Immobilized Yeast for Production of Ethanol from Molasses

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Abstract

The production of ethanol from cane molasses by Ca-alginate immobilized *Saccharomyces cerevisiae* in shaking incubator was studied. The temperature was 30C and the shaking rate was 150 r/m. Maximum ethanol (4.62%), theoretical yield (82.9%) and volumetric productivity (10.16 gl/1) were obtained from the cane molasses medium containing 10.90% total sugar with 2.0-2.4 mm diameter beads prepared from 2% (w/v) sodium alginate solution added dropwise to 1000 ml of 2% CaCl2 . Lower ethanol concentration (3.94%), theoretical yield (70.7%) and productivity (8.67 g/1) were obtained.

استعمال الخميرة المسكنة لانتاج الايثانول من المولاس

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الخلاصة

تمت دراسة انتاج الايثانول من مولاس القصب بواسطة الخميرة *Saccharomyces cerevisiae* المسكنة بالجينات الكالسيوم في حاضنة هزازة . كانت درجة الحرارة 30 م ^{0} وسرعة الهز 150 دورة / دقيقة . كانت اعلى نسبة للايثانول هي (4.62 %) والحاصل النظري (82.9 %) والانتاجية (10.16 غم / لتر) في وسط يحتوي على (10.90 % سكريات كلية) وقطر حبيبات الجينات الكالسيوم 2.42.0 ملم حضرت من 2% (وزن / حجم) من محلول الجينات الصوديوم قطرت على $CaCl_2$% (وزن / حجم) . اقل تركيز للايثانول كان (3.94 %) والحاصل النظري (70.7 %) والانتاجية 8.67 غم / لتر) .

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 (4-9).

The aim of the present study was to investigate continuous ethanol production from sugar molasses in a packed-bed incubation. 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 with beads of Ca-alginate in which Saccharomyces cerevisiae cells were immobilized. The effects of pH, bead diameter, and alginate concentration were determined.

Materials and Methods

Microoganism and substrate Compressed bakers yeast, *Saccharomyces cerevisiae* (Pakmaya Yeast Co., Üzmir), was used throughout this investigation. The organisms were maintained on Potato Dextrose Agar (Oxoid) and transferred to fresh medium every month. Cane molasses used throughout the study was obtained by Sugar Factory, Misan-Iraq. The molsses 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. 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; (NH4)2SO4, 5.19; KH2PO4, 1.53; MgSO4, 0.55. Unless otherwise stated, pH was adjusted to 3.9 with 1 N HCl.

Prior to the immobilization step, *S. cerevisiae* cells were grown at 30C for 36 hours in a temperature controlled shaker (B Braun Certomat). The composition of the growth medium was (grams per liter): glucose, 15; (NH4)2SO4, 18; (NH4)2HPO4, 10; KH2PO4, 5; MgSO4, 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% $CaCl_2$. 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 107 cfu /g bead. To increase the entrapped cell population, the beads were incubated overnight in the production medium at 30C with continuous shaking and the number of entrapped bacterial cells increased to 9.31 x 107 cfu /g bead. The beads were stored at 4C in 0.2% yeast extract solution until use.

Equipment and experimental procedures Continuous ethanol fermentation experiments were carried out using 500 ml-flasks with Ca-alginate beads. The temperature of the system was maintained at 30C . The sugar concentration of the medium was 10.90%. Fermentation was started by inoculating 25 g of Ca-alginate beads. Before fermentation, the process was carried out in batch mode during the first 18 hours.

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 30C for 48 h. Total sugar was determined by refractometer index method using sucrose as the standard (5).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 160C, 250C and 200C respectively. All experiments were done in triplicate samples and mean values were calculated.

Results and Discussion

Pretreated cane 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.

Roukas (6) 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 (6), 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. 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
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

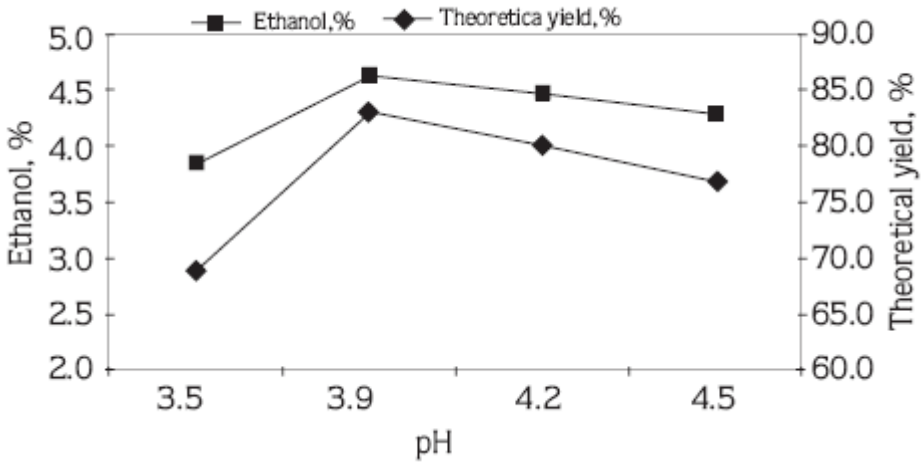

Figure 1. The effect of pH on ethanol production from molasses by immobilized yeast (T=30C, sugar conc.= 10.90%) .

lactic acid production from cane molasses by Ca-alginate immobilized lactic acid bacteria. Gilson and Thomas (7) 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.
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 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 fermentation. 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 (7) 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.

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 was 3.9. After steady state was achieved for each sugar concentration, samples were taken. Before increasing the sugar concentration of the substrate , 2% $CaCl_2$ solution was passed through the column to prevent deformation and maintain the integrity of the beads.

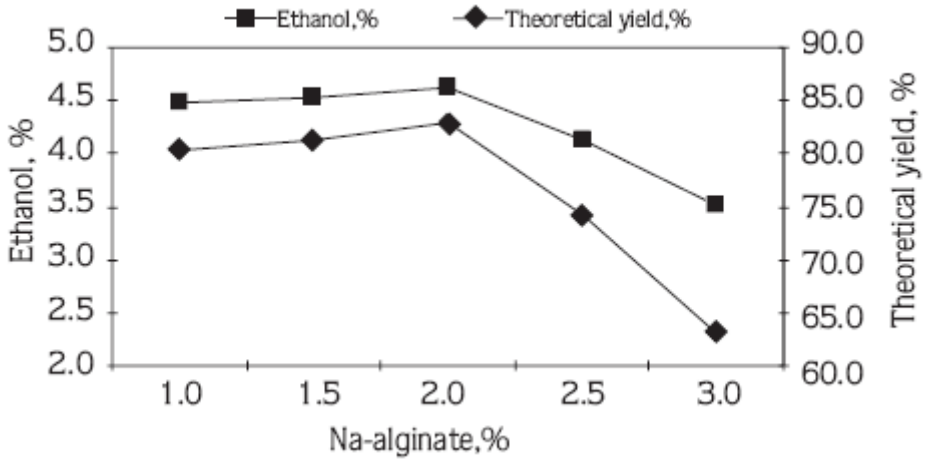

Figure 2. The effect of Na-alginate concentration on ethanol production from cane molasses by immobilized yeast cells (T=30C, pH=3.9, (T=30C, sugar conc.= 10.90%).

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. Roukas (6) 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) 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 ethanol productivity values were obtained, respectively. To increase productivity, higher dilution rates were employed for continuous ethanol production.

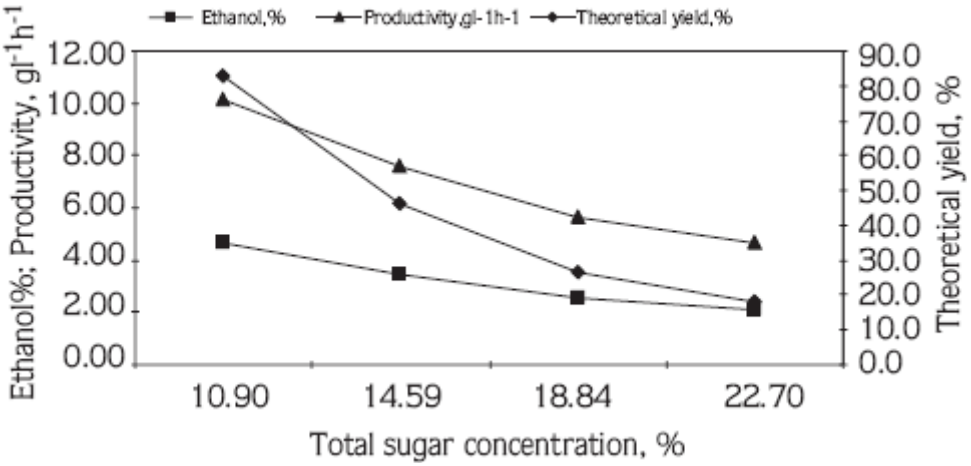

Figure 3. The effect of initial sugar concentration on ethanol production from cane molasses by immobilized yeast cells

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 are given in Table 1.

As seen in Table 1, molasses medium containing 10.90, 14.59 and 18.84% of initial sugar were tested. 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. 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, ethanol

concentration and yield values could be increased and unutilized residual sugar concentration could be lowered.

Colak and Hamamci (8) stated that Ca-alginate gels were somewhat compressible and the production of CO2 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.

Substrate conc., % Residual sugar, Ethanol yield, Theoretical yield, concentration, 9.11% of sugar was not utilized and both ethanol yield (28.3%) and theoretical yield (55.3%) values were low. 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, 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 beds, the system was run continuously for 25 days. Cane molasses medium (pH=3.9) containing 10.90% initial

Table 1. Ethanol (%), residual sugar (%), ethanol yield (%) and theoretical yield (%) obtained .

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

sugar was used as the production medium and the temperature was maintained at 30C. The ethanol concentration was 4.2-4.6% during the 25 days operation of the packed-bed system. 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 (6), and for 55 days by Bravo and Gonzales (4), for ethanol production.

References

1. G.ksungur, Y. and G.ven • , U. Production of lactic acid from beet molasses by calcium alginate immobilized Lactobacillus delbrueckii IFO 3202. J. Chem. Technol. Biotechnol. 74: 131-136, 1999.
2. Rosevear, A. Immobilised biocatalysts-a critical review. J. Chem. Technol. Biotechnol. 34B:127-150,1984
3. Skjak-Braek, G. Alginates: biosynthesis and some structure-function relationships relevant to biomedical and biotechnological applications. Biochem. Soc. Transactions. 20: 27-33, 1992
4. Bravo, P. and Gonzales, G. Continuous ethanol fermentation by immobilized yeast cells in a fluidized-bed reactor.J.Chem.Technol. Biotechnol.52:127- 134, 1991.
5. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28: 350-356, 1956.
6. Roukas, T. Continuous ethanol production from carob pod extract by immobilized *Saccharomyces cerevisiae* in a packed-bed bioreactor. J. Chem. Technol. Biotechnol. 59: 387-393, 1994.
7. Gilson, C. D. and Thomas, A. Ethanol production by alginate immobilized yeast in a fluidised bed bioreactor. J.Chem. Technol. Biotechnol. 62: 38-45, 1995.
8. Colak,T.and HamamcÝ, H.The optimum taper angle for ethanol fermentation in a packed-bed column reactor. J.Chem. Technol. Biotechnol. 52: 445-455, 1991.