observed.

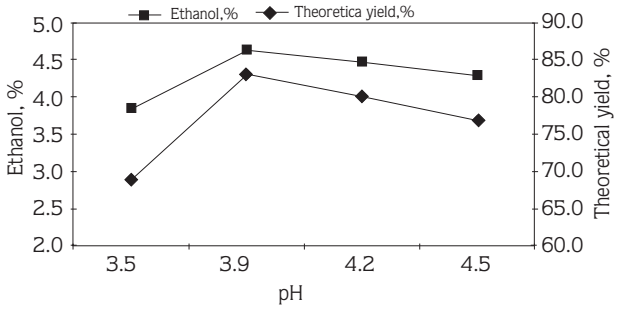

Figure 1. The effect of pH of medium on ethanol production from beet molasses by immobilized yeast cells in a packed-bed bioreactor (T=30°C, initial substrate concentration= 10.90%)

Roukas (9) found that the optimum pH range for ethanol production from carob pod extract by Ca-alginate immobilized *S. cerevisiae* was 3.5-5.5 and reported that this was due to the good yeast growth over the pH range of 3.5-6.5. In contrast to the findings of Roukas (9), we found a sharp decrease in ethanol fermentation when the initial pH of the medium was decreased to 3.5. Also at pH 3.5 white, gray deposits were seen on the surface of the beads, which could have been alginic acid formed by the disruption of Ca-alginate in acidic medium.

Effect of bead diameter

In order to determine the effect of bead diameter on ethanol production, beads with diameters of 1.3-1.7 mm, 2.0-2.4 mm and 2.8-3.2 mm were prepared by using different nozzles and used for continuous ethanol production in the packed-bed bioreactor at a dilution rate of 0.22 h^{-1}. Similar ethanol production was obtained with 1.3-1.7 mm and 2.0-2.4 mm beads. Further increases in bead diameter decreased ethanol production. The highest ethanol production (4.62%) was obtained with cells entrapped in 2.0-2.4 mm Ca-alginate beads; 4.27% and 3.81% ethanol was produced with 1.3-1.7 mm and 2.8-3.2 mm diameter beads,

respectively. Smaller beads yielded more ethanol, probably due to an increase in surface-volume ratio. A similar result was reported in a previous study (1) on lactic acid production from beet molasses by Ca-alginate immobilized lactic acid bacteria. Gilson and Thomas (5) also found that D-glucose consumption and ethanol production fell with increasing bead diameter in ethanol production by Ca-alginate immobilized yeast cells in a fluidized-bed bioreactor. They attributed this to the fact that a given volume of larger beads has less surface area available for mass transfer of substrate into and through the beads.

Effect of Na-alginate concentration

Yeast cells were immobilized in Ca-alginate gel beads prepared from different concentrations of Na-alginate (1.0, 1.5, 2.0, 2.5, 3.0, 3.5% w/v) and continuous ethanol fermentation (D=0.22 h^{-1}) was carried out in molasses medium containing 10.90% total sugar at pH 3.9. As seen in Figure 2, the highest ethanol production (4.62%) and theoretical yield (82.9%) were obtained with beads prepared from 2% Na-alginate. Although similar ethanol production and yield values were obtained in beads obtained from 1.0% and 1.5% Na-alginate, these beads were highly susceptible to compaction and disintegration during the operation of packed-bed bioreactor. Especially after 24-30 hours of bioreactor operation, the evolution of CO_2 caused an internal mechanical loading on the beads which led to disintegration of most of these Ca-alginate beads. Above 2% Na-alginate concentration, ethanol production decreased probably due to the lower diffusion efficiency of the beads. Gilson and Thomas (5) used 0.56% $CaCl_2$ with the production medium to minimize bead degradation and found that 1% alginate beads of 4 mm diameter suffered some breakage, whereas 2-5% alginate beads suffered no damage. They proposed to increase alginate concentration if breakage occurs in any particular application.

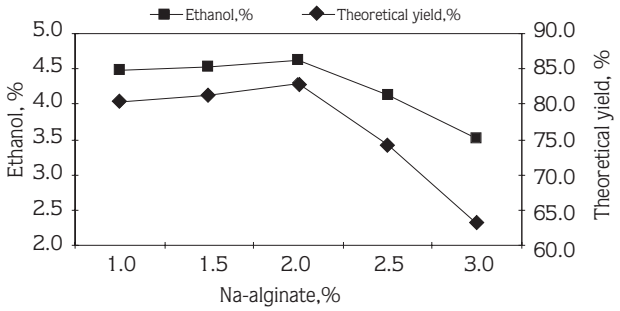

Figure 2. The effect of Na-alginate concentration on ethanol production from beet molasses by immobilized yeast cells in a packed-bed bioreactor (T=30°C, pH=3.9, initial substrate concentration=10.90%)

Effect of initial sugar concentration

In order to determine the effect of sucrose concentration on continuous ethanol production by Ca-alginate immobilized yeast cells, diluted molasses containing 10.90, 14.59, 18.84 and 22.70% of total sugar were used. The pH of the medium and dilution rate was 3.9 and 0.22 h^{-1}, respectively. After steady state was achieved for each sugar concentration, samples were taken. Before increasing the sugar concentration of the feed, 2% $CaCl_2$ solution was passed through the column to prevent deformation and maintain the integrity of the beads. As seen in Figure 3, ethanol concentration, productivity and theoretical yield values decreased as sugar concentration of the medium was increased. The reason for this decrease in the overall fermentation performance was possibly product and substrate inhibition.

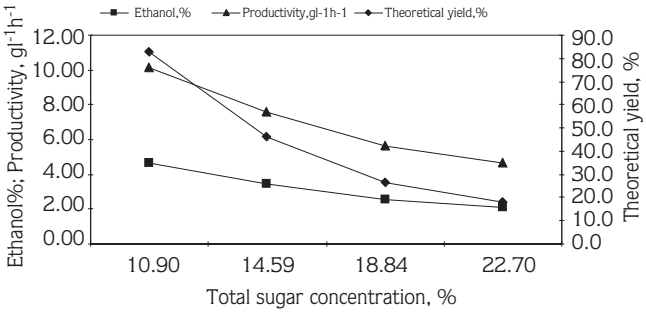

Figure 3. The effect of initial sugar concentration on ethanol production from beet molasses by immobilized yeast cells in a packed-bed bioreactor.

Roukas (9) studied continuous ethanol production from carob pod extract in a packed-bed bioreactor and found that ethanol concentration and ethanol productivity increased significantly with the increase in initial sugar concentration up to 20%, but theoretical ethanol yield decreased with the increase of initial sugar concentration from 10% to 25%. They stated that above a critical substrate concentration, decreased water activity and the proceeding of plasmolysis caused a decrease in the rates of fermentation.

Maximum ethanol productivity (10.16 $gl^{-1}h^{-1}$) was achieved at 10.90% initial sugar concentration. At 14.59%, 18.84% and 22.70% of initial sugar concentrations, 7.57, 5.63 and 4.62 $gl^{-1}h^{-1}$ ethanol productivity values were obtained, respectively. To increase productivity, higher dilution rates were employed in the packed-bed bioreactor for continuous ethanol production, but at dilution rates higher than 0.22 h^{-1} , the column was inoperable due to pressure buildup and bead compression. When flow rate was increased in order to get a higher dilution rate, the peristaltic feed pump was found to be incapable of withstanding the level of backpressure caused by the CO_2 gas and the reactor was therefore shut down. Using

columns with a certain taper angle or using specially designed columns might be a solution to this problem. Such behavior of packed-bed bioreactors was also reported by other researchers (7,12,13). Colak and Hamamci (12) stated that Ca-alginate gels were somewhat compressible and the production of CO_2 during ethanol production caused the beads to be compressed and the result was a high pressure drop and phase separation together with a decrease in ethanol productivity. They used tapered columns successfully to decrease accumulation of CO_2 in the column and to prevent excessive pressure buildup.

Recirculation of substrate

In order to increase ethanol concentration and decrease residual sugar in the effluent stream, substrate was recirculated through the packed-bed column. For each substrate concentration, ethanol, residual sugar, ethanol yield (percentage of ethanol produced per initial quantity of sugar present in the medium) and theoretical yield values obtained after each circulation of the effluent are given in Table 2. The first circulation was the normal continuous operation of the packed-bed bioreactor. The effluent was then collected and passed through the column again and this was repeated until the increase in ethanol concentration and decrease in residual sugar in the effluent came to a constant value.

As seen in Table 2, molasses medium containing 10.90, 14.59 and 18.84% of initial sugar were recirculated 2, 3 and 4 times respectively through the packed-bed bioreactor and 82-

Table 2. Ethanol (%), residual sugar (%), ethanol yield (%) and theoretical yield (%) values obtained with the recirculation of substrate containing different initial sugar concentrations through the packed-bed bioreactor.

Substrate conc., %	Recirculation number	Ethanol, %	Residual sugar, %	Ethanol yield, %	Theoretical yield, %
10.90	1	4.62	0.87	42.4	82.9
10.90	2	4.76	0.56	43.7	85.4
14.59	1	3.44	7.27	23.6	46.1
14.59	2	5.99	1.04	41.1	80.3
14.59	3	6.20	0.60	42.5	83.1
18.84	1	2.56	12.80	13.6	26.6
18.84	2	5.93	6.16	31.5	61.6
18.84	3	8.21	1.63	43.6	85.3
18.84	4	8.39	1.23	44.5	87.1
22.70	1	2.10	18.23	9.3	18.1
22.70	2	3.11	16.17	13.7	26.8
22.70	3	4.18	14.03	18.4	36.0
22.70	4	5.34	11.43	23.5	46.0
22.70	5	6.21	9.61	27.4	53.5
22.70	6	6.42	9.11	28.3	55.3

87% theoretical yield and 42-44% ethanol yield values were obtained and 0.56, 0.60 and 1.23% of unutilized residual sugar was present in the medium. At 22.70% initial sugar concentration, 9.11% of sugar was not utilized and both ethanol yield (28.3%) and theoretical yield (55.3%) values were low, even after 6 recirculations of the medium. These unsuccessful results obtained at initial sugar concentrations higher than 19-20% were attributed to both substrate inhibition and accumulation of toxic materials which might be originally present in molasses medium. Therefore, it was concluded that, up to a certain substrate concentration, with the recirculation of the effluent stream through the packed-bed bioreactor, ethanol concentration and yield values could be increased and unutilized residual sugar concentration could be lowered.

Long-term continuous ethanol production

In order to study the operational stability of the immobilized packed-bed bioreactor, the system was run continuously for 25 days at a constant dilution rate of 0.22 h^{-1}. Beet molasses medium (pH=3.9) containing 10.90% initial sugar was used as the production medium and the temperature was maintained at 30°C. $CaCl_2$ solution (2%) was passed through the column every 7 days to prevent disruption and maintain the mechanical structure of Ca-alginate beads, and air was given to the column every 2-3 days to remove accumulated CO_2. The ethanol concentration was 4.2-4.6% during the 25 days' operation of the packed-bed bioreactor. At the end of 25 days, 4.43% ethanol concentration and 79.5% theoretical yield were obtained. During the continuous fermentation, the structure of the Ca-alginate beads was not destroyed. Ca-alginate beads with immobilized yeast cells were also used continuously for 30 days by Roukas (9), and for 55 days by Bravo and Gonzales (4), for ethanol production.

Comparison of the packed-bed bioreactor with a continuous stirred bioreactor

For comparison of the packed-bed bioreactor with a stirred tank bioreactor, an aspirator-type bioreactor was used. The substrate concentration was 10.90% and the temperature was maintained at 30°C. The fermentation was initially carried out in batch operation for 18 hours after which continuous operation was started at a dilution rate of 0.22 h^{-1}. In the stirred tank reactor, the ethanol concentration, theoretical yield and productivity values obtained were 3.94%, 70.7% and 8.67 $gl^{-1}h^{-1}$ respectively. These values were lower than the previously stated values obtained in the packed-bed bioreactor for 10.90% initial sugar concentration (ethanol conc., 4.62%; theoretical yield, 82.9%; productivity, 10.16 $gl^{-1}h^{-1}$). Sitton and Gaddy (14) immobilized *S. cerevisiae* in the Raschig rings and compared fermentation efficiencies of packed-bed and continuous stirred reactors. They obtained 4.2 times better productivity (7.4 $gl^{-1}h^{-1}$) with the packed-bed bioreactor and observed reduced inhibition from both product and toxic materials in the substrate with this type of bioreactor.

Conclusion

When beet molasses was used as substrate, the highest ethanol production and theoretical yield for ethanol production in a packed-bed bioreactor was obtained with 10.90% initial sugar concentration (pH=3.9) and with 2.0-2.4 mm diameter Ca-alginate beads prepared from 2% Na-alginate. Dilution rates higher than 0.22 h^{-1} could not be used due to pressure buildup and concomitant plugging of the bioreactor. Recirculation of substrate seems to be an efficient process for almost complete utilization of sugar at higher substrate concentrations. The packed-bed bioreactor worked efficiently and was stable for a period of 25 days without bead disintegration. Compared to the continuous stirred bioreactor, higher ethanol production, theoretical yield and productivity were obtained with the packed-bed bioreactor. In the light of the present study, packed-bed bioreactors seem to be efficient and stable bioreactors for ethanol fermentation with lower power input and operation costs. The major drawback of this type of reactor is the existence of pressure drop or resistance to flow across the reactor bed and deviation of the bioreactor from plug-flow behavior. This problem can be solved by using tapered columns or special column configurations.

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