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EL CRECIMIENTO CONTROLADO Y SU APLICACION AL ESTUDIO DE LA REGULACION DE LA GLUTAMINO SINTETASA

T E S I S

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C O N T E N I D O

I N T R O D U C C I O N

La regulación del crecimiento tiene gran significado para reducir o eliminar variables en el estudio de los sistemas microbianos, lo que ha motivado el diseño de equipos especializados para lograr crecimiento regulado en estos sistemas. Tal es el caso del quimiostato, originalmente descrito por Monod (1950) con el nombre de "bactogen".

El quimiostato es un fermentador continuo que opera en un equilibrio entre la entrada de nutriente y el crecimiento celular resultante, en un medio controlado. Su principio radica en que, en condiciones de equilibrio, la velocidad de dilución es igual a la velocidad específica de crecimiento del microorganismo (Herbert, 1961). Esto nos lleva a definir el quimiostato como un aparato que proveé un ambiente para el microorganismo que está bajo el control del experimentador en términos de los parámetros físicos, y que es independiente del tiempo (Salomons, 1972).

El quimiostato ha sido empleado exitosamente para evaluar el efecto de concentraciones limitantes de las fuentes de carbono y nitrógeno en la regulación de la glutamino sintetasa (E.C.6.3.1.2) en organismos procarióticos (Senior, 1975), pero no había sido posible hacer esto mismo en el

hongo *Neurospora crassa* pues este organismo miceliar, al crecer en un quimiostato convencional causa problemas de taponamiento con matas de micelio en las tuberías del sistema, por lo cual no es posible mantener el estado estacionario por un tiempo prolongado. Esto es especialmente cierto en equipos de volumen pequeño como los usados para investigación, lo que nos llevó a estudiar diseños novedosos de quimiostatos e incluso sistemas alternativos de crecimiento regulado como es el cultivo alimentado en el estudio del metabolismo nitrogenado en *Neurospora*. Puede decirse que este trabajo representa el esfuerzo de un grupo de investigación aplicada por aportar conocimientos del área de ingeniería bioquímica a la solución de problemas de investigación fundamental enfrentados por otro grupo de enfoque más básico.

La presente tesis presenta primero el trabajo en cultivo continuo, pues este enfoque se utilizó primero para lograr crecimiento regulado en *N. crassa*, con miras a estudiar la regulación de la glutamino sintetasa en este microorganismo, pero por diversos problemas que adelante se mencionan, esta técnica se abandonó. En seguida se discute el caso del cultivo alimentado, el cual se revisa más extensamente por ser esta metodología la que constituye la aportación fundamental de este trabajo. A con-

tinuación se revisa el caso de la glutamino sintetasa, y se pone el trabajo en contexto con los avances realizados en el área. En seguida se presenta un resumen del artículo que se originó de este trabajo y que lleva por título "Regulación de la Glutamino Sintetasa en Cultivos Alimentados de Neurospora Crassa", de Jorge Limón-Lason, Miguel Lara, Berta Resendiz y Jaime Mora; que salió publicado en Biochemical and Biophysical Research Communications en 1977.

El siguiente capítulo de esta tesis está formado por tres publicaciones del autor. La primera es la arriba mencionada, mientras las otras dos representan aplicaciones en otras áreas, que serán tratadas someramente en el capítulo de Conclusiones y Perspectivas, con objeto de poner de relieve el sustrato común de tres investigaciones tan disímbolas en apariencia. Para terminar, se describen las últimas investigaciones del autor y es interesante observar que la visión de cultivo alimentado vuelve a aparecer, pero ahora en relación con el crecimiento de peces.

EL CASO DEL CULTIVO CONTINUO

Todos los quimiostatos son fermentadores continuos, pero algunos son más continuos que otros. Esto ha llevado a establecer un tiempo mínimo de crecimiento continuo y así, Solomons (1972) opina que "experimentos donde condiciones estacionarias fueron mantenidas por 8 horas" no constituyen cultivo continuo y arbitrariamente elige un mínimo de 1000 h de tiempo de operación. Nosotros consideramos que 100 h de operación ya constituye cultivo continuo, pues en este lapso, a una velocidad de dilución de 0.14 h^{-1} tenemos un tiempo de duplicación de 5 h y por lo tanto 20 generaciones.

El problema está en que en un sistema convencional de laboratorio ni siquiera las 8 h a que hace alusión este autor son alcanzables. Por ello realizamos un diseño propio, basado en la unidad de columna ciclónica de Dawson (1963) y tomando ciertos elementos del fermentador de domo del mismo autor (Dawson *et al*, 1971) y del fermentador de torre (Greenshields y Smith, 1971). El aparato resultante consistía en una matraz con entrada ciclónica en su cuello y tenía uma bomba de levantamiento de aire (air-lift) para alimentar el ciclón, con lo que se obtenía un circuito de recirculación y aeración. Las ventajas derivadas de este diseño eran mantener un cultivo -

homogéneo y bien aerado, evitando crecimiento en la pared y áreas de estancamiento. Al no permitir crecimiento en la pared se evitaba la formación de matas de micelio y por tanto no había taponamiento de las tuberías. Esto permitió obtener crecimientos continuos por 100 h..

Sin embargo, el equipo resultaba extraordinariamente sensible a problemas externos como fallas en el suministro eléctrico, muy comunes en esa época, por lo que el cultivo continuo fué abandonado y los esfuerzos centrados en el cultivo alimentado que para esos momentos estaba proporcionando resultados muy alentadores.

EL CASO DEL CULTIVO ALIMENTADO

El cultivo alimentado ha sido desarrollado para lograr altas productividades en células microbianas y metabolitos secundarios en la industria fermentativa. Por ejemplo, este procedimiento ha sido usado en la producción de levadura de pan, *Saccharomyces cerevisiae* (Dairaku *et al*, 1981; Aiba *et al*, 1976; Wang *et al*, 1977): *Candida utilis* para proteína unicelular (Nyriri *et al*, 1975); penicilina, donde el precursor, ácido fenilacético inhibe el crecimiento celular (Chain, 1971; Fishman y Biryukov, 1974; Hegewold *et al*, 1981; Bajpai y Reuß, 1981) y Cefalosporina (Trilli *et al*, 1977).

El interés en el cultivo alimentado radica en que esta técnica permite evitar la represión catabólica. Así, la levadura de pan se produce añadiendo incrementalmente melaza al fermentador, con lo que se mantiene baja la concentración de sustrato y se elimina la represión por glucosa. Sin embargo, el sistema no carece de interés teórico, pues si se sobrealimenta glucosa se produce etanol, y si no se alimenta suficiente cae la productividad -- (Wochrer y Rochr, 1981).

El cultivo alimentado es una operación semicontinua de cultivo en lote en la cual un sustrato limitante al crecimiento se suministra de modo continuo. El término "cultivo alimentado" fue acuñado por Yoshida y colaboradores (1973), quienes reportaron que el rendimiento de biomasa a sustrato en un cultivo creciendo linealmente fue el doble del encontrado en crecimiento exponencial, lo que en su opinión implica que cuando se limita el crecimiento por accesibilidad de sustrato, los microorganismos pueden convertir éste más eficientemente a biomasa.

La cinética de este tipo de cultivo ha sido extensamente desarrollada por Dunn y Mor (1975); Jones y Anthony (1977) y Yamané y Hirano (1977) y es interesante notar que es posible lograr en cultivo alimentado un estado cinéticamente análogo al estacionario dinámico que se establece en cultivo continuo (Dunn y Mor, 1975). Este ha sido llamado "estado cuasiestacionario", ya que no es un estacionario verdadero pero imita muchas de sus características, como veremos más adelante. De esto se desprende que el efecto fisiológico sobre el metabolismo celular no debe ser muy diferente entre el cultivo alimentado y el continuo.

Debido a esto, y dado que en cultivo alimentado no se provee una salida para la biomasa producida, con lo cual se evitan los problemas de taponamiento, se centró nuestra atención en el cultivo alimentado.

Es posible simplificar notablemente la cinética del cultivo alimentado para el caso de volumen constante. Esta situación se puede aproximar bastante usando una bomba microinyectora capaz de mantener flujos constantes muy pequeños, y en estas condiciones en que el cambio de volumen producido es pequeño, la velocidad de crecimiento es constante y es una función lineal de la tasa de alimentación del sustrato limitante.

Para analizar la cinética del cultivo alimentado hacemos un balance de masa del sustrato sobre el sistema:

ACUMULACION = ENTRADA-SALIDA - CONSUMO PRODUCCION

(1) $\frac{d(SV)}{dt} = FSo - \emptyset - \mu VX/Y \quad \emptyset$

So = Concentración de sustrato limitante en la alimentación.

S = Concentración de sustrato limitante en el fermentador.

F = Flujo volumétrico de alimentación.

V = Volumen del fermentador

T = Tiempo de fermentación

μ = Velocidad específica de crecimiento

Y = Rendimiento de biomasa en función del sustrato

X = Biomasa

El término de acumulación está representado por la tasa de cambio de volumen y de la concentración de sustrato, y el de entrada, por el flujo de masa de sustrato.

A su vez, el término de consumo es proporcional a la biomasa presente (su concentración por el volumen) e inversamente proporcional a la constante de rendimiento. La constante de proporcionalidad es precisamente μ, la velocidad específica de crecimiento. Al no haber salida ni producción de sustrato, los términos respectivos son cero y pueden eliminarse.

Haciendo ahora un balance sobre la biomasa, tenemos:

$$(2) \quad \frac{d\,(XV)}{dt} = \mu VX$$

El término de la izquierda es el de acumulación, y el de la derecha representa la producción. No hay entrada, salida ó consumo de la biomasa.

Si asumimos que el volumen se mantendrá constante (y más adelante veremos que en las condiciones de operación el volumen cambia menos de un 10%) - sustituímos la ecuación 2 en la 1 y rearreglamos para obtener:

$$(3) \quad DSo = \frac{1}{Y}\frac{dX}{dt} - \frac{dS}{dt}$$

D es la velocidad de dilución, o sea la relación entre flujo y volumen. Es interesante notar en este punto que el error cometido al asumir volumen constante se puede corregir usando la relación V/V' (V' = V aparente) para el caso de cultivo lineal (Keller y Dunn, 1978).

En las condiciones de operación S es muy pequeño y no se puede acumular, o sea que el sustrato limitante es consumido de inmediato y se mantiene abajo de los límites de detección. Por tanto el segunto término del lado derecho de la ecuación (3) se puede despreciar, con lo que llegamos a:

$$(4) \quad \frac{dX}{dt} = DSoY$$

La ecuación (4) nos dice que la velocidad de crecimiento bajo estas condiciones es constante y es función del flujo de masa de sustrato (DSo) y de la constante de rendimiento Y. Esta es la ecuación de diseño del sistema, y nospermite fijar las condiciones de operación para obtener un crecimiento dado, o

bien calcular la constante de rendimiento Y.

(5) $X = Xo + DSoYt$

Siendo Xo el inóculo. En la gráfica 1 del primer artículo considerando (Limón-Lason *et al*, 1977) se ve que efectivamente dx/dt es constante (representa la pendiente) y se puede calcular Y. Esto se muestra en la tabla A.

TABLA A. Rendimiento de biomasa en función de sustrato calculado según la ecuación 4, en cultivo alimentado de la cepa salvaje 74A de *N.crassa*, limitada de amonio o sacarosa a una velocidad de dilución de 3.2×10^{-3} h^{-1}.

Sustrato limitante	Concentración del sustrato (mol)	Y (g proteína/mol)	Y (g proteína/g)
Sacarosa	14.6	58	.322
NH_4 Cl	9.2	60.8	1.14

El rendimiento expresado en términos molares es prácticamente igual en ambos casos, lo que es muy interesante y valdría la pena investigar más. El rendimiento para sacarosa es aproximadamente el doble del calculado teóricamente para cultivo ex

ponencial, lo cual está de acuerdo con lo encontrado por Yoshida *et al* (1973), y apuntaría al hecho de que Y en realidad es una "pseudo constante", es decir, es contante solo bajo condiciones muy restringidas. De hecho, Y es una función de μ (Abott y Clamen, 1973). El rendimiento teórico se calculó de acuerdo al contenido disponible de electrones (e^- / mol) del sustrato (Abott, 1973) y es de .164 g proteína/g sacarosa. El cambio de volumen es del 7.7% pasando de 3 a 3.23ℓ en 24 hs. En otras palabras, el cultivo se suspende cuando el cambio de volumen apenas empieza a volverse significativo.

Continuemos nuestro análisis de la cinética del sistema para obtener algunas otras conclusiones.

Sabemos que el crecimiento microbiano es exponencial y el crecimiento lineal resulta difícil de entender, pues implicaría que el crecimiento es independiente de la biomasa ó del número de células. Ahora bien, si las células en el cultivo se dividen por fisión celular, la tasa media de fisión por célula debe ser inversamente proporcional a la concentración de biomasa (Jackson y Edwards, 1972). Esto nos lleva a preguntar cómo es que en este sistema se obtiene un

crecimiento lineal. Para contestar esta pregunta sustituímos la ecuación (4) en la ecuación de Monod (Monod, 1949; Levenspiel, 1980) que describe el crecimiento de microorganismos cuando un único requerimiento nutricional es el factor limitante al crecimiento:

$$(6) \qquad \frac{1}{X}\frac{dx}{dt} = \frac{\mu max \; S}{Ks + S}$$

μmax es la velocidad específica máxima de crecimiento, y Ks es la concentración de sustrato a la cual la velocidad de crecimiento es la mitad de la máxima. Efectuando la sustitución y rearreglando:

$$(7) \qquad DSoY = \frac{\mu max \; S \; X}{Ks + S}$$

Sustituyendo ahora por X en la ecuación 5 y despejando S, llegamos a:

$$(8) \qquad S = \frac{KsDSoY}{DSoY\,(1-\mu max\; t) - \mu max\; Xo}$$

Consideremos el caso de un inóculo despreciable. En este caso el término μmax Xo se puede eliminar con lo que la ecuación se simplifica a:

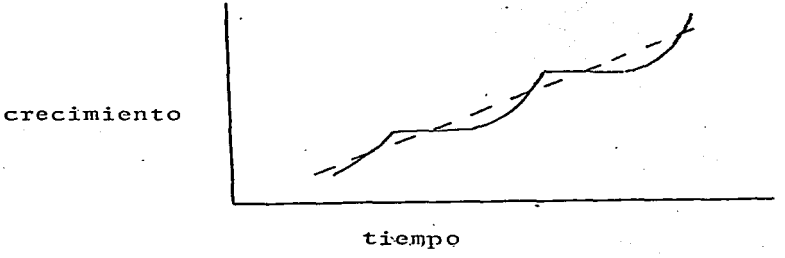
$$(9) \qquad S = \frac{Ks}{1-\mu max\; t}$$

Y a tiempos muy cortos ($t \rightarrow o$) $S = Ks$

Lo que nos dice esto último es que el crecimiento es exponencial a tiempos microscopicamente pequeños hasta que el sustrato se agota en el microambiente y el crecimiento cesa, en espera de que el sustrato se vuelva a acumular a un nivel tal que pueda ser consumido.

El efecto acumulativo es de crecimiento lineal sostenido, y la tendencia observada se manifiesta como una línea recta. Esto se muestra esquemáticamente en la figura A.

Figura A.- Crecimiento en cultivo alimentado de un microorganismo hipotético, con la escala del tiempo muy amplificado.



Sin embargo, no debe pensarse que pudiera observarse este fenómeno simplemente muestreando muy frecuentemente un cul

tivo alimentado, pués se trataría de una manifestación local del modo de crecimiento que en el mejor de los casos pudiera resultar en conducta levemente oscilatoria de un detector del sustrato contínuo y en tiempo real.

Otra conclusión que puede obtenerse del modelo cinético descrito (que podría llamarse "cultivo alimentado de volumen constante") es que puede obtenerse crecimiento lineal manteniendo constante el sustrato por control de la alimentación. Una forma simple de hacer esto es controlar y mantener constante el oxígeno disuelto. Esto se hizo con *Neurospora* en un fermentador New Brunswick equipado con un controlador de oxígeno obteniéndose efectivamente crecimiento lineal (no se muestra).

Esto último es de mucho interés pues nos habla de un sistema de control por retroalimentación del cultivo alimentado, en el que una señal proporcional a la concentración del sustrato controlado abre o cierra la válvula de oxígeno. Este es un campo en plena expansión, como veremos en seguida, a la vez que se trata de una metodología que pudiera dar información sobre la manera que la célular controla niveles de metabolitos.

Las múltiples aplicaciones industriales del cultivo alimentado, a que previamente hemos hecho referencia, más el hecho de que puede servir para arrancar o incluso substituir en algunas aplicaciones al cultivo contínuo, justifican la gran excitación que hay en el campo. En efecto, la producción industrial continua a gran escala de proteína unicelular o levadura por tiempos muy prolongados se ve impedida por problemas de contaminación o degeneración del cultivo, y dado que el cultivo alimentado puede usarse para arrancar rápidamente el cultivo continuo (alcanzar rápidamente el estacionario) se han desarrollado varias técnicas para optimizar este proceso, lo que debe redundar en mayor productividad y calidad más constante del producto (Yamané *et al*, 1979; Ohno *et al*, 1976)

Weigand (1981) relata las ventajas que puede tener el cultivo alimentado cíclico sobre el cultivo en lote o contínuo. Se trata de retirar parte del cultivo al terminar un ciclo, para que pueda continuarse el cultivo, que a su vez se retira parcialmente reiniciando el ciclo. Se logra así un proceso cíclico o semi-contínuo. En este sentido cabe mencionar que Pirt (1974) describió el estado cuasiestacionario (ECE), donde X y S se mantienen constantes, $\mu = D$, y V aumenta, por lo que D disminuye en el tiempo. Al quitar parte del cultivo D aumenta (se restablece) lo que hace al cultivo alimentado cí

clico en ECE cinéticamente análogo al cultivo contínuo, de lo que se desprende que no hay mucha diferencia entre ambas técnicas pero la primera puede ser más fácil de mantener y controlar (y usarse con organismos miceliales). Incluso se ha desarrollado un sistema de control que hace que un cultivo alimentado alcance el ECE rapidamente, manipulando D y So mediante un microprocesador (Kalogerakis y Boyle, 1981). Se ha simulado el cultivo alimentado cíclico (Keller y Dunn, 1978 b) y se han hecho predicciones de costos (Trilli, 1977), pero sobre todo se han visto incrementos del 30% en la productividad usando cultivo alimentado cíclico en ECE (Trilli *et al*, 1977).

El cultivo alimentado también puede hacerse exponencial y Martín y Felsenfeld (1964), Lim *et al* (1977) y Kishimoto *et al* (1976) describen la cinética, los equipos que hay que utilizar y las condiciones de operación necesarias para mantener los estacionarios y controlar más exitosamente el proceso. Este consiste escencialmente en la alimentación del sustrato limitante a una tasa que se está incrementando exponencialmente. El crecimiento resultante es exponencial y μ es constante. Usando - cultivo alimentado exponencial con un control PID (porporcional, integrativo y derivativo) de retroalimentación con etanol a $\mu = 0.3\ hr^{-1}$ se obtuvo un rendimiento de 0.5 g de biomasa por

gramo de glucosa en la producción de levadura de pan (Dairaku *et al*, 1981). Un estudio equivalente logró una producción de 64 g/l a las 24 hs (Huang y Chu, 1981).

Es indudable que técnicas como el cultivo alimentado cíclico en ECE y cultivo alimentado exponencial pueden ser usadas ventajosamente para estudios metabólicos en microorganismos, al proveer métodos muy finos para la regulación del crecimiento. En este sentido cabe mencionar que Toda y colaboradores (1980) desarrollan un modelo que describe el crecimiento bifásico de levadura en glucosa y etanol, en cultivo alimentado, y predice los parámetros de control óptimo para cosechar un alto rendimeinto de células con elevada actividad de invertasa; mientras que Yoon y Blanch (1977) proponen la competencia de sustratos limitantes para observar las interacciones de sistemas microbianos mixtos bajo condiciones bien definidas en el estudio de la ecología microbiana. En esta misma línea, un trabajo reciente (Esener *et al*, 1981) muestra el cultivo alimentado como una herramienta poderosa en el estudio simultáneo de cinética y energética microbianas, y se muestran y evalúan métodos para determinar los requerimientos y coeficientes de mantenimiento.

De todo lo anterior cabe concluir que el entusiasmo actual en el cultivo alimentado está plenamente justificado.

El Caso de la Glutamino Sintetasa

La glutamino sintetasa (G.S.) cataliza la síntesis de glutamina a partir de glutamato y amonio, a expensas de energía del ATP y con un balance energético de - 3.9 kcal mol^{-1}. El gran interés que existe por esta enzima radica en que se encuentra en posición central con respecto al metabolismo nitrogenado. En efecto, glutamina es un precursor o donador de grupos amino en muchos productos metabólicos, tales como triptofano, histidina, carbamil fosfato, glucosamina 6 fosfato, etc. (Meister, 1968). No debe entonces resultar extraño que la G.S. es una enzima con una regulación extremadamente compleja.

Así, en *E. coli* todos los metabolitos arriba mencionados más glicina, alanina, CTP y AMP sirven como inhibidores alostéricos independientes (Stadtman,1968). En *Neurospora* es interesante observar que el ácido antranílico y no el triptofano es inhibidor, siendo el primero el compuesto inicial para la biosíntesis del segundo. También son inhibidores glicina, histidina, CTP, AMP, GTP, carbamil fosfato y NAD, este último probablemente debido a que el donador de nitrógeno para la síntesis de nicotinamida en este organismo es la glutamina y

no el amonio (Kapour y Bray, 1968). Por otra parte el α-cetoglutarato (αcg) activa la G.S. tanto en prokariotes (Magasanik, *et al*, 1974) como en organismos superiores (Tate y Meister, 1973).

Este último efecto es muy interesante, y apunta al hecho de que la glutamina es no sólo donador de grupos amino sino también de esqueletos de carbono a tráves del αcg, y aquel sistema que provea a la célula de una fuente de carbono y de nitrógeno estará sujeto a regulación por ambos compuestos (Vaca *et al*, 1974).

Por otra parte, en *E. coli* y otras bacterias gram-negativas la G.S. puede existir en dos formas catalíticamente distintas pero interconvertibles: una adenilada y otra no. De hecho, la enzima consta de 12 sub-unidades, cada una de las cuales se puede adenilar a nivel de una tirosina para reducir la actividad y hacer aparecer la sensibilidad a la regulación alostérica previamente señalada (Shapiro y Stadtman, 1968).

El efecto de limitación de amonio en cultivos contínuos de *Escherichia coli* y *Klebsiella acrogenes*, fue estudiado por Senior (1975), quien encontró que estas condiciones inducían la G.S. la que se encontraba desadenilada (activa), mientras que la limitación de glucosa en *E. coli* reprimía la G.S. y

la inactivaba por adenilación. Además en *Klebsiella* la limitación de amonio resultaba en una inducción de la glutamato sintetasa (E.C.1.4.7.1) (GOGAT). Estos resultados nos llevaron a plantear la investigación que aquí se relata, en colaboración con el grupo del Dr. Jaime Mora, en ese tiempo del Instituto de Investigaciones Biomédicas de la UNAM. Mora y sus colaboradores han estudiado extensamente el metabolismo nitrogenado en *N. crassa*, y en particular la regulación de la G.S. En este organismo pueden coexistir dos formas oligoméricas diferentes de la enzima. Se encuentra un octámero al crecer al hongo exponencialmente en glutamato, glutamina o amonio como fuente de nitrógeno (Vichido *et al*, 1978), mientras que en condiciones de limitación de amonio, en una mutante deficiente de deshidrogenasa (Limón-Lason *et al*, 1977) o en mutantes deficientes en actividades de G.S. (Dávila *et al*, 1978) se encuentra un tetrámero, lo que hace pensar que esto constituye una respuesta regulatoria del hongo *N. crassa* y que existe una relación entre la capacidad de fijar amonio a bajas o altas concentraciones de sustrato y el estado oligomérico de la enzima.

Además, es importante señalar que en este organismo, la regulación de la G.S. por la fuente de nitrógeno se expresa a nivel de la síntesis específica de la enzima (Quinto *et al*, 1977; Espin y Mora 1978) y que esta regulación está mediada por un ajuste de los niveles del mRNA específico (Sánchez *et al*,1978)

Marzluf (1981) ha realizado una excelente revisión del metabolismo nitrogenado y expresión génica en hongos, en donde se pone en evidencia que sí bien diferentes organismos modulan la actividad de la G.S. por distintos mecanismos, *Neurospora*, según datos del grupo de Jaime Mora, controla esta enzima primariamente, si no de forma exclusiva, cambiando su tasa de síntesis (Vichido *et al*, 1978). Sin embargo, la regulación fina parece basarse en el hecho de que la G.S. en *N. crassa* se compone de dos polipéptidos diferentes, α y β, estructurados respectivamente en un tetrámero y en un octámero (Dávila *et al*, 1978 ; Sánchez *et al*, 1978).

Otro resultado que conviene discutir es el relacionado con la actividad de GOGAT, que sugerimos podría ser la enzima responsable de la fijación de amonio en condiciones de limitación de éste (Limón-Lason *et al*, 1977). En efecto, Hummelt y Mora (1980a) encontraron esta actividad y purificaron una GOGAT NADH-dependiente, que junto con la G.S. tetramérica constituye el sistema de fijación de amonio a bajas concentraciones. Los mismos autores (Hummelt y Mora, 1980b) estudiaron la regulación de esta enzima, la purificaron a homogeneidad y encontraron una mutante sin actividad de GOGAT, y concluyen que esta enzima se comporta de modo similar a la de nódulos de plantas.

Todo lo anterior lleva a la conclusión de que en *Neurospora* coexisten dos rutas para la asimilación de amonio. Una opera a altas concentraciones de este compuesto, como en crecimiento exponencial en exceso de amonio, el que es fijado por una deshidrogenasa glutámica (DHG) y una G.S. octamérica cuya síntesis (a nivel del polipéptido β) se regula por la fuente de nitrógeno en exceso: se eleva en glutamato, es intermedia en amonio y es baja en glutamina. Por otra parte, en condiciones de limitación de amonio (como en cultivo alimentado limitado de amonio) ocurre una G.S. con un polipéptido diferente, el α que se organiza como tetrámero; esta α G.S. y la glutamato sintasa fijan amonio a bajas contraciones (Lara *et al*, 1982).

I. DHG + β G.S.: Fijación de amonio a altas concentraciones.

II. αG.S. + GOGAT : Fijación de amonio a bajas concentraciones.

Esto tiene su lógica, pues si bien la ruta I es energéticamente más conveniente (no requiere de energía del ATP), la glutamino sintetasa tiene mayor afinidad por amonio que la deshidrogenasa glutámica, por lo que la ruta II es más eficiente a bajas concentraciones de amonio.

SUMARIO DEL ARTICULO

Limón-Lason, J., M. Lara, B. Rosendiz y J. Mora
REGULACION DE LA GLUTAMINO SINTETASA EN CULTIVOS ALIMENTADOS DE *NEUROSPORA CRASSA*.
Biochem. Biophys. Res. Comm. 78 (1977) 1234.

El efecto de las fuentes de nitrógeno y carbono en la regulación de la glutamino sintetasa se ha estudiado en cultivos alimentados de *Neurospora crassa*. La limitación de amonio en un exceso de la fuente de carbono lleva a una acumulación de α-cetoglutarato y una elevación de la actividad de la glutamino sintetasa. La limitación de sacarosa en un exceso de la fuente de nitrógeno resulta en una caída de la actividad de la enzima. Una mutante (AM-1) deficiente de deshidrogenasa glutámica biosintética y por ello incapaz de hacer glutamato a partir de α-cetoglutarato se comporta igual a la cepa salvaje 74A bajo limitación de amonio, lo que hace pensar que la glutamato sintasa participa en la asimilación de amonio en estas condiciones.

Estos datos sugieren que la fuente de carbono ejerce un control positivo en la regulación de la glutamino sintetasa, por las siguientes razones:

a) No hay efecto del amonio *per se*, pues la AM-1 en exceso de amonio sigue comportándose como si estuviese en condiciones de limitación de la fuente de nitrógeno.

b) No se observa efecto de la glutamina, pues la poza de este aminoácido se reduce tanto en limitación de amonio como de sacarosa.

c) Siempre hay coincidencias entre el aumento de α-cetoglutarato y la glutamino sintetasa.

Por otra parte, la glutamino sintetasa se encuentra conformada como un octámero cuando *Neurospora* está creciendo en un exceso de amonio, tanto en cultivo en lote como en cultivo alimentado limitado de sacarosa. Sin embargo, en condiciones de limitación de amonio la enzima se conforma como un tetrámero. Es interesante notar que la AM-1 siempre presenta una glutamino sintetasa tetramérica, ya sea que esté creciendo en exceso o limitada de amonio. Esto es evidencia adicional de que la vía de la glutamato sintasa se utiliza para fijar amonio a bajas concentraciones de este metabolito, o cuando la deshidrogenasa glutámica biosintética está ausente, pues la diferente forma oligomérica de la glutamino sintetasa facilitaría su participación en

dos vías metabólicas diferentes, permitiendo una regulación también diferente.

Los resultados además claramente muestran la ventaja del uso de técnicas de crecimiento controlado en el estudio de la regulación enzimática en organismos miceliares.

PUBLICACIONES

Vol. 78, No. 4, 1977 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

REGULATION OF GLUTAMINE SYNTHETASE IN FED-BATCH CULTURES OF NEUROSPORA CRASSA.

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Summary

The effect of the nitrogen and carbon sources in the regulation of glutamine synthetase has been studied in fed-batch cultures of Neurospora crassa. The limitation of ammonium in an excess of the carbon source, leads to an accumulation of α-ketoglutarate and elevation of glutamine synthetase. The limitation of sucrose in an excess of ammonium results in a decrease in glutamine synthetase activity. These results indicate that the carbon source exerts a positive control in the regulation of glutamine synthetase.

Introduction

It is known that in bacteria α-ketoglutarate and glutamine regulate the activity of glutamine synthetase (EC 6.3.1.2) (1). Although the effect of different nitrogen sources on the activity of this enzyme has been studied in eukaryots (2, 3), the difficulties encountered in growing filamentous fungi under conditions of regulated growth, as in a chemostat (4, 5), have not made possible to evaluate the effect of limiting concentration of the carbon and nitrogen sources in the regulation of glutamine synthetase, as has been done in prokaryotic organisms (6). We have been able to regulate the growth of Neurospora crassa by continuously adding to the medium the limiting nitrogen or carbon source. This instrumentation, known as fed-batch culture (7), has allowed us to study the effect of the carbon and the nitrogen sources on the activity and oligomeric state of the enzyme glutamine synthetase.

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Material and Methods

Strains and Chemicals

Neurospora crassa wild-type strain 74-A and the glutamic acid dehydrogenase deficient mutant am-1, obtained from the Fungal Genetics Stock Center at the Humboldt State University Foundation, Arcata, Calif. U.S.A. All chemicals used were analytical grade.

Growth Conditions

Batch cultures of N. crassa were grown after inoculating conidia in Vogel's minimal medium (8) with 25 mM NH_4Cl as nitrogen source and 1.5% sucrose as carbon source. Fed-Batch cultures were achieved for up to 24 hs by pumping the limiting substrate (9.2 mM NH_4Cl or 14.6 mM sucrose) at a dilution rate of 3.2×10^{-3} hr^{-1}, into an agitated and aerated reactor vessel held at 25°C containing Vogel's medium lacking the nitrogen or carbon source used as limiting substrate. The NH_4Cl was pumped after incubating the conidia for 3 hs in Vogel's without nitrogen source, and the sucrose after germinating the conidia 4 hs in substrate excess. Under these conditions the change in reactor volume is negligible and it can be shown that the growth rate is constant and equals the product of the dilution rate, the feedstock concentration and the substrate yield constant.

Growth was determined by collecting mycelium samples on 0.45 μ membrane filters, washing with distilled water and placing in 5% trichloro acetic acid. After centrifugation the acid precipitate was resuspended in 1.0 N NaOH and protein was determined by the method of Lowry et al (9), using bovine serum albumin as standard.

Preparation of soluble extracts for amino acid analysis

Mycelium samples were harvested by filtration. Glutamic acid and glutamine were extracted by homogenizing the cells in 80% ethanol in the presence of L-U-^{14}C glutamine and L-U-^{14}C glutamic acid, separated using the method of Yemm and Cocking (10) as modified by Ferguson and Simms (11), and quantified by isotopic dilution. α-ketoglutarate was assayed accordingly to Bergmeyer and Bernt (12), after resuspending the washed mycelium in 0.6 N $HClO_4$ and neutralizing the supernatant with K_3PO_4.

Determination of glutamine synthetase activity

Glutamine synthetase measured as transferase activity was assayed as described by Ferguson and Simms (11). Units of activity are expressed as micromoles of γ-glutamyl hydroxamate produced per min at 30°C per mg of protein.

Sucrose Gradient Sedimentation

The samples, in a final volume of 0.3 ml were layered over a 5 to 20% continuous sucrose gradient and centrifuged at 4° for 12 hs at 248,000 x g in the Beckman SW 40 rotor. After centrifugation, fractions were ob-

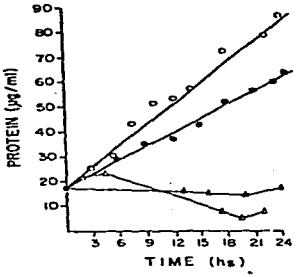

Fig. 1 Growth curves of the wild-tipe strain 74A in limiting ammonium (●) or sucrose (○) in fed-batch culture. Without ammonium (▲) or sucrose(△) also shown.

tained from the top of the tube and glutamine synthetase activity was determined in each fraction. Details are given in ref. 13.

Results

Neurospora crassa wild-type 74-A exhibits exponential growth with a doubling time of 2 to 3 hs, when grown under conditions of excess substrate. However, in fed-batch culture linear growth is found as shown in fig. 1.

Under conditions of ammonium limitation we found, after 12 hs, an almost 12 fold increase in the intracellular concentration of α-ketoglutarate, while glutamine and glutamate decrease ten and two times, respectively, compared to the control growing in excess substrate. However, when sucrose is the limiting substrate, α-ketoglutarate does not vary appreciably and glutamine decreases three times (Table 1).

Under ammonium limitation a ten-fold rise in glutamine synthetase activity is found. However, when sucrose is limiting the activity of this

TABLE 1.- Intracellular concentrations of ∝-ketoglutarate, glutamate and glutamine, and glutamine synthetase activity in the wild-type strain 74-A, under nitrogen or carbon source limitation*.

Condition	hs	kg	glu	gln	G.S.
Batch, ammonium plus carbon excess	12	0.002	0.183	0.480	0.09
Fed-batch, ammonium limited	12	0.025	0.069	0.040	0.42
	18	0.020	0.084	0.045	0.77
	24	0.015	0.049	0.040	0.99
Fed-batch, sucrose limited	12	0.003	0.169	0.320	0.02
	18	0.003	0.120	0.270	0.02
	24	0.002	0.145	0.190	0.02

*∝-ketoglutarate (kg), glutamate (glu) and glutamine (gln) concentrations expressed as μmoles/mg of protein. Glutamine synthetase activity (G.S.) expressed as μmoles of product per min per mg of protein.

enzyme falls four times compared to that found in an excess of substrate (Table 1). To ascertain whether these effects are due to ammonium per-se or are mediated by the trapping of ∝-ketoglutarate and/or its conversion to glutamate or glutamine, the mutant strain am-1, which lacks the activity of the biosynthetic glutamic acid dehydrogenase and grows very slowly in excess ammonium, was grown under the same conditions. We found that this strain, when limited of ammonium, has the same growth rate as the wild-type grown under the same conditions. The activity of glutamine synthetase and the intracellular concentrations of ∝-ketoglutarate and glutamate are quite similar to those found in the wild-type strain; glutamine is not detectable in comparison with the wild-type, where this amino acid is just in the limits of detection. Furthermore, when there is an excess of ammonium present, the above parameters still resemble those found in the wild-type in ammonium limitation (Table 2).

When the wild-type strain is grown in glutamate as the nitrogen

TABLE 2.- Intracellular concentrations of α-ketoglutarate, glutamate and glutamine, and glutamine synthetase activity in the mutant strain am-1, under nitrogen limitation*.

Condition	hs	kg	glu	gln	G.S.
Batch, ammonium plus carbon excess	12	0.012	0.027	0.045	0.55
Fed-batch, ammonium limited	12	0.023	0.031	N.D.	0.62
	18	0.022	0.055	N.D.	0.86
	24	0.020	0.079	N.D.	0.81

Abbreviations as in Table I. Not detected (N.D.)

source, glutamine synthetase is found as an octamer (13). We have found that a lower oligomeric form of this enzyme, possibly a tetramer, is found in the wild-type strain when ammonium is limiting, but not when it is in excess concentration (fig. 2). The mutant strain am-1, however, exhibits only the lower oligomeric state, regardless of the way ammonium is administered (data not shown).

Discussion

It is not possible to regulate the growth rate of Neurospora crassa in batch cultures just by changing the substrate concentration. However, this has been achieved by the use of fed-batch cultures.

We have shown that glutamine synthetase activity rises when ammonium is limited. This rise with time corresponds to the increase in limitation that occurs as result of growth, addition of substrate remaining constant. Coinciding with this effect, we found an important rise in α-ketoglutarate together with a decrease in glutamate and glutamine. We would like to propose that the carbon source exerts a positive control in the induction of glutamine synthetase in Neurospora crassa since: a) ammonium does not

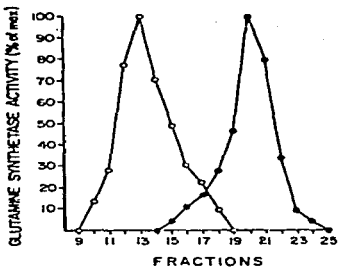

Fig. 2 Sedimentation in sucrose gradients of glutamine synthetase from the wild-tape strain 74A grown in limiting (○) and excess (●) ammonium.

have an effect per se, as shown by the increase in enzyme activity in the glutamic acid dehydrogenase deficient mutant grown in excess ammonium, as compared with the wild type and b) by the decrease of the activity under sucrose limitation in the wild-type strain where ammonium is in excess. This data indicates that ammonium does not exert its main effect through its conversion to glutamine, for if this were the case, a rise in activity would also be expected when carbon is limiting and glutamine falls (Table 1). It is suggestive that a rise in α-ketoglutarate is found whenever the enzyme is induced (Tables 1 and 2). This elevation is to be expected if ammonia is limited or if a block exists in the conversion of ammonium and α-ketoglutarate to glutamic acid. On the other hand we have recently reported that an excess of ammonium or glutamine represses the de novo synthesis of glutamine synthetase (4).

Very possibly a relationship exists between the oligomeric state of glutamine synthetase and its capacity to fix ammonium at low or high sub-

strate concentrations. The ability of the tetramer to synthesize glutamine at low substrate concentration is being studied.

The fact that a strain completely lacking the biosynthetic glutamic acid dehydrogenase grows as well as the wild-type in limited ammonium makes worthy to look for the activity of glutamate synthase in Neurospora crassa. The catabolic glutamic acid dehydrogenase has low affinity for ammonium (15), and thus cannot be expected to be fixing ammonium under these conditions.

The results clearly show the advantage of the use of growth controlled cultures in the study of enzyme regulation in filamentous organisms.

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Immobilization of Glucose Isomerase to Ion-Exchange Materials

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Summary

Glucose isomerase (D-xylose ketol-isomerase EC 5.3.1.5) from *Bacillus coagulans* was partially purified and immobilized by adsorption to anion exchangers. The highest activities were obtained when the enzyme was adsorbed to DEAE-cellulose. On immobilization to DEAE-cellulose the measured optimum pH value for enzyme activity shifted from 7.2 to 6.8. There was no appreciable difference between the heat stabilities of soluble and immobilized enzyme. The $K_{m\ app}$ values for the immobilized enzyme were found to be 0.25*M* in the presence of 0.01*M* Mg^{2+} and 0.19*M* with 0.005*M* Mg^{2+}, while those for the soluble enzyme were 0.11 and 0.17*M*, respectively. Under conditions of continuous isomerization of D-glucose, a decrease of activity with time was observed, but this decrease was less at a low Mg^{2+} concentration and was affected by column geometry. There were no appreciable diffusional limitation effects in packed-bed columns.

INTRODUCTION

The enzyme glucose isomerase (D-xylose ketol-isomerase EC 5.3.1.5) catalyzes the reversible isomerization of D-glucose to D-fructose. This enzyme is now used in the food-processing industry to produce a sugar mixture as sweet as sucrose. Initially the large-scale production of fructose-containing syrups was accomplished by using either whole cells or a soluble enzyme preparation as catalyst. Recently a commercial process for continuous conversion of D-fructose using immobilized cell-free glucose isomerase has been described.[1] The enzyme has previously been immobilized by covalent binding,[2,3] entrapment,[4,5] and adsorption.[6] Enzyme immobilization by adsorption is simple, mild, and reversible, permitting reuse of both the enzyme and the support, and can give immobilized enzyme preparations with high activity per unit of weight of support.[7] Since both the substrate and product of glucose isomerase are small molecules without charge, this should allow the

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adsorbed enzyme to be used at high substrate concentrations. Although glucose isomerase adsorbed to DEAE-cellulose is now used commercially, the preparation and properties of adsorbed cell-free glucose isomerase have not been described in detail. In this paper the preparation of an immobilized partially purified glucose isomerase by adsorption to DEAE-cellulose or other anion exchangers and its utilization in the continuous production of D-fructose are described.

MATERIALS AND METHODS

Materials

A crude preparation of glucose isomerase from *Bacillus coagulans* was provided by Novo Industri A/S, Copenhagen, Denmark. The DEAE-cellulose (grade DE23) was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, England. The DEAE-Sephadex A-25 and A-50 (40–120 μm particle diam, 3.5 ± 0.5 mequiv/g) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Glucose (Analar grade), maleic acid, and L-cysteine hydrochloride were obtained from BDH Biochemicals Ltd., Poole, Dorset, England, and carbazole from Hopkin and Williams Ltd., Chadwell Heath, Essex, England. All inorganic chemicals were Analar grade where available.

Purification of Glucose Isomerase

All purification procedures were carried out at 4°C. The crude preparation of glucose isomerase (10 g dry basis) was suspended in 0.05*M* maleate buffer, pH 6.5, containing 0.02*M* $MgSO_4 \cdot 7H_2O$ to give a final volume of 250 ml.

The suspension was centrifuged at 12,000 *g* for 30 min. The supernatant (*I*) yielded 80–90% of the original activity. Manganese chloride solution (1.0*M*) was added slowly to the supernatant with stirring to give a final concentration of 0.05*M*. After 3 hr, the precipitate was removed by centrifugation and discarded. The supernatant solution was brought to 70% saturation with solid ammonium sulfate. The precipitate obtained by centrifugation at 12,000 *g* was dissolved in 0.01*M* maleate buffer, pH 6.5. The solution was dialyzed against the same buffer for 3 hr. Dialyzed enzyme solution (18 ml) was placed on a column (2.5 cm diam × 30 cm) containing DEAE-Sephadex A-50 previously equilibrated with 0.01*M* maleate buffer, pH 6.5. After passage of 1000 ml of the same buffer, the enzyme was eluted by stepwise increasing the potassium chloride concentration. Ten ml aliquots of eluate were collected (Fig. 1). The activity recovered after elution was about 90% of that loaded on the column. The results for specific activity and yield of glucose isomerase during the purification steps are given in Table I. Despite the low purification factor (4–5 times) the specific activity after DEAE-Sephadex chromatography was relatively high compared with preparations obtained from *Streptomyces phaeochromogenes*,[8] *Lactobacillus brevis*,[9] *Bacillus coagulans*,[10] and *Streptomyces griseolus*.[4]

Immobilization of Glucose Isomerase

A suspension of 500 mg (dry weight) of precycled DEAE-cellulose or activated DEAE-Sephadex in distilled water was stirred gently at 4°C with a magnetic stirrer. Glucose isomerase solution (4 ml), which was obtained after precipitation with ammonium sulfate, was added to the above and stirred for 30 min at the same temperature, the final volume being 50 ml. The enzyme–support complex obtained was washed with 500 ml distilled water and suspended in 50 ml water. There were no appreciable differences in activity retention when either the DEAE-Sephadex A-25 or A-50 were pretreated with 0.10*M* phosphate buffer, pH 7.0, or 0.10*M* maleate buffer, pH 7.0

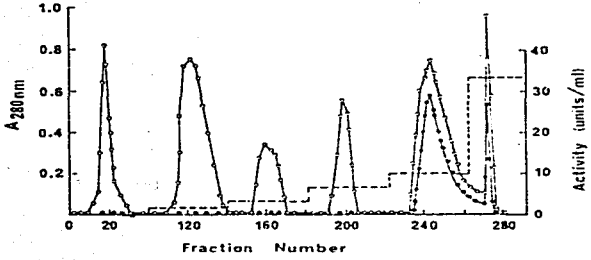

Fig. 1. Chromatography on DEAE-Sephadex A-50 of the enzyme preparation obtained by precipitation with ammonium sulfate. Column (2.5 cm diam × 30 cm) was eluted stepwise with 0.05, 0.1, 0.2, 0.3, and 1.0*M* KCl in 0.01*M* maleate buffer, pH 6.5, at a flow rate of 15 ml/hr. Fraction volumes were 10 ml. (○) Absorbance at 280 nm; (●) glucose isomerase activity; (- - - -) KCl concentration in effluent fed to column.

TABLE I
Purification of Glucose Isomerase Activity

	Protein (mg)	Activity* (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant (1)	2200	16,400	7.5	100
Mn^{2+} treatment	1600	10,300	6.5	63
Ammonium sulfate	660	10,200	15.5	62
Dialysis	650	9,200	14.2	56
DEAE-Sephadex A-50 chromatography	270	8,150	30.2	50

* Units = μmol D-fructose/min.

Assay of Glucose Isomerase Activity

Glucose isomerase activity was determined by measuring the formed D-fructose using a modified version of the method reported by Lloyd et al.[11] The enzyme activity during the purification steps was determined in a system containing 0.25*M* maleate buffer, pH 6.5; 0.10*M* $MgSO_4 \cdot 7H_2O$; 0.001*M* $CoCl_2 \cdot 6H_2O$, and 0.275*M* D-glucose. The final volume was 2.0 ml. The reaction mixture was incubated in a water bath at 65°C and the reaction stopped by the addition of 10 ml 0.10*M* $HClO_4$.

For the assay of soluble and immobilized enzyme in a batch reactor the reaction mixture contained: enzyme solution; 0.01*M* $MgSO_4 \cdot 7H_2O$; 0.001*M* $CoCl_2 \cdot 6H_2O$, and 0.275 or 1.0*M* D-glucose adjusted to pH 6.8. The final volume was 20 ml. The reaction mixture was incubated at 65°C, stirred at 250 rpm with a paddle stirrer (Varilab type, Citenco Ltd., Borehamwood, Herts., England), and the rate of isomerization was determined by taking 2 ml samples of the reaction mixture at intervals from the batch reactor. Each withdrawn sample was added immediately to 10 ml 0.01*M* $HClO_4$ to stop the reaction.

The D-fructose formed was determined by the modified cysteine–carbazole sulfuric acid method of Dische and Borenfreund.[12] One unit of enzyme activity was defined as that which produced 1 μmol D-fructose/min.

Enzyme activity was linear for up to 15 min reaction time. The assay was usually taken to 6 min at which time conversion was between 4 to 9% in any assay condition.

Protein Measurements

Protein concentrations were determined by the Folin method of Lowry et al.,[13] except for chromatographic column eluates where the optical density at 280 nm was monitored.

Continuous Isomerization of D-Glucose by DEAE-Cellulose–Glucose Isomerase Column

Packed columns of immobilized glucose isomerase were continuously operated at 65°C. Column lengths varied from 3 cm (500 mg DEAE-cellulose–enzyme complex as dry weight) to 12 cm (2000 mg enzyme complex). Substrate concentrations were from 0.275 to 2.5*M* D-glucose. Data on the actual length and substrate and metallic ions concentrations of each column are shown in the figures. The substrate solution was pumped through the column at a flow rate of 10 ml/hr, except where otherwise stated. The effluent was collected as 1.9–2.0 ml fractions in tubes containing 5 ml 0.1*M* $HClO_4$, and the fructose production was evaluated.

Film diffusion was found not to be limiting, as shown by assuming film diffusion to be limiting and calculating the required column height using Satterfield's correlation,[14] and by a plot of conversion versus reciprocal flow rate which gave a straight line (Fig. 2). The absence of pore diffusional limitation was verified by calculating Pitcher's modulus,[15] using an effective diffusivity 1/5 that of the bulk diffusivity.[16] Effectiveness factors were essentially 1.0 for substrate concentrations between 0.275 and 2.5*M*. Furthermore, grinding the immobilized enzyme did not change $K_{m\ app}$ or the specific activity (Table II).

TABLE II
Specific Activity and K_m' of the Free and Immobilized Enzyme Before and After Grinding*

Condition	Specific activity (units/mg protein)	$K_{m\ app}$ (*M*)	$K_{m\ app}$* (*M*)
Free enzyme	9.32	0.17 ± 0.056 (14)	0.11 ± 0.01 (6)
Immobilized enzyme			
Nonground	10.91	0.19 ± 0.033 (7)	0.25 ± 0.017 (6)
Ground	10.79	0.18 ± 0.028 (8)	–

* Mg^{2+} concentration is 0.005*M* except in *, which was 0.01*M*. $K_{m\ app}$ values given as mean ± standard deviation (number of points).

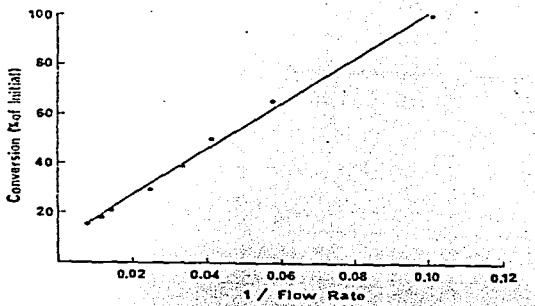

Fig. 2. Dependence of conversion on reciprocal flow rate. Substrate solution passing through the column contained 0.275*M* D-glucose, 0.001*M* Co^{2+}, and 0.01*M* Mg^{2+} at pH 6.9.

Residence-time distribution analysis using tracers showed that the flow pattern for a 1.6 × 3.0 cm packed bed corresponded to more than 20 stirred tanks in series, essentially plug-flow behavior.

RESULTS

Preparation of Immobilized Glucose Isomerase

An enzyme preparation obtained after ammonium sulfate precipitation was used to prepare immobilized glucose isomerase since it could be prepared quickly and the glucose isomerase activity was more stable than that obtained after the chromatographic step. As can be seen in Table III, DEAE-cellulose retains more activity and protein than the other ionic supports. In order to determine the maximum protein and activity that DEAE-cellulose could adsorb, different concentrations of protein were added to the ion-exchange support. Figure 3 shows that up to 170 mg protein/g support the activity retained was proportional to the protein added. Throughout this range DEAE-cellulose adsorbed 80% of the protein added and retained between 93 and 100% of the initial activity. No activity and only 20% of the added protein was found in filtrates made in this range. The maximum values for protein and activity bound occurred

TABLE III
Preparation of Immobilized Glucose Isomerase Activity

Support	Activity[a] (units/g support)			Protein (mg/g support)		Specific activity (units/mg protein)	
	added (*A*)	bound (*B*)	Activity retained (*B*/*A* × 100)	added (*C*)	bound (*D*)	added (*A*/*C*)	bound[b] (*B*/*D*)
AE-cellulose	317	73	23	ND[c]	ND	ND	ND
DEAE-Sephadex A-25	317	133	42	34	11	9.32	12.1
	634	128	20	68	11	9.32	11.6
	950	140	14	102	11	9.31	12.7
DEAE-Sephadex A-50	856	453	53	ND	ND	ND	ND
DEAE-cellulose	634	590	93	68	55	9.32	10.7
	1268	1186	94	136	106	9.32	11.2
	2400	1700	70	272	152	8.82	11.2

[a] Units = μmol D-fructose/min.
[b] Determined after elution with 1.0*M* NaCl.
[c] ND is not determined.

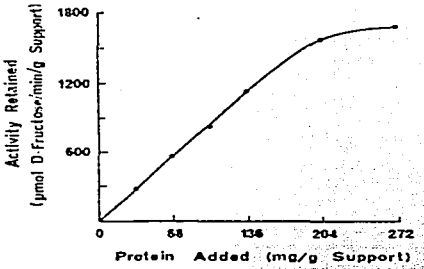

Fig. 3. Immobilization of glucose isomerase to DEAE-cellulose. Enzyme immobilization and assays were carried out under standard conditions except for the concentration of protein added.

when 272 mg protein/g support were added, being 152 mg/g and 1700 units/g support, respectively. The specific activities of immobilized glucose isomerase preparations remained practically constant and were higher than the specific activity of the soluble enzyme (Table III).

The activity adsorbed on DEAE-cellulose was not desorbed by up to 2.5*M* D-glucose in the presence of 0.001*M* $CoCl_2$ but without Mg^{2+}. However, only 10% of both protein and activity remained adsorbed after incubation for 15 min with 0.10*M* Mg^{2+}, while 95% of protein and activity remained adsorbed after incubation for the same period with 0.01*M* Mg^{2+}, as measured after elution in a column with 1.0*M* NaCl.

Effect of pH

The effect of pH on glucose isomerase activity is illustrated in Figure 4. The optimum pH values were found to be about 7.2 and 6.8 for the soluble and immobilized enzyme, respectively.

Effect of Substrate Concentration

The effect of substrate concentration on the rate of isomerization of D-glucose was investigated in the presence of 0.01 or 0.005*M* Mg^{2+}. Apparent Michaelis constants for glucose were determined for both soluble and immobilized glucose isomerase by using

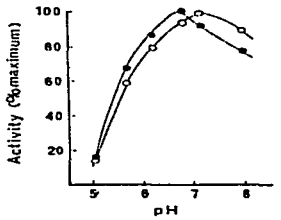

Fig. 4. Effect of pH on glucose isomerase activity in solution (○), and when immobilized to DEAE-cellulose (●). Enzyme assays were carried out under standard conditions except for the pH of the reaction mixture.

Lineweaver–Burk plots, which gave a reasonable spread of the data, and are shown in Table II. The difference between soluble and immobilized enzyme for the low Mg^{2+} concentration was not significant ($P > 0.05$), but at the high Mg^{2+} there was a significant difference ($P < 0.001$). Grinding did not change K_m ($P > 0.05$).

Heat Stability

The heat stability of free and immobilized glucose isomerase for various temperatures in the presence of Mg^{2+} is shown in Figure 5.

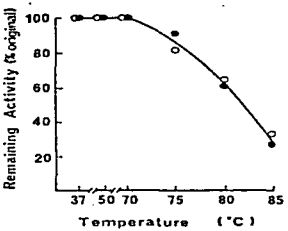

Fig. 5. Heat stability of glucose isomerase. Ten ml enzyme solution or immobilized enzyme suspended in distilled water were preincubated for 15 min at the specified temperatures, rapidly cooled, and the remaining activity assayed at 37°C. (○) Soluble enzyme; (●) immobilized enzyme.

It can be seen that, at least for the 15 min periods studied, there is no difference between the preparations. First-order decay occurred at the three temperatures studied, with half-lives of 528 ± 3.9 hr at 40°C; 192 ± 10.1 hr at 60°C; and 55 ± 11.4 hr at 70°C. The decay curve for 60°C was the same for free and immobilized enzyme. From the above data, the activation energy for heat inactivation was 81,000 J·mol^{-1}.

Continuous Isomerization of D-Glucose by DEAE-Cellulose–Glucose Isomerase Complex—Operational Stability

The results for conversion in a 1.6 × 3.0 cm bed at a constant flow rate show that when a substrate solution containing 0.01*M* Mg^{2+} at pH 6.9 was passed through the column, the activity decreased during continuous operation, reaching 50% of the initial activity after 15 hr (Fig. 6). This decrease seems to be mainly due to desorption of the enzyme during operation, because after 48 hr the conversion was only 8% of the initial value and 11% of protein remained in the column. At pH 8.0 there was only a small decrease in the desorption rate (not shown). To assess whether Mg^{2+} was responsible for this high desorption rate, substrate solution at pH 8.0 and 0.005*M* Mg^{2+} was passed through the column. The glucose

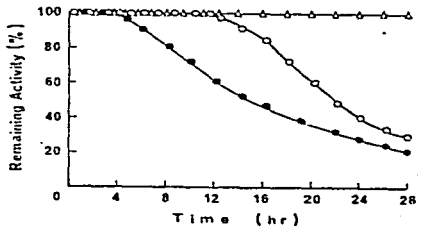

Fig. 6. Stability of DEAE-cellulose–glucose isomerase when used for the continuous isomerization of D-glucose in a column system: Immobilized enzyme preparation (500 mg) was packed into a jacketed column held at 65°C and substrate was passed through the column at a flow rate of 10 ml/hr. (●) 1.6 × 3.0 cm column receiving 0.275*M* D-glucose, 0.001*M* Co^{2+}, 0.01*M* Mg^{2+}; (○) 1.1 × 6.0 cm column receiving 1.0*M* D-glucose, 0.001*M* Co^{2+}, 0.01*M* Mg^{2+}; (△) 1.6 × 3.0 cm column receiving 0.86*M* glucose, 0.001*M* Co^{2+}, and 0.005*M* Mg^{2+}. Initial isomerization values for runs were 54, 45, and 30%, respectively.

conversion remained the same during the first 28 hr of operation, but decreased to 50% of the initial value after 60 hr, and to 12% after 120 hr. The amount of protein still adsorbed after this time was 20% of the initial concentration. A 1.15 × 6.0 cm packed bed was then evaluated and the results show what seems to be a decrease in desorption when the bed length is increased at the expense of cross-sectional area (Fig. 6). While the rate of desorption does not decrease, the time lag before the onset of desorption increases. When a larger column bed (1.6 × 12 cm) was operated continuously, there was no loss of activity for 200 hr (Fig. 7).

DISCUSSION

The use of immobilized enzymes in industrial processes depends on many factors. The ability to produce, by simple means, a relatively low cost immobilized enzyme product with high activity per weight of support, with adequate operational stability, and efficient use of that product, is an important technical consideration. Here we have examined the preparation of immobilized glucose isomerase by adsorption to DEAE-cellulose and other anion exchangers. The retained activity of the DEAE-cellulose–glucose isomerase complex was higher than that obtained with either AE-cellulose or DEAE-Sephadex A-25 and A-50. The capacity of DEAE-Sephadex A-25 to adsorb protein and glucose isomerase activity could be limited by the porosity of the ion-exchanger beads. Although

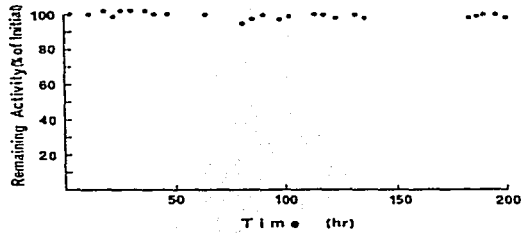

Fig. 7. Stability of DEAE-cellulose–glucose isomerase in a 1.6 × 12.0 cm column, fed a substrate solution containing 2.5*M* glucose, 0.001*M* Co^{2+}, and 0.005*M* Mg^{2+} at 10 ml/hr.

DEAE-Sephadex A-50 adsorbed more activity than DEAE-Sephadex A-25, it still adsorbed less than DEAE-cellulose. On the other hand, DEAE-Sephadex A-50 has more swollen beads and therefore not as good flow properties as the A-25 form.

On immobilization of glucose isomerase an increase in specific activity took place because it was utilized as an impure preparation. The maximum activity per gram of support bound to DEAE-cellulose was 1700 μmol/min/g, which is higher than those reported for other immobilized glucose isomerase preparations, such as glucose isomerase from *Streptomyces phaeochromogenes* bound to silanized coated porous glass,[3] to glass beads,[2] and entrapped in cellulose triacetate fibers.[4] Also it is higher than those reported for immobilized cells of *Bacillus coagulans*,[17,18] and entrapped cells of *Streptomyces phaeochromogenes*.[19] Moreover, the retention of activity on adsorption to DEAE-cellulose was higher than that reported for other enzymes adsorbed to DEAE-cellulose.[20,21]

The optimum pH value of soluble glucose isomerase was found to be 7.2, which agrees well with the pH 7.0 value reported by workers from Novo Industri A/S for soluble enzyme produced by *Bacillus coagulans*,[18] and with pH 7.0 reported for purified enzyme from *Bacillus coagulans* strain HN-68.[22]

The optimum pH of DEAE-cellulose–glucose isomerase shifted slightly toward the acid side in comparison to the soluble enzyme. However, a shift from 7.0 for soluble glucose isomerase, to 8.5 for immobilized cell debris of *Bacillus coagulans*[18] has been reported. Displacements of pH curves toward a more acid pH for other enzymes adsorbed to DEAE-cellulose, such as aminoacylase,[23] ATP-deaminase,[24] and invertase,[25] have also been reported. These displacements may be explained by the difference in the electrostatic field of both enzyme molecules and the surrounding aqueous medium caused by the net positive charge of the support.[26]

The $K_{m\ app}$ value of soluble glucose isomerase was slightly higher than that of 0.09*M* observed by Danno[10] for a purified enzyme from *Bacillus coagulans*, when measured at 40°C and pH 7.0, but was lower than that of 0.4*M* reported for the enzyme from *Streptomyces* sp.[27] $K_{m\ app}$ for our immobilized glucose isomerase was also lower than that of 2.25*M* reported by workers from Novo Industri[18] for immobilized cell debris of *Bacillus coagulans*. We observed a slight increase in $K_{m\ app}$ on adsorption to our enzyme to DEAE-cellulose. However, upon grinding of the complex a lower K_m is not observed. Thus we do not believe this effect to be due to diffusional resistance, but to microenvironmental effects. The effect, however, is more

marked in 0.01*M* Mg^{2+}, which is what would be expected if caused by the density of charges in the microenvironment.

Desorption of adsorbed enzymes under operational conditions has been reported for several immobilized enzymes. Desorption of aminoacylase from DEAE-Sephadex columns occurred at concentrations of acetyl-DL-methionine greater than 0.2*M*.[28] Invertase adsorbed on DEAE-cellulose was desorbed rapidly at low pH and high sucrose concentration.[25] Amyloglucosidase was desorbed from DEAE-cellulose at temperatures above 55°C.[21] We have found that our glucose isomerase preparation is desorbed at 0.1*M* Mg^{2+}, but much less so at 0.005*M* Mg^{2+}. Accordingly, the operational half-lives of the columns are a function of the Mg^{2+} concentration. They also seem to depend upon the geometry of the columns. Thus at 0.01*M* Mg^{2+} level when using a packed bed of 3 cm length and total volume of 6.03 cm^3, the operational half-life was only 15 hr. A bed of similar volume but of double height had an operational half-life of 21 hr. A column of 24.12 cm^3 with geometrical similarity to the latter could be operated for 200 hr without loss of activity with 0.005*M* Mg^{2+}.

In the first two cases a striking feature is that the residual activity plots show two distinct phases: at first there is no loss of activity and then decay is observed showing what appears to be first-order kinetics (a plot of log residual activity versus time yields a straight line). We believe that since adsorption–desorption is a reversible process, the desorbed enzyme is again adsorbed distally in the column until it is finally lost in the effluent. Thus a time lag would be expected for the desorption process to be apparent and this time lag would be greater in a longer column. Alternatively, it could be said that diffusional effects could mask the instability for the initial hours of operation.[29] However, we have not detected film or pore diffusional resistance by several different approaches even though DEAE-cellulose is a porous support. This is in agreement with that found for glucose isomerase immobilized on glass beads, but not for whole trapped cells, where there is marked diffusional limitation through the membranes.[30]

Aminoacylase was reported[31] to have increased thermal stability when adsorbed to DEAE-Sephadex A-25 or to DEAE-cellulose. Also the thermal stability of glucose isomerase from *Streptomyces* has been reported[30] to improve very strikingly when bound to DEAE-cellulose. Although our data for the free enzyme are in agreement with published results,[30] we found no improvement in thermal stability upon adsorption to DEAE-cellulose.

The use of immobilized glucose isomerase has recently been reported[17,18] by workers from Novo Industri A/S. The reported activity (150 μmol/min/g at 65°C) was lower than the one we found because they seem to have used immobilized cell debris.

The long-term stability of the above preparation, using 150 ml columns, seems to be better than that for the enzyme bound to DEAE-cellulose. However, it must be noted that we ran the columns in the presence of oxygen. It has been reported that glucose isomerase from *Bacillus coagulans* is sensitive to oxygen, especially at elevated temperatures where the exposure to oxygen decreases the operational stability of the enzyme.[17]

The method we have described is a simple and efficient procedure for obtaining a preparation with a high glucose isomerase activity. Since the support may be reutilized, the method should also be economical. Thus it is not surprising that a process using DEAE-cellulose–immobilized glucose isomerase is presently under commercial exploitation.

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Reactor Properties of a High-Speed Bead Mill for Microbial Cell Rupture

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Summary

Laboratory and pilot-plant high-speed bead mills of 0.6 and 5 liter capacity and consisting of four and five impellers in series, respectively, were used to follow the batch and continuous disruption of bakers' yeast (*Saccharomyces cerevisiae*). The mills are not scaled equivalents. Throughputs ranging from 1×10^{-6} m³/sec to 12×10^{-6} m³/sec for the 0.6 liter mill and from 16×10^{-6} m³/sec to 100×10^{-6} m³/sec for the 5 liter mill were used for continuous disruption studies. Variables studied included the effect of impeller tip speed, temperature, and packed yeast concentration (ranging from 15 to 75% by weight packed yeast). Disruption kinetics, as measured by the release of soluble protein, followed a first-order rate equation, the rate constant being a function of impeller tip speed and yeast concentration. For continuous disruption studies the bead mills behaved as a series of continuous stirred-tank reactors, each impeller forming a reactor. In the smaller mill a considerable degree of backflow between the reactors was evident. For certain mixing conditions the maximum amount of releasable protein was dependent on the impeller geometry, construction material, and also the concentration of packed yeast. The relative power efficiencies of the two mills are discussed along with possible criteria for scaling of bead mills.

INTRODUCTION

In 1972 we described yeast cell rupture in a vertical bead mill.[1] Protein release appeared to be a first-order process with respect to time and we were able to show how disruption efficiency varied with bead size, bead load, cell concentration, agitator speed, and temperature. Subsequently, we briefly described the behavior of a

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more advanced horizontal bead mill.[2] Other workers[3,4] have examined this horizontal machine.

Studies to date have not indicated the nature of the flow and mixing patterns involved within the agitator chamber. Since an understanding of these is important in terms of the design of agitator mills for microbial cell rupture, we have extended our earlier work.

We report here a study of laboratory and pilot-scale horizontal agitator mills of similar design. As in the past, we have used bakers' yeast (*Saccharomyces cerevisiae*) as test material.

MATERIALS AND METHODS

Microorganism

All experiments were carried out using commercial packed bakers' yeast, *Saccharomyces cerevisiae*, obtained from the Distillers Co., Ltd., London, England. It was kept refrigerated and used within a week of its arrival. During this storage period there was no change in the amount of releasable protein as measured by disruption in a bead mill.

Bead Mill

Two sizes of horizontal bead mills (Willy A. Bachofen Maschinenfabrik, Basel, Switzerland) were used (Table I): a laboratory machine (model KDL) with disruption chamber volume of 0.6 liter and a pilot-plant machine (model KD-5) of 5 liter disruption chamber volume (Fig. 1(a)). The two models are not scaled equivalents. Two types of impellers are available for both machines as shown in Figure 1(b).

Glass beads of 0.42–0.5 mm diam were used. During continuous

TABLE I
Details of Laboratory and Pilot-Plant Bead Mills

Model	KDL	KD-5
Chamber volume (liter)	0.6	5
Bulk bead volume (liter)	0.505	4.15
Liquid volume (liter)	0.32	2.45
Number of impellers	4	5
Impeller diam (m)	0.064	0.126
Chamber diam (m)	0.077	0.154
Motor rating (kW)	1.85	10

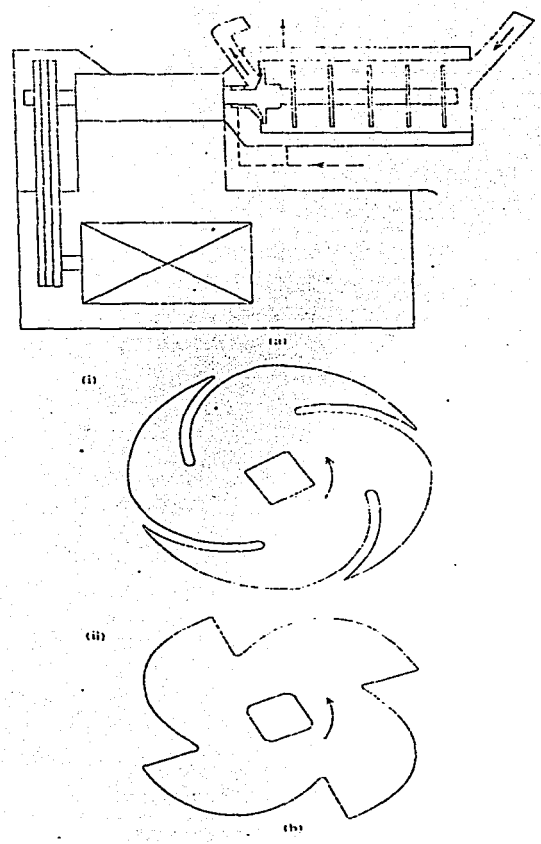

Fig. 1. Horizontal bead mill: (a) general view of mill; (b) details of (i) stainless-steel and (ii) polyurethane impellers.

operation the beads are retained in the mill by adjusting the gap between the rotor and stator to 0.07 mm.

Both mills are equipped with a cooling jacket. For the 0.6 liter mill a glass chamber was used for direct observations of the flow patterns and bead distribution during mixing studies. Also, for the 0.6 liter mill a port between the second and third impeller allowed access for sampling or injection.

Disruption Procedure

Bakers' yeast (45% wet weight/volume) was suspended with a Silverson stirrer-homogenizer in medium containing 0.15*M* NaCl and 0.004*M* K_2HPO_4, as previously reported.[5]

The mill was filled with this suspension for batch trials, and cooled to 5°C. The 0.6 liter mill was operated for the duration of the batch run and sampled at timed intervals with a syringe fitted with a polycarbonate tube of 4 mm diam. The 5 liter mill was operated in a stop-and-go manner, because sampling from the machine during operation was not feasible.

Continuous trials were carried out by pumping the yeast suspension (using Mono pumps types SB14 or SB15) through the mill until steady-state conditions were achieved as noted by analysis of the exit stream. Except where otherwise stated, the yeast suspension was cooled to 5°C prior to disruption.

Protein Assay

The disrupted yeast suspension was centrifuged at 36,000 *g* for 30 min in a MSE-L centrifuge and filtered through Whatman filter paper. Protein was assayed by adapting the method of Lowry et al.[6] to a Technicon Auto-Analyser.

Protein concentration was corrected for solids content of the yeast suspension as described previously.[5] All reagents used in the assay procedure were analytical grade.

Tracer Studies

These were carried out by injection of a pulse of a dye in the inlet stream of a continuously operating machine, and measuring its concentration in the outlet by colorimetry. Technicon ink was found to give the best results and was used as the test dye.

Normalized exit age distribution curves were calculated as described by Levenspiel.[7]

RESULTS

Batch Disruption of Yeast

For first-order disruption kinetics the rate of protein release is directly proportional to the amount of unreleased protein

$$\frac{dR}{dt} = k(R_M - R) \tag{1}$$

where R is the weight of protein released per unit weight of packed yeast, R_M is the maximum measured protein release, and k is the first-order rate constant.

Integration of eq. (1) between $t = 0$ and $t = t$ gives

$$\log_e[R_M/(R_M - R)] = \log_e D = kt \tag{2}$$

where D is the reciprocal of the fraction of unreleased protein.

Using the 0.6 liter mill R_M values of 0.100 ± 0.005 kg protein release/kg packed yeast were measured. For both stainless-steel and polyurethane impellers first-order kinetics were noted (Fig. 2).

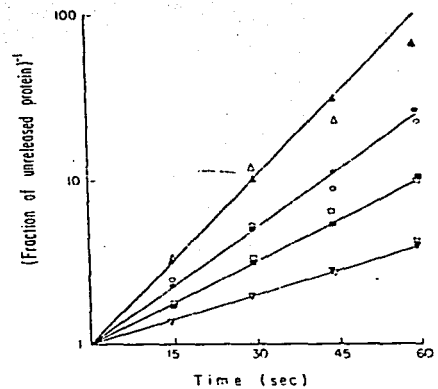

Fig. 2. Batch disruption of yeast in 0.6 liter mill ($V = 0.45$, $T = 5$°C, closed symbols stainless-steel impellers, open symbols polyurethane impellers). (○) u_t = 20 m/sec, $k = 0.0747$ sec^{-1}; (□) u_t = 15 m/sec, $k = 0.0544$ sec^{-1}; (△) u_t = 10 m/sec, $k = 0.0384$ sec^{-1}; (▽) u_t = 6.7 m/sec, $k = 0.0223$ sec^{-1}.

Problems of temperature rise in runs carried out over extended periods in the 5 liter mill prevented the direct measurement of the R_M value. Thus, this value was obtained by determining the R_M' value, which yields a best data fit for a first-order process. The corresponding D value is shown as D'.

Disruption data obtained for the 5 liter mill operating at low impeller tip speeds (u_t = 10 m/sec) can be represented by first-order kinetics (Fig. 3). For higher impeller tip speeds this is not possible. There is little justification for using higher orders of reaction to fit the data as this deviation from first-order kinetics may

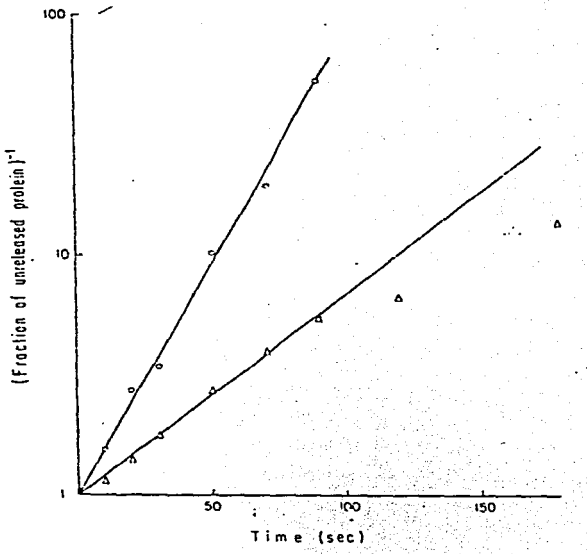

Fig. 3. Batch disruption of yeast in 5 liter mill. u_t = 10 m/sec, Y = 0.45, I = 5°C: (○) polyurethane impellers, R_M' = 0.084, k = 0.0440 sec⁻¹; (△) stainless-steel impellers, R_M' = 0.096, k = 0.0192 sec⁻¹.

be due in part to difficulties experienced in controlling the temperature of the disruption medium. Furthermore, in contrast to the 0.6 liter machine, sampling of the disruption medium required that the impeller drive be stopped. Even in cases where fresh medium was used for each kinetic datum point, the acceleration and deceleration of the beads could well be responsible for the measured higher rates of protein release compared with those expected from a first-order process.

Effect of Temperature on Disruption Kinetics

Owing to the size and geometry of the 5 liter mill, the cooling jacket around the disruption chamber cannot readily remove heat dissipated during rotation of the impellers (modifications to the commercial machine have since been made to improve cooling). Therefore high-temperature rises occur and it is necessary to determine the effects of temperature on disruption kinetics. Table II gives some typical temperature rises in a 45% packed yeast dispersion in a 5 liter machine equipped with a cooling jacket held at −10°C.

The effect of these temperature rises on yield was studied by following the disruption of yeast slurries at average temperatures ranging from 5 to 40°C (Fig. 4). Over this temperature range there is a decrease of 18% in the protein released. However, over the 5 to 20°C range a decrease in yield of only 2% was noted. In all other disruption experiments the feed was maintained at 5°C, therefore no correction is made for the effect of temperature rise on yield. The cooling system on the 0.6 liter machine maintained temperature rises at less than 6°C.

TABLE II
Effect of Throughput on Temperature Rise*

Throughput, Q (m³/sec)	Temperature rise (°C)	
	stainless-steel impeller	polyurethane impeller
83×10^{-6}	16.5	17.0
55×10^{-6}	17.0	19.5
42×10^{-6}	18.0	22.0
22×10^{-6}	20.0	28.0

* Five liter mill, u_t = 10 m/sec; inlet temperature of yeast dispersion = 5°C.

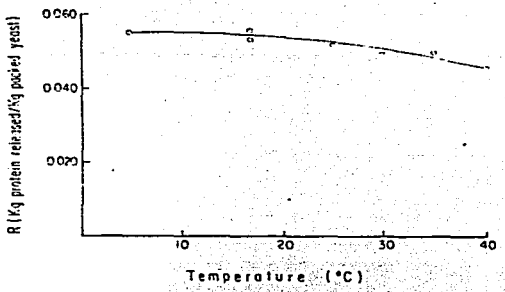

Fig. 4. Effect of temperature on the disruption kinetics in a 5 liter mill with stainless-steel impellers: $Q = 66.7 \times 10^{-6}$ m³/sec, $u_t = 8$ m/sec, $Y = 0.45$.

Tracer Studies

Some typical tracer curves for a pulse injection technique are presented in terms of the normalized exit age distribution function (E_θ) and the reduced residence time (θ) (Figs. 5–7). Several models can be used to describe the mixing or flow patterns within the mill. In the 5 liter mill the tracer curve matches well with a continuous stirred-tank reactor (CSTR) in-series model, the equation for which is

$$E_\theta = \frac{j^j \cdot \theta^{j-1} \cdot e^{-j\theta}}{(j-1)!} \qquad (3)$$

and where in this case the number of CSTRs (j) is found to be five (Fig. 5). The 5 liter mill consists of five impellers, therefore the concept of an impeller forming a single CSTR gives a satisfactory picture of the flow patterns in the mill. Similar tracer profiles were obtained for both polyurethane and stainless-steel blades operating at tip speeds of 10 m/sec. Tracer studies at higher tip speeds were not possible owing to the appearance of colloidal glass in the outlet tracer samples.

The tracer curves obtained in the 0.6 liter mill (Figs. 6 and 7) do not correspond to a four CSTR in-series model, although this mill consists of four impellers. The peak position (θ_m) may be used to characterize a normalized exit age distribution curve in terms of an equivalent number of CSTRs in series.[8] As shown in Table III the

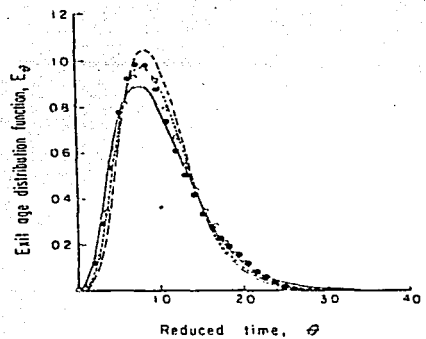

Fig. 5. Tracer studies—continuous flow-through 5 liter mill. $u_t = 10$ m/sec. $Q = 54 \times 10^{-6}$ m³/sec: (○) polyurethane impellers; (●) stainless steel impellers; (——) theoretical curve for 4 CSTRs in series; (- - - -) theoretical curve for 5 CSTRs in series; (— — —) theoretical curve for 6 CSTRs in series.

0.6 liter mill may be represented as a series of between one and two CSTRs for the range of flow rates and impeller tip speeds studied (calculation of the equivalent number of CSTRs based on variance of the distribution curves is not reliable for these wide distributions). Reasonable agreement between measured and calculated tracer distribution curves is obtained using a CSTR in-series model where j is not an integer[8] and also using a model based on the dispersion number (Table III) for a closed vessel.[7]

A model that gives a better physical picture of the flow patterns in the mill is based on partial backflow from any one CSTR to the previous CSTR in the series (Fig. 8). This view is supported by experiments where the tracer was injected between the first and second impellers. Backflow of tracer to the region of the first impeller was observed. The tracer curve for this model is given by the following relationship:[9–11]

$$E_\theta = \sum_{i=1}^{\infty} \frac{p(i) \cdot t^{i-1} \cdot e^{-t/\tau}}{\tau^i \cdot (i-1)!} \qquad (4)$$

$$\tau = 1/j(1 + 2b/Q)$$

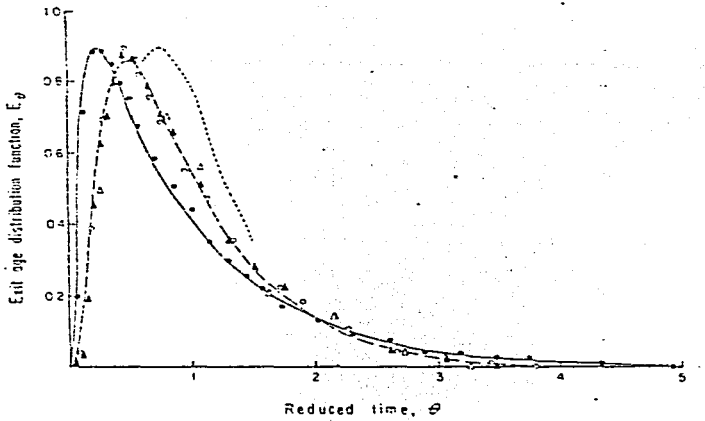

Fig. 6. Tracer studies—continuous flow-through 0.6 liter mill with stainless-steel impellers: (▲) $Q = 1.00 \times 10^{-5}$ m³/sec; (○) $Q = 1.80 \times 10^{-5}$ m³/sec; (△) $Q = 5.87 \times 10^{-5}$ m³/sec, $u_t = 10$ m/sec; (●) $Q = 1.80 \times 10^{-5}$ m³/sec, $u_t = 15$ m/sec; (——) $b/Q = 5.0$; (— —) $b/Q = 1.0$; (- - - -) $b/Q = 0$; all for 4 CSTRs in series.

where $p(i)$ is the probability of appearance of tracer in the outlet stream after passing through i CSTRs. This probability function depends on the ratio of backflow between CSTRs (b) to the overall flow rate through the mill (Q). The probability of transfer of tracer from a CSTR to another CSTR or the outlet stream is governed by several conditions; for example, tracer can only move directly from a CSTR to an adjacent CSTR; rates of tracer movement are determined by relative flow rates within the mill; tracer enters the outlet stream irreversibly and only from the last CSTR in the series, and all the tracer initially starts in the first CSTR of the series.

For the case where the time constants (ratio of volume of vessel to the total flow through that vessel that comprises part of the bead mill reactor model) are the same, Retallick[9] gives a simple, approximate probabilistic method for calculating $p(i)$. This has been developed into a more general matrix method by Buffham et al.[10] In the bead mill, however, the time constants for the end vessels are not the same as for the middle vessels, but the above models may be applied with reasonable accuracy for low b/Q values. Buffham and Kropholler[11] have shown how the general matrix method may be modified to the case where time constants vary through the overall reactor vessel and this is the method used for modeling the flow patterns in the bead mill.

Retaining the concept of an impeller forming a single CSTR ($j = 4$) and using the peak position (θ_m) as the major parameter for fitting the theoretical curve to the experimental points, it was found that b/Q varies from 4 to 5 for the stainless-steel impellers and from 2.5 to 8 for the polyurethane impellers, both impellers operating at 10 and 15 m/sec, respectively. The curves show very good agreement with experimental values (Figs. 6 and 7).

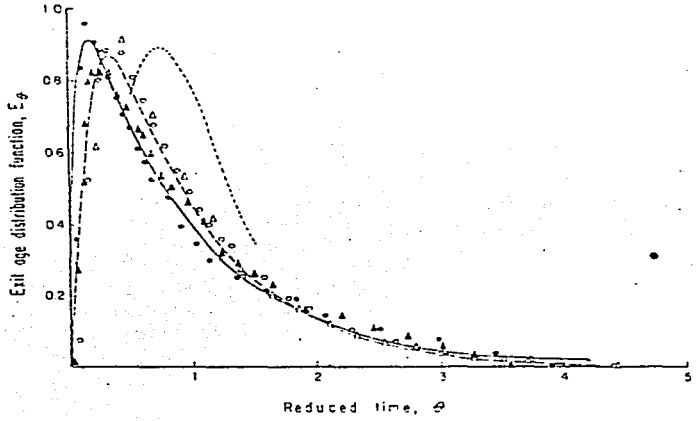

Fig. 7. Tracer studies—continuous flow-through 0.6 liter mill with polyurethane impellers: (▲) $Q = 0.75 \times 10^{-5}$ m³/sec; (○) $Q = 1.90 \times 10^{-5}$ m³/sec; (△) $Q = 6.00 \times 10^{-5}$ m³/sec, $u_t = 10$ m/sec; (●) $Q = 1.90 \times 10^{-5}$ m³/sec, $u_t = 15$ m/sec; (——) $b/Q = 8.0$; (— —) $b/Q = 2.5$; (- - - -) $b/Q = 0$; all for 4 CSTRs in series.

TABLE III
Bead Mill Tracer Studies

Throughput, Q (m^3/sec)	Impeller tip speed, u, (m/sec)	Variance	Dispersion No.	Peak position (θ_m)	No. of CSTRs $j = 1/(1 - \theta_m)$
0.6 liter mill		*Stainless-steel impellers*			
5.86×10^{-6}	10	0.36	0.23	0.5	2.0
1.80×10^{-6}	10	0.43	0.30		
1.00×10^{-6}	10	0.39	0.26		
1.80×10^{-6}	15	0.61	0.58	0.25	1.3
		Polyurethane impellers			
6.60×10^{-6}	10	0.54	0.46	0.35	1.5
1.90×10^{-6}	10	0.54	0.45		
0.75×10^{-6}	10	0.57	0.50		
1.90×10^{-6}	15	0.70	0.84	0.15	1.2
5 liter mill		*Stainless-steel impellers*			
89.4×10^{-6}	10	0.22	0.13	0.8	5.0
55.0×10^{-6}	10	0.20	0.12		
55.0×10^{-6} [a]	10	0.23	0.13		
		Polyurethane impellers			
53.1×10^{-6}	10	0.19	0.11	0.8	5.0

[a] Viscosities are 1.0×10^{-3} N sec/m² except for this measurement where the viscosity was increased to 6.6×10^{-3} N sec/m².

Continuous Disruption of Yeast

For a first-order reaction and a CSTR in-series model, cell disruption is described by the following equation:

$$D = \frac{R_M}{R_M - R} = \left(1 + \frac{k\tau}{j}\right)^j \tag{5}$$

where τ is the mean residence time in the mill (total volume of the mill, V, divided by the total throughput Q) and j is the number of

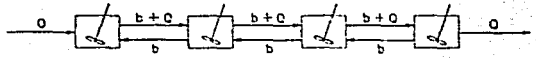

Fig. 8. CSTR in-series backflow model.

CSTRs in series; this may include fractions of CSTRs.[5] The remaining terms k, R_M, R, and D are as defined for eq. (1).

The extension of this CSTR in-series model to incorporate backflow may be solved by completing mass balances across the individual reactors:

$$D^{-1} = \frac{R_M - R}{R_M}$$
$$= \{p^3(2p-1)/[z^4 - 2(1-p)z^3 + (1-p)(1-4p)z^2 + 4p(1-p)^2 z + p^2(1-p)^2 - p(1-p^3)]\} \tag{6}$$

$$z = 1 + \tau k, \qquad p = \frac{1 + b/Q}{1 + 2b/Q}$$

and

$$\tau = \frac{V/Q}{4(1 + 2b/Q)}$$

where b/Q is the ratio of backflow between CSTRs to the overall flow rate (Q). First-order rate constants derived from the batch studies (Fig. 2) have been assumed to apply to the continuous disruption process in the 0.6 liter mill. Good agreement is noted between calculated and experimentally measured yields of soluble protein (Table IV). Calculated yields (not given in this paper) based on the dispersion model,[12] the CSTR in-series model (incorporating fractions of CSTRs),[5] and by integration of the combined first-order disruption eq. (2) and the experimental tracer curve[7] all show reasonably good agreement with experimental values.

The first set of studies on the 5 liter machine involved the use of a 45% by weight packed yeast suspension at throughputs of 16×10^{-6}–100×10^{-6} m³/sec and feed temperature of 5°C. As with batch studies it was not possible to measure maximum protein yields. Equation (5) was solved by altering both R_M and k until a minimum variance from the theoretical straight line was achieved. The R_M' values obtained were consistent for each impeller giving $R_M' = 0.105 \pm 0.005$ kg protein released/kg packed yeast for stainless-steel impellers and $R_M' = 0.090 \pm 0.005$ kg protein released/kg packed yeast for polyurethane impellers. The disruption data are shown in Figure 9. The results obtained at impeller tip speeds of 10 m/sec are consistent with the equivalent batch studies (Fig. 3).

The second set of disruption experiments using the 5 liter machine involved variations in the disruption medium from 15 to 75% by

TABLE IV
Continuous Disruption of Yeast in a 0.6 Liter Mill with Stainless-Steel Impellers: Comparison of Predicted and Measured Conversions[a]

Q (m³/sec) u_t = 10 m/sec	R measured (kg/kg) k = 0.0384 sec⁻¹ [b]	R predicted (kg/kg) backflow model (eq. (6)) b/Q = 1
1.00 × 10⁻⁶	0.120	0.117
2.94 × 10⁻⁶	0.105	0.107
4.69 × 10⁻⁶	0.091	0.097
7.03 × 10⁻⁶	0.068	0.085
9.67 × 10⁻⁶	0.068	0.074
12.00 × 10⁻⁶	0.056	0.067
1.86 × 10⁻⁶	0.119	0.117
2.92 × 10⁻⁶	0.109	0.111
7.22 × 10⁻⁶	0.070	0.087
12.22 × 10⁻⁶	0.056	0.068
u_t = 15 m/sec	k = 0.0544 sec⁻¹ [b]	b/Q = 5
1.86 × 10⁻⁶	0.120	0.117
2.92 × 10⁻⁶	0.114	0.111
7.22 × 10⁻⁶	0.094	0.092
9.58 × 10⁻⁶	0.087	0.084
12.22 × 10⁻⁶	0.074	0.076

[a] R_M = 0.120 ± 0.002 kg/kg.
[b] See Figure 1.

weight packed yeast dispersion. All the trials were carried out using a feed temperature of 5°C, impeller tip speed of 10 m/sec, and throughputs ranging from 16 × 10⁻⁶ to 100 × 10⁻⁶ m³/sec. For packed yeast concentrations of 30% by weight or greater, the same R_M' values were obtained as in previous trials (Fig. 9), but for packed yeast concentrations of 15% by weight the R_M' value obtained was much lower. The results using these R_M' values are plotted in Figure 10.

Enzyme Release

The disruption of yeast is also of interest in processes for the extraction of enzymes. The rate of release of an enzyme may depend on its location within a cell.[13] For a soluble cytoplasmic enzyme such as alcohol dehydrogenase (ADH), enzyme release should directly parallel the release of soluble protein. This assumes that there is no loss of enzyme activity due to overheating or shear. Similarly, using the same assumptions, an unambiguous R_M value for the enzyme may be measured. If enzyme damage occurs, a kinetic model is needed to describe both the rate of release and the rate of inactivation of the enzyme.[14,15]

The continuous release of ADH from the yeast was followed in the 5 liter mill using stainless-steel impellers operating at 15 m/sec. No enzyme inactivation was evident from the extended disruption

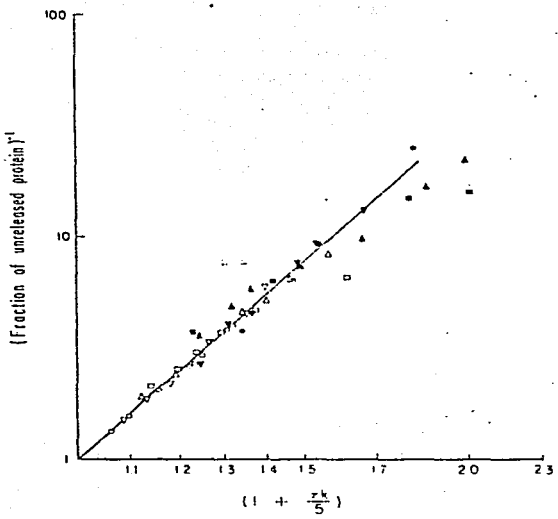

Fig. 9. Continuous disruption of yeast in a 5 liter mill, effect of impeller tip speed (Y = 0.45, T = 5°C). Stainless-steel impellers, R_M' = 0.105 kg/kg: (○) u_t = 8 m/sec, k = 0.0113 sec⁻¹; (▽) u_t = 10 m/sec, k = 0.0183 sec⁻¹; (△) u_t = 15 m/sec, k = 0.0238 sec⁻¹; (□) u_t = 20 m/sec, k = 0.0267 sec⁻¹. Polyurethane impellers, R_M' = 0.090 kg/kg: (●) u_t = 8 m/sec, k = 0.0380 sec⁻¹; (▼) u_t = 10 m/sec, k = 0.0488 sec⁻¹; (▲) u_t = 15 m/sec, k = 0.0494 sec⁻¹; (■) u_t = 20 m/sec, k = 0.0466 sec⁻¹; (——) theoretical line for 5 equal-volume CSTRs in series.

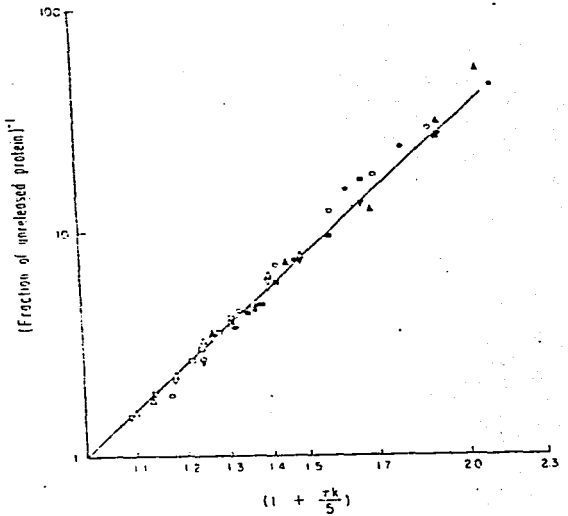

Fig. 10. Continuous disruption of yeast in a 5 liter mill; effect of yeast concentration (u_t = 10 m/sec, T = 5°C). Stainless-steel impellers: (○) Y = 0.15, R_M' = 0.081 kg/kg, k = 0.033 sec^{-1}; (△) Y = 0.30, R_M' = 0.105 kg/kg, k = 0.0179 sec^{-1}; (▽) Y = 0.45, R_M' = 0.105 kg/kg, k = 0.0183 sec^{-1}; (□) Y = 0.75, R_M' = 0.105 kg/kg, k = 0.0161 sec^{-1}. Polyurethane impellers: (●) Y = 0.15, R_M' = 0.076 kg/kg, k = 0.0647 sec^{-1}; (▲) Y = 0.30, R_M' = 0.090 kg/kg, k = 0.0608 sec^{-1}; (▼) Y = 0.45, R_M' = 0.090 kg/kg, k = 0.0488 sec^{-1}; (■) Y = 0.75, R_M' = 0.090 kg/kg, k = 0.0350 sec^{-1}. (——) Theoretical line for 5 equal-volume CSTRs in series.

runs. The rate of soluble protein release (Fig. 9) was used as a basis for a model of ADH release as shown in Figure 11: the experimental data and 5 CSTR kinetic model give good agreement.

Evaluation of Kinetic Data

The first-order rate constants measured for protein release using the 0.6 or 5 liter mill are summarized in Figure 12. The kinetics of

yeast disruption in the 0.6 liter mill show no variation with the type of impeller used (Fig. 2). This is in contrast to the results obtained using the 5 liter mill, where consistently higher rates of disruption (but lower values of maximum releasable protein) were achieved when using polyurethane impellers as opposed to stainless-steel impellers.

These observations raise the question of the mode of mixing in the 5 liter mill. Rotation of the impellers will cause movement of the beads about the central axis of the mill. Therefore near the impeller there is a region of high shear. The farther away from the

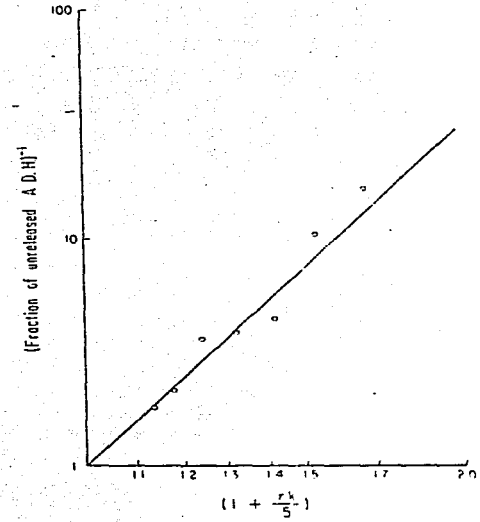

Fig. 11. Continuous release of alcohol dehydrogenase from yeast using a 5 liter mill with stainless-steel impellers: u_t = 15 m/sec, Y = 0.45, R_M = 250 units ADH/kg packed yeast, Q = 16 × 10^{-6}–100 × 10^{-6} m^3/sec; (——) theoretical line for 5 equal-volume CSTRs in series and k = 0.0238 sec^{-1}.

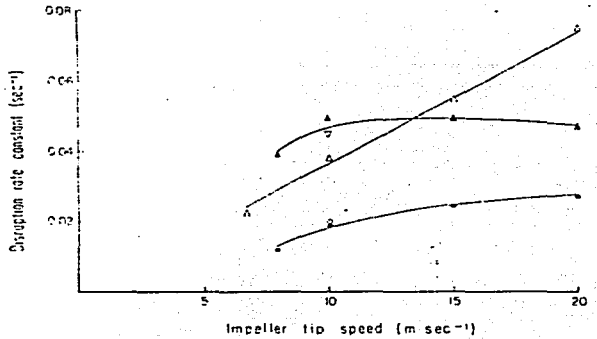

Fig. 12. Variation in the rate constant (k) with disruption conditions: 0.6 liter mill stainless-steel or polyurethane impellers: (△) batch. 5 liter mill stainless-steel impellers: (○) batch; (●) continuous. 5 liter mill polyurethane impellers: (▽) batch; (▲) continuous.

impeller, the slower will be the motion of the beads. The more open structure of the polyurethane impellers will promote a greater degree of agitation at a distance from the impeller, but consequently there will be relatively low shear rates in the vicinity of the impeller. Conversely, with the stainless-steel impellers there will be a high degree of shear near the impeller, rapidly dying out in the disruption medium due to the lower level of dispersion.

These higher shear rates experienced with the stainless-steel impellers would explain the higher maximum yields of protein, while the greater degree of mixing experienced with polyurethane impellers would explain the increased rates of disruption. Owing to the greater number of blades per unit volume in the 0.6 liter mill, this effect of different mixing patterns on the disruption kinetics is not significant.

The effect of initial yeast concentration on the rate of disruption is summarized in Figure 13. For a first-order reaction the rate constant should be independent of cell concentration (eq. (1)). This is achieved for stainless-steel impellers at higher yeast concentrations ($Y' \geq 0.30$) but for the polyurethane impellers the rate constant continuously decreases with increasing yeast concentrations.

Therefore the rheology of the medium directly affects the kinetics of disruption.

The rheology of a whole yeast dispersion is Newtonian, the viscosity varying from 2.2×10^{-3} N sec/m² at 15% by weight packed yeast to approximately 16×10^{-3} N sec/m² at 75% by weight packed yeast. Disrupted yeast suspensions show pseudoplastic behavior.[16] The flow properties of a highly disrupted 45% by weight yeast dispersion may be described using a power law relationship between the shear stress (τ') and shear ($\dot{\gamma}$):

$$\tau' = k' \dot{\gamma}^{0.82} \quad (7)$$

where

k' = consistency index
= 39.37 N sec$^{0.82}$/m²

and

$$\dot{\gamma} = 0\text{–}400 \text{ sec}^{-1}$$

The viscosity prior to disruption for a 45% by weight yeast dispersion is 6.6×10^{-3} N sec/m². Assuming that eq. (7) can be extrapolated to high shear rates (such as would occur in a bead mill), then

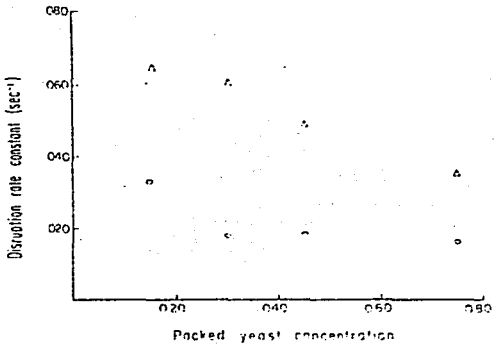

Fig. 13. Variation in the rate constant (k) with yeast concentration, continuous disruption in 5 liter mill: (○) stainless-steel impellers, $R_w' = 0.081$ kg/kg ($Y' = 0.15$), $R_w' = 0.105$ kg/kg ($Y' = 0.30$); (△) polyurethane impellers, $R_w' = 0.076$ kg kg ($Y' = 0.15$), $R_w' = 0.090$ kg/kg ($Y' = 0.30$).

it may be seen that the apparent viscosity ($\tau'/\dot{\gamma}$) will be approximately the same as that for the undisrupted yeast in the shear rate range 10^1–10^3 sec^{-1}. Therefore during disruption in high shear regions there will be no appreciable change in the apparent viscosity of the medium.

As the yeast concentration and the viscosity, is increased, this in turn will increase the rate at which the mixing is damped out at finite distances from the impeller. Therefore the rate of disruption will decrease. At low yeast concentrations ($Y = 0.15$) a very low maximum yield of protein (R_M) was observed. In a low viscosity medium the energy from the impeller will be dissipated very rapidly through the medium, thereby diminishing the region of high shear near the impeller. Thus at low yeast concentrations, although high rates of disruption are noted, there are poor yields of soluble protein especially at low throughputs or high residence times (Fig. 14).

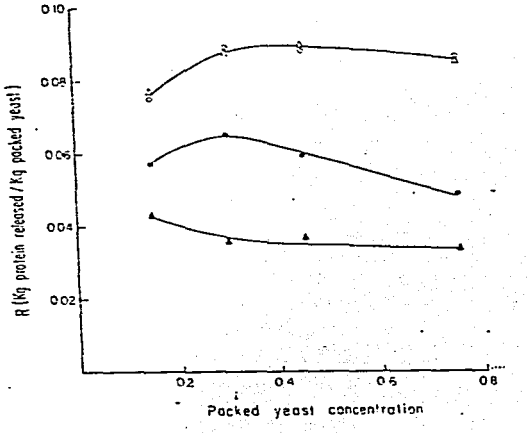

Fig. 14. Effect of yeast concentration on protein released (R) – u_t = 10 m/sec, 5 liter mill. Stainless-steel impellers: (Δ) $Q = 20 \times 10^{-6}$ m³/sec; (▲) $Q = 100 \times 10^{-6}$ m³/sec. Polyurethane impellers: (○) $Q = 20 \times 10^{-6}$ m³/sec; (●) $Q = 100 \times 10^{-6}$ m³/sec.

One other way of altering the viscosity of the disruption medium is by varying its temperature. The results presented in Figure 4 show that, for the same mean residence time in the mill, more protein is released at a lower temperature and hence a higher viscosity (6.6×10^{-3} N sec/m² at 5°C) than at a higher temperature (the viscosity drops to 2×10^{-3} N sec/m² at 40°C). The variation in R_M' values was not measured for different disruption temperatures. If the lower R_M' values obtained from the disruption of 15% by weight yeast slurries are assumed to apply to the higher temperature disruption studies, then it is found that similar trends in the rates of disruption are found for different viscosities, whether varied by temperature or yeast concentration.

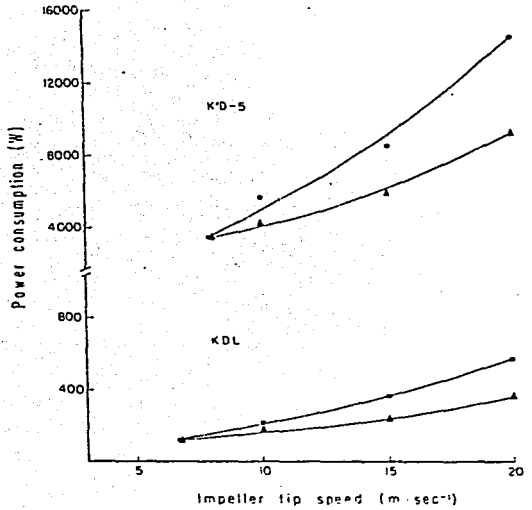

Fig. 15. Effect of impeller type, tip speed, and bead-mill capacity on power consumption: $Y = 0.45$, $T = 5$ C: (●) polyurethane impellers; (▲) stainless-steel impellers.

Efficiency of Disruption

The major source of power consumption of the mill is in the drive to the impellers. The effect of impeller tip speed on power input for both mills is presented in Figure 15.

For a certain throughput Q, impeller tip speed u_t and yeast concentration Y, it is possible to calculate the amount of protein released per kg packed yeast (R) on the basis of the kinetic data summarized in Figure 12. The efficiency of the mill is given by

$$E_m = RYQ/P \qquad \text{where } P = \text{power consumption} \qquad (8)$$

Plots of efficiency versus yield are presented in Figures 16–18; these relationships will be evaluated in the following section.

It should be noted that the power consumption for the 0.6 liter mill was measured with an ammeter. This method overestimates the power consumption but this does not affect the distinctions between the efficiencies of the two machines. True values of the power consumption for the 5 liter mill were measured using a wattmeter.

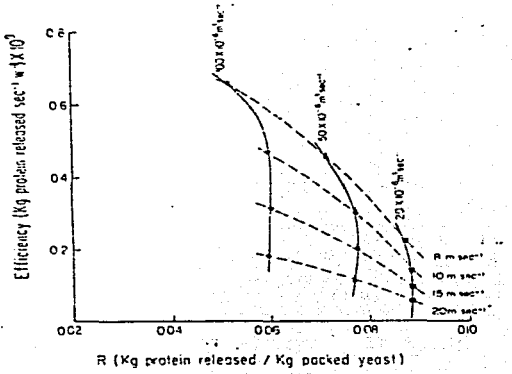

Fig. 16. Efficiency of 5 liter mill operating with polyurethane impellers, Y = 0.45.

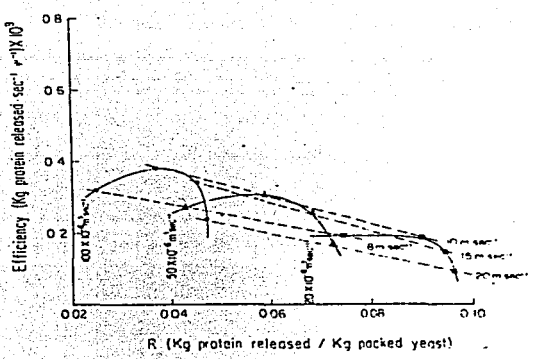

Fig. 17. Efficiency of 5 liter mill operating with stainless-steel impellers, Y = 0.45.

DISCUSSION

The continuous disruption of yeast in a high-speed bead mill has been adequately represented by a first-order process occurring in a series of continuous stirred-tank reactors. Each impeller in the mill forms the basis of a single reactor. In the 0.6 liter mill a significant degree of backflow occurs between consecutive reactors reducing the number of effective CSTRs. In the 5 liter mill backflow is negligible, presumably due to the greater relative spacing of the blades. The rate of disruption of yeast and the maximum yield of soluble protein are both dependent on impeller geometry (and possibly the material of construction), impeller tip speed, yeast concentration, and the number of impellers per unit volume of the mill. Other factors that affect the kinetics of disruption, but which have not been extensively studied in this paper, are temperature, viscosity, and bead size and weight.[1,6,17] Before discussion of the effect of mill characteristics on cell disruption is possible, an understanding of the mechanism of protein release is required, with particular reference to the term maximum yield of soluble protein (R_M).

The question arises as to what is the maximum amount of releas-

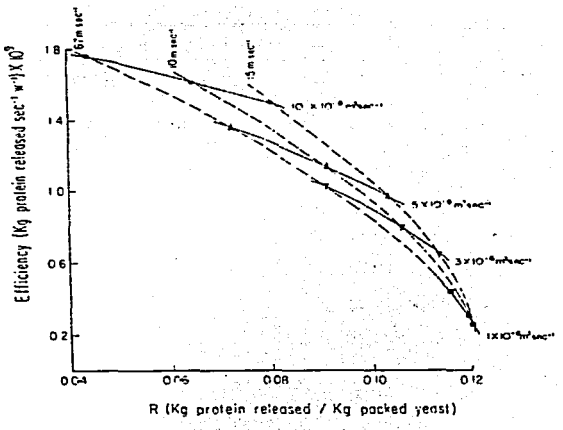

Fig. 18. Efficiency of 0.6 liter mill with stainless-steel impellers, $Y = 0.45$.

able protein? In yeast homogenization studies, Hetherington et al.[5] reported maximum yields of 0.096 kg protein/kg packed yeast, while values varying from 0.090 to 0.120 kg protein/kg packed yeast have been reported in this paper. Fresh packed yeast was used for all the disruption trials. This yeast will consist of a narrow cell size distribution although there may be a considerable variation in the wall thickness of the cells. The maximum shear stresses applied within the mill are probably well above the cell wall yield stress, therefore the mechanism of yeast disruption should follow a straightforward first-order relationship; i.e., the rate of breakage of cells is directly proportional to the number of unbroken cells. This of course assumes that the release of the cell contents does not affect the disruption mechanism. A single fracture of the cell wall will release all the cytoplasmic protein and this will constitute most of the total releasable protein. One possible explanation for changes in R_M values is the release of insoluble complexed protein and other Folin-positive material such as peptide, glycopeptide, and amino acids by micronization of the cell debris. Disruption trials not reported here have shown the release of 0.150 kg equivalent protein (Folin-positive material)/kg packed yeast. It must be stressed that some of this increased yield may be nonproteinaceous.

The micronization of cell debris is again probably a first-order process. For all purposes, after a relatively short period of disruption the cell debris content may be said to be constant for a wide range of the mean residence times used. Therefore, the following equations represent a simple disruption and micronization process:

$$\text{whole cells} \xrightarrow{k_1} \text{protein} + \text{cell debris}$$

$$\text{cell debris} \xrightarrow{k_2} \text{folin-positive material} + \text{cell debris}$$

$$\frac{dR}{dt} = k_1[\text{whole cells}] + k_2[\text{cell debris}]$$

$$= k_1[\text{whole cells}] + k_2 C$$

where C is a constant, and R is the total Folin-positive material released. Relating these quantities back to the disruption kinetics for the release of soluble protein (eq. (1)):

$$\frac{dR}{dt} = k_1[R_{M_A} - R] + k_2 C$$

where R_{M_A} is the actual amount of protein released from a cell by a single fracture (this should be a constant for all bead mill conditions)

$$\frac{dR}{dt} = k_1[R_{M_M} - R]$$

where R_{M_M} is the measured R_M value

$$R_{M_M} = R_{M_A} + k_2/k_1 K$$

Therefore the measured R_M values represent the amount of easily accessible protein plus the relative rate of release of Folin-positive material by micronization of the cell debris. For the purposes of cytoplasmic protein or enzyme release lower R_M values than those presented will be more representative of the kinetics of the process. On the basis of yeast homogenization results, where little cell debris micronization has been brought about, there is probably 0.096 kg cytoplasmic protein[5,18,19] while there is approximately 0.120 kg total protein/kg packed yeast.[20]

The open design of the polyurethane impellers promotes more mixing and hence higher rates of disruption. This design is suitable for the purpose of simple breakdown of whole cells and probably would be ideal for the production of enzymes. If the aim of a process is to produce as much soluble protein as possible, then rapid disruption by polyurethane impellers followed by micronization using stainless-steel impellers (even in the same machine) may lead to an improved mill performance. Changes in the number and surface of the blades and the bead size and type may also prove advantageous.

The 0.6 and 5 liter mills are not scaled equivalents, therefore, it is interesting to study the differences in mixing patterns and disruption kinetics obtained with the two mills. An increase in the longitudinal dispersion or backflow and consequently a decrease in the number of effective CSTRs in the mill will lead to a decrease in the yield of the mill and hence its efficiency especially at low throughputs or long residence times. This obviously supposes that the factors leading to increased dispersion do not increase the rate of disruption. In the 0.6 liter mill there is a sharp decline in the power efficiency with decreasing throughput (Fig. 18). Increasing impeller tip speeds lead to increased rates of disruption, but any improvement in yield can be partially or fully negated by the increased dispersion. This is more apparent in the case of the 5 liter mill using polyurethane impellers. The rate constant k reaches a maximum at impeller tip speeds of 10 m/sec (Fig. 12). At greater tip speeds it is likely that the actual rate constant for disruption is increasing but simultaneously there is an increased level of dispersion, these two effects acting in opposition. The decrease in the apparent rate constant at the highest tip speed (u_t = 20 m/sec) may be a real phenomenon where the effect of increased longitudinal dispersion is predominant. In a 5 liter mill operating with polyurethane impellers, no advantage is gained by disrupting at tip speeds greater than 10 m/sec (Fig. 16). In the 5 liter mill operating with stainless-steel impellers the rate constant tends to a maximum value with increasing tip speed; again dispersion and increasing rates of disruption having conflicting effects. Owing to lower levels of dispersion, the dependence of efficiency on throughput or degree of conversion is not so significant as when using polyurethane blades (Figs. 16 and 17). There is an optimal impeller tip speed of 10–15 m/sec for yeast disruption with stainless-steel impellers.

Disruption rate constants as measured in batch and continuous studies on the 0.6 liter mill (Fig. 12) may be summarized as follows:

$$k = Ku_t \qquad \text{where} \quad K = 0.0036\ \text{m}^{-1}$$

This direct relationship of rate constant with impeller tip speed probably also applies to the 5 liter mill. Tracer studies are required at the higher impeller tip speeds to analyze the degree of dispersion, these will be possible only when the problem of the formation of colloidal glass has been overcome.

In the 0.6 liter mill tracer studies showed a significant increase in dispersion or backflow when using polyurethane blades. This may either be due to the more open geometry of the impeller or the flexible structure of the blade (the polyurethane blades for the 0.6 liter mill do not have a rigid steel core as in the 5 liter mill). Continuous disruption studies with polyurethane blades in the 0.6 liter mill gave very high maximum yields of Folin-positive material (R_M = 0.150 kg/kg) and further trials are being carried out to examine this effect. In the 5 liter mill the polyurethane blades gave significantly higher release rates but also lower maximum yields of soluble protein. These results have already been discussed in the section summarizing the kinetic data. Briefly the more open structure and greater width of the polyurethane blades gave higher degrees of dispersion but lower maximum shear rates than for the more closed structure of the stainless-steel blades.

The full analysis of the kinetic data obtained at high impeller tip speeds in the 5 liter mill was not possible owing to the inability to carry out tracer studies in this region. However, it should be noted that only the values of the rate constant k and not the values of R_M' are dependent on the number of CSTRs (j) used in evaluating eq. (5). One curious aspect of the results not explained by the mixing model is the fact that the R_M' values for the polyurethane blades do not approach the values obtained when using the stainless-steel impellers, even at the highest tip speeds. This observation leads to the conclusion that the R_M' values may also be controlled by the nature of the blade surface. The stainless-steel impeller surfaces are more abrasive and harder than the polyurethane surfaces. For the 0.6 liter mill the R_M value is independent of the blade surface and type. In this case it is probable that the intensity of mixing is so high that other factors, such as bead-to-bead impact, control the final R_M value.

One further area of interest is the observed independence of backflow ratio or longitudinal dispersion with variations in the over-

all mill throughput. Only a narrow range of throughputs was covered and this work needs to be extended to take full advantage of the various combinations of flow patterns and disruption rate constants available.

The final subject requiring comment is the relative efficiencies of the 0.6 and 5 liter mills. For equivalent mean residence times in the mills higher efficiencies of disruption are observed in the 0.6 liter mill; this is contrary to what would be expected on the basis of dispersion studies. The most-likely explanation is that there is a more efficient utilization of the beads in the smaller mill owing to the greater number of blades per unit volume of the disruption chamber. Evidently scale-up criteria for a bead mill require a complex compromise between power input per unit volume and mill geometry. Also relevant to this problem are some of the recent developments in ball mill design[17] where longitudinal dispersion or backflow is increased by incorporating blades mounted obliquely to the drive shaft. Decreasing the angle of the blade to the shaft gives an apparent greater rate of release of protein. Here again there are probably conflicting factors that contribute to the overall disruption model. Decreased blade angle gives increased dispersion, which will lead to relatively less disruption in continuous flow studies. But this is compensated for by increased power transfer to the yeast suspension and hence greater rates of disruption. Therefore, although an overall increased rate of disruption is achieved, the power efficiency may be considerably decreased, especially at high shaft speeds. As with the polyurethane impellers operating at high speeds in the 5 liter mill, further increase of dispersion (by using even smaller blade angles) may lead to an overall decrease in the apparent rate of disruption.

One of the major problems in operating the type of bead mill described in this paper is the temperature control of the disruption medium. Recent improvements in the design of the cooling jacket should reduce this problem. Incorporation of a cooling system in the agitator shaft and blades has been reported by Rehacheck and Schafer.[17] The formation of colloidal glass is particularly undesirable if the process stream is to be used as a food component. The use of plastic or steel beads and their effect on the flow patterns and kinetics of disruption are being further studied by Limon-Lason.

The ability to apply mixing and kinetic models to yeast disruption in a high-speed bead mill will allow the development of scale-up criteria for the design of bead mills for this purpose. The optimization of the running conditions for a bead mill involves complex considerations of the flow and mixing patterns in the mill and the actual rate constants for the breakage of the microorganism. The ranges of flow rates, viscosities, and tip speeds used to develop flow models in the mill as described in this paper, are not sufficient for a complete evaluation of the 0.6 and 5 liter mills. The present study does indicate the design and operating factors to which particular attention should be given in the future.

Nomenclature

b	backflow between CSTRs (m³/sec)
D	(fraction of unreleased protein) $= 1 - R_M/(R_M - R)$ [$D = R_M'/(R_M' - R)$]
E_n	exit age distribution function
E_H	efficiency of mill (kg protein released/sec W)
j	number of CSTRs in series
k	first-order rate constant (sec⁻¹)
P	power input—impeller drive only (W)
Q	volumetric throughput (m³/sec)
R	amount of protein or enzyme released (kg protein/kg packed yeast)
R_M	measured maximum yield of soluble protein (kg protein/kg packed yeast)
R_M'	extrapolated R_M value based on best fit of a first-order reaction (kg protein/kg packed yeast)
t	residence time (sec)
T	temperature (°C)
u_t	impeller tip speed (m/sec)
V	disruption volume (volume of disruption chamber − bead volume) (m³)
Y	weight of packed yeast/unit volume (kg/m³)
θ_m	peak position of normalized exit age distribution curve
θ	normalized reduced time
τ	mean residence time in mill $= V/Q$ (sec)

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CONCLUSIONES Y PERSPECTIVAS

Los resultados alcanzados en esta investigación muestran como la ciencia aplicada puede orientarse a la solución de problemas de ciencia básica, y proveer a esa última de metodologías útiles que quedan en su repertorio y pueden seguirse utilizando (p. ej. ver Vichido *et al*, 1978; Dávila *et al*, 1978 y 1980; Hummelt y Mora, 1980 a y b; Lara *et al*, 1982).

Es interesante en este punto relatar que en *E. coli* crecida en cultivo contínuo, el grado de adenilación (y por lo tanto de inactividad de la G.S.) es una función lineal de la velocidad específica de crecimiento (μ), es también función de la relación entre α-cetoglutarato y glutamato pero es independiente de la concentración de amonio (Senior, 1975). Valdría la pena buscar esta modulación fina en *Neurospora*, para lo cual se podría emplear el cultivo alimentado exponencial operando en el cuasiestacionario, con lo que sería posible fijar valores de μ sin incurrir en los problemas de cultivo contínuo, y medir las proporciones e incluso tasas de aparición de las dos formas de la enzima.

Puede decirse que en este trabajo, se ha visualizado el sistema de cultivo como un reactor para la transformación de sustrato en biomasa, lo que ha permitido estudiar algunos aspectos de las relaciones participantes. Esta visualización es el pri

mer paso a la elaboración de un modelo, que en este caso se queda a un nivel conceptual. Lo mismo puede decirse de una segunda investigación que se realizó sobre la inmovilización de la glucosa isomerasa a materiales de intercambio iónico (Huitrón y Limón-Lason, 1978), con la diferencia de que este trabajo es de aplicación en el sector productivo. En él, en colaboración con un grupo de enzimología aplicada, estudiamos los efectos difusionales, utilizando para ello modelos matemáticos que describen los diferentes tipos de limitación difusional y que no pueden entenderse sin el apoyo de un modelo cinético que describe el reactor.

Sin embargo, en la sigueinte investigación ya se describe un modelo simbólico de un reactor contínuo para la desintegración celular. En el estudio de las propiedades de reactor de un molino de esférulas de alta velocidad para la desintegración celular (Limón-Lason *et al*, 1979), se describe un aparato comercial de este tipo (Dynamill) como un reactor contínuo bien agitado, en serie de cuatro cámaras con retromezclado; y se desarrollan las ecuaciones que describen su comportamiento cinético, su productividad y su eficiencia.

La siguiente investigación pretendió ya la elaboración de un modelo determinístico que fuera la base de una simulación

por computadora del proceso en cuestión. Para ello se modeló el reactor de isomerización contínua de glucosa a fructosa - usando glucosa isomerasa - y se formuló un programa de cómputo para recrear en la computadora (simular) el proceso. Los resultados fueron presentados en la XIII Reunión Nacional de la Sociedad Mexicana de Bioquímica y en el XIII Congreso Nacional de Microbiología (Alcaántara y Limón-Lason, 1980 y 1981), y son interesantes por el método usado para describir la reversibilidad de la enzima, que al reducirse a una ecuación formalmente similar a la de Michaelis-Menten simplifica el manejo del modelo.

Por otro lado, conviene enfatizar que al hacerse una simulación se aprovecha íntegramente el modelo, pues ésta facilita la comprobación del mismo y permite hacer inferencias de costos y aplicar técnicas de optimización, todo lo cual lleva a su utilización en el diseño, al que hace más científico.

En la actualidad se trabaja en la elaboración de un modelo de acuacultura intensiva, visualizando una técnica conocida como "raceway" como un reactor de flujo contínuo cerrado para catalizador (peces), en el que se convierte sustrato en biomasa. Este puede modelarse como un caso especial de cultivo alimentado de volumen constante en el que la tasa de desaparición de sustrato (-ds/dt) sí es significativa y en

el que se supone que el paso limitante es el consumo de oxígeno (Burrows y Chenoweth, 1970), en una reacción catalizada por enzima. Esto justifica el uso de la ecuación de Monod, la que describe (con una cinética de Michalis-Menten) la utilización de un sustrato escencial para la nutrición celular (Kargl, 1977) y esta ecuación constituye el fundamento conceptual de un programa de simulación probabilística tipo Monte Carlo, que se basa en la construcción de muchos modelos determinísticos que asumen diferentes valores, y que rinden una familia de resultados sobre los cuales pueden hacerse inferencias estadísticas. De esta manera se obtiene un modelo estocástico, en el que muchos grados de incertidumbre pueden ser introducidos.

Este modelo, y la simulación del proceso, se utilizarán para diseñar y optimizar los parámetros operativos de un sistema de raceways para la preengorda de alevines en una estación intensiva de reproducción piscícola.

De todo lo anterior se puede concluir que las técnicas de crecimiento regulado contribuyen notablemente a incrementar nuestro conocimiento sobre los sistemas vivos, y que este conocimiento puede emplearse ventajosamente en aplicaciones útiles al hombre.

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