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A New Kinetic Approach to the Fermentation of Multisubstrate Complex Media

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ABSTRACT

Ethanol production from natural complex media has been studied in this work. A new kinetic approach is presented for two-substrate media, such as hardwood hemicellulose hydrolysate, which predominantly consists of a mixture of xylose and glucose. It has been founded on the supposition that the whole ethanol production and biomass growth can be subdivided into two separated components imputable to glucose and xylose consumptions, respectively. A model describing the continuous fermentation in CSTR by *Pachysolen tannophilus* has been also presented, and experimentally verified; it takes into account the different substrate consumption rates of these sugars contained in both natural and synthetic complex media.

Index Entries: Hemicellulose hydrolysate; batch and continuous fermentations; multisubstrate kinetics; *Pachysolen tannophilus*.

NOMENCLATURE

P	ethanol concentration, g/L
S	substrate concentration, g/L
Xyl	xylose concentration, g/L
Glu	glucose concentration, g/L
Pen	pentose concentration, g/L
Hex	hexose concentration, g/L

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X	cell mass concentration, g/L
I	inhibitor concentration, g/L
t	time, h
v	specific productivity, $g_P/g_X h$
μ	specific growth rate, h^{-1}
K_s	saturation constant of Monod equation, g_S/L
K_I	inhibition constant, g/L
$Y_{P/S}$	product yield, g_P/g_S
r	specific rate of substrate consumption, $g_S/g_X h$
D	dilution rate, h^{-1}

SUBSCRIPTS

°	initial values
max	maximum values
P	values referred to the product
X	values referred to the biomass
S	values referred to the substrate
1	values referred to glucose or hexoses
2	values referred to xylose or pentoses

INTRODUCTION

The kinetic study of fermentations through the analysis of the variations of specific kinetic parameters is usually carried out for both natural and synthetic single-substrate media.

However, in the case of multisubstrate complex media, such as hardwood hemicellulose hydrolysate, all the efforts to pursue such an aim end in failure, mainly because of the difficulty in obtaining hydrolysates having exactly the same composition, in consequence of the inevitable unconstancy of the conditions during the acid hydrolysis. This problem has been overwhelmed in this study by using the same batch of hemicellulose hydrolyzate for all the runs considered for calculating the kinetic parameters.

A further cause of failure of the traditional kinetic approaches in the study of complex media is the simultaneous presence of more than one substrate that are consumed at the same time. In this particular case, glucose and xylose are fermented separately with different reaction rates.

The experimental data previously obtained using hardwood hemicellulose hydrolysate (*1*) have been compared with those obtained in this work using both complex and pure synthetic media, which allowed us to evaluate variations of the kinetic parameters ascribable to the presence of inhibiting substances, such as furfural, hydroxymethylfurfural (*2*), their precursors, and phenolic compounds (*3*).

The new kinetic approach presented in this paper enables one to predict ethanol productivity from hemicellulose hydrolysate in CSTR, with a reasonable agreement between calculated and experimental values.

MATERIALS AND METHODS

Synthetic Media

Three synthetic media have been prepared for studying the conversion rates of different sugars to ethanol by *Pachysolen tannophilus* in the presence of no inhibiting substance. They contained glucose 12.5 g/L, xylose 40.0 g/L, or both as carbon sources, and the following amounts of additional nutrients: 0.2% urea (w/v), 0.2% yeast extract (w/v), and 0.05% KH_2PO_4 (w/v).

Natural Medium

The hemicellulose hydrolysate used in this study, kindly supplied by Tennessee Valley Authority (T.V.A.), has been prepared from wood chips, predominantly oak, according to the procedure of Beck and Strickland (*4*), pretreated with heat (100°C), lime (up to pH 10.0), and sulfite (0.1% v/v), and finally supplemented with 0.2% urea (w/v), 0.2% yeast extract (w/v), and 0.05% KH_2PO_4 (w/v). Some additional operations with respect to the original T.V.A. procedure have been successfully proposed in a previous work (*1*) to improve fermentation efficiency: in particular, the nutrient solution has been warm-filtered after overliming, was not boiled after Na_2SO_3 addition, and finally filtered through 0.45 μm filters. Final hemicellulose hydrolysate composition has been reported in Table 1.

Although this substrate consists of several pentoses and hexoses, the fermentation behaviors are so uniform within each class of these sugars that hemicellulose hydrolysate has been assumed in this work as a mixture of two uniform classes. For this reason, reference will be made in the text simply to pentoses and hexoses rather than each single sugar. This assumption has been successfully made by the authors in a previous work (*1*) and by Beck and Strickland (*4*) when hemicellulose hydrolysates particularly rich in pentoses were tested.

Microorganism

The strain of *Pachysolen tannophilus* (NRRL Y 2460) was maintained on agar slants containing 0.1% (w/v) yeast extract and 50.0% (v/v) hemicellulose hydrolysate. The cells were grown aerobically at 32°C for 72 h on a medium containing 50.0 g/L D-xylose, 5.0 g/L KH_2PO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$, using loosely capped 250 mL Erlenmeyer flasks on an orbital shaker set at 100 rpm. After separation from the growth media

Table 1
Average Composition of Hardwood Hemicellulose Hydrolysate

Component	Concentration (g/l)
Xylose	43.5
Glucose	9.0
Galactose	3.3
Arabinose	2.9
Mannose	3.4
Acetic acid	10.9
Furfural	0.3
Hydroxymethylfurfural	0.9
Minerals:	
Cr	0.002
Ni	0.003
Fe	0.038
Mn	0.014
Density	1,024

by centrifugation, the cells were washed twice and resuspended in sterile water to give the selected yeast density of 1.0 g/L (dry wt) before the addition to the hydrolysate.

Fermentation Conditions and Experimental Set-up

A 5.0-L Gallenkamp FBL-195 chemostat with working volume of 3.0 L, stirred at 300 rpm, was employed for both batch and continuous fermentations. Microaerophilic conditions were maintained during all runs by covering the gas output of the fermentor with sterile cotton caps. The pH of the fermentation broth was automatically regulated, to an accuracy of 0.1 pH units, by a pH control module FBL-725, provided with a peristaltic pump that injected a fine stream of 1*N* NaOH solution. The temperature was kept at a constant value of 32 ± 0.5°C by a temperature control module FBL-360. The fermentor and the media were sterilized by autoclaving at 120°C for 20 min, unless specified otherwise.

Analytical Measurements

The hydrolysate sugar consumption during the fermentations has been followed using a HPLC, Type Waters ALC 201 with R.I. detector. A Bio-Rad HPX-42 column was used with 70:30 acetonitrile/water as the mobile phase at a flow rate of 0.8 mL/min.

Ethanol concentration was measured by a gas chromatograph (Fractovap model C Type ATC/t Carlo Erba Milano), with a column packed with Cromosorb W coated with Carbowax 1500. The column was kept at 187 mA. Helium at 1.5 atm was used as a carrier gas. The gas chromatograph was calibrated several times during each run by means of standard ethanol–water solutions.

The cell concentration was determined by filtering a known volume of culture broth on 0.45-μm autoclavable filters. The filters were dried at 105°C until no weight change between consecutive measurements was observed. In order to obtain exactly the selected starting biomass concentration, a turbidimetric assay described in details in a previous work (*5*) has been utilized, using a Bausch & Lomb colorimeter at 595 nm wavelength.

RESULTS AND DISCUSSION

The Traditional Approach

As mentioned above, it is not possible to carry out, for multisubstrate complex media, a conventional kinetic study referring to only one substrate through the well-known Monod equation (*6*):

$$v = v_{max} S/(K_s + S) \quad (1)$$

where K_s is the saturation constant while v and v_{max} are the specific ethanol productivity and its maximum value, respectively.

Furthermore, the modified equation proposed to take into account eventual factors of competitive inhibition:

$$v = v_{max} S/[K_s (1 + I/K_I) + S] \quad (2)$$

has been demonstrated to be valid only in the case of whole cell reactions where the product behaves as a competitive inhibitor (*7–9*).

In the presence of only one substrate, the kinetic constants (v_{max} and K_s) can be calculated by plotting $1/v$ vs $1/S$, according to Lineweaver-Burk (*10,11*). In the particular case of simultaneous presence of two substrates, on the contrary, this graphical extrapolation cannot be utilized because of the influence of each sugar on the biodegradation of the other, and of the different fermentation rates.

As a consequence, the kinetic behaviors of both degradations cannot be described by a sole equation containing global process parameters, whose physical meaning would be, what's more, unlikely explainable. Furthermore, if the total sugar concentration would be used in Monod equation, the presence of high ethanol levels related to the simultaneous conversion of both sugars at the beginning of the fermentation would not allow us to calculate v_{max} as a result of the absence of the lag phase.

The New Kinetic Approach

Synthetic Media

Owing to the above difficulties, ethanol curve has been subdivided into two independent curves related to the consumptions of glucose and xylose, respectively.

In order to know which fractions of alcohol are related to the degradation of each substrate, batch fermentations of three synthetic media, containing 40.0 g/L xylose, 12.5 g/L glucose or both, respectively, have been carried out to ascertain whether the simultaneous presence of two substrates in the same medium (as in the complex medium under consideration) could, anyhow, influence the fermentation kinetics on the whole. The mixed synthetic medium simulates the composition of hemicellulose hydrolysate, but in the presence of neither inhibiting or stimulating factors. The experimental values of these batch fermentations, listed in Table 2 together with those previously obtained using hemicellulose hydrolysate (*1*), show that the two-substrate medium yielded ethanol levels in satisfactory agreement with the algebraic sum of those obtained with single-substrate media, but the fermentation appears to be quicker than in the presence of pure xylose. This suggests the existence of an activating role of glucose in the fermentation of xylose, which has been early observed by Beck and Strickland (*12*) for hydrolysates containing predominantly glucose, and by Jeffries et al. when glucose was added in small doses to xylose (*13*). These observations were subsequently confirmed by us (*1,14*) also in the case of hemicellulose fraction constituted by predominantly xylose.

A more rigorous approach to the problem could imply the supposition of a similar stimulation of glucose conversion to ethanol by xylose. The existence of this effect, never observed up to now, will be confirmed later on by comparing the starting specific productivities (v_o) listed in Table 3.

Since the fermentation yields were not significantly influenced by the simultaneous presence of more than one substrate, the same value of $Y_{P/S}$ obtained with pure glucose has also been hypothesized in the mixed nutrient. The remaining fraction of alcohol produced has then been related to the biodegradation of xylose, thus allowing us to separate the two curves imputable to each production.

The values of cell mass concentration observed for the above three fermentations show that cell growth in two-substrate media is lower than the algebraic sum of the curves obtained in the presence of single-substrate media. This behavior in the complex medium is likely the result of the repressing action of increased ethanol levels on the biomass growth with respect to the single-substrate media, that is in agreement with previous observations of other workers (*7–9*). As a consequence, the calculation of separate specific productivities from glucose and xylose in complex media requires the use of a correction factor. As an example, it assumes a value of 1.18 for the two-substrate synthetic medium, which

Table 2
Experimental Results of Batch Fermentations of Both Synthetic and Natural Media

				Xylose 40.0 g/l			
t (h)	0	12.0	24.0	36.0	48.0	96.0	144.0
P (g/l)	0	0.56	1.44	2.70	4.40	11.50	12.60
Xyl (g/l)	40.0	38.3	35.6	31.8	26.5	5.0	0.1
X (g/l)	1.0	1.5	2.3	3.4	4.5	5.50	5.60
				Glucose 12.5 g/l			
t (h)	0	1.5	3.0	6.0	12.0	24.0	48.0
P (g/l)	0	0.34	0.75	1.90	3.65	4.05	3.20
Glu (g/l)	12.5	11.5	10.2	6.7	1.2	0	0
X (g/l)	1.0	1.3	1.6	2.5	3.9	4.5	4.0
				Xylose 40.0 g/l + Glucose 12.5 g/l			
t(h)	0	3.0	9.0	24.0	36.0	48.0	72.0	120.0
P (g/l)	0	1.02	4.19	7.13	9.88	12.23	15.20	14.85
Xyl (g/l)	40.0	39.2	37.0	29.5	21.6	13.7	1.6	0
Glu (g/l)	12.5	10.2	3.35	0	0	0	0	0
X (g/l)	1.0	1.3	2.2	4.8	5.9	6.4	6.5	5.5
				Hardwood Hemicellulose Hydrolysate (ref. 1)				
t (h)	0	24.0	48.0	72.0	96.0	120.0	144.0	168.0
P (g/l)	0	5.9	9.5	13.5	16.6	18.0	17.9	14.7
Pen (g/l)	46.0	37.8	26.8	16.9	9.8	3.2	2.6	1.7
Hex (g/l)	15.6	3.1	1.9	1.5	0.3	0	0	0
X (g/l)	1.0	2.9	4.8	5.2	5.2	5.1	4.7	3.8

P = Product concentration
Xyl = Xylose concentration
Pen = Pentose concentration
Glu = Glucose concentration
X = Biomass concentration
Hex = Hexose concentration

Table 3
Comparison of the Kinetic Parameters Calculated
for Both Synthetic and Natural Substrates Through Batch Fermentations

SUBSTRATE	SYNTHETIC				NATURAL	
TYPE	PURE		MIXED		HYDROLYSATE	
SUGARS	GLUCOSE	XYLOSE	GLUCOSE	XYLOSE	HEXOSES	PENTOSES
S_o (g/l)	12.5	40.0	12.5	40.0	10.1	43.5
X_o (g/l)	1.0	1.0	0.5	0.5	0.5	0.5
v_o (g/gh)	0.214	0.039	0.437	0.196	0.360	0.142
μ_o (h^{-1})	0.160	0.034	0.101	0.030	0.133	0.024

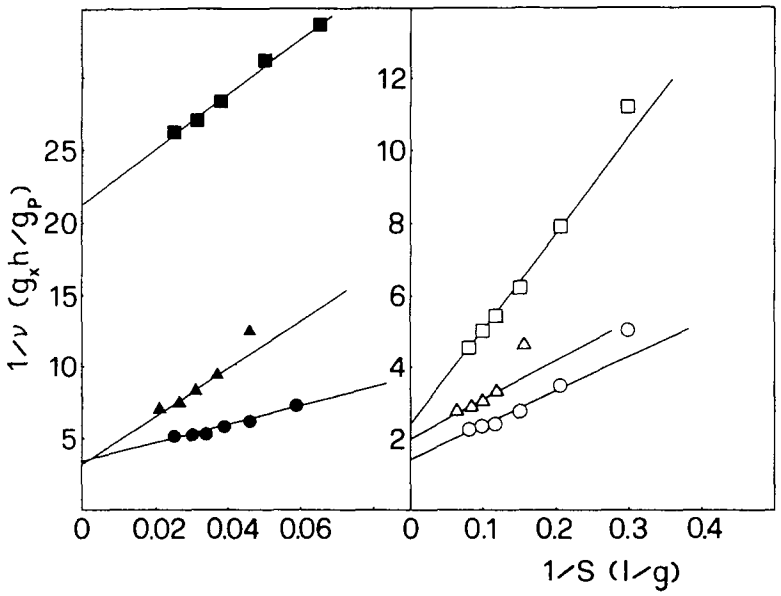

Fig. 1. Lineweaver-Burk plots for the calculation of maximum specific productivity for both natural and synthetic substrates. Pure substrates: (□) glucose; (■) xylose. Mixed synthetic substrate: (○) glucose; (●) xylose. Hemicellulose hydrolysate: (△) hexoses; (▲) pentoses.

means that both separated experimental curves of biomass have to be reduced by 18% for the calculation of the respective specific productivities in this medium.

In order to give confirmation of the above suggestions, a kinetic study has been carried out using the data of Table 2 and calculating the product yields for pure substrates by means of the well-known equation:

$$dP = Y_{P/S}\, dS \tag{3}$$

whereas more different product yields have been supposed for the complex media:

$$dP = Y_{P/S1}\, dS_1 + Y_{P/S2}\, dS_2 + \ldots + Y_{P/Sn}\, dS_n \tag{4}$$

The subdivision of the fermentation curves into two components allowed us to calculate the values of v_{max} and μ_{max} in reference to each substrate, as follows: by plotting in Fig. 1 the respective values of $1/v$ vs $1/S$ according to Lineweaver-Burk (*10,11*), one or two straight lines have been obtained for each pure or mixed substrate, respectively, whose slopes and intercepts on the ordinate axis allowed the calculation of the values of the maximum productivity (v_{max}) and the saturation constant (K_s), extrapolated for an infinite value of the substrate concentration.

Since v_{max} is a purely theoretical parameter, it has no practical significance, therefore, we have preferred, for kinetic purposes, to consider the

values of v_o calculated at the beginning of each fermentation, where the substrate concentration is equal to the feed value (S_o). Decreases or increases of v_o can be imputed to the presence of inhibitions of the noncompetitive nature or activation phenomena, respectively. The same considerations can be made also for the maximum values of the specific growth rate (μ_{max}), calculated by deriving the curves of the cell density vs the fermentation time. The values of these kinetic parameters, calculated at the beginning phase of each fermentation for both synthetic and natural media, have been listed in Table 3.

A comparison of v_o values shows that ethanol production form xylose in mixed substrate proceeds with a specific rate (0.196 $g_P/g_X h$), about five times higher than in pure xylose (0.039 $g_P/g_X h$), whereas ethanol production from glucose redoubles, which gives confirmation of the above supposed reciprocal stimulations in the fermentation of these sugars. Furthermore, the supposed preferential fermentation of glucose with respect to xylose is confirmed by the higher value of v_o from glucose (0.437 $g_P/g_X h$) also in case xylose fermentation is stimulated by the presence of glucose itself (0.196 $g_P/g_X h$).

Another interesting observation comes from the data of Table 2: the behavior of alcohol production from glucose shows a maximum ethanol yield ($Y_{P/S} = 0.32\ g_P/g_S$) unexpectedly lower with respect to the one observed with yeasts usually degrading hexoses, such as *Saccharomyces cerevisiae* ($Y_{P/S} \sim 0.50\ g_P/g_S$) (*5,15*). This suggests that the metabolism of these sugars in the pentose fermenting yeasts is not likely as essential as in the strains able to ferment only hexoses. Furthermore, the decreasing concentration of ethanol before the total consumption of nutrients confirms also for pure substrates the simultaneous consumption of xylose and ethanol, with ethanol disappearing at a faster rate than xylose, already evidenced in a previous work (*1*).

Hemicellulose Hydrolysate

The same approach has been followed for hemicellulose hydrolysate to subdivide ethanol and biomass concentrations in two separate fractions ascribable to hexoses and pentoses metabolizations, respectively. To this purpose, the same rate of glucose consumption as the one observed for the mixed synthetic medium has been supposed; this can be justified only when glucose concentration in the medium is very lower than xylose concentration. The results of hemicellulose hydrolysate batch fermentations, presented in a previous work (*1*), have been listed in Table 2 together with the ones previously discussed for synthetic media. For cell mass curves, the same correction factor (1.18) of the synthetic medium has been employed because the starting glucose and xylose levels are exactly the same, and then no kinetic variation is reasonably proposable.

The substrate consumption rates ($-dS/dt$) of all three synthetic media have been reported in Fig. 2 vs the unfermented sugar level (S) while the

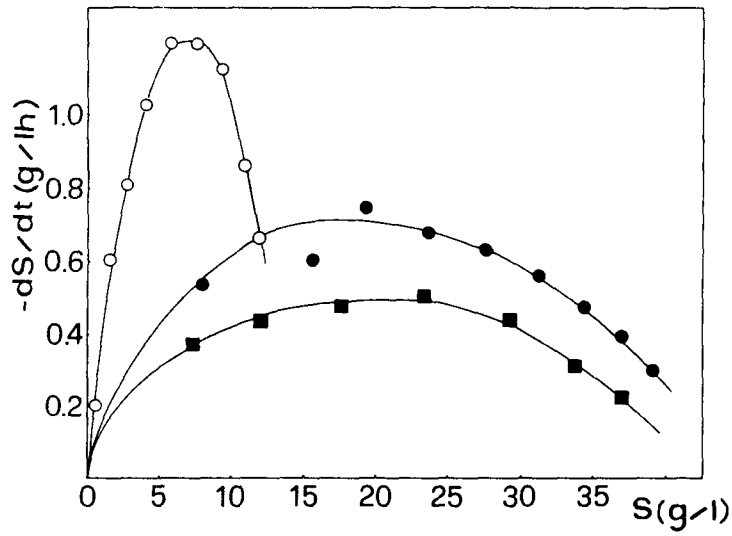

Fig. 2. Substrate consumption rates of batch fermentations of synthetic substrates. Glucose: (○). Xylose: (■) pure substrate; (●) mixed substrate.

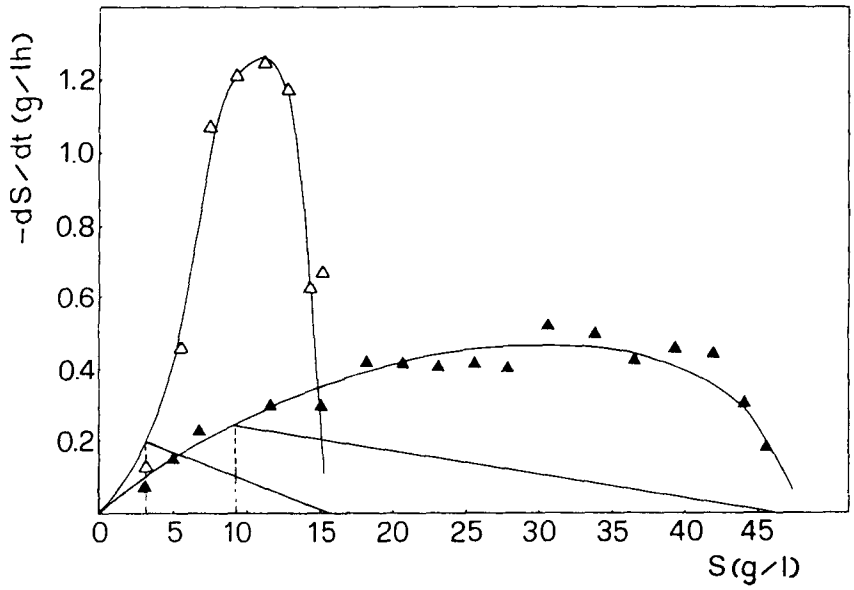

Fig. 3. Substrate consumption rates of batch fermentations of hemicellulose hydrolysate: (△) hexoses; (▲) pentoses.

behavior of the natural substrate is shown in Fig. 3. These curves will be very useful in the next section where the fundamental kinetic parameters of continuous fermentations will be graphically extrapolated for hemicellulose hydrolysate. Mixed media show, obviously, two separate curves for xylose and glucose consumptions. From these values, one can notice that xylose is consumed at a much higher rate in the mixed substrate

rather than in a pure solution, while, as expected, a sole curve for glucose is present.

The values of v_o and μ_o, calculated for hemicellulose hydrolysate using the same procedure described for mixed synthetic media, have been listed in Table 3 together with those calculated for synthetic substrates. Consistent decreases in starting ethanol productivity from either hexoses (–27%) or pentoses (–18%) provide the kinetic confirmation of the inhibiting action of a certain number of substances contained in hemicellulose hydrolysate, which negatively influence the fermentation kinetics with respect to the corresponding synthetic mixed substrate.

Continuous Fermentations

The graphical technique of Deindoerfer and Humphrey (*16*) has been utilized in this section to determinate the optimal conditions for the continuous fermentation of hemicellulose hydrolysate in CSTR from the experimental results of batch runs. In particular, this technique is used to calculate the dilution rate value of a continuous process corresponding to a desired unfermented sugar level if an experimental curve of the substrate consumption rate vs S is available from batch data. For the complex medium under consideration, each substrate refers to a separate curve.

As shown in Fig. 3, for both substrates, a straight line is drawn starting from S_o and touching the relative curve in correspondence to a value of S, assuring a satisfactory conversion yield. Since the substrate consumption rate in a CSTR is defined as:

$$rX = D(S_o - S) \tag{5}$$

the slope of each straight line corresponds to the dilution rate necessary to obtain the desired value of S. In the specific case under consideration, dilution rates of 0.012/h and 0.0065/h should be used for hexoses and pentoses continuous fermentations, respectively, to obtain a conversion yield of at least 80%. Then, in order to assure a reasonable conversion of the substrate consumed more slowly (pentoses), the lower calculated dilution rate should be employed for the whole process.

By substituting the selected values of unfermented pentoses and hexoses concentrations and the corresponding value of D, calculated as described above, into the general expression of the global productivity for a multisubstrate medium:

$$PD = Y_{P/S1} D(S_{o1} - S_1) + Y_{P/S2} D(S_{o2} - S_2) + \ldots + Y_{P/Sn} D(S_{on} - S_n) \tag{6}$$

the theoretical dashed curve of Fig. 4 has been obtained.

The use of stoichiometric instead of differential expressions in Eqs. (5) and (6) is justified by the steady-state conditions assumed in the continuous cultures, during which no variation of the involved parameters occurs.

The satisfactory agreement between calculated and experimental results has been verified only at relatively low dilution rates because the

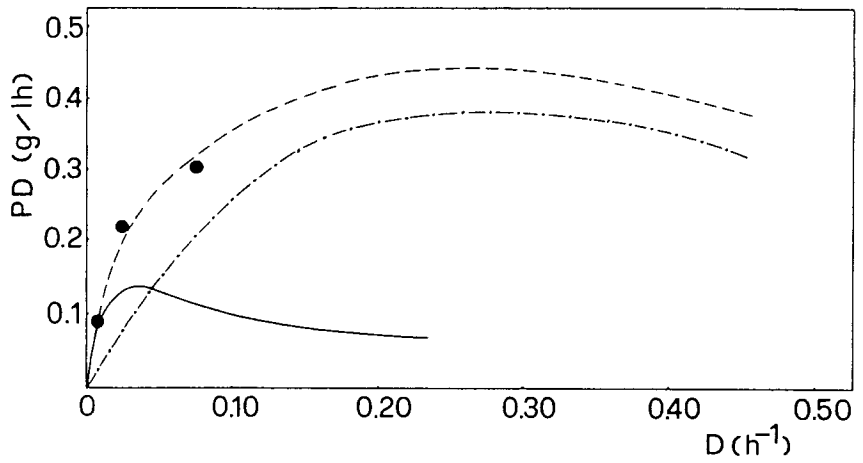

Fig. 4. Graphical determination of the optimal dilution rate for the continuous fermentation of hemicellulose hydrolysate in CSTR. Theoretical productivities: (—·—) from hexoses; (——) from pentoses; (--) total; experimental values of total productivity: (●).

degradation of pentoses, that are the most aboundant sugars in hemicellulose hydrolysate, becomes nearly negligible at dilution rates exceeding 0.01/h. From the industrial point of view, this means that, although the highest productivity (0.45 g_P/lh) is theoretically attainable with hemicellulose hydrolysate around 0.25/h, the majority of ethanol would be produced under these conditions from hexoses, whereas pentoses would nearly be completely wasted.

In conclusion, to assure a satisfactory conversion of pentoses (>80%), it is necessary to select, for continuous fermentation of hemicellulose hydrolysate, a very low dilution rate (0.0065/h) at which the simultaneous conversion of hexoses is nearly complete (~90%).

CONCLUSIONS

The new methodology presented in this report constitutes one of the few attempts made to carry out rigorous kinetic studies on multisubstrate media. A satisfactory agreement between the model and preliminary experimental continuous data gives confirmation of the validity of the assumptions made toward this aim.

In the authors' opinion, this methodology is of general significance and could also be successfully applied to more complex situations, such as the biological wastewater treatment. To this purpose, further experimental confirmations and the formulation of a more general model are the objectives of the next studies.

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