A STUDY ON THE ETHANOL PRODUCTION BY IMMOBILIZED CELLS OF *ZYMOMONAS MOBILIS*

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Abstract—Cells of *Zymomonas mobilis* immobilized in Ca-alginate matrix were used for ethanol production under various conditions.

Immobilized cells showed broad optimum pH profile and their operational optimum temperature shifted from 30°C to 40°C upon immobilization. As reportedly *Z. mobilis* did get the substrate inhibition by glucose, but at high concentration level of glucose the reduction of activity for ethanol production was less severe than that for yeast.

The used beads of the immobilized *Z. mobilis* were reactivated by incubating them in the activation medium. The increase in cell number and the enhancement of the specific activity per each cell are considered the two major factors responsible for the overall activation.

A packed-bed reactor with the feed glucose concentration of 20% (W/V) gave an ethanol productivity as high as 33.0 g/l.hr at the flow rate of 58.5 ml/hr.

A comparison between the experimental results from the real packed-bed reactor and the simulation results of an ideal case showed a two-fold inferior performance by the real reactor and this is at least partly attributed to the CO_2 gas effect.

INTRODUCTION

The hope for the energy production from the renewable resources has culminated in the upsurge of the research interest in the ethanol fermentation, and especially techniques such as recycle fermentation[1,2], rapid fermentation[3], immobilized yeast fermentation[4-6] and vacuum fermentation[1] have been devised to improve the overall economics of the process.

More recently another ethanol producing bacterium, the anaerobe *Z. mobilis*, began to get a new attention[7-12] due to its promising levels of the specific glucose uptake rate[9,10] and the specific ethanol productivities, and also due to its desirable immunity to high glucose concentration.

In order for the continuous fermentative production of ethanol as a fuel[13,14,21,26] or a chemical feedstock to be of practical significance, the following criteria must be met:

(1) high conversion of substrate to ethanol

(2) high concentration of ethanol in the product stream

(3) high productivity

The criteria outlined above mostly depend on cell concentration and ethanol toxicity[3,15,16]. A number of different strategies on yeast fermentation have been tested in an effort to overcome these limitations. As one candidate to cope with these criteria the immobilized-cell method[22-24] has been widely practiced because of its desirable characteristics:

(1) high cell density

(2) extraction and purification of enzyme is unnecessary

(3) immobilized whole-cells maintain the multienzyme system stable.

In many cases these merits override some inherent drawbacks such as the difficulty of keeping the integrity of cells, the loss of enzyme activity in nongrowing cells, side reactions, and the mass transfer limitations. In the ethanol fermentation with the immobilized whole-cell technique the CO_2 gas accompanying the production of ethanol is known to have an adverse effect on the overall reactor performance[17].

In this study *Z. mobilis* cells are immobilized in the Ca-alginate gel matrix[4,7,18] in order to have a still closer look at the CO_2 gas effect as well as the basic reaction kinetics of this immobilized system and also to

make a more specific comparison with a best possible situation as a standard.

A similar study using the immobilized yeast cells had already been published [29].

MATERIALS AND METHODS

Culture and Media

Z. mobilis ATCC 10988 was cultured at 30°C in liquid medium[9] containing 10% glucose, 1% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 7H_2O$. For ethanol production studies in the immobilized cell bioreactors, the only ingredients in the aqueous solution were glucose and 10mM $CaCl_2$ as gel stabilizer.

Assay Methods

Ethanol: The modified enzymatic method]19] was used to determine the ethanol concentration. 3ml of standard ethanol assay buffer at pH 8.7 (75mM semicarbazide, 21mM glycine), 0.1ml of 24mM NAD and 0.1ml of alcohol dehydrogenase (30 units/test) were added to 0.1ml of diluted ethanol sample and then the mixture was incubated at 35°C for 40 minutes. In the presence of alcohol dehydrogenase, ethanol reacting with NAD gives rise to the production of NADH and the absorbance at 340nm is measured.

Glucose: Glucose concentration was determined by the dinitrosalicylic acid method[20]. 3ml of dinitrosalicylic acid reagent (dinitrosalicylic acid 10%, phenol 0.2%, sodium bisulfite 0.05%, NaOH 1.0%) was added to 2ml of diluted glucose sample, the mixture was heated in the boiling water bath for 10 minutes, cooled under running tap water, and the absorbance was observed at 575nm.

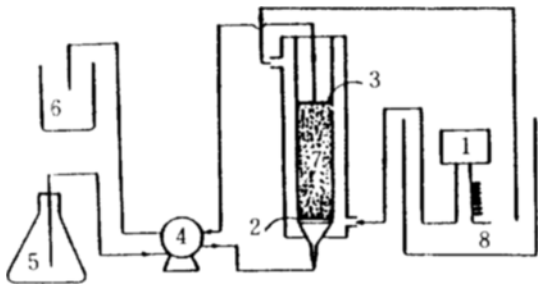

Fig. 1. A detailed configuration of immobilized *Z. mobilis* packed bed reactor

1. Thermostat
2. Supporter (sintered glass filter)
3. Supporter (stainless steel sieve)
4. Peristaltic pump 5. Substrate reservoir
6. Product reservoir 7. Immobilized cell
8. Water bath

Apparatus

Cylindrical glass column with the glass filter bottom as catalyst support was prepared for packed bed reactor operation in a constant temperature bath. Substrate was pumped into the bottom of the column using a peristaltic pump, the detailed configuration of which is shown in Fig. 1.

Immobilization Procedure[18]

100ml of 4% (W/V) sodium alginate solution was carefully mixed with 100ml of a suspension of *Z. mobilis* cells (100g wet wt./100ml) in water. The *Z. mobilis* cell mixture was extruded from the hypodermic needle attached to a syringe into a stirred 0.1M calcium chloride solution and bead diameter was adjusted to 2.5-3.3mm (Fig. 2).

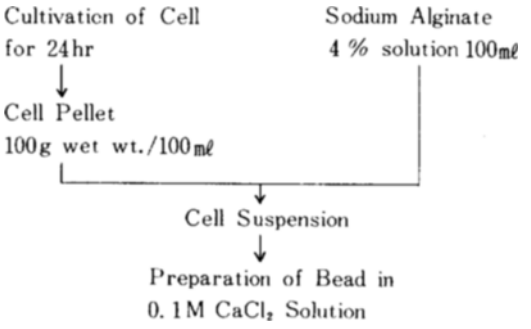

Fig. 2. Immobilization procedure

Determination of the Activity of Immobilized *Z. mobilis*

200 beads were reacted with 25ml of substrate mixture containing 10% (W/V) glucose, 10mM calcium chloride and 50mM succinate buffer, pH 5.0, at 30°C for 45 min. in the batch system so that the conversion is held under 50%. 0.1ml of substrate mixture was sampled every 15 minutes. The activity of the immobilized *Z. mobilis* bead was determined by measuring the initial alcohol production rate.

Counting the Number of Cells in the Immobilized Beads

10 beads were dissolved in 15ml of 0.1M Sørensen's citrate buffer, pH 5.5, for 2 hours and released cells were counted by the hemacytometer.

Achieving the Steady State

In a continuous system, it is important to check for every run whether a steady state is attained at various concentrations of substrate and flow rates. The effluent product concentration was determined at various time intervals. At steady state the product concentration would stay constant as far as the inactivation of immobilized Zymomonas cells does not occur. The conversion was determined at this steady state.

Effect of pH

For the study of pH effect, different buffers were used for various ranges of pH; 50mM glycine-HCl buffer for pH 3.0, 50mM succinate buffer for pH 4.5, 50mM malate buffer for pH 6.0 and 50mM tris buffer for pH 7.0. The effect of pH on immobilized *Zymomonas* cells was determined by measuring the initial rate of ethanol production in a batch reactor.

Effect of Temperature

The activity of Zymomonas cells was determined between 15°C and 45°C by measuring the initial rate of ethanol production in a batch reactor. For this purpose glucose concentration was kept at 10% (W/V) in 50mM succinate buffer solution, pH 5.0.

Effect of Substrate Concentration

Using a batch reactor the effect of glucose concentration in the range of 0.5%-25% on immobilized Zymomonas cells was determined by measuring the initial rate of ethanol production in 50mM succinate buffer solution, pH 5.0, at 30°C.

Activation of Immobilized *Z. mobilis* cells

The activity of the immobilized cells gradually declined when they were used in the reaction solution containing glucose alone. The immobilized cells with declined activity were reactivated[4,25] by incubating in the medium containing growth factors. A constant number of beads and a given quantity of reaction solution were sampled at each of the regular time intervals while maintaining a constant ratio of beads to the medium so that the relative ratio of the components of the reaction volume can remain constant. After washing the sampled beads with water, the initial rate of ethanol production was examined and cell numbers were counted for those beads.

Operation of Packed-bed Reactor

As the substrate solution containing 10% (W/V) glucose as the only active ingredient was fed upward through the reactor, the immobilized cell did not grow and glucose uptake was used for the production of ethanol only. In the packed-bed reactor (3cmI.D.×6cm), the effects of flow rate and glucose concentration on the ethanol productivity were examined.

Modeling and Simulation

During the operation of a packed-bed column reactor, the CO_2 gas diffusing out from the inside of the bead adversely affects the reactor performance[17] and the following equations were derived to investigate the CO_2 gas effect. The ethanol production by *Z. mobilis* is known to have the following stoichiometry[28]:

1 Glucose $\longrightarrow$ 1.8 Ethanol + 1.9 CO_2 + 0.15 Lactic acid

This reaction was assumed to have a multi-enzyme system and follow the Michaelis-Menten type kinetics.

$$r = -\frac{ds}{dt} = \frac{dp}{1.8\,dt} = \frac{Vm.\,S}{Km + S + S^2/Kis} \tag{1}$$

In eqn.(1), the product inhibition term is not included, because the specific ethanol production rate was not so sensitive to the ethanol concentration up to 5% (W/V)[10]. In the case where neither the CO_2 gas is generated nor the mass transfer resistance affects the reactor performance, the plug flow reactor performance equation for this ideal situation becomes

$$\tau = \int_{s_o}^{s} \frac{ds}{-r \cdot N} \tag{2}$$

Integrating the eqn. (2), one obtains:

$$\tau = \frac{1}{N \cdot Vm}\left\{(Km.\ln S_o/S) + (S_o - S) + \frac{1}{2Ki_s}(S_o^2 - S^2)\right\} \tag{3}$$

A batch reaction under vigorous stirring was used to determine the kinetic parameters in the absence of the CO_2 gas bubble formation and these parameters were used to calculate the glucose conversion at various residence times by computer as a standard indication of the best possible reactor performance for this idealized case. Data used in the simulation are shown in Table 1. The glucose conversions obtained from experiment and simulation were compared in Fig. 10.

Table 1. Parameter Values Used for Simulation

K_m (Michaelis constant)	:	41 mM
Ki_s (substrate inhibition constant)	:	10.0 M
S (initial feed concentration)	:	0.555 M
V_m (maximum velocity)	:	9.6 (umole/bead.hr)
N (number of beads/unit volume of reactor)	:	21450 (beads/l)
ε (voidage)	:	0.41

RESULTS AND DISCUSSION

Effect of pH

As shown in Fig. 3, immobilized cells had the optimum pH around 6. The pH-activity profile was broad indicating that the activity was barely influenced by pH of the external medium. In the case of alginate gel, internal diffusional limitations can probably explain the broad activity-pH profile[30] and it was also reported that diffusion[18] becomes the rate determining step which was unaffected when a higher sucrose concentration or higher alginate concentration was used. It is also known that the proteinous support such as the living cell can be quite insensitive to external pH change due to the buffering effect of proteins, which can apparently result in a broad pH-activity profile[31].

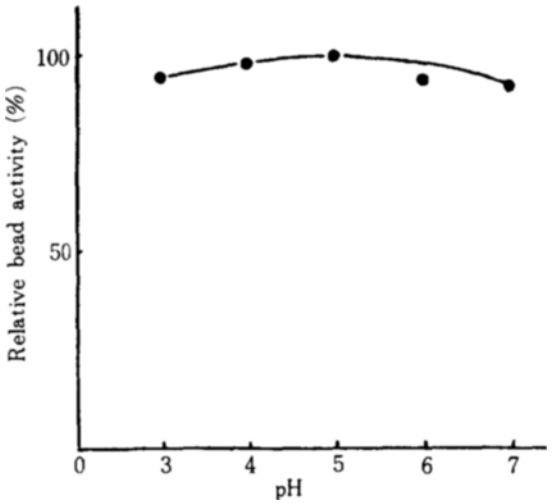

Fig. 3. Relative bead activity profile at various pH. Different buffer components were used for different ranges of pH, i.e. 50mM glycine- HCl for pH 3.0, 50mM succinate for pH 4.0 and 5.0, 50mM malate for pH 6.0 and 50mM tris for pH 7.0.

Effect of Temperature

The temperature-activity profile in Fig. 4 shows that the immobilized *Z. mobilis* cells have the highest activity around 40°C. The result suggests that optimum temperature of immobilized cells have shifted to a level higher than that of free cell by about 10°C and the immobilized cell kept the relatively high activity up to the temperature of 45°C. The effect of temperature on fermentation was plotted according to the Arrhenius equation:

$$\ln r = \ln A - \frac{Ea}{RT} \qquad (4)$$

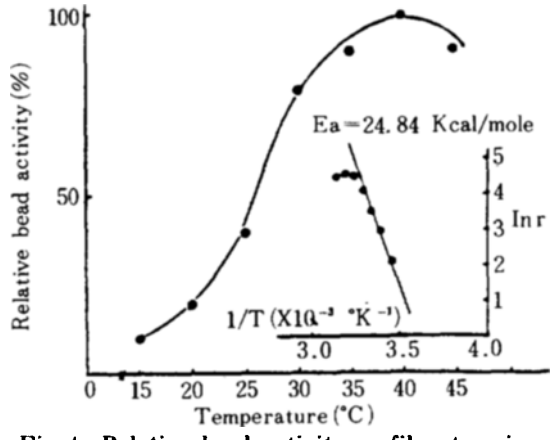

Fig. 4. Relative bead activity profile at various temperature

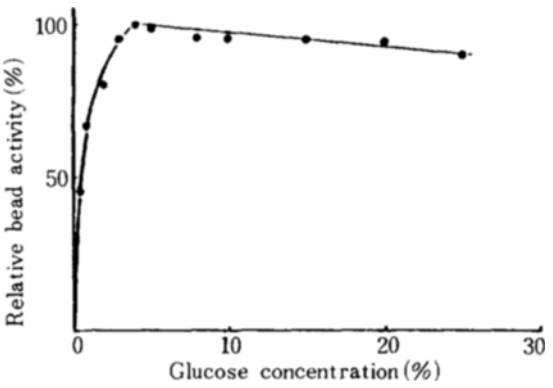

Fig. 5. Effect of substrate concentration on bead activity

The activation energy Ea of immobilized *Z. mobilis* cells was calculated to be 24.84 Kcal/mole in the low temperature range.

Effect of Substrate Concentration

The Michaelis-Menten constant, the maximum reaction rate and the substrate inhibition constant were examined in a stirred batch reactor. In Fig. 5 one sees the substrate inhibition by glucose at the concentration level higher than 8% (W/V). However the extent of the substrate inhibition is rather meager and the immobilized cells retained 90% of their maximum activity at quite a high value of the glucose concentration.

From the Lineweaver-Burk plot, the values of Km, Vm and Kis are found to be about 41mM, 9.6μmole/bead.hr and 10.0M respectively. Our results demonstrate that in the case of the immobilized *Z. mobilis* cells, the effect of substrate inhibition may be neglected. (Fig. 6,7)

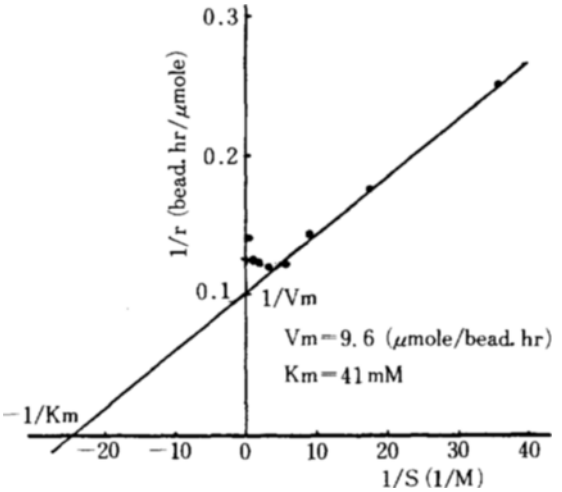

Fig. 6. Lineweaver-Burk plot for K_m value

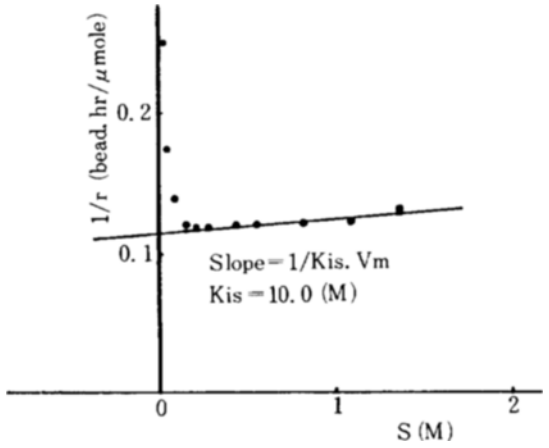

Fig. 7. A plot of 1/r vs. S to determine the substrate inhibition constant

Activation of Immobilized *Z. mobilis* cells

Immobilized microorganisms can grow in the medium containing nutrients for the cell growth. The cofactor potential and the activity of enzymes participating in the ethanol fermentation inside the cells can also be increased. These all contribute to the high activity of the immobilized cells. As shown in Fig. 8 such a steady increase in activity was observed with incubation in the presence of nutrients. After 8 hours, the activity became gradually declined. It was considered that cells in the matrix were confined and were autolysed within the gel, or they leaked out into the broth. With increase in the overall activity, the number of living cells also got increased. However the relative bead activity increased more than the number of living cells did. Our data showed that not only the cell number but also the specific activity per cell increased during activation. If we accept the observation that specific activity of immobilized cell increases, then the cell activation may further be related to the generation or the activity increase of many enzymes and cofactors such as ATP and NADP.

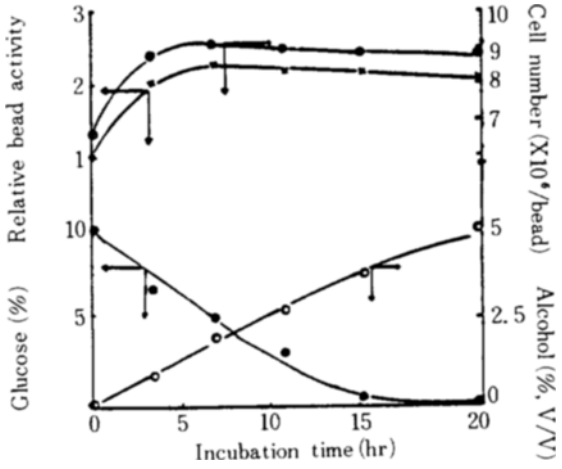

Fig. 8. Time course behavior of *Z. mobilis* bead activation in the the medium

Operation of Packed-bed Reactor

As shown in Fig. 9, the flow rate increase accompanies the increase in the ethanol productivity in the packed-bed reactor. The productivity with 10% (W/V) glucose feed reached as high as 26.3 gr/l.hr at 58.5ml/hr of flow rate, the maximum flow rate that we employed. In the case of 20% (W/V) glucose solution, the productivity reached 33.0gr/l.hr at 58.5ml/hr, which showed that high glucose concentration was more advantageous than low glucose concentration for ethanol production in a packed-bed reactor, but with sacrifice in conversion

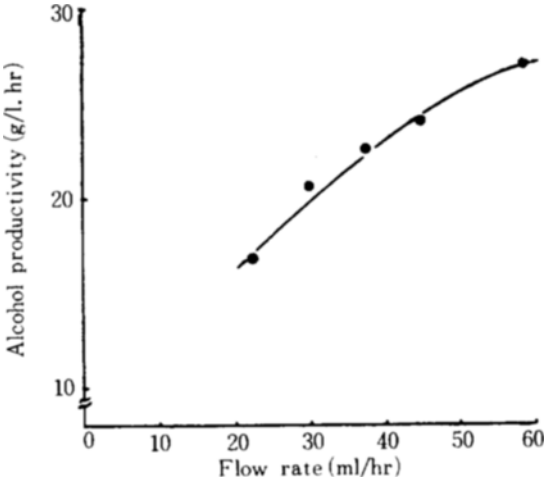

Fig. 9. Effect of flow rate on alcohol productivity in the packed bed column reactor(3cm × 6cm) with 10% glucose feed concentration

Table 2. Effect of glucose concentration

a) Effect on alcohol productivity

Conc. (%)	22.5 (ml/hr)	58.5 (ml/hr)
10	16.9 (g/l.hr)	26.3 (g/l.hr)
20	25.4 (g/l.hr)	33.0 (g/l.hr)

b) Effect on conversion

Conc. (%)	22.5 (ml/hr)	58.5 (ml/hr)
10	91(%)	50(%)
20	62(%)	34(%)

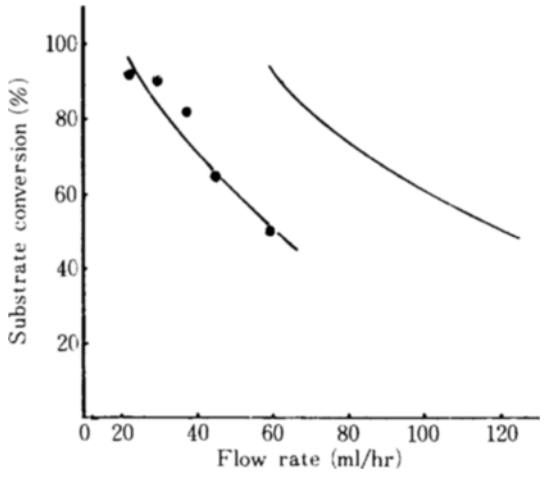

Fig. 10. Experimental data and simulation results for an ideal case for the conversion of the glucose to ethanol at various flow rates in packed bed column reactor with dimension of 3cm inside diameter and 6cm bed height

level, e.g. 90% and 50% conversions at 10% glucose concentration, and 60% and 30% conversions at 20% glucose concentration (Table 2). From an economic standpoint one may have to make compromise among several economic factors such as the glucose cost, the equipment cost and the desired ethanol concentration level. In order to see to which extent the CO_2 gas generated *in situ* in the packed column affects the overall reactor performance, the simulation result from eqn. (3) for the idealized reactor without the CO_2 gas effect is compared with the experimental result in Fig. 10. One can see a very adverse effect of CO_2 gas in the packed-bed reactor. Similar results had been reported on yeast by one of the authors[29]. The reduction in ethanol productivity can be explained in several ways. If the CO_2 gas diffusion[17] is slow compared with CO_2 production, the CO_2 gas will be accumulated in the gel matrix. The product diffusion in the gel becomes slower and the reaction rate will decrease. Due to the CO_2 gas remaining in the reactor as bubbles, the contact area between the substrate and the catalyst surface as well as the effective reactor volume may decrease causing the increase in the actual linear velocity of the substrate solution. This increase in linear velocity may lessen the external mass transfer resistance to some extent. However it is clear that CO_2 gas is involved in many of these factors and the net result has turned out to be a rather adverse one as our experimental and theoretical data indicate.

CONCLUSION

The *Z. mobilis* cells (ATCC 10988) were immobilized successfully by entrappment in the Ca-alginate gel. The average size of resulting bead was in the range of 2.5 to 3.3mm.

The optimum temperature of immobilized Zymomonas cells was around 40°C, a shift of 10°C above the optimum temperature for free cell. The activation energy calculated by Arrhenius plot was 24.84 Kcal/mole in the low temperature range.

Although the substrate inhibition phenomena appeared above 8% (W/v) of glucose concentration, its effect was negligible. The values of Km, Vm and K_{is} obtained by Lineweaver-Burk plot were 41mM, 9.6μmole/bead.hr and 10.0M respectively.

The immobilized Zymomonas cells had a broad activity-pH profile that was barely influenced by the pH in the outer medium with optimum level around pH 5 similar to the case of free cell.

The immobilized *Z. mobilis* was so unstable that a reactivation for about 24 hours was carried out to give about twice the original activity. This activation effect can be ascribed to the increase in the number of cells and the increase in the specific activity per cell.

In order to quantitatively characterize the CO_2 gas problem in the packed-bed reactor, the ideal system modeling and simulation was carried out. The comparison between the simulation result and the real reactor performance shows as high as 50% decrease in the overall reactor performance as indicated by the difference in conversions at the flow rate of 60ml/hr. In the packed-bed reactor, high glucose concentration (20%) was more advantageous than low glucose concentration (10%) when one is concerned about the ethanol production alone at the expense of conversion and the increase in the flow rate resulted in enhanced productivity as one knows from a usual type of reactor analysis.

NOMENCLATURE

S_0 : initial concentration of glucose (M)
S : concentration of glucose (M)
V_m : maximum reaction velocity (umole/hr.bead)
K_m : Michaelis Menten constant (M)
K_{is} : substrate inhibition constant (M)
ε : void fraction, dimensionless
τ : residence time (sec)
N : number of beads per unit volume of reactor (bead/l)
r : reaction velocity per each bead (μmole/bead.hr)
A : a constant in the activation energy equation
R : gas constant
T : absolute temperature (°K)

E_a : activation energy (Kcal/mole)

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