The Society for Bioscience and Bioengineering, Japan

[J. Ferment. Technol., Vol. 55, No. 5, p. 516~524, 1977]

Continuous Enzymatic Production of Invert Sugar

H. GOLDSTEIN,* P.W. BARRY, A.B. RIZZUTO, K. VENKATASUBRAMANIAN,**† and W. R. VIETH†

Amstar Corporation, Corporate Research and Development, 266 Kent Avenue, Brooklyn, New York 1121 and †Department of Chemical and Biochemical Engineering, Rutgers University-The State University of New Jersey, New Brunswick, New Jersey 08903

Continuous inversion of sucrose by invertase immobilized on collagen membranes in semi-pilot scale reactors was investigated using industrial feed streams. Several commercially available invertase preparations were evaluated. The highest activity obtained was 2,400 IU per g of collagen-invertase membrane. In continuous reactor (capacity 1.2 *l*) operation performed at a temperature of 55°C using 1.52 M sucrose solution as substrate, the operational half-life of the catalyst was estimated to be 1,870 hr. At lower substrate feed concentrations, the reactor operational stability was even better. The color of the process stream remained unchanged during the inversion process. Product quality compared favorably to acid hydrolyzed invert sugar. At high reactor residence times, greater than 90% sucrose conversion could be achieved without any objectionable color formation.

Kinetic data from both batch and continuous reactors indicate the presence of mass transfer resistances which disguise the overall reaction kinetics. The sucrose concentration at which substrate inhibition is observed ranged from 0.285 M in the case of the soluble enzyme to 0.564 M in the case of the immobilized enzyme. At low substrate concentrations, sucrose hydrolysis could be described by Michaelis Menten kinetics. The significance of the above results are discussed with respect to reactor scale-up.

Invert sugar is the hydrolysis product of sucrose and is a mixture of glucose, fructose, and unhydrolyzed sucrose. Sucrose inversion can be effected either by acid hydrolysis or enzymatically using the enzyme invertase (β-fructofuranosidase EC 3.2.1.26). Acid hydrolysis using hydrochloric acid at pH 2 is the preferred commercial process to produce invert sugar in the United States, while enzymatic conversion is reported to be practiced by a few processors in Europe and Japan. Although invertase is a relatively inexpensive enzyme, its use in the free form necessitates a batch operation, which reduces process efficiencies. Acid hydrolysis is a simple process which can be carried out continuously in a tubular reactor but the practical limit of conversion is about 65 to 70%. Higher conversions often require pre-treatment and high reactor residence times which also result in undesirable side reactions and excessive color formation in the product stream. Enzymatic hydrolysis, on the other hand, leads to very high conversions (above 90%) without the above problems.

The development of immobilized enzyme technology offers an alternate means of continuous processing. There have been many reports in the literature on the immobilization of invertase on a variety of carriers.[1~9] However, most of these papers are concerned with test conditions which do not typify commercial process conditions, *i.e.*, low substrate concentration, low operating temperature, etc. In this report, results on the

* Present Address: Miller Brewing Company, 4000 West State Street Milwaukee, Wisconsin 53208.
** Also with: H. J. Heinz Company, Pittsburgh, Pennsylvania.

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

performance of continuous reactors for sucrose inversion are discussed. The work was a joint effort between Amstar Corporation's Research and Development Division and Rutgers University. The Rutgers laboratory has expended a rather extensive research effort in the development of reconstituted collagen as a carrier matrix for enzyme and whole cell immobilization and these results have been summarized recently.[10~12] Attachment of invertase to collagen membranes[13,14] and collagen-polyurethane sponges[15] have been described previously. The major purpose of the work reported here was to examine the feasibility of the immobilized enzyme process for industrial operation, and to evaluate the potential of reconstituted collagen as a carrier for other applications as well.

Materials and Methods

Enzyme Several commercially available invertase preparations were evaluated in this study. They were purchased from Schwarz-Mann Laboratories, New York; Sigma Chemicals, St. Louis; G. D. Searle and Co., Chicago; and Nutritional Biochemicals, Cleveland, Ohio. Specific activities of these preparations assayed under standard conditions (25°C, pH 5.0, and an initial sucrose concentration of 0.285 M) are tabulated in Table 1. In addition to these preparations, whole yeast cells (*Saccharomyces cerevisiae*, National Yeast Company) were also immobilized on collagen.

Substrate A solution of commercial sucrose (Domino Brand, Amstar Corp., New York) in city water was used as the substrate in all experiments. Continuous reactor studies were carried out at 55°C, with pH adjusted to 5.0±0.1. Standard assays in batch reactors were conducted at 30°C, unless otherwise indicated.

Immobilization of invertase on collagen Invertase was attached to reconstituted collagen (kindly supplied by the Eastern Regional Research Laboratories, U. S. Department of Agriculture, Philadelphia, Pa.) by either impregnation of the enzyme on to pre-formed membranes or by direct complexation. These procedures have been described in detail elsewhere.[12] The latter method, *viz.* direct complexation was the preferred method used in this work. Briefly, 6 g of invertase were added to a hide collagen dispersion containing 21 g (dry weight) of collagen. The pH of the dispersion was adjusted to 4.3–4.5 with dilute lactic acid. The enzyme-collagen mixture was thoroughly mixed, deaerated under 747 mm Hg of vacuum and cast on a Mylar sheet to form a membrane. A Gardner knife was used to ensure the formation of a membrane of uniform thickness. Following air drying at room temperature for about 24 hr, the membrane was peeled off the Mylar support and tanned with 5% glutaraldehyde solution at pH 4.0 for 1 min. The tanned membrane was washed thoroughly in fresh water for 1 hr and again air dried.

Spirally wound collagen-invertase reactors Spirally wound biocatalytic modules have proven to be excellent reactor configurations for the use of collagen-enzyme membrane systems.[11] Collagen-invertase membranes were also tested in a spirally wound reactor. The desired amount of collagen-invertase membrane was layered on Vexar netting (E. I. DuPont, Buffalo, New York) which served as a backing material. It separated the successive layers of invertase-collagen membrane, thus preventing overlapping of the membrane layers. A stainless steel rod was used as a central core element. A spiral reactor configuration was formed by coiling alternate layers of the membrane and the backing around the central spacer element. The spiral cartridge was fitted into a Plexiglass or jacketted stainless steel outer shell. The housing was affixed to two threaded Plexiglass or stainless steel end plates provided with an inlet and outlet for the flow of the substrate over the membrane surface. A uniform axial distribution of the substrate was achieved by metering the flow through a distrubutor plate containing a number of 0.5 mm diameter holes.

Assay of immobilized invertase Catalytic activities of collagen-invertase membranes were rapidly determined by testing them in a stirred batch reactor, provided with temperature control. 1 to 2 g of membrane, cut into small chips, was used in a reactor volume of 25–50 ml, at 30°C and pH 5.0. The initial substrate concentration was 0.285 M. Glucose formed by the inversion reaction was determined by the Glucostat method[16] using Glucostat Special reagent (Worthington Biochemicals, Freehold, N.J.). One ml sample of this reaction mixture was removed and placed in a test tube immersed in a 110–115°C oil bath for 1 min. The samples were then refrigerated until assayed for glucose. Alternatively, the catalytic activity was measured by the Lane-Eynon volumetric method[17] for the determination of reducing sugars. There was a very good correlation between the results obtained by these two assay procedures.

Amount of enzyme bound to collagen The proteinaceous nature of the carrier material, collagen,

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

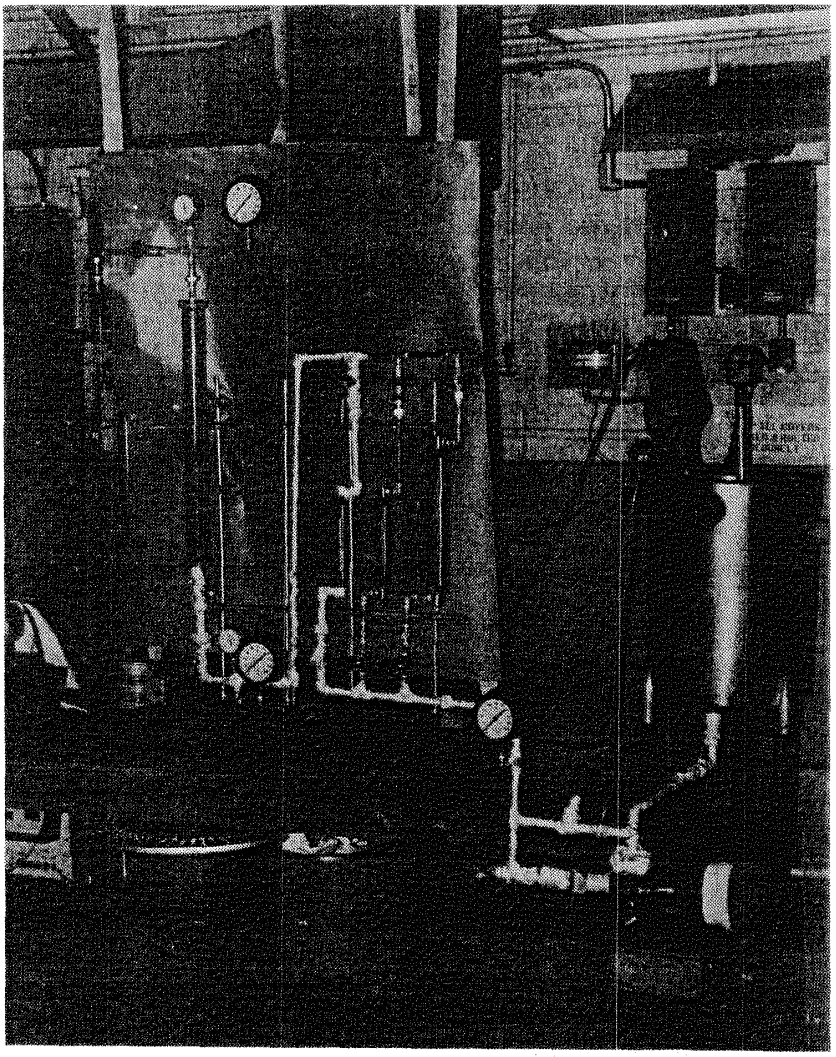

Fig. 1. Photograph of the experimental immobilized enzyme reactor unit.

renders most of the common methods of enzyme determination inapplicable. Therefore, a suitable method had to be devised which would distinguish between the enzyme protein and the carrier protein. This was accomplished by taking advantage of the fact that invertase contains tryptophan, while collagen does not. The method of Blackburn[18] for tryptophan determination was suitably modified to estimate the tryptophan content of collagen-enzyme membranes. This procedure has been described in detail elsewhere.[19]

Continuous sucrose inversion Different reactor columns were used for the continuous inversion of sucrose. The experimental set up is shown in Fig. 1. The sucrose feed solution, (containing 0.1% methyl paraben as a bacteriostat when substrate solutions were dilute and at room temperature) was prepared daily and stored in a stainless steel tank equipped with a sweep agitator. A constant head was maintained in an intermediate overflow pot (supplied with a peristalic pump for recirculation) which served as the column feed vessel. A positive displacement Moyno pump was used to supply feed material to the column. In experiments where the Plexiglass column was employed, the feed material was preheated in the column delivery lines and the column was insulated. When the stainless steel column was used, a constant supply of thermostatically-controlled hot water at the operating temperature was recirculated through the jacket. The system was equipped with thermometers, pressure gauges and relief valves where necessary and all feed and product lines were made of stainless steel. An automatic sampler was connected to the column effluent line which sampled the product at selected time intervals.

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

Results and Discussion

Comparison of the catalytic activities of different collagen-invertase preparations Several commercially available invertase preparations were evaluated as potential enzyme sources for immobilization. The catalytic activities of the free enzymes and the corresponding immobilized enzymes are compared in Table 1. The least expensive enzyme was Fermvertase supplied by Searle Biochemics; however, the specific activity of this enzyme was too low. In addition, this preparation contained a rather high proteolytic activity as evidenced by the required mechanical strengthening of the carrier membrane prior to immobilization. (This was accomplished by tanning collagen membrane with 0.5% glutaraldehyde solution for 1 min prior to immobilization). Of all invertase preparations tested, the best specific activity was obtained with the one purchased from Nutritional Biochemicals; this preparation was used for reactor stability tests. Different levels of glutaraldehyde tanning (aldehyde concentration and time of contact) were examined. The best conditions were found to be a combination of 5% glutaraldehyde and 1 min contact time. Reactor columns were successfully tanned in situ by pumping glutaraldehyde solution through the pre-formed cartridge at sufficiently high flow rates to achieve a reactor residence time of 1 min. The implications of this procedure to reactor scale-up are obvious.

Whole cells of commercially available yeast (*Saccharomyces cerevisiae*) were also immobilized on collagen membranes by the method of direct macromolecular complexation.[14] A stable activity of 73 IU/g complex was obtained. Since whole cells might present an additional transport resistance to substrate and product diffusion, the observed activity under the test conditions employed may not be the true activity of the preparation.

Continuous reactor studies Several runs were made on reactors I and II at different initial substrate concentrations and flow rates. Data from these runs are presented in Tables 2 and 3. Conversion levels in excess of 90% could be achieved when sucrose was fed to the column at low concentrations (<0.3 M) at reactor residence times of 8 to 12 min. At higher substrate feed concentrations, the steady state conversion levels decrease at comparable reactor residence times.

The other objectives in making these runs were to examine the quality of the final

Table 1. Comparison of the catalytic activities of different collagen-invertase preparations.

Enzyme source	Free enzyme specific activity (IU/mg protein)	Enzyme loading (g invertase per g collagen-invertase membrane)	Expressed activity[a] (IU/g catalyst)	Apparent specific activity (IU/g enzyme)
Schwarz-Mann Laboratories	108	0.20	550	2,750
Sigma Chemicals	170	0.20	863	4,315
Searle Biochemics[b] (Fermvertase)	64	0.071	170	2,430
Nutritional Biochemicals	420	0.20	2,400	12,000
Yeast whole cells	26,000[c]	0.25	73	292[c]

[a] Observed activity based on a batch assay. Activity expressed is on the basis of 1 g of catalyst, *i. e.*, collagen and enzyme.
[b] Prepared by impregnation of the enzyme on to pre-formed collagen membrane; all other membranes were prepared by the method of macromolecular complexation.
[c] IU/g Yeast whole cells.

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

Table 2. Continuous sucrose inversion at low (<0.3 M) feed concentrations (Reactor I).

Feed concentration (M)	Flow rate (ml/min)	Residence time (min)	Conversion (%)
0.285	720	0.875	42.0
0.285	72	8.75	93.7
0.285	23	27.4	96.8
0.196	720	0.875	48.0
0.196	210	3.0	83.6
0.196	77	8.2	92.7
0.196	17	37	97.7
0.098	720	0.875	53.5
0.098	210	3.0	85.7
0.098	74	8.5	95.2

Details of Reactor I: Plexiglass construction; inner diameter, 4.45 cm; length, 76.2 cm; total volume, 1.18 *l*; void volume, 0.63 *l*; membrane surface, 3.03 m^2; total catalyst, 100 g; membrane thickness, 0.1 mm.
All runs were made at 27–28°C.

product and to determine which microbiological problem might be encountered, if any. Substrate and product stream color data are tabulated in Table 4. In contrast to acid hydrolysis, sucrose inversion catalyzed by the immobilized enzyme does not lead to any color formation during the reaction; actually, in some instances a reduction in substrate color has been experienced. This is indeed an additional favorable feature since decolorization requirements subsequent to the inversion process would be simplified. Mechanisms responsible for the decreased presence of color bodies in the final product are not clear.

Table 3. Continuous sucrose inversion at high feed concentrations (Reactor II).

Run No.	Solids (%)	Feed concentration (M)	Residence time (min)	Conversion (%)
1	10.2	0.310	11.3	95.6
2	10.2	0.310	11.3	92.6
3	21.1	0.670	11.3	74.3
4	21.3	0.675	11.3	83.2
5	21.5	0.684	11.3	83.7
6	30.4	1.004	11.3	69.5
7	40.6	1.403	11.3	53.3
8	51.2	1.851	11.3	34.0
9	21.4	0.680	11.3	80.0
10	51.0	1.842	21.5	53.9
11	51.6	1.868	8.1	27.1
12	21.2	0.639	8.1	71.1
13	50.9	1.837	5.6	20.5
14	50.5	1.819	4.2	17.0
15	21.8	0.670	11.3	73.3

Constructional details for Reactor II are the same as that of Reactor I except for void volume, which was 0.85 *l* for Reactor II.
Runs 1 through 3 were at 30°C; the rest of the runs were at 45–50°C.

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

Table 4. Color of substrate and product streams.

Run No.[a]	Approximate solids (%)	Color comparator units[b]	
		Inlet	Outlet
3	20	78	59
4	20	66	56
5	20	37	37
6	30	50	42
7	40	67	65
8	50	76	72
9	20	24	39
10	50	87	85
11	50	136	123
12	20	72	52
13	50	52	52
14	50	59	50

[a] Run numbers correspond to those cited in Table 3.
[b] Color comparator unit: A unit of color measurement in which absorption of visible radiation determined spectrophotometrically is expressed in terms of arbitrary color units in common company use.
A higher value of the color comparator unit corresponds to a higher color intensity, and vice versa.

Sugar solutions are known to undergo complex (non-enzymatic) reactions some of which might lead to the formation of colored products. The relatively mild conditions and small reactor residence times employed for the immobilized enzyme system apparently minimize these side reactions. Product quality with respect to taste and odor was also found to be satisfactory.

When operated at temperatures above 45°C at solid concentrations above 1 M, there were no problems with respect to microbial growth in the reactor. Apparently, this temperature-concentration combination is sufficient to inhibit any significant microbial proliferation. For operation at lower temperature, 0.1% methyl paraben was added to the substrate feed and microbial contamination was effectively eliminated. When not in use, the columns were stored under dry conditions in the refrigerator.

Another variable monitored in these runs was the pressure drop across the reactor column. In all cases, the pressure drop was less than 0.07 kg/cm^2. The low pressure drop is attributable to the spirally wound reactor configuration which minimizes pressure drop by routing the substrate through a number of narrow flow channels of essentially equal hydraulic resistance.[20]

Operational stability Collagen-invertase reactors were operated continuously with a constant feed concentration and flow rate in order to investigate the operational stability of collagen-enzyme membranes. When operated with a feed sucrose concentration of 0.285 M, no significant decrease in activity was observed over a period of 7 days. At a reactor residence time of 23 min, the steady state conversion was 90% in this case.

Operational stability data for reactors II and III are shown in Fig. 2. In these cases, the reactors operated at 55°C with a feed concentration of 1.52 to 1.55 M. Assuming that the decrease in catalytic activity is exponential with time, the operational half-life of the catalyst can be estimated. Linear regression of reactor operating data (Fig. 2) yields catalyst half-life of 1870 and 1050 hr for reactors II and III respectively. Presented in

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

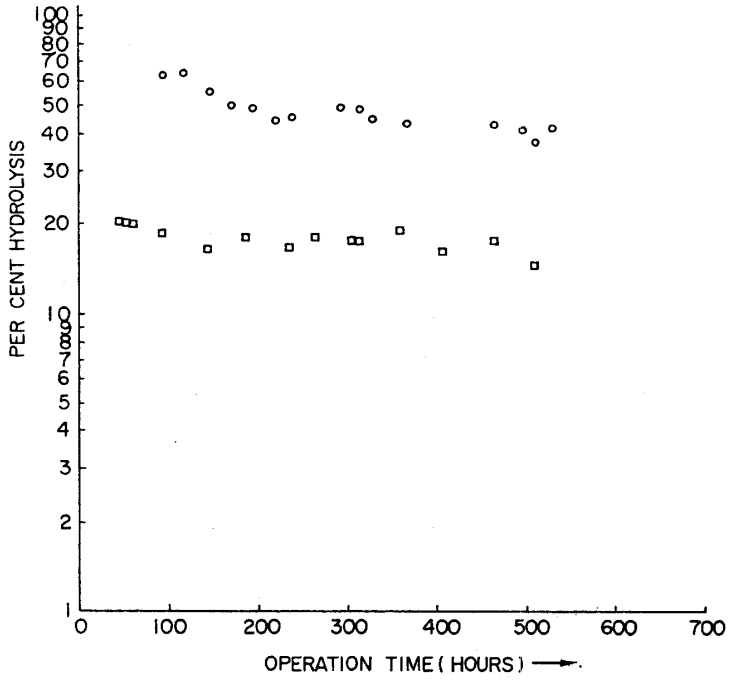

Fig. 2. Operational stability of collagen-invertase reactors.
—□— Reactor II
—○— Reactor III

Table 5 are actual operating data obtained using reactor III. These data show fluctuations in reactor operating temperature, feed concentration and flow rate. Reactor operation

Table 5. Decay of enzyme activity during continuous operation (Reactor III).

On stream (hr)	Feed concentration (M)	Temperature (°C)	Feed rate (ml/min)	Sucrose processed (kg)	Conversion (%)
22	1.89	54	52.5	46	84.4
94	1.93	53	53.5	198	62.6
118	1.91		48.0	244	64.8
146	1.90		50.0	298	55.4
170	1.90	52	50.0	345	49.8
190	1.90	53	51.0	384	48.2
219	1.87	52	51.5	441	44.1
238	1.89	53	52.5	480	45.5
292	1.92	53	50.0	587	49.2
		Column refrigerated			
319	1.94	52	50.0	631	44.4
367	1.94	54	50.0	726	42.9
464	1.80	54	51.0	816	42.8
488	1.92	55	52.5	924	41.1
510	1.97	55	50.0	1038	37.6
530	1.97	53	45.0	1090	41.8

Reactor details: Stainless steel jacketted construction; inner diameter, 5.1 cm; length, 76.2 cm; total volume, 1.54 *l*; void volume, 1 *l*.
Other details are the same as those for Reactor I.

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

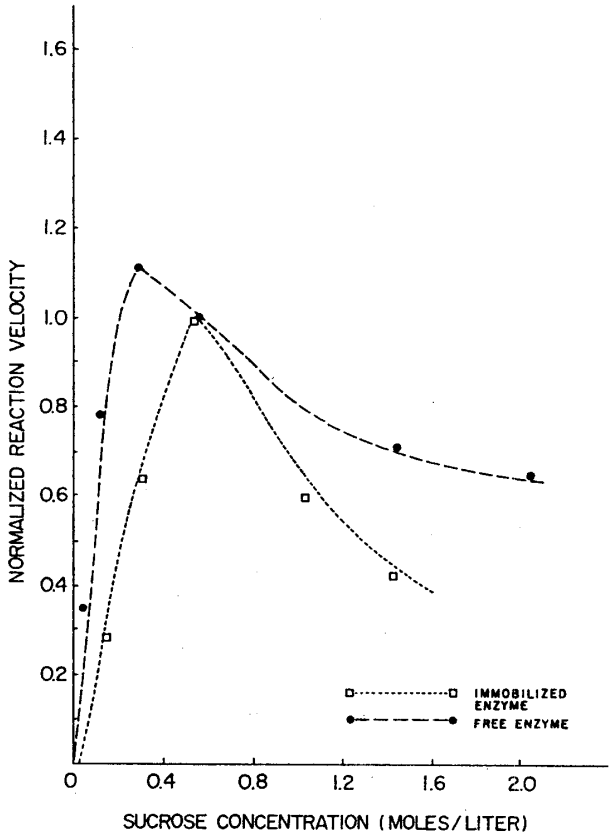

Fig. 3. Variation of reaction rate with substrate concentration.

was interrupted, as indicated, simulating the type of operating schedule typically encountered in industrial practice. During these periods, the reactor was stored in a refrigerator. In 530 hr of total operation, the reactor processed about 1,100 kg of sucrose. This corresponds to a productivity of 11 kg of product produced per g of catalyst during this period.

Reaction kinetics and mass transfer Batch data on the kinetics of sucrose inversion by free and collagen-bound invertase are shown in Fig. 3. Since the enzyme concentration levels were different for the soluble and the immobilized enzymes, the kinetic data have been normalized to account for this variation. While both forms are subjected to substrate inhibition, the concentration of sucrose above which the inhibition occurs (*i.e.*, threshold inhibition concentration) is different. This concentration is 0.285 and 0.564 M for the free and bound enzyme, respectively. The shift in the maximum reaction rate to higher concentration in the case of the bound enzyme might be due to bulk mass transfer problems. Significant transport resistances in the bulk phase would cause appreciable concentration drop. Thus, at the immobilized enzyme surface the substrate concentration would be much lower than at the solution bulk, requiring an apparently higher bulk concentration for the onset of the inhibitory phenomenon.

At the low substrate concentration, kinetic data could be represented by the classical Michaelis-Menten kinetics for both soluble and bound enzyme forms. Continuous reactor data at different flow rates and initial substrate concentrations were described by the integrated form of the Michaelis-Menten equation. Based on the data of Table 2, apparent Michaelis constants for different flow rates were calculated and tabulated (Table 6). As the linear velocity through the column is increased, the value of the Michaelis constant

NII-Electronic Library Service

The Society for Bioscience and Bioengineering, Japan

Table 6. Kinetic data.

Residence time (min)	Flow rate (ml/min)	*Km* (M)	*Vm* (moles/*l.* min)
0.32	1970	0.135	0.213
0.875	720	0.290	0.315
8.75	72	0.680	0.233

decreases, approaching a value close to the free enzyme *Km* value (0.12 M). This implies the presence of significant external film diffusional resistance. From separate experiments, the effectiveness factor for a 0.1 mm thick collagen-invertase membrane was found to be 0.45.[21] Thus, a rigorous representation of the observed reaction kinetics should consider both the intrinsic inhibition kinetics as well as external and internal mass transfer impedances. A kinetic model for sucrose hydrolysis by soluble invertase has been developed by the Rutgers Laboratory.[16] Efforts are now underway to develop a detailed mass transfer-kinetic model for the immobilized enzyme and they would be described in future communication.

Acknowledgement

The technical assistance of Messrs. Nicholas Bosko, John Fisher, Sheldon Pollack, and Ray Franklin is gratefully acknowledged. Work done at Rutgers University was supported in part by the National Science Foundation Grant-in-Aid GI-34287.

References

1) Fillippusson, H., Hornby, W.E.: *Biochem. J.*, **120**, 215 (1970).
2) Negoro, H.: *J. Ferment. Technol.* **48**, 689 (1970).
3) Monsaw, P., Durand, G.: *C.R. Acad. Sci* (Paris)., **273**, 33 (1971).
4) Usami, S., Noda, J., Goto, K.: *J. Ferment. Technol.*, **49**, 598 (1971).
5) Mason, R.D., Weetall, H.H.: *Biotechnol. Bioeng.*, **14**, 637 (1972).
6) Maeda, H., Suzuki, H.: *Biotechnol. Bioeng.*, **15**, 403 (1973).
7) Marconi, W., Gulinelli, S., Morisi, F.: *Biotechnol. Bioeng.*, **16**, 501 (1974).
8) Boudrant, J., Cheftel, C.: *Biotechnol. Bioeng.*, **17**, 827 (1975).
9) Toda, K., Shoda, M.: *Biotechnol. Bioeng.*, **17**, 401 (1975).
10) Bernath, F.R., Vieth, W.R. 1974.: *Immobilized Enzymes in Food and Microbial Processes,* (Olson, A. C., Cooney, C. L.) 157, Plenum Press, New York (1974).
11) Vieth, W. R., Venkatasubramanian, K.: "*Enzyme Engineering,*" *ACS Monograph,* (1973).
12) Vieth, W. R., Venkatasubramanian, K.: *Methods in Enzymology,* **45**, 243 (1976).
13) Saini, R., Vieth, W. R., Wang, S. S.: *Trans. N. Y. Acad. Sci.* Series II., **34**, 664 (1972).
14) Venkatasubramanian, K., Vieth, W. R.: *Biotechnol. Bioeng.*, **15**, 583 (1973).
15) Maldonado, O., Rolz, C.: *Enzyme Immobilization in Collagen Films Adhered to Semi-rigid Supports: The Enzymatic Sponge Reactor.* (submitted for publication).
16) Bowski, L., Saini, R., Ryu, D.Y., Vieth, W.R.: *Biotechnol. Bioeng.*, **13**, 641 (1971).
17) Lane-Eynon, W.H.: *Official Methods of Analysis,* 494, Association of Official Agricultural Chemists, Washington, D. C. (1965).
18) Blackburn, H.: *Amino Acid Determination,* 193, Marcel Dekker Inc., New York (1968).
19) Venkatasubramanian, K., Saini, R., Vieth, W.R.: *J. Food Sci.*, **40**, 109 (1975).
20) Vieth, W. R., Gilbert, S. G., Wang, S. S., Venkatasubramanian, K.: Biocatalytic modules, U. S. Patent 3,809, 613 (1974).
21) Fernandes, P. M.: *Modeling and Optimization of Multienzyme Systems,* Ph. D. Thesis, Rutgers University (1975).

(Received May 9, 1977)

NII-Electronic Library Service