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CONTINUOUS ETHANOL PRODUCTION BY IMMOBILIZED *SACCHAROMYCES CEREVISIAE*: EFFECTS OF CELL CONCENTRATION AND GLUCOSE FLOW RATE[1]

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ABSTRACT

Aqueous glucose (10% wt/wt) was converted to ethanol by *Saccharomyces cerevisiae* NRRL Y-2034 entrapped in calcium alginate gel in continuous-flow fermentors. Different glucose feed-solution flow-rates [3 ml/hour (dilution rate 0.026/hour), 5 ml/hour (dilution rate 0.043/hour), and 7 ml/hour (dilution rate 0.060/hour)], and yeast cell concentrations (20 g 10 g, 5 g, and 1 g, wet wt per 115 ml) were used to determine their effect on the glucose conversion to ethanol. Entrapped 30-hour (late log phase) and 96-hour (beginning death phase) *S. cerevisiae* cells were compared for each experimental condition. The results confirm our previous data where the older alginate-entrapped cells (96 hour) ferment over a much longer perid than do younger cells (30 hour). The highest quantities of cells (20 g and 10 g) at the lowest glucose flow rate (3 ml/hour) produced the most ethanol.

INTRODUCTION

Ethanol is used as a solvent, in beverages, in food and feed via single cell protein, in hydrogen synthesis (via ethylene), as gasoline dilutant (gasohol), for biological energy (ATP) and as an intermediate in the production of chemicals. The ancient art of converting sugar to alcohol by yeast fermentation has been well documented in the literature (Del Rosario *et al.*, 1979).

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Continuous heterogeneous catalysis by immobilization techniques may be the most efficient approach to fermentation processes. There is a surge of research activity in this novel and rapidly growing field (Chibata and Tosa, 1981; Wada *et al.*, 1981; Arcuri *et al.*, 1980, Ghose and Bandyopadhyay, 1980). We have investigated the use of immobilized cell technology for ethanol production (Bagby, 1983). Our previous research showed that calcium alginate gel is an efficient matrix for entrapment of yeast cells. Further, we demonstrated that *Saccharomyces cerevisiae* produces alcohol from glucose for as long as three months in a continuous-flow fermentor. The fact that young immobilized yeast cultures (24-48 hour old, late log phase) have a shorter glucose fermentation lifespan than older cultures (72-96 hour, early death phase) was also shown in our previously reported data (McGhee *et al.*, 1982 & 1982a).

The data presented here elucidate the quantity of *Saccharomyces cerevisiae* (NRRL Y-2034) and the optimum glucose feed solution flow rate needed for continuous maximum ethanol production. Aqueous glucose (10% wt/wt) fermentation to ethanol by 30-hour (late log phase) and 96-hour (beginning death phase) calcium alginate-immobilized (entrapped) *S. cerevisiae* at 20 g, 10 g, 5 g, and 1 g (per 115 ml fermentor vol of glucose feed) are compared. Ethanol production results are given for each of the different quantities of entrapped yeast as they are fed the glucose solution continuously at flow rates of 3 ml, 5 ml, and 7 ml per hour (dilution rates of 0.026 $hour^{-1}$, 0.043 $hour^{-1}$, 0.060 $hour^{-1}$, respectively).

MATERIALS AND METHODS

Microorganism and culture conditions. S. cerevisiae NRRL Y-2034 was obtained from the Agricultural Research Service Culture Collection and maintained in liquid YMG medium (pH 6.8), which contains (per liter) 5 g of yeast extract, 5 g of malt extract, 5 g of peptone, and 20 g of glucose. The yeast cells were grown as previously described (McGhee *et al.*, 1982).

Immobilization of yeast cells. 20 g, 10 g, 5 g, or 1 g (wet wt) of 30- or 96-hour *S. cerevisiae* cells were mixed with 1 g of sodium alginate and encapsulated into beads as previously described (McGhee *et al.*, 1982). Each calcium alginate bead contained an average of 1×10^9 cells (range 1×10^8 to 2×10^9) when the 20 g yeast paste was entrapped; 4×10^8 cells/bead (range 1×10^6 to 8×10^8) for the 10 g yeast paste; 9×10^5 cells/bead for the 5 g yeast paste (range 1×10^5 to 2×10^8) and 1×10^4 cells/bead (range 0 to 1×10^6) for the 1-g yeast paste. Viable cell counts were made prior to immobilizing the yeast cells, after the cells were entrapped in the alginate bead, periodically during the fermentation experiments and again at the end of the experiments.

Fermentation. The continuous-flow fermentor is depicted in a simplified diagram in Figure 1; in practice four to six columns were operated simultaneously during each experiment. A detailed description of the continuous-flow system has been presented previously (McGhee et al, 1982). In full operation, the fermentor column contains 1,200 calcium-alginate beads and 115 ml of the glucose feed solution. The glucose solution was pumped from the reservoir at flow rates of either 3 ml, 5 ml, or 7 ml/hour (dilution rates of 0.026 $hour^{-1}$, 0.043 $hour^{-1}$, 0.060 $hour^{-1}$, respectively) upward into the bottom of the column through the bed of calcium alginate-entrapped yeast cells and finally into the collecting flask. Ethanol concentrations

were determined by gas-liquid chromatography. Glucose control and residual glucose in the product solution were determined by high-pressure liquid chromatography. Ethanol and glucose were calculated on a wt/wt basis and are reported as g/100 g solution. All data presented are average values calculated from 3-5 separate experiments for each set of parameters. The fermentations were carried out at 28-30°C.

RESULTS

Figures 2 and 3 both show ethanol production with a glucose feed solution flow rate of 3 ml per hour. Figure 2 shows glucose fermentation by 30-hour cells, and Figure 3 shows results with 96-hour cells. Although the patterns of alcohol production were similar, the duration of glucose conversion to ethanol was different. The 20 g of 30-hour cells produced the maximum theoretical yield of ethanol (5.11 g/10 g of glucose) from day 7 to 16 of the continuous fermentation. The 10 g of 30-hour cells produced 4.9 g of ethanol (about 96% glucose conversion) continuously from day 7 to 16. The 5 g of 30-hour cells produced 4.7-5.0 g of ethanol (92% to 98% conversion) from day 3 to day 7. A continuous 4.5 g (about 88% conversion) of ethanol production occurred from day 8 to 14. In each of the cell quantities (except 1 g of cells) ethanol production gradually decreased and ended at day 30 (Fig. 2). The 1 g of 30-hour cells (as well as all other tests with 1 g of cells) never was capable of extended ethanol production.

The 20 g of 96-hour cells with a glucose feed solution flow rate of 3 ml/hour converted 100% of the glucose to ethanol beginning at day 3 and continuing for about 3 months (Fig. 3). The 10 g of 96-hour cells (3 ml/hour flow rate) converted 100% of the glucose to ethanol continuously from day 4 to day 50 of the fermentation. Peak ethanol production (5.0 g, 98% glucose conversion) occured only for two days with the 5 g of entrapped yeast cells. As can be seen in Figure 3, ethanol production abruptly declined at day 92 for the 96-hour cells and ceased at day 94. The 10 g and 5 g of cells (3 ml/hour flow rate) ended ethanol production at day 72 and 62, respectively.

Figures 4 and 5 depict ethanol production with a glucose feed solution flow rate of 5 ml per hour (0.043 hour^{-1} dilution rate). These figures compare 30-hour yeast cells (Fig. 4) with 96-hour cells (Fig. 5). The 20 g of 30-hour cells gradually increased their ethanol production efficiency to a high of 4.5 g (88% conversion) from day 5 to 7 and then gradually dropped to a low of less than 0.4 g ethanol by day 16. The 10 g of 30-hour cells peaked ethanol production for one day at 4.5 g (88% conversion) and then continuously descreased daily to minimal production by day 12. The 5 g of 30-hour cells never produced more than 2.4 g of ethanol and that amount for only one day (Fig. 4). The 96-hour cells sustained ethanol production levels for longer peroids of time than did the 30-hour cells. For example, at the 20 g and 10 g quantities 96-hour cells converted 88% and 82% of the glucose for 11 and 8 days, respectively (Fig. 5).

Figures 6 and 7 present data regarding 30-hour or 96-hour cells at 7 ml/hour (0.060 hour^{-1} dilution rate) glucose feed solution flow rate. At this fastest flow rate, the 20 g of 30-hour cells daily increased their ethanol production to a high of 4.8 g (94% conversion) on day 3, then the amount of ethanol produced diminished daily to 1.0 g (20% conversion) by day 12. The 10 g of cells (30-hour old) followed

the ethanol production pattern of the highest cell quantity but produced less ethanol. The 5 g of cells produced significantly less ethanol, and no ethanol was produced by the 1 g of cells (Fig. 6). In contrast, the 96-hour cells demonstrated the capability to sustain a continuous level of ethanol production for several days. As can be seen in Figure 7, the 20 g of cells produced a high of 4.2 g (82% conversion) ethanol for 4 days and the 10 g of cells produced 3.0 g (59% conversion) ethanol for 9 days.

DISCUSSION

Colony counts were used to calculate the number of cells viable in the total cell population. Initially about 90% of the centrifuged 30-hour yeast cells were viable as were nearly 40% of the 96-hour cells. However, the alginate-bound 30-hour *S. cerevisiae* maintained a 25% viability for one week with a gradual decrease to 3% viability by day 16. The 96-hour alginate-entrapped cells maintained about 10% viability for one week and decreased to 1% viability by day 12 of the continuous-flow fermentation. Obviously, the ability of the yeasts to reproduce (viability) is not a prerequisite to their converting glucose to ethanol.

The obvious general conclusion that can be garnered from Figures 2-7 is that cell quantity, glucose feed solution flow rate, yeast age, total amount of ethanol produced, and the duration of the fermentation are all important considerations for developing a continuous-flow system. The most important result is the 20 g of 96-hour *S. cerevisiae* cells produced the most total ethanol (470.9 g) at a 3 ml/hour flow rate. Also, these data again confirm that older cells (96 hour) make for a column with a much longer lifespan than do younger cells (30 hour).

However, what is not readily apparent from the figures is that usually the larger concentration of immobilized yeast cells (20 g/115 ml glucose feed solution) produced less ethanol per gram of cells than did the smaller number of cells (the exception being the 1 g of cells/115 ml). This phenomenon occurs regardless of the glucose feed solution flow rate or the age of the cells as can be seen in Table 1. For example, at 3 ml/hour flow rate 20 g of 96-hour cells produced a total of 23.5 g of ethanol/g of cells, whereas 10 g of cells produced a total of 34.3 g of ethanol/g of cells and 5 g of cells produced 43.1 g total ethanol/g of cells (Table 1).

The fermentations containing 20 g of 96-hour cells (3 ml/hour feed solution flow rate) continued for 94 days, the fermentations with 10 g of cells continued for 72 days and the fermentations with 5 g of cells continued for 62 days. Therefore, the maximal number of cells should be used in the continuous-flow system if maximum total quantity of ethanol is the priority. However, if shorter duration of the fermentation is desirable along with maximal ethanol production per gram of cells, then a smaller amount of cells should be used.

Although glucose feed solution flow rate does not affect the relationship of cell numbers to grams of ethanol produced, it clearly affects the total amount of ethanol produced. Invariably the longer the glucose solution was retained in the fermentor column, the more complete its conversion to ethanol. The slowest flow rate yielded a total of 24 g ethanol per gram of cells in the 20 g cell concentration, whereas the 5 ml/hour flow rate caused 8 g of total ethanol to be produced per gram of yeasts, and 20 g of cells produced only 2 g ethanol/g of cells at the 7 ml/hour flow rate. The other cell concentrations gave similar decreasing ethanol totals with increasing feed solution flow rate. Apparently, the feed solution must make a certain sustained

contact time with the yeast to enable glucose incorporation into the metabolic pathway.

The extended duration of ethanol production by the highest quantity of cells does indicate that a larger pool of enzymes necessary for the conversion of glucose to ethanol is present for a longer period of time. The fact that less ethanol per gram of cells is produced by the larger quantity of cells may be partially explained by the research of Lee and Woodward regarding properties and application of immobilized ß-D-glucosidase (Lee and Woodward, 1983). Their data suggest that a concentration gradient exists between the glucose (10.0% w/w) in the feed solution and the glucose within the calcium aglinate bead, so that glucose concentration per yeast cell is less than optimal. Also it may be simply that some yeast cells are physically crowded together in such a manner as to never come into contact with any significant glucose concentration. The abrupt cessation of alcohol production in many of the experiments might imply the depletion of the enzyme systems needed to maintain glycolysis. Other possibilities for the loss of alcohol production include intracellular pH change, osmolarity imbalance, cofactors, and cell structure integrity.

ACKNOWLEDGMENT

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REFERENCES

Arcuri, E.J., Worden, R.M., and Shumate, S.W. II. (1980) Ethanol production by immobilized cells of *Zymomonas mobilis*. Biotechnology Letters 2, 499-504.

Bagby, M.O. (1983) Research at the Northern Agricultural Energy Center. Proceedings of the 3rd Annual Solar Biomass Workshop, Atlanta, GA, April 26-28.

Chibata, I., and Tosa, T. (1981) Use of immobilized cells. Annual Review of Biophysics and Bioengineering 10, 197-216.

Del Rosario, E.J., Lee, K.J., and Rogers, P.L. (1979) Kinetics of alcohol fermentation at high yeast levels. Biotechnology and Bioengineering 21, 1477-1482.

Ghose, T.K., and Bandyopadhyay, K.K. (1980) Rapid ethanol fermentation in immobilized yeast cell reactor. Biotechnology and Bioengineering 22, 1489-1496.

Lee, J.M., and Woodward, J. (1983) Properties and application of immobilized ß-D-glucosidase coentrapped with *Zymomonas mobilis* in calcium-alginate. Biotechnology and Bioengineering 25, 2441-2444.

McGhee, J.E., St. Julian, G., Detroy, R.W., and Bothast, R.J. (1982) Ethanol production by immobilized *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, and *Zymomonas mobilis*. Biotechnology and Bioengineering 24, 1155-1163.

McGhee, J.E., St. Julian, G., and Detroy, R.W. (1982a) Continuous and static fermentation of glucose to ethanol by immobilized *Saccharomyces cerevisiae* cells of different ages. Applied and Environmental Microbiology 44(1), 19-22.

Wada, M., Kato, J., and Chibata, I. (1981) Continuous production of ethanol in high concentration using immoblized growing yeast cells. European Journal of Applied Microbiology and Biotechnology 11, 67-71.

Table 1.
Continuous Ethanol Production by *Saccharomyces cerevisiae*

Immobilized yeast/115 ml glucose feed solution	Glucose feed solution flow rate/hour					
	30-hour culture			96-hour culture		
	3 ml	5 ml	7 ml	3 ml	5 ml	7 ml
(g)	(total g ethanol/g yeast)					
1	14.2	1.8	0.0	31.0	9.0	0.0
5	20.4	2.9	2.2	43.1	8.2	2.0
10	11.6	2.7	1.9	34.3	11.7	3.9
20	5.7	2.1	1.8	23.5	8.2	1.7

See Figures 2-7 for the duration of each fermentation.

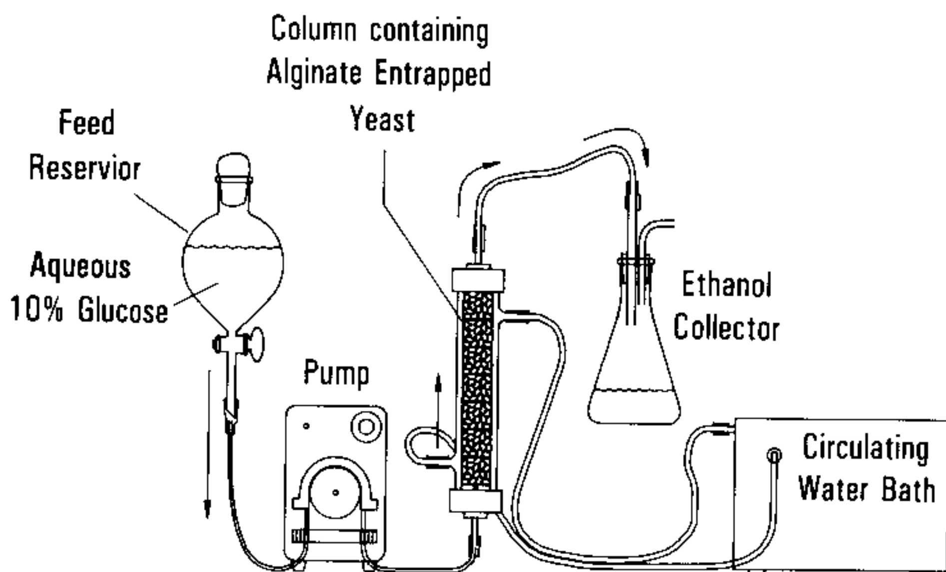

Fig. 1 Flow-sheet diagram depicting the continuous-flow fermentor system for glucose fermentation to ethanol via calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034.

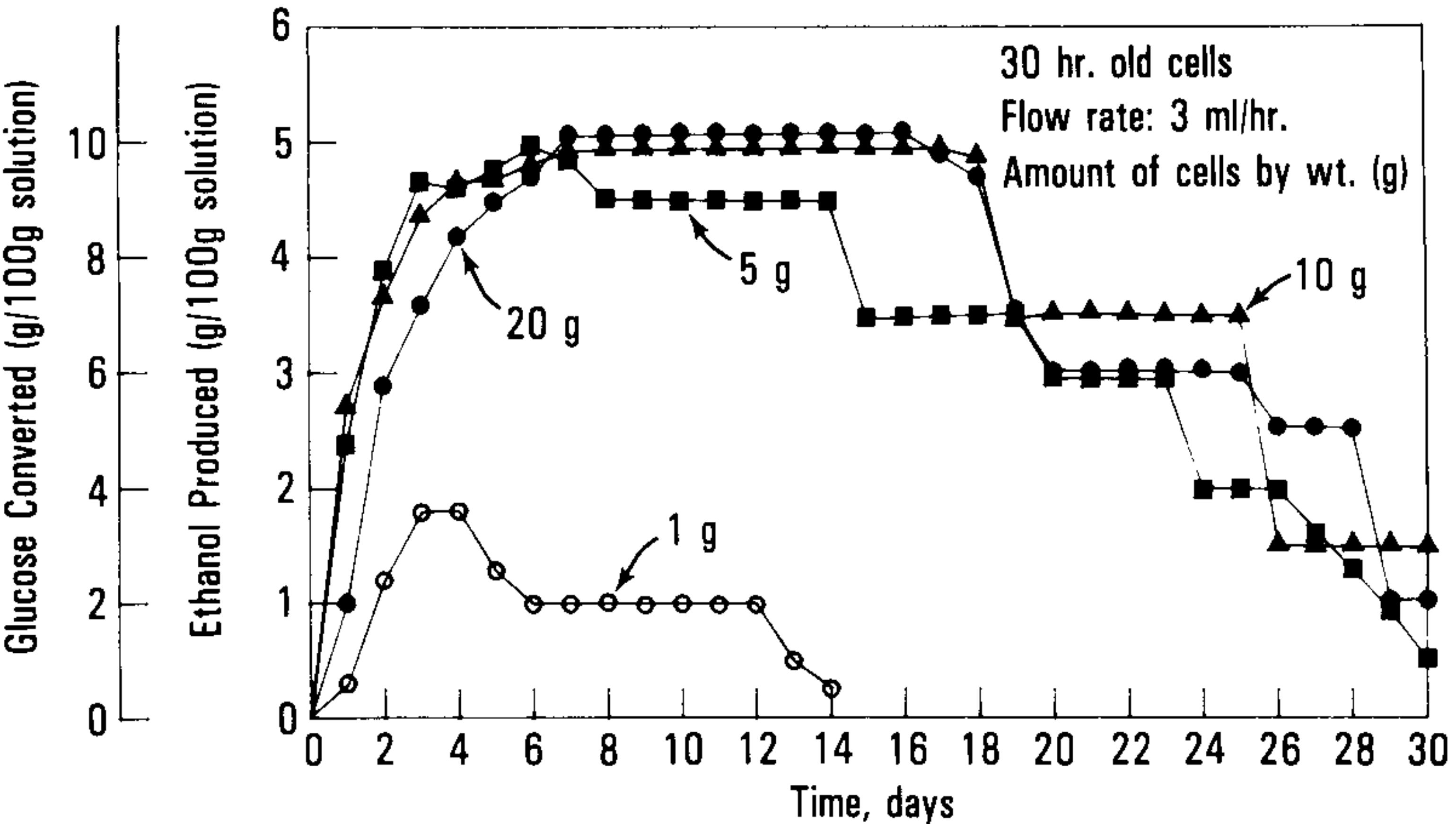

Fig. 2. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 30 hours old (late log phase) and the glucose feed solution flow rate is 3 ml/hour (0.026 $hour^{-1}$ dilution rate). The fermentation is carried out at 28-30°C.

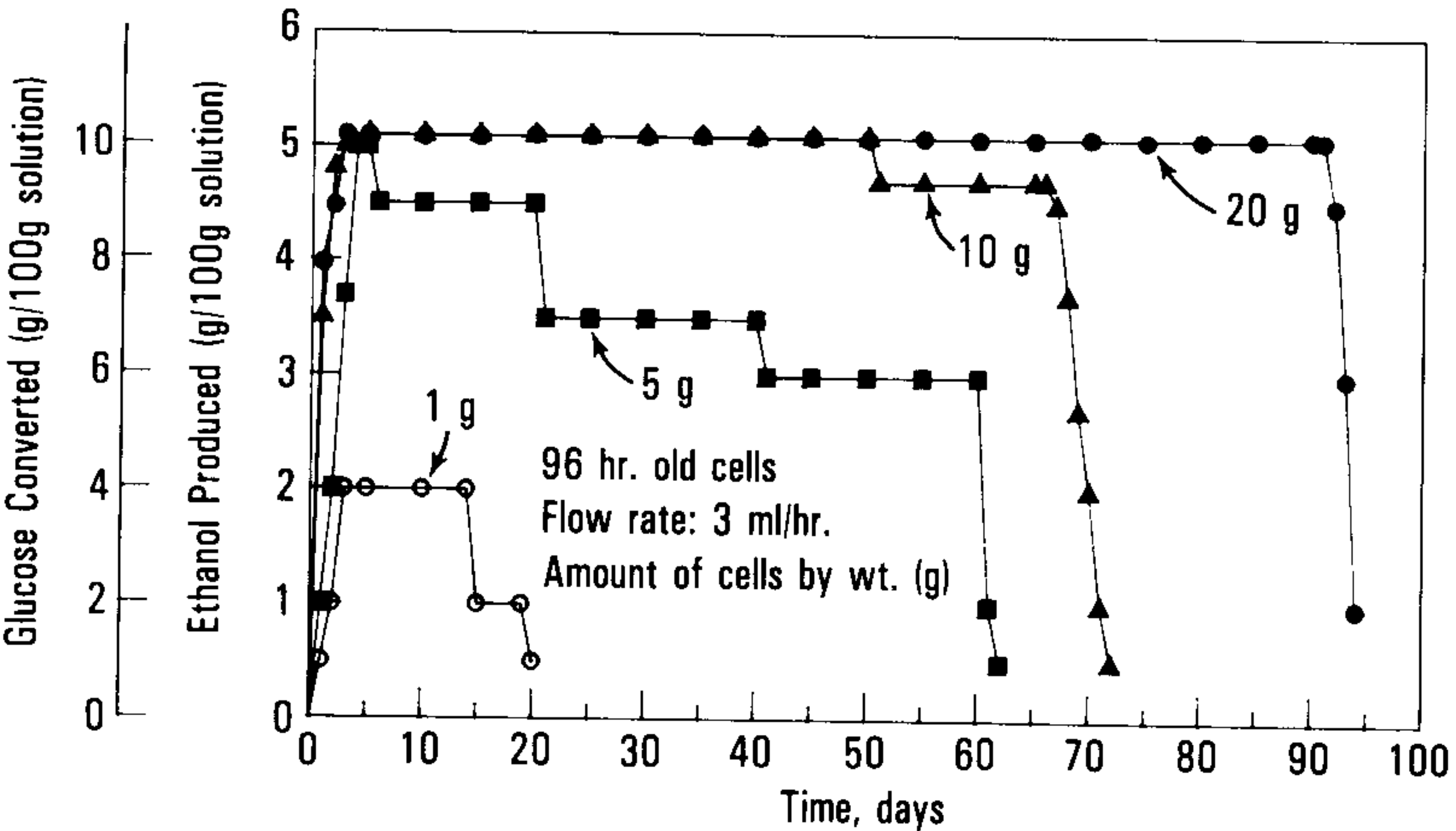

Fig. 3. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 96 hours old (beginning death phase) and the glucose feed solution flow rate is 3 ml/hour (0.026 $hour^{-1}$ dilution rate). The fermentation is carried out at 28-30°C.

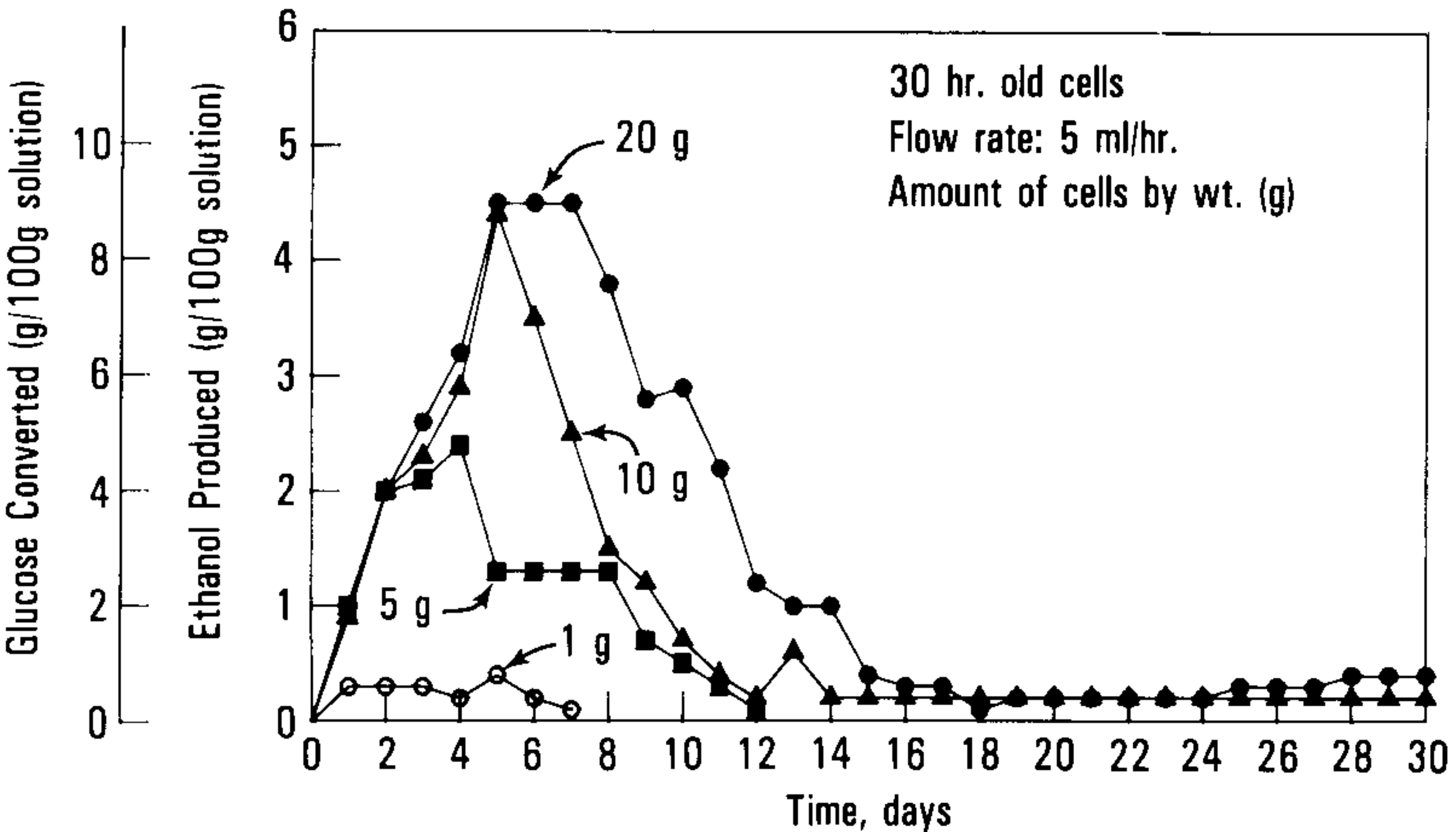

Fig. 4. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 30 hours old (late log phase) and the glucose feed solution flow rate is 5 ml/hour (0.043 $hour^{-1}$ dilution rate). The fermentation is carried out at 28-30°C.

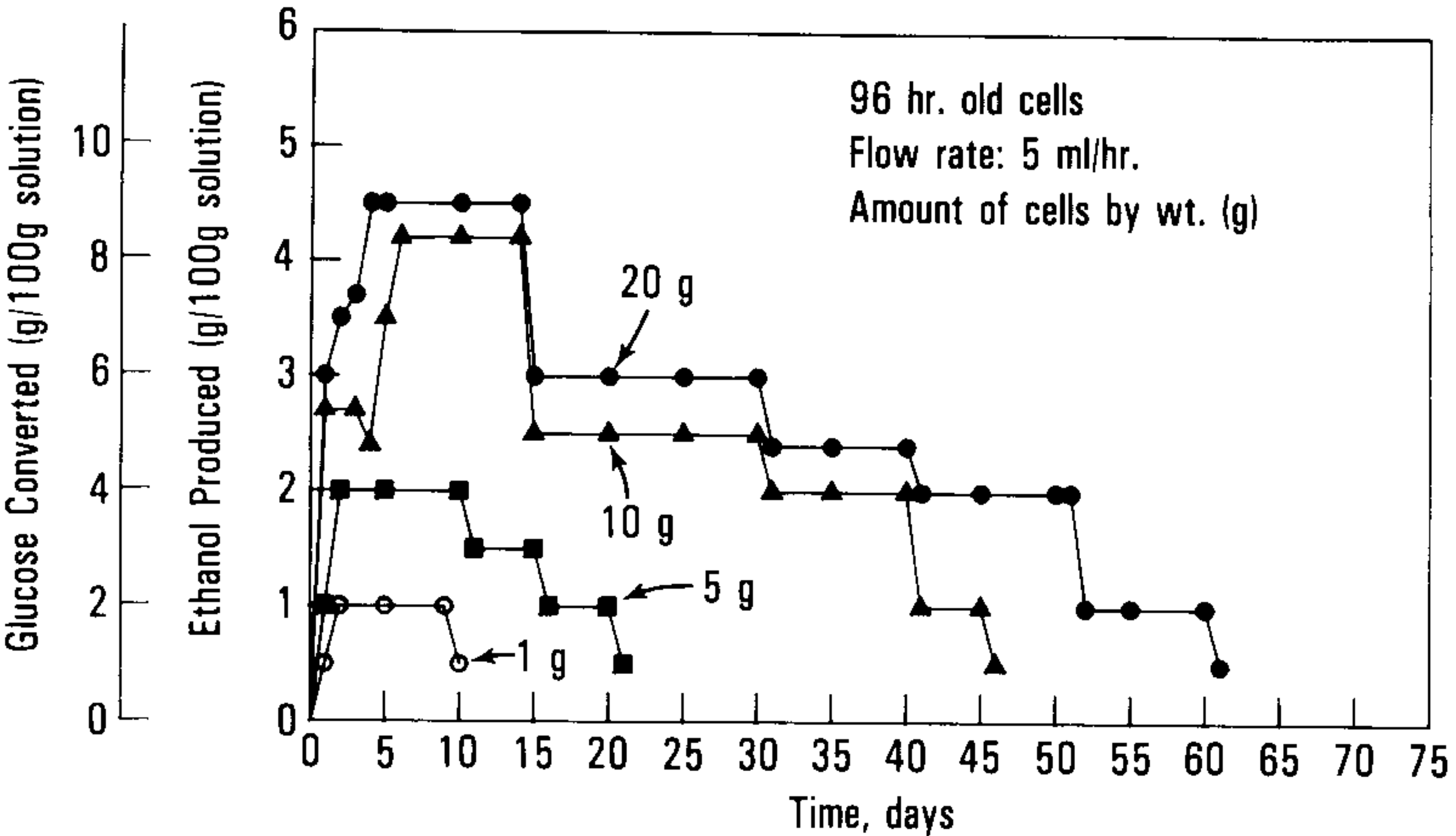

Fig. 5. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 96 hours old (beginning death phase) and the glucose feed solution flow rate is 5 ml/hour (0.043 $hour^{-1}$ dilution rate). The fermentation is carried out at 28-30°C.

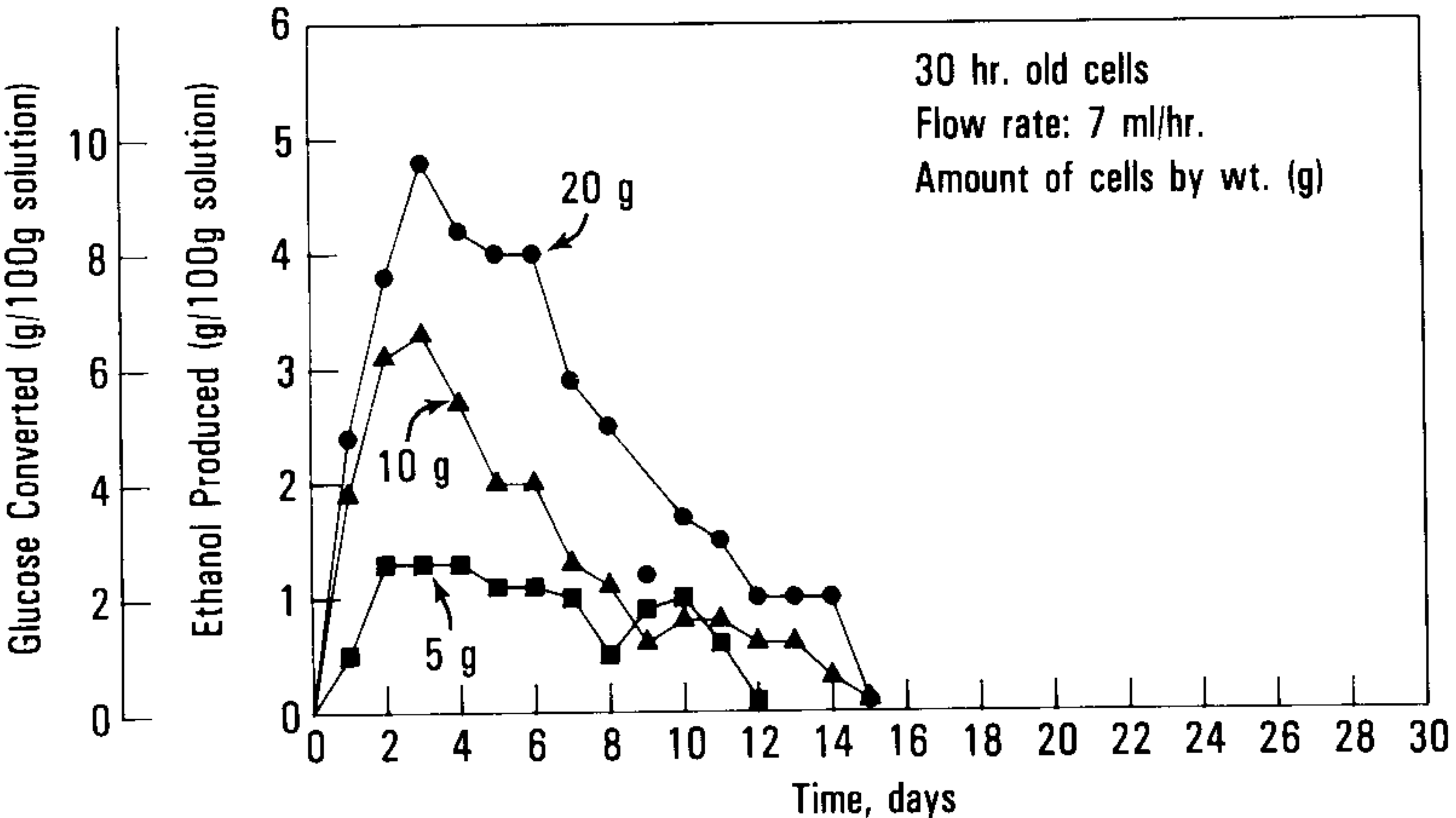

Fig. 6. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 30 hours old (late log phase) and the glucose feed solution flow rate is 7 ml/hour (0.060 $hour^{-1}$ dilution rate). The fermentation is carried out at 28-30°C.

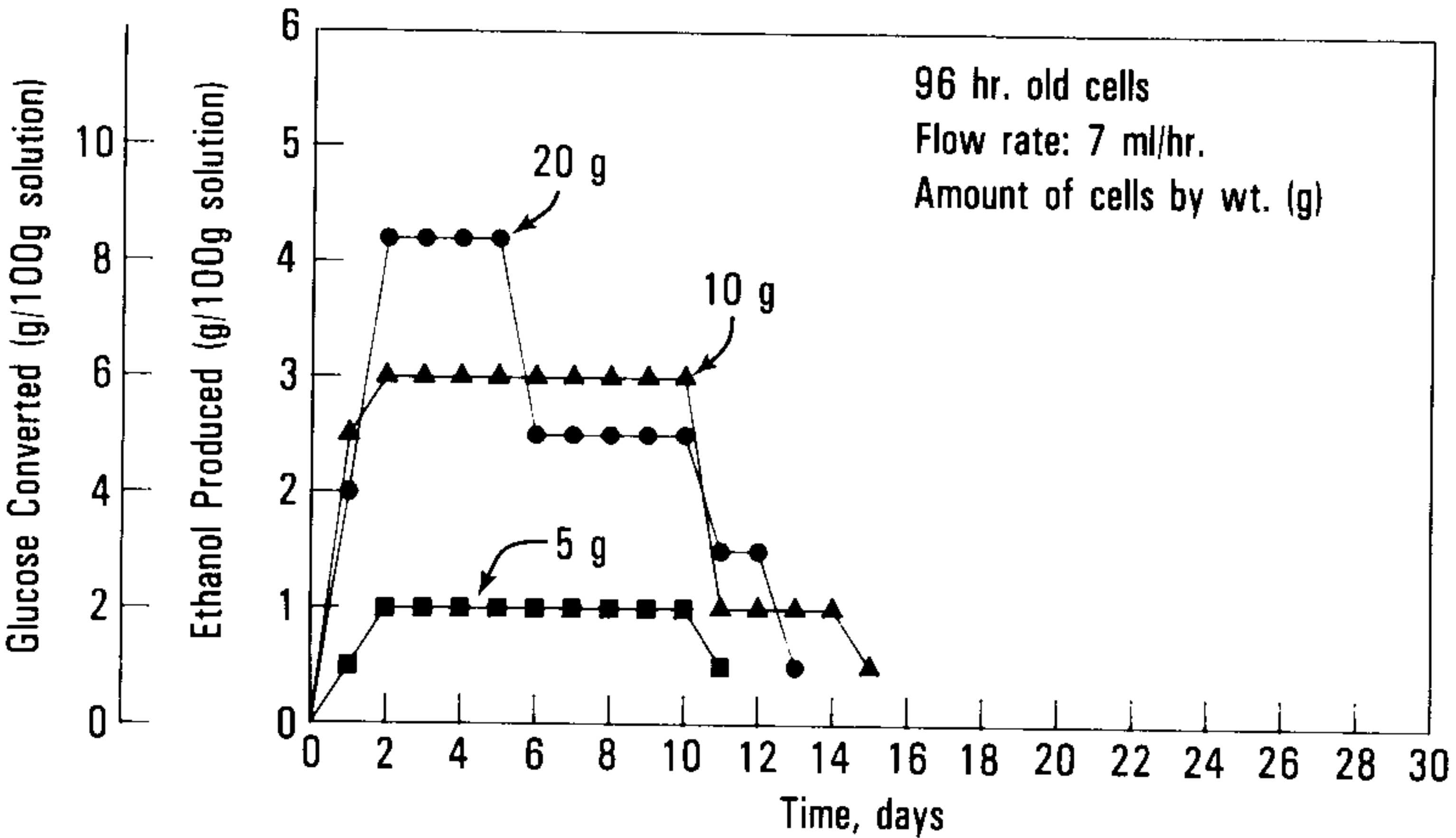

Fig. 7. Continuous aqueous glucose (10% wt/wt) fermentation to ethanol by different gram (wet wt) quantities of calcium-alginate entrapped *Saccharomyces cerevisiae* NRRL Y-2034. The yeast cells are 96 hours old (beginning death phase) and the glucose feed solution is 7 ml/hour (0.060 hour^{-1} dilution rate). The fermentation is carried out at 28-30°C.