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회분 배양 및 포도당 유가 배양에 의한 최적 에타놀 발효

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Optimal Ethanol Fermention by Batch and Glucose Fed-Batch Culture

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요 약

S. cerevisiae 균주를 사용하여 혐기조건 하에서의 에타놀 발효를 pH 4 및 30°C에서 회분 및 반회분 방법으로 조사하였다. 후라스크내에서 발효를 수행한 결과 최적 중량 포도당 농도가 10%로 측정되었다. 초기 포도당 농도를 증가 시켰을 때 초기 lag phase 및 기질저해가 증가하였다. 회분 발효에서는 초기 접종량 및 포도당 농도와 같은 인자들의 발효기간과 에타놀 생성 속도에 대한 영향을 조사하였다. 실험결과와 수치모형을 사용하여 포도당 유가에 대한 최적 정책을 찾아 내었다.
일정한 포도당 유속에서 50% 포도당 용액을 사용하여 발효를 수행하였다. 이 유가 발효에 의한 생산성이 회분 발효에 의한 것 보다 균체 단위 접종량 당 30% 더 높은 값을 나타내었다. 유가하여 주는 포도당은 용액 상태보다도 가루 형태로 해 주는 것이 희석 효과를 줄여 주게 될 것이고 따라서 등일한 최종 에타놀 농도에 도달하는 데 필요한 발효기간도 축소시킬 수 있을 것이다.

ABSTRACT

The ethanol fermentation under anaerobic condition by a strain of S. cerevisiae was investigated in batch and fed-batch modes at pH4.0 and 30°C. The flask fermentation showed the optimal glucose concentration to be 10% by weight. With the increase in the initial glucose concentration, the inital lag phase as well as substrate inhibition increased. In batch fermentation, the effect of some fermentation parameters such as the inoculum size and the glucose concentration on the fermentation time and the ethanol production rate was investigated.

The experimental results together with the mathematical model were utilized for finding the optimal strategy for the glucose addition rate.
Fermentations with constant glucose feed rate were undertaken using 50% glucose solution. The productivity of fed-batch fermentation per cell inoculated was about 30% higher than that of the batch fermentation. The use of the glucose powder instead of the solution may help reduce the dilution effect and hence the fermentation period required to arrive at the same final ethanol concentration.

Ⅰ. Introduction

The recent oil shortage has created a surge of interest in fermentation of cellulosic waste materials for bulk production of ethanol both as an alternative energy souergy source and as an alternative chemical feed stock.

The ethanol fermentation process has been in operation for a long time and attracted the attention of many research workers.[1-10] The major recent topics of research by many of these workers can be categorized into two main areas, i.e. the development of new strains and the improvement by process modifications. Thus the search for and artificial generation of the high temperature strains and the strains with more versatile capabilities have resulted in substantial improvement in both productivity and yield,[16,17] and the new process developments like those in the vacuum process, the cell recycle recycle process and the process with immobilzed whole-cells added up to the improved overall performance.

Fed-batch method is another tool to be employed in the development of proesses with improved performance and has been applied to fermentation for relatiely long period of time. Its application of special significance was to bakers' yeast[11-13] and penicillin fermentations[14,15] and was known to be very effective when high sugar concentration inhibits the product formation. In ethanol fermention fed-batch method was not reported yet but high sugar concentration is known to inhibit the ethanol production as well as cell growth.

In this study, the effects of glucose concentrations and cell inocula on fermentation were investigated and the glucose concentration which maximizes ethanol prodution rate was determined. Computer simulation using kinetic equations related to ethanol and cell production was performed and optimum feed rate was determined The results of batch and fed-batch fermentations were also compared.

Ⅱ. Theory

The effort to model the fermentation processes have been in practice for many years and is considered essential for a more sophisticated operation of fermentors.[21-23] Recently bakers' yeast and penicillin fermentation were studied in depth in this respect. As microorganisms, however, are different from chemical catalysts and as the physiological state cannot be precisely described, the establishment of the kinetic equations consistent with real fermentations throughout the fermentation period is unrealistic.

In this study, the balance equations among the fermentation variables are set up to ma-

intain the glucose concentration in the fermentor at the value corresponding to the maximun specific production rate and the glucose feed rate is chosen as a control variable. For cell biomass, ethanol production and substrate consumption, the following equations are derived from material balance;

$$\frac{d(VX)}{dt} = \mu VX \tag{1}$$

$$\frac{d(VP)}{dt} = \nu VX \tag{2}$$

$$\frac{d(VS)}{dt} = FS_F - \frac{\nu VX}{Y_{P/S}} \tag{3}$$

$$\frac{dV}{dt} = F \tag{4}$$

In equation (3) maintenance term was deleted for simplicity. In the same equation the term accounting for the substrate consumption in the cell growth is not separately included because the magnitude of the product yield coefficient taken from Ghose's work[2] is an apparent overall value allowing for both the cell growth and the product formation. Of course for a more exact evaluation the terms accounting for cell growth, product formation and maintenance respectively with the true yield coefficients and maintenance coefficients will have to be used and such a work is currently under progress. μ and ν are influenced by glucose and ethanol concentrations; these are expressed as the function of S and P. Although many equations are documented in the literature,[2,24,25] Ghose's equations are chosen because of the simplicity.[2] The equations are:

$$\mu = \mu_m\left(1 - \frac{P}{P_m}\right)\frac{S}{S + K_S + S^2/K_S W} \tag{5}$$

$$\nu = \nu_m\left(1 - \frac{P}{P'_m}\right)\frac{S}{S + K_S + S^2/K_S' W} \tag{6}$$

In the above six equations, there are seven dependent variables, i.e. X, S, P, V, F, μ and ν, and the constants which must be determined by experiment are S_F, μ_m, ν_m, K_S, K'_S, P_n, P'_m, $Y_{P/S}$, W and W'. Thus in order to solve these equations one more equation is required, If S is fixed at a constant value for maximum ethanol production, the equations from (1) to (4) can be rearranged as below;

$$\frac{dX}{dt} = -\frac{X^2}{Y_{P/S}(S_F - S)} + \mu X \tag{7}$$

$$\frac{dV}{dt} = \frac{\nu VX}{Y_{P/S}(S_F - S)} \tag{8}$$

$$\frac{dP}{dt} = -\frac{\nu PX}{Y_{P/S}(S_F - S)} + \nu X \tag{9}$$

In the equations (7) and (9) the first term on the right hand side expresses the dilution effect by glucose solution feed. Also equations (10) and (11) are obtained from (5) and (6) ;

$$\mu \times \mu_m\left(1 - \frac{P}{P_m}\right) \tag{10}$$

$$\nu \times \nu_m\left(1 - \frac{P}{P'_m}\right) \tag{11}$$

These equations are non-linear and analytical solutions can not be obtained, necessitating the solution via numerical method. If S is maintained at a predetermined value up to the final moment of the total fermentation period, much glucose will remain at the end of run and this would not be an economical operation in the industrial fermentation. The time to stop the feed, the switching point should be determined based on the substrate cost, the fermentation time, and the reactor volume. When feed is stopped at a predetermined time, the equations from (1) to (4) simply become batch system equations. These are ;

$$\frac{dX}{dt} = \mu X \tag{12}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}}\frac{dP}{dt} \tag{13}$$

$$\frac{dP}{dt} = \nu X \tag{14}$$

Table 1. Base Medium for Fermentation

component	Per liter
glucose(convertedto 100% purity)	50.0g
yeast extract (Difco)	2.5g
KH_2PO_4	0.5g
$(NH_4)_2SO_4$	0.75g
$MgSO_4.7H_2O$	0.06g
$CaCl_2.2H_2O$	0.03g
Tap water to	1.0 *l*

Ⅲ. Materials and Methods

1. Organism

Saccharomyces cerevisiae, NRRL Y-132, was obtained from the NRRL, U.S. Department of Agriculture, Peoria, Illinois.

2. Medium

The standard medium employed in fermentation throughout this study is listed in *Table 1*. Glucose was obtained from Miwon Co., Ltd. and had a purity of 90.6 g glucose in 100 g powder. When the glucose concentration of the medium was varied, all other components were also changed by the same ratio. pH was adjusted to 4.0 before sterilization using 4.0 N NaOH.

3. Inoculation

The culture was maintained on malt extract-yeast extract-glucose-pepton (MYGP) slants. For ethanol production plenty of cells were required, and thus the medium listed in *Table 1* was inoculated from the slants and incubated in reciprocal shaker at 30°C for 24 hrs. This seed culture was used for inoculation into the 10 *l* jar fermentor. After 24 hrs of culturing, the cells were harvested, centrifuged at 4000 rpm for 30 min, and maintained in a refrigerator at 0—4°C. These cells were used within one week.

4. Analytical Methods

For cell biomass assay 10 ml samples were withdrawn, the cells were cenrifuged at 4000 rpm for 30 min and the supernatent was stored under refrigeraticn. The optical density of the cell suspension was measured at 560 nm. Cellls were washed twice with distilled water and then dried at 100°C to constant weight.

Ethanol contents were determined by gas chromatographic[2] and enzymatic method.[18] A 2.0m long and 3.2 mm outside diameter steel column packed with Chromosorb 101, 60—80 mesh, was used with flame ionization detector. Both the injector and the detector were kept at 160°C and column oven operated isothermally at 150°C. Helium was used as carrier gas at a flow rate of 25ml/min. The combustion gases were hydrogen and air. For enzymatic method, alcohol dehydrogenase (ADH) was used. Assay buffer solution, NAD solution and ADH solution were prepared in every assay procedure. Glucose concentrations were determined by Somogyi-Nelson.[19]

5. Apparatus

Experiments were carried out in 2.6 l and 10 l jar fermentors equipped with pH and D.O. controllers with 1 *l* and 4 *l* working volume respectively (L.E. Marubishi Co., Ltd. Japan). The fermentors and their contents were sterilized in autoclave at 15 psig and 120°C for 20 min. For glucose feed 50% glucose solution were prepared in 4.0 *l* reservoir and supplied to fermentor by a peristaltic pump. During fermentation the pH was maintained constant at 4.0 by automatic addition of 4.0N NaOH solution to fermentor. Sa-

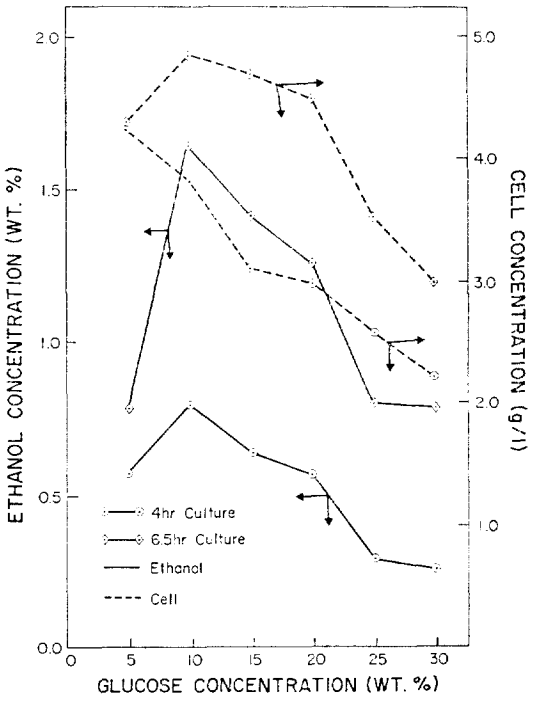

Fig. 1. Dependence of Final Cell and Ethanol Concentrations on the Initial Glucose Concentration in Flask Culture

mples were withdrawn through a sampling port located on the bottom of the vessel. The soy bean oil was used as antifoam.

Ⅳ. Results and Disussion

1. Flask Culture

The effect of initial glucose concentraton on ethanol fermentation were investigated in flask(*Fig. 1*). In anaerobic condition each flask was inoculated with 1.35 g cell (converted to dry cell weight) and the glucose concentrations in the initial media were 5%, 10%, 15%, 20%, 25%, and 30% respectively. In order to investigate the glucose inhibition in early phase of fermentation, fermentation times 4 hr and 6.5 hr were chosen as observation times. *Fig. 1* shows that at a glucose concentration of 10% the maximum ethanol production was obtained for both fermentation periods. In *Fig. 2* the ethanol concentration during the course of fermentation is plotted against time. For the flasks initially charged with 10% glucose, additional glucose solution (50 wt. %) was added at predetermined time intervals (6, 12 and 18 hr) during the course of fermentation

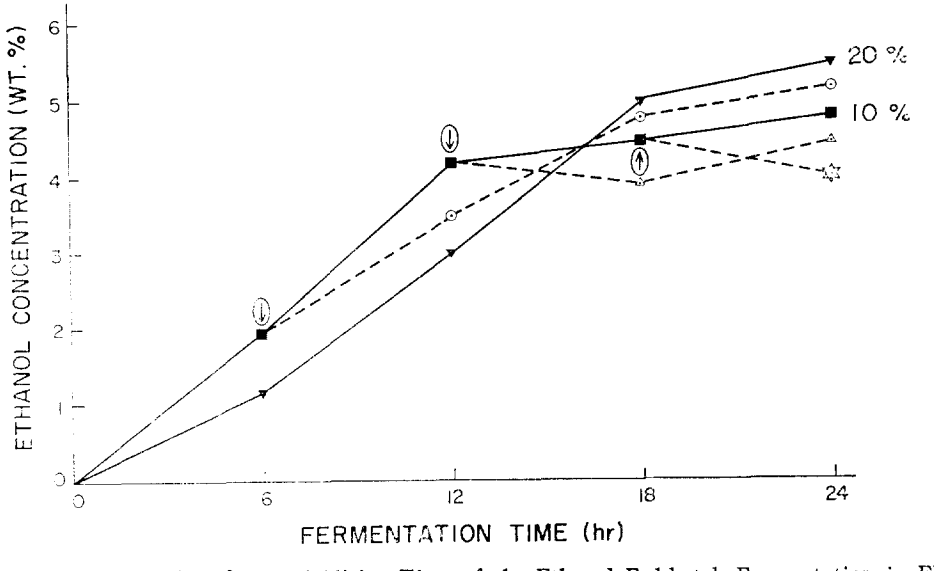

Fig. 2. Depence on the Glucose Addition Time of the Ethanol Fed-batch Fermentation in Flask

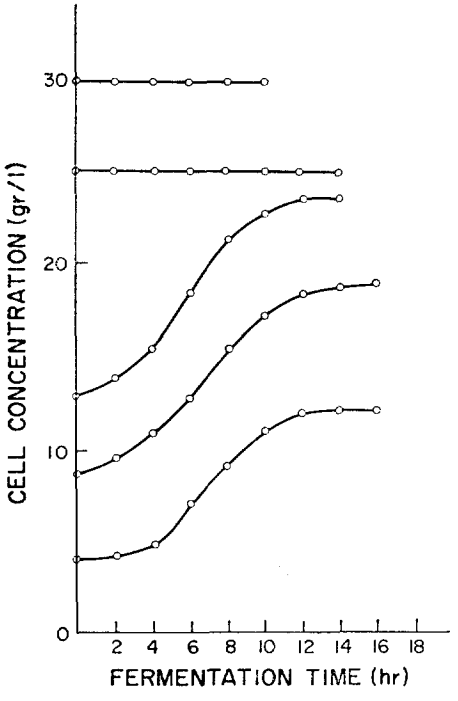

Fig. 3. Effect of Initial Cell Concentration on Cell Growth during Batch Fermentation

such that the total glucose concentration at the time of each addition was 20% and the results from these flasks were compared with that from the control flask which contains 20% glucose right from the beginning and hence needs no more glucose addition. As was the case with *Fig. 1*, here also during the initial 12 hr the fermentation with 10% glucose was superior to that with 20% glucose in ethanol production. When 50% glucose solutions were added later on, however, the final ethanol concentration at 24 hr were lower than that of control. This might be explained by two factors, i.e., the dilution of the fermentation volume by the solution added and the lag time for cells to adapt to new environment. If glucose powder is added instead of liquid the dilution effect is presumed to be prevented. Also continuous feeding will overcome the problem of adaptation brought about by instaneous feeding. Thus from the above results, one can observe that high glucose concentrtaion inhibits the ethanol prdouction as well as growth and maintaining proper glucose concentration during the fermentation period will increase the ethanol production rate.

2. Batch Experiment

In flask culture the controls on pH and temperature are not accurate, and it is very difficult to observe time course behaviour of fermentation variable. Using jar fermentors these defects can be overcome. *Fig. 3* shows the effect of inital cell concentration on cell growth during batch fermentation using the jar fermentor. It was observed that as the inoculum cell concentration increased in the fermentor, the cell yield decreased, and at 25 g/l or above, the cell growh stopped shows concentrations completely. This shows that the cell concentrations higher than 25.0 g/l

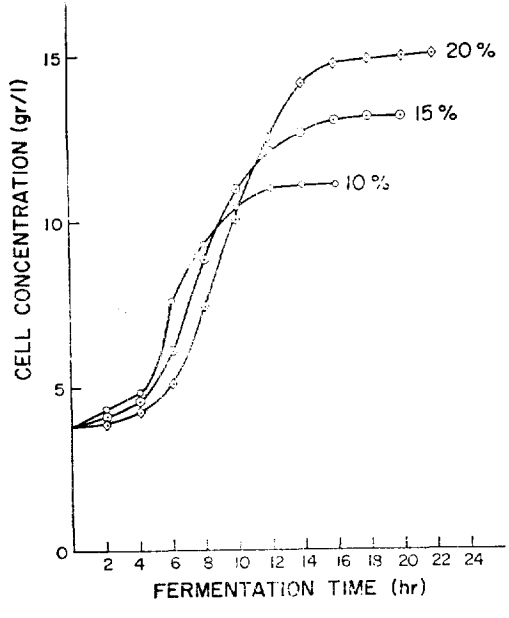

Fig. 4. Cell Concentration Variation with Various Initial Glucose Concentration for Batch Ethanol Fermentation

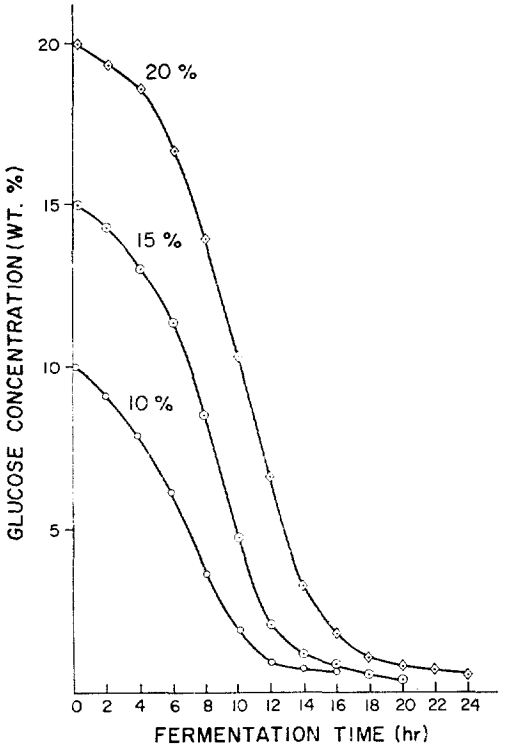

Fig. 5. Glucose Concentration Change withFermentation Time

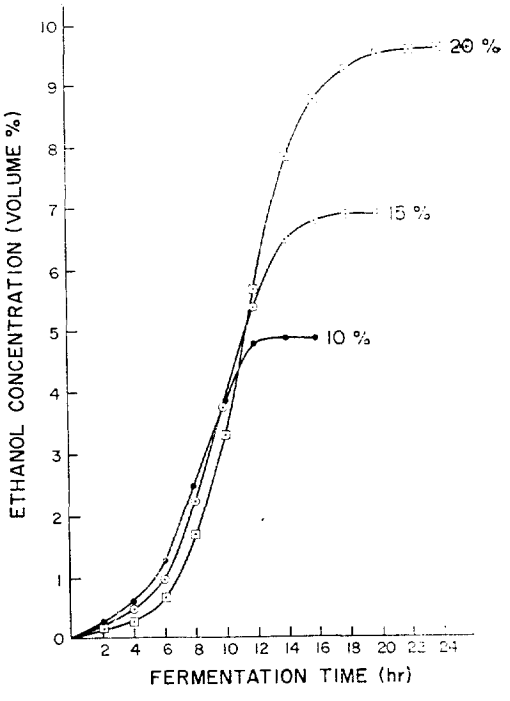

Fig. 6. Ethanol Concentration Variation with Various Intial Glucose Concentration for Batch Ethanol Fermentation

were not obtained by starting with smaller inoculum, and if higher cell biomass is required for rapid ethanol production, an excessive number of cells must be added cells harvested with elsewhere separately.

Typical batch runs with various glucose concentrations carried out with constant amount of inoculum are shown in *Fig. 4—6*. In these experiments the glucose concentration ranged from 100 to 200 g/l and air was supplied at 0.12 VVM.[2] The general pattern of cell growth, ethanol production, and substrate consumption changed with glucose concentration. The maximum growth rate decreases as the glucose concentration increases even at very low ethanol concentration. The ethanol production rate as well as sugar consumption rate was also found to decrease, and the time of fermentation was prolonged with increased initial substrate concentrations in the fermentor. In this study, all rates were obtained from the tangent lines in *Fig. 4—6*. From these results it is apparent that high sugar concentration has inhibitory effect on cell growth, ethanol production and substrare utilization. The rates of glucose consumption and alcohol production were higher in the negative acceleration growth phase. At glucose concentration of 200 g/l, in spite of a sufficient amount of nutrients present in the medium, the cell growth stops at an ethanol concentration of 80 to 90 g/l, even though a large amount of glucose(60 g/l) remained in the medium.

However, the cells continue to produce ethanol even after complete cessation of cell growth until the energy source is exhausted and ultimately the fermentation was limited by the availability of energy source. The ethanol concentration at 24 hr was about 10 vol. %, and glucose which was not consumed was about 15 g/l. This shows that two ethanol concentrations are involved in the metabolic pathway of this yeast strain: one for complete cessation of cell growth at about 85 g/l and the other with the ability to produce alcohol maintained above 10% ethanol concentration. These are shown in some other literatures and by various experiments.[3,20] But even when the same strain is used, different experimental methods gave different ethanol concent-ration inhibiting the pathway, depending on whether the ethanol was added externally or produced by the ye-

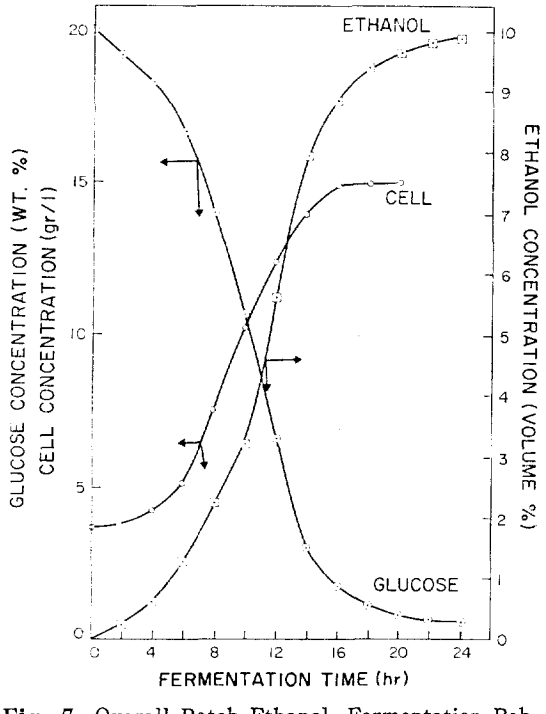

Fig. 7. Overall Batch Ethanol Fermentation Behaviour with 20% Initial Glucose Concentration

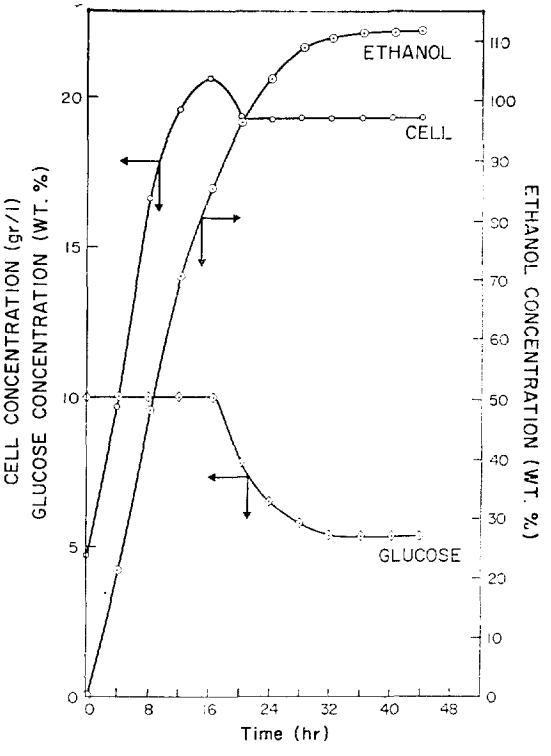

Fig. 8. Computer Simulation of the Fed-batch Fermentation: Time Course Behavior of Concentration of Ethanol, Cell and Glucose

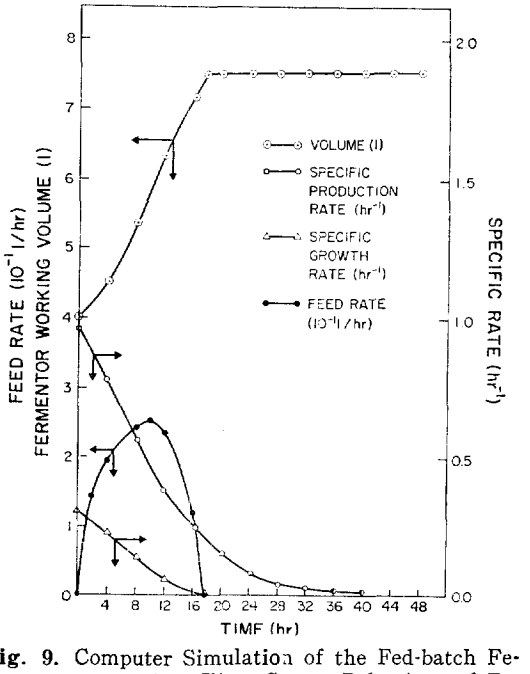

Fig. 9. Computer Simulation of the Fed-batch Fermentation: Time Course Behaviour of Fermentor Volume, Specific Production Rate, Specific Growth Rate and Feed Rate

ast cell itself.[20] This apparent discrepancy may be caused by the difference between the intracellular and extracellular ethanol concentrations. The conversion efficiency varied from 85% of theoretical at glucose concentration of 100g/l to 90% of theoretical at 200 g/l. Maximum specific growth rate of cells was 0.30 hr^{-1} and maximum specific production rate of ethanol was 0.95 hr^{-1}. When jar fermentor with proper controls was used, the high glucose concentration caused less inhibition as compared with flask culture. This shows that pH and temperature have an important effect on metabolic pathway. *Fig. 7* shows the changes with time of cell biomass, ethanol and giucose.

3. Results of Computer Silmulation

Using the equations in the theory section, the ethanol fermentation which is initiated as fed-batch and then switched to batch mode can be modeled. The switching point was chosen as the time when no more cells can grow, i.e.the ethanol concentration reaches P_m. Glucose concentration during fed-batch mode is determined to be 100 g/l. The initial values are given below;

$X_o = 3.8$g/l $V_o = 4.0l$ $P_o = 0.0$g/l

and the constants used are obtained from batch results and literature[2];

$\mu_m = 0.37hr^{-1}$ $\nu_m = 1.3hr^{-1}$ $K_S = 0.476$g/l

$K'_S = 0.666$g/l $P_m = 85$g/l $P'_m = 110$g/l

$W = 1000$ $W' = 1000$ $S = 100$g/l

$S_F = 500$g/l $Y_{P'S} = 0.47$ $Y_{X'S} = 0.09$

To solve these equations fourth order Runge-Kutta method was used.[26] *Fig. 8* and *9* show the results of computer simulation of fed-batch fermentation: *Fig. 9* shows the time course behaviour of concentration of ethanol, cell biomass and glucose, *Fig. 9* shows that of fermentor volume, specific

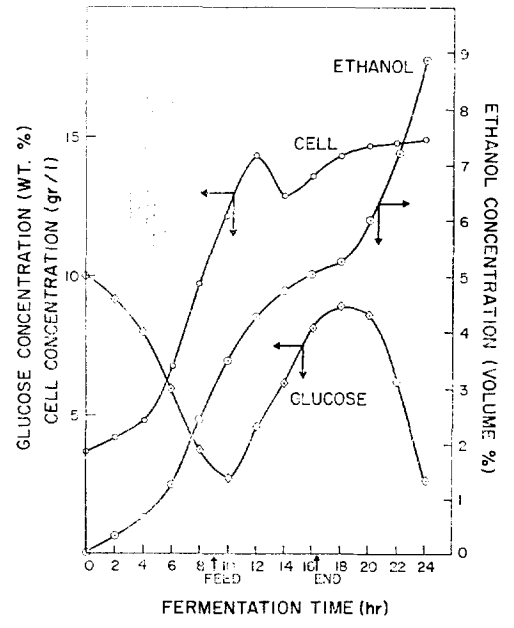

Fig. 10. Constant Feed Fed-batch Fermentation for Ethanol Production (Late Fed-batch)

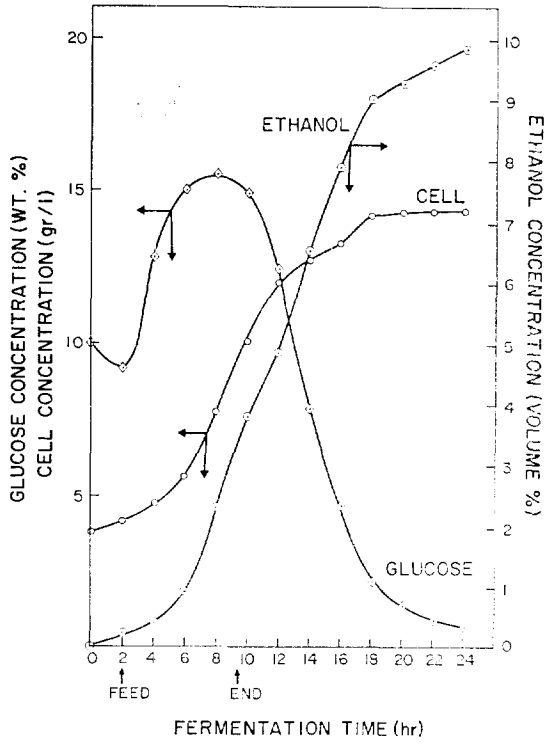

Fig. 11. Constant Feed Fed-batch Fermentation for Ethanol Production (Early Fed Fed-btach)

production rate, specific growth rate and feed rate. Upon the passage of 17 hrs from the start of run, μ became near zero, P reached P_m the feed is stopped. At this point, the system becomes batch type and the fermentor volume is about 7.5 *l*. Maximum cell concentration is obtained at 14 hrs and after 14 hrs the the dilution due to glucose feed overrides the cell growth, thus causing the decrease in cell concentration until the feed is stopped. Glucose feed rate increases to 0.25 l/hr at 11 hrs, and then decreases to zero at 17 hrs. The glucose concentration is maintained constant for 17 hrs and decreases to 28 g/l at 32 hr. After 32 hr, ethanol produced inhibits glucose conversion to ethanol completely and glucose cannot be utilized for ethanol production. In order to decrease the glucose remaining at the end of fermentation the glucose feed must be stopped at an earlier time and this time can be calculated from the above equations.

4. Fed-Batch Experiment with Constant Feed

Although many assumptions are made, the model described previously gives insight into the ethanol fermentation. However, controlling the feed rate in accordance with the results of modelling involves technical difficulties, and the fermentation with constant feed rate was performed as a preliminary step. In order to compare with simple batch fermentation, the total glucose added during the fementation was kept equal to that added initially to simple batch fermentation. *Fig. 10* shows the time course behaviour of constant feed fed-batch fermentation in which the feed (2.88 ml/min) was started at ninth hour and ended at 16.5 hr. During the initial 9 hrs, fermentation was performed in the batch type with initial glucose concentration of 100 g/l. In this case the final glucose concentration was relatively high, 15g/l,because the feeding was started too late. The variations of cell and glucose concentrations with time show the similarity to the transition phase from batch to contin uous fermentation. In *Fig. 11*, glucose feeding was started after 2 hrs which was considered the end of lag time and constant feed rate was maintained at 2.88 ml/min for 7.5 hrs. The glucose concentration increased during the period of feed and decreased rapidly. But cell and ethanol concentrations continuously increased as was the case with the batch fermentation in *Fig. 7* and the time required to reach the same level of ethanol concentration was almost the same. The fermentor volume, however, increased to 6.5 *l* and the productivity per cell inoculated increased by about 30%. This results hows that fed-batch system is a better one in ethanol fermentation than batch system in spite of the detrimental dilution effect by the added glucose solution causing the increase in fermentor volume. This means that glucose powder instead of solution can be utilized to prevent the dilution effect. This will reduce the final fermentor volume and the fermentation time necessary to arrive at the same final ethanol concentraton. This will certainly contribute to several beneficial results including the enhanced productivities per unit time and per unit volume basis as well as the saving in pumping cost. Also the feed rate corresponding to the simulation results derived in the previous section will further enhance the ethanol productivity.

V. Conclusion

Ethanol was produced through the batch

and fed-batch fermentation using **S. cerevisiae**. The flask fermentation showed the optimal glucose concentration to be 10%. With increase in the initial glucose concentration the lag period increased. The production of ethanol and cell biomass was higher until 6 hr with the initial glucose concentration of 10% compared with higher initial glucose concentration. A successful computer simulation was also carried cut. The productivity of fed-batch fermentaction per cell biomass inculated was at least 30% higher than that of batch fermentation.

Adnowledcement

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NOMENCLAURE

X cell concentration in the fermentor (g/l)
S glucose concentrationin the fermentor (g/l)
P ethanol concentration in the fermentor (g/l)
V fermentor volume (l)
F feed rate of glucose solution (l/hr)
S_F glucose concentration in the feed (g/l)
μ specific growth rate of cell (hr^{-1})
ν specific ethanol production rate (hr^{-1})
$Y_{P'S}$ yield coefficient on glucose (g ethanol/g glucose)
μ_m maximum specific growth rate (hr^{-1})
μ_{ms} maximum specific growth rate at given glucose concentration (hr^{-1})
ν_m maximum specific ethanol production rate (hr^{-1})
ν_{ms} maximum specific ethanol production rate at given glucose concentration (hr^{-1})
P_m maximum ethanol concentration above which cells do not grow (g/l)
P'_m maximum ethanol concentration above which cells do not produce ethanol (g/l)
K_S saturation costant for growth (g/l)
K'_S saturarion ccnstant fcr product formation
W degree of saturation inhibition for growth
W' degree of saturation inhibition for product formation

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