UNIVERSIDAD NACIONAL AUTONOMA

DE MEXICO

INSTITUTO DE INVESTIGACIONES BIOMEDICAS

U A C P y P del C C H

APLICACION DEL CULTIVO ALIMENTADO EN LA PRODUCCION DE PECTINASAS EXTRACELULARES DE Aspergillus sp. Y ESTIMULACION POR ADICION DE ACIDO GALACTURONICO Y GLUCOSA

T E S I S

QUE PARA OBTENER EL GRADO DE:

MAESTRO EN INVESTIGACION BIOMEDICA BASICA

P R E S E N T A

Q.F.B. GUILLERMO AGUILAR OSORIO

MEXICO, D.F. 1986

JURADO

Presidente	Dr. Josef Herz Priggen
Vocal	Dr. Ramón Crúz Camarillo
Secretario	Dr. Carlos Huitrón Vargas
Suplente	Dr. Guillermo Alfaro Martínez
Suplente	Dr. Lino Díaz de León Hernández

A Blanca por su constante apoyo, estímulo,comprensión y confianza

A mis hijos: Carlos Gustavo, Diana y Mariana

A mi mami quien siempre me ha brindado su apoyo, a la memória de mi papi y a todos mis hermanos por la confianza que han depositado en mí.

Al Dr. Carlos Huitrón cuya guía, apoyo y confianza fueron esenciales para el desarrollo de este trabajo.

Al Dr. Manuel Jiménez y a la M.en C. María Elena Flores por su amplia colaboración.

A todos mis compañeros de trabajo y a todos aquellos que de una u otra forma colaboraron para la realización de este trabajo.

Finalmente quisiera expresar mi agradecimiento a la Organización de Estados Americanos (OEA) y al Consejo Nacional de Ciencia y Tecnología (CONACyT) por el apoyo parcial otorgado para la realización de este trabajo.

GRACIAS.

INDICE

RESUMEN

Las enzimas pécticas son un grupo de enzimas ampliamente distribuidas en la naturaleza que se caracterizan por su capacidad para degradar pectina y substancias relacionadas. Estas enzimas juegan un papel importante en la naturaleza, ya que la degradación de la pectina esta asociada al desarrollo de muchos procesos biológicos en las plantas, entre los cuales se encuentran: el crecimiento de las plantas, la elongación celular, la maduración de las frutas y la absición de las hojas. Además de encontrarse en las plantas,las enzimas pécticas tambien son producidas por bacterias, hongos, levaduras,y algunos insectos, nemátodos y protozoarios.La degradación microbiana de la pectina de las plantas está estrechamente ligada con la patogénesis, la simbiosis y la descomposición de frutas, vegetales y residuos de plantas. Las pectinasas se utilizan industrialmente para el procesamiento de frutas y vegetales en la despectinización y clarificación de jugos, y para aumentar los rendimientos de extracción, reducir el tiempo de filtración y la extracción de aceites esenciales. Dada la naturalerza inducible de estas enzimas, para su producción es necesaria la adición de pectina o materiales que la contengan, como la pulpa de remolacha y de henequén, el bagazo de manzana, o la cáscara de algunos cítricos. Actualmente la producción industrial de pectinasas se lleva a cabo a partir de bagazo de manzana o pulpa de remolacha

como sustratos, suplementados con glucosa, maltosa, almidón o cereales molidos, y utilizando hongos, principalmente del género **Aspergillus**.

Nuestro grupo de investigación ha estado trabajando en la producción de pectinasas extracelulares de **Aspergillus** sp. a partir de pulpa de henequén y cáscara de limón, principalmente.Este microorganismo fue aislado de muestras de suelo de una desfibradora de henequén en el estado de Yucatán y fue selecionado por los altos niveles de pectinasas extracelulares producidas cuando crece a 37°C en pulpa de henequén, pectina, ácido poligalacturónico, goma de tragacanto y otros desechos agroindustriales.

En este trabajo se presentan los resultados de la aplicación del cultivo alimentado en la producción de pectinasas extracelulares de **Aspergillus** sp. en condiciones en las cuales la fuente de carbono (pectina citrica), la fuente de nitrogeno (sulfato de amonio) o de ambas fueron adicionadas en concentraciones limitantes. Asímismo,se presentan los resultados del efecto estimulatorio del ácido galacturónico y glucosa sobre la produccón de las pectinasas, cuando son adicionados a diferentes tiempos a cultivos de **Aspergillus** sp. creciendo en pectina.Tambien se analizan algunos de los factores involucrados en la biosíntesis de estas enzimas en **Aspergillus** sp.

INTRODUCCION

El uso de enzimas pécticas en el procesamiento de frutas, fué introducido por Kertesz en los Estados Unidos alrededor de 1930, siendo las principales, aunque no las únicas, ya que en algunos casos se emplean también enzimas celulolíticas [1]. Una de las principales causas de la opalescencia en jugos de frutas y vinos se debe a la presencia de substancias pécticas y particularmente pectina, que se encuentran localizadas en las paredes celulares vegetales, y son liberadas cuando la fruta es exprimida, por lo cual durante la producción de jugos, extractos o concentrados de frutas, es conveniente la adición de enzimas pécticas, para acelerar las operaciones de maceración y solubilización de la fruta, así como para incrementar los rendimientos de extracción y reducir el tiempo de proceso. Actualmente el uso de enzimas pectinolíticas en el procesamiento de frutas, está muy difundido, sobre todo en la industria de jugos ya que la pectina insoluble tiene efecto negativo sobre la viscosidad, estabilidad y color [1-3].

Las propiedades coloidales de la pectina impiden la sedimentación de partículas en el jugo. En presencia de enzimas pécticas las moléculas de pectina son hidrolizadas y los productos formados no retienen las propiedades

coloidales de la pectina, lo que facilita la rápida sedimentación de las partículas responsables de la opalescencia, que pueden ser fácilmente separadas por centrifugación, decantación o filtración [1-5].

Es importante mencionar que hay casos en que la opalescencia en los jugos es comercialmente deseable, tal es el caso del jugo y del puré de tomate.En estos procesos es importante precalentar los jugos para destruir las pectinasas naturalmente presentes y así garantizar la opalescencia del jugo[3].

Las enzimas pécticas son un grupo de enzimas ampliamente distribuídas en la naturaleza, normalmente presentes en frutas y vegetales, así como en bacterias, levaduras y hongos, capaces de degradar substancias pécticas de diversos origenes,(Tabla I)[6]. Sín embargo, un solo organismo no es capaz de producir todos los diferentes tipos de actividades encontradas. De hecho, los hongos producen preferencialmente poligalacturonasas y pectinesterasas, siendo las primeras las que representan la mayor importancia desde el punto de vista industrial, lo que ha favorecido la utilización de hongos para la obtención industrial de estas enzimas [7,8].

Actualmente la producción de enzimas pécticas se lleva a cabo utilizando sistemas de fermentación sumergida [7] o semisólida [9],utilizando principalmente hongos del género

Aspergillus como A. niger , A. oryzae y A. wentii en medios de composición compleja y usualmente

TABLA I

PRINCIPALES FUENTES NATURALES DE PECTINASAS

Organismo	PE	PG	PGL	PMG	PMGL	OG	OGL
Plantas superiores:							
Cítricos	+						
Tomate	+	+					
Manzana	+	+					
Hongos:							
Aspergillus niger	+	+		+	+		
Penicillium digitatum	+			+	+		
Fusarium solani			+		+		
Levaduras:							
Kluyveromices		+					
Bacterias:							
Bacillus sp.			+			+	
Erwinia aroideae		+	+		+		+
Pseudomonas marginalis	+	+	+				

PE, pectinesterasa;PG, poligalacturonasa;PGL,poligalacturonato-liasa;PMG, polimetilgalacturonasa; PMGL, polimetilgalacturonato-liasa;OG, oligogalacturonasa;OGL, oligogalacturonato-liasa.
Adaptado de Fogarty y Kelly [4] y Pilnik y Rombouts [6]

indefinida utilizando sustratos como glucosa, maltosa, almidones y cereales molidos, entre otros, adicionados de materiales que contengan pectina, generalmente desperdicios del procesamiento de frutas como bagazo de manzana, cáscara de cítricos y otros como la pulpa de remolacha.

Las preparaciones comerciales de pectinasas no contienen todas las actividades pectinolíticas encontradas, están formadas principalmente por poligalacturonasas y pectinesterasas obtenidas de varios hongos, principalmente de **Aspergillus** y **Penicillium** [3].

Muchas, pero no todas las pectinasas extracelulares son sintetizadas en respuesta a la presencia de inductores los cuales son generalmente, insolubles o por lo menos incapaces de ser transportados al interior de la membrana celular por lo que no pueden ser los inductores directos a menos que la inducción se lleve a cabo en la periferia externa de la célula. Los inductores directos son probablemente productos intermedios de la degradación de los primeros como por ejemplo, el ácido digalacturónico para la pectinliasa de **Erwinia carotovora**[10].

Debido a la naturaleza inducible de la mayoría de las enzimas pectinolíticas, su producción está estrechamente ligada al sustrato utilizado como inductor, por lo que el rendimiento y tipo de enzimas que se obtenga dependerá fundamentalmente de: a)Del microorganismo utilizado (bacteria, hongo o levadura), b) De la naturaleza y concentración del sustrato inductor,c)De la eficiencia del proceso de producción y d) Del tipo de proceso que se utilice (lote, continuo o alimentado).

Aunque todos los inductores de las pectinasas son urónidos, productos derivados de la pectina o del ácido poligalacturónico, dependiendo de la concentración y tipo de inductor presente,el microorganismo puede producir cierta actividad pectinolítica preferencialmente sobre otras [10,11].

ENZIMAS PECTICAS

Las enzimas que actuan sobre las substancias pécticas pueden ser divididas en dos grandes grupos [1,2,12]; las enzimas despolimerizantes y las pectinesterasas, las primeras pueden ser clasificadas a su vez de acuerdo a los siguientes criterios:

a) Si la ruptura de los enlaces alfa-1,4 se lleva a cabo por hidrólisis o transeliminación.
b) De acuerdo al sitio de iniciación de la ruptura: aleatoriamente dentro de la cadena (de tipo endo),o en el extremo de la misma (de tipo exo), y
c) Por el tipo de sustrato sobre el que preferentemente actuan (pectina,ácido péctico u oligo-D-galacturonatos).

Actualmente se han encontrado, en diversos organismos, doce diferentes actividades pectinolíticas (Tabla II). De todas éstas la de mayor importancia desde el punto de vista de su

aplicación en la industrialización de frutas y vegetales son las enzimas despolimerizantes de tipo endo, ya que reducen rápidamente la viscosidad de la pectina presente en estos materiales, permitiendo aumentar los rendimientos de extracción y reduciendo los tiempos de filtración de los jugos [1]. Las despolimerasas rompen los enlaces glucosídicos alfa-1,4 del sustrato de preferencia

TABLA II

CLASIFICACION DE ENZIMAS PECTICAS

Enzima	Mecanismo de acción
I. Pectinasas despolimerizantes	
1. Actuan sobre pectina	
a) Polimetilgalactouronasas (PMG)	
i) Endo-PMG	Hidrólisis
ii) Exo-PMG	Hidrólisis
b) Polimetilgalacturonato-liasas (PMGL)	
i) Endo-PMGL	Transeliminación
ii) Exo-PMGL	Transeliminación
2. Actuan sobre ácido péctico	
a) Poligalacturonasas (PG)	
i)Endo-PG	Hidrólisis
ii)Exo-PG-1	Hidrólisis
iii)Exo-PG-2	Hidrólisis
b) Poligalacturonato-liasas (PGL)	
i) Endo-PGL	Transelimicación
ii) Exo-PGL	Transeliminación
3. Actuan sobre oligo-D-galactosiduronatos	
a) Oligogalacturonasas (OG)	Hidrólisis
b) Oligogalacturonato-liasas (OGL)	
II. Esterasas	
a) Pectinmetilesterasa o Pectinesterasa (PE)	Hidrólisis

Adaptado de Kilara [1] y Fogarty y Kelly [4]

por hidrólisis (hidrolasas) o por beta- eliminación (liasas).El rompimiento hidrolítico puede iniciarse por el

extremo de la cadena de sustrato (enzimas de tipo exo), rindiendo productos de bajo peso molecular, principalmente monomeros. Si el rompimiento es de manera aleatoria (enzimas de tipo endo),los productos principales son oligómeros con menores proporciones de monomeros y dímeros, que con las de tipo exo [13]. El ataque por beta-eliminación da como resultado la formación de un doble enlace entre los carbonos 4 y 5, y al igual que las hidrolasas puede haber de tipo exo y endo.

Las pectin-liasas (PMGL) son producidas por hongos y no han sido encontradas en bacterias ni plantas superiores. Las pectato-liasas (endo y exo-PGL) son enzimas típicas de de bacterias, pocos hongos las producen y no han sido encontradas en plantas superiores. Las endo y exo poligalacturonasas son producidas por la mayoría de los hongos, por algunas bacterias y se encuentran frecuentemente en plantas superiores. Las pectinesterasas se encuetran en plantas, hongos y algunas bacterias. Por último la exo-PMG y la exo-PGL no han sido encontradas en la naturaleza [6].Las oligogalacturonasas se han encontrado en algunas bacterias de los géneros **Bacillus** y **Erwinia**. Estas enzimas, generalmente unidas a la célula, convierten oligourónidos en monómeros y atacan al sustrato solo por los extremos (reductor y no-reductor) por lo que son de tipo exo y se han descrito tanto oligogalacturonato-hidrolasas como oligogalacturonato-liasas [4,13].

SUSTANCIAS PECTICAS

Las substancias pécticas están localizadas en la pared celular primaria y en la lámina intermedia de tejidos vegetales, donde funcionan como cemento intercelular y se encuentran también en algunos microorganismos [3]. Según la nomenclatura de la American Chemical Society [14], las substancias pécticas son carbohidratos complejos de propiedades coloidales formados principalmente por unidades de ácido anhidrogalacturónico unidas por enlaces alfa-1,4, cuyos grupos carboxilo pueden estar esterficados parcial o totalmente con grupos metilo, o neutralizados por una o más bases (Fig.1). Dentro de las substancias pécticas se encuentran: a) La protopectina, precursor insoluble de la pectina; b) ácidos pectínicos, son ácidos poligalacturónicos coloidales parcialmente esterificados; c) pectina,ácido poligalacturónico soluble en agua con grado de esterificación y neutralización variables, pero mayores a los ácidos pectínicos, y d) ácidos pécticos, son ácidos poligalacturónicos coloidales prácticamente libres de ésteres [4].

A pesar de que el principal componente de las substancias pécticas es el ácido anhidro galacturónico, también se han encontrado otros azúcares como L-ramnosa, L-arabinosa, D-galactosa, D-xilosa y L-fucosa, formando parte de la pectina [12]. El tipo y cantidad de éstos así como el grado de esterificación y contenido de ésteres metílicos

[illegible] del gen de [illegible] pectinas se [illegible] encuentran originalmente en forma de

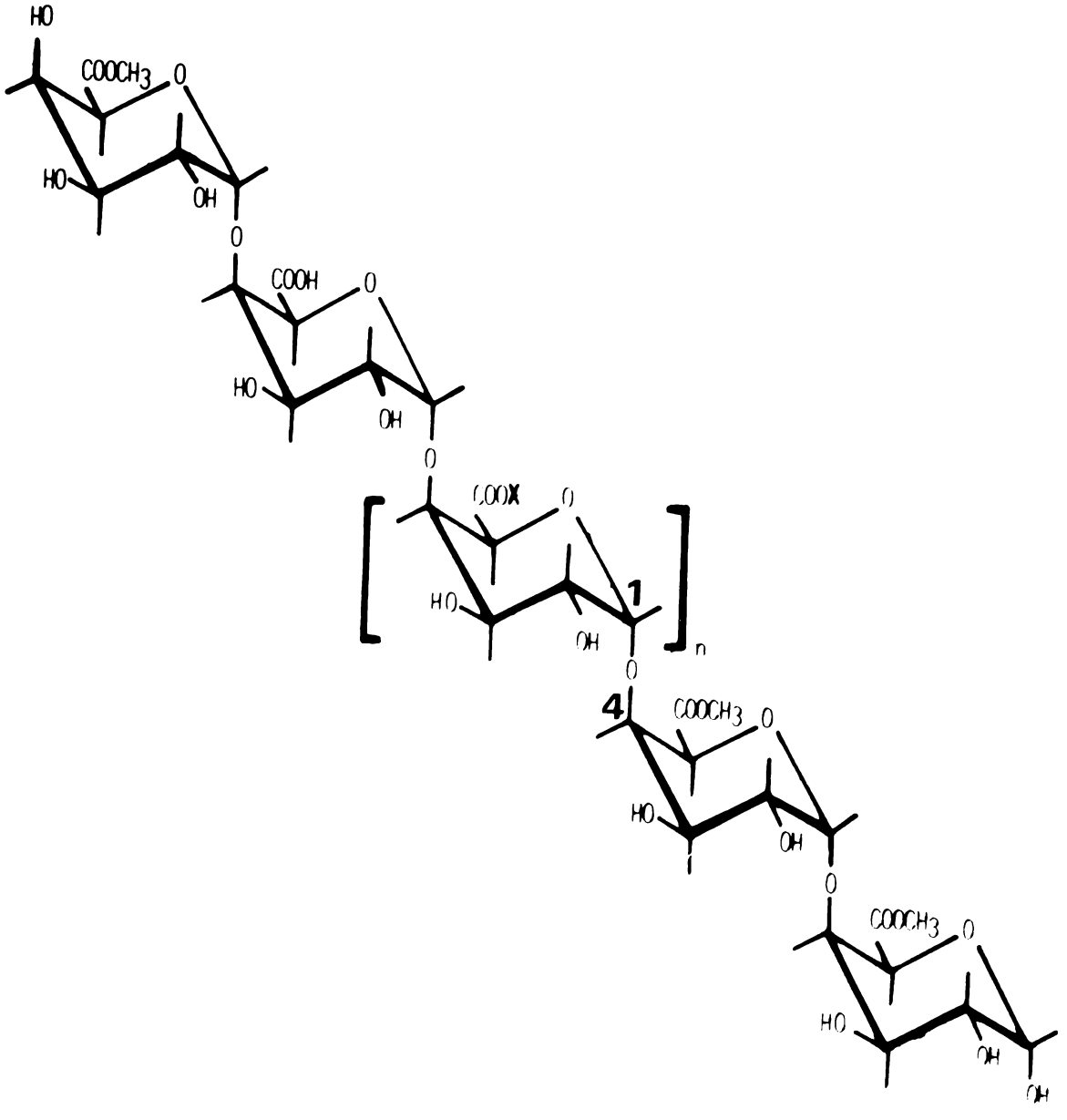


Fig.1.Estructura de la pectina, donde se muestran los enlaces alfa-1,4 del ácido galacturonico. [illegible] representa H CH_3 de acuerdo al grado de esterificación.

protopectina cuya síntesis ocurre en las etapas tempranas de crecimiento, cuando el área de la pared celular aumenta

[3]. La transformación de la protopectina en pectina soluble, está directamente relacionada con el proceso de maduración y envejecimiento de frutas y vegetales. Este fenómeno tiene una importancia económica considerable, ya que la textura de éstos está profundamente influída por el tipo y cantidad de pectina presente.

El uso comercial más importante de la pectina es en la fabricación de mermeladas y jaleas, ya que es el ingrediente esencial que permite la gelificación de estos productos [3]. La pectina también se utiliza en la industria farmaceútica como lubricante entre algunos alimentos y la pared intestinal, ya que promueve el peristaltismo sin ocasionar irritación. Asimismo, la pectina se utiliza en el tratamiento de diarrea y disenteria, y su gran efectividad en estos casos, puede deberse a que los productos de su degradación tienen propiedades bactericidas [3,4].

APLICACIONES DE LAS PECTINASAS

La principal aplicación comercial de las pectinasas es en la industrialización de frutas y vegetales ya que aumentan el rendimiento de jugo y solidos, reducen la viscosidad en concentrados de frutas y modifican y solubilizan las substancias pécticas, que dificultan la sedimentación de

particulas indeseables y la clarificación de jugos y concentrados [4]. Sin embargo, estas enzimas tienen otras aplicaciones comercialmente importantes como, la clarificación de vinos particularmente en vinos tintos, ya que favorecen la eliminación de la turbiedad producida por la pectina y aumentan la extracción del colorante de la piel de las uvas [4], y el enrriado de fibras textiles [3].

ASPECTOS REGULATORIOS DE LA PRODUCCION DE PECTINASAS

Las pectinasas son producidas por varios géneros de bacterias que incluyen , **Bacillus** , **Clostridium** , **Pseudomonas** , **Xantomonas** y **Erwinia** [4] **Bacillus subtilis** produce poligalacturonato-liasa (PGL) en forma constitutiva, utilizando diferentes fuentes de carbono; sin embargo, cuando **B. subtilis** crece en presencia de pectina, el crecimiento y la producción de (PGL) se ven disminuidos debido a que el microorganismo no produce pectin esterasa responsable de la desmetilación de la pectina [15]. De las bacterias conviene destacar al género **Erwinia**, que produce prácticamente todas las actividades reportadas [4].

La síntesis de endo-poligalacturonato liasa es constitutiva en **E. carotovora** y **E. aroidae**,y debido a la velocidad diferencial de sintesis en glucosa, glicerol y pectato de

sodio, se ha concluido que esta enzima está sujeta a represión catabólica. **E. carotovora**, **E. chrysantheni** y **E. aroidae**, secretan una parte de la poligalacturonato-liasa que producen al medio de cultivo, y otra parte permanece asociada a la célula [16]. Se ha observado también que en **E. carotovora** la adición de AMPc revierte la represión catabólica producida por glucosa, y aumenta la síntesis de endopolimetilgalacturonato-liasa [4]. Por otro lado, **Xantomonas spp.** produce pectinesterasa en forma inducible y poligalacturonato-liasa en forma constitutiva [13]. Así mismo, se ha descrito que la síntesis de endopoligalacturonato-liasa extracelular de **Aeromonas liquefaciens**, es constitutiva y sensible a la represión catabólica [17].

En hongos, los fenómenos regulatorios asociados a la producción de pectinasas han sido menos estudiados que en bacterias, a pesar de la gran importancia que esta información representa a nivel industrial. Sin embargo, se sabe que la síntesis de pectinasas en hongos de los generos **Aspergillus**, **Fusarium** y **Verticillium** es , en general, inducida por substancias que contienen pectina [2-4,6-8,12,13,18], y que están sujetas a represión catabólica como se ha descrito para **Aspergillus niger**[19] y **Pyrenochaeta terrestris** [20], entre otros.

La síntesis de poligalacturonasa de **A. niger** está sujeta a represión catabólica por glucosa,y se ha determinado que se presenta tanto a nivel de traducción como de transcripción [19,21]. Asimismo, se ha descrito que la síntesis de endopoligalacturonasa de **Pyrenochaeta terrestris** se reprime cuando medios de cultivo que contienen pectina, son suplementados con glucosa u otras hexosas en concentraciones mayores de 0.05 M pero se estimula a concentraciones de 0.005 M [20]. Se sabe que la mayoría de las enzimas pécticas son inducibles. Sin embargo, se han descrito algunos ejemplos de enzimas constitutivas,principalmente en bacterias y un caso en hongos [4].**Bacillus subtilis** y algunas especies de **Pseudomonas** producen respectivamente, poligalacturonato-liasa y endo-poligalacturonato-liasa en forma constitutiva, [4,22].En hongos la producción constitutiva de pectinasas, sólo se ha descrito para la endo-polimetil- galacturonato-liasa de **Penicillium digitatum** [23].

Actualmente no se sabe con precisión cual es el inductor directo de la actividad pectinolítica en hongos, de entre los diferentes compuestos producidos durante la degradación de las substancias pécticas [4]. Sin embargo, se ha descrito que el ácido galacturónico induce la síntesis de endo-poligalacturonasa y endo-pectin-transeliminasa, en **Verticillium** y que el D-galactarato (ácido múcico) parece

ser el único compuesto estimulante (inductor directo) de la poligalacturonasa y la pectinesterasa en **Penicillium chrysogenum** [24].Por otro lado, se ha observado que en **Fusarium roseum** se presenta un fenómeno de inducción diferencial relacionado con el pH. Se ha descrito que a pH de 3.5 en medios que contienen pectina como fuente de carbono, se inducen la endo-poligalacturonasa y pectinesterasa. Esto no ocurre a pH de 6.5 [18].

PRODUCCION DE PECTINASAS DE Aspergillus sp.

La producción de pectinasas de **Aspergillus** sp., ha sido estudiada en cultivo en lote en matraces agitados, y en fermentador de 14 litros, utilizando pectina, pulpa de henequén y otros subproductos agroindustriales como fuente de carbono [25,26]. Este microorganismo, aislado del suelo de una desfibradora de henequén en el estado de Yucatán,crece a 37°C, produce pectinasas extracelulares que pueden ser utilizadas en la despectinización del jugo de manzana, tiene bajos requerimientos nutricionales, es una cepa silvestre que puede ser manipulada genéticamente con grandes perspectivas, puede crecer y producir actividad pectinolítica extracelular a partir de pectina o materiales que la contengan, como pulpa de henequén, cáscara de limón y piña, entre, otros y no parece ser patógeno ni producir aflatoxinas [25,26].

La capacidad de producción de actividad pectinolítica de **Aspergillus** sp. ha sido evaluada y comparada con cepas de colección como, **Aspergillus niger** 20107 ATCC, tanto en matraces agitados como en fermentador de 14 l,encontrandose que a 37°C, la cepa de colección produce alrededor de cinco veces menos que **Aspergillus** sp. , cuando la actividad se mide como grupos reductores (1.1 mg/ml para **A. niger** y 6.0 mg/ml para **Aspergillus** sp.), y cerca de 18 veces menos actividad de acuerdo al ensayo viscosimétrico (0.006 U para **A. niger** y 0.11 para **Aspergillus** sp.) en matraces agitados [27]. En fermentador se obtienen resultados similares, con aumentos de 7 y 15 veces en la producción de pectinasas para la actividad medida por grupos reductores y por viscosidad, respectivamente para **Aspergillus** sp. en relación con **A. niger** 20107 ATCC [28]. Asimismo, se ha determinado la capacidad del microorganismo para crecer y producir pectinasas utilizando pectina, ácido galacturónico, glucosa, glicerol, celobiosa y pulpa de henequén como sustratos, encontrandose que crece en todas estas fuentes de carbono, pero sólo produce pectinasas en presencia de pectina o de algún material que la contenga [27]. La producción de la actividad también se ve afectada por la fuente de nitrógeno. Cuando se utiliza sulfato de amonio,la producción de la actividad disminuye en un 15-20% en relación con la actividad obtenida cuando se utiliza fosfato de amonio [28]. Esta disminución no parece deberse

solo a la disponibilidad de la fuente de nitrógeno **per se**, sino tambien a la mayor concentración de fosfato en el medio de cultivo [28].

Por otro lado, se ha obtenido un medio de cultivo simple y de bajo costo a base de pulpa de henequén, sales de grado industrial y agua de la llave [26,29]. Este medio simplificado es capaz de promover el crecimiento y la producción de la actividad pectinolítica de **Aspergillus** sp., obteniendose un rendimiento del doble para la actividad medida por viscosimetría, en relación al obtenido en medios más complejos, con sales de grado analítico y agua destilada. La actividad medida por grupos reductores se redujo en el medio simplificado en un 11.0%. Sin embargo, desde el punto de vista práctico el incremento en la actividad viscosimétrica es muy deseable para su aplicación industrial [29].

Por otro lado se ha demostrado que la adición de Tween 80 y goma de tragacanto (**Astragalus gumifer**), tienen un efecto estimulatorio sobre la producción de pectinasas en **Aspergillus** sp., y se ha determinado que las condiciones más apropiadas para la producción de pectinasas en fermentador de 14 l son: temperatura, 37° C ; pH, 3.5; agitación, 200 rpm y aireación 1.0 vvm [26].

Las pectinasas producidas por **Aspergillus** son potencialmente interesantes para su aplicación industrial, ya que se ha demostrado que son capaces de clarificar jugos de manzana con un rendimiento relativo de 0.85, que es muy similar al de preparaciones comerciales como Clarex y Ultrazym 100 [26], y por que además, se pueden producir en medios de fermentación simples y de bajo costo utilizando pulpa de henequén, cáscara de limón, cáscara de naranja u otros subproductos similares, obtenidos en la industrialización de frutas y vegetales.

CULTIVO ALIMENTADO

El cultivo alimentado puede definirse como un cultivo en lote con adición continua de uno o varios nutrientes [30]. Esta alimentación se puede utilizar para controlar la velocidad de crecimiento y la actividad metabólica de un microorganismo, cuando el nutriente alimentado es capaz de limitar la velocidad de crecimiento [30]. Se ha demostrado además, que el cultivo alimentado es superior al cultivo tradicional en lote, especialmente cuando la presencia de altas concentraciones de algún nutriente afecta negativamente el rendimiento y la productividad del metabolito deseado [17,31-37]. Este efecto ha sido observado para la producción de la poligalacturonato-liasa de **Aeromonas liquefaciens**, la cual se estimula por concentraciones limitantes de la fuente de carbono [17].

Así mismo, es posible eliminar o por lo menos reducir, problemas como la inhibición por sustrato o la represión catabólica, y aumentar el tiempo de operación de la fermentación. El cultivo alimentado ha sido utilizado con éxito para incrementar los rendimientos de celulasas de **Myrothecium verrucaria** [31] y de **Trichoderma viride** [32], la producción de beta-glucanasa de **Bacillus subtilis**, [33] y también ha sido utilizado para estudios de la regulación de la producción de celulasas de **M. verracaria** [34], de pectinasas y xilanasas de **Verticillium albo-atrum** [35], de cefalosporina de **Cephalosporium acremorium** [36] y la glutamino sintetasa de **Neurospora crassa** [37].

Es posible también afectar cambios en la permeabilidad celular por manipulación del nutriente limitante, y se ha descrito que la limitación de Mg^{++} afecta la composición química de la pared celular en especies de **Bacillus** [38]. En la Tabla III se mencionan los efectos más probables ocasionados por la limitación de diferentes nutrientes, sobre los microorganismos.

De forma general, el cultivo alimentado tiene ventajas sobre el cultivo en lote. Estas ventajas son similares a aquellas obtenidas con el cultivo continuo [40,41], y son:

1) La velocidad de crecimiento puede ser controlada y mantenida en un valor predeterminado durante el proceso de fermentación.
2) Permite establecer condiciones de crecimiento bajo limitación de nutrientes.
3) El microorganismo puede crecer bajo condiciones constantes y controladas durante el proceso.
4) Se obtienen productividades normalmente más altas.
5) Se pueden eliminar o reducir la inhibición por sustrato y la represión catabólica, y
6) Se puede aumentar el tiempo de operación.

TABLA III

LIMITACION DE NUTRIENTES Y SU POSIBLE MODO DE ACCION

Nutriente limitante	Modo de acción
Carbón-energía	Restringe el suministro de carbono para la biosintesis de energía.
Nitrógeno o Azufre	Restringe la síntesis de proteína.
Fosfato	Restringe la síntesis de ac. nucleicos y/o la producción de energía.
Magnesio o potasio	Restringe la síntesis de ac. nucleicos o pared celular y/o tiene efecto sobre la estructura de la membrana o su permeabilidad.

Tomado de Wang,et al.[39]

El gran beneficio que ofrece el sistema de cultivo alimentado es el de poder ejercer un control muy fino sobre las actividades de los microorganismos en crecimiento

controlado, lo que permite realizar estudios de tipo fisiológico y morfológico sobre producción, regulación e inducción enzimática [41].

Para la producción de enzimas en cultivo alimentado es importante considerar los siguientes factores [42]:

a) Velocidad de alimentación
b) Concentración y tipo de nutrientes limitantes
c) Formación de represores o inductores internos
d) Dependencia de la síntesis enzimática sobre la velocidad y crecimiento, y
e) La velocidad de síntesis de otros compuestos ya sea intra o extracelulares.

Estos factores pueden determinar la calidad de los resultados obtenidos, así como su adecuada interpretación.

El cultivo alimentado ha sido utilizado para la producción de una gran variedad de productos con el objeto de aumentar los rendimientos. Dichos productos incluyen, levadura de panificación, proteína unicelular, antibióticos, vitaminas, enzimas y aminoácidos, entre otros. Sín embargo, no se dispone de información precisa sobre la utilización de este cultivo a nivel industrial, ya que normalmente esta información es de caracter confidencial [41].

OBJETIVO

El interés del presente trabajo está centrado en la utilización del cultivo alimentado, para evaluar la producción de las actividades pectinolíticas del hongo **Aspergillus** sp., en condiciones de limitación de nutrientes, tratando de minimizar o eliminar los posibles efectos de represión catabólica o inhibición, y poder aumentar el rendimiento en la producción de estas enzimas. Asimismo, se evaluará el efecto de la adición de ácido galacturónico y de la glucosa, sobre cultivos en lote de **Aspergillus** sp. creciendo en pectina, a fin de determinar algunos de los factores involucrados en la inducción del sistema enzimático pectinolítico de este microorganismo.

Este enfoque puede ser interesante, ya que el cultivo alimentado es más productivo que los cultivos de lote, y puede aplicarse en la industria con ciertas ventajas sobre el proceso tradicional de lote. Por otro, lado es de gran utilidad para el estudio de fenómenos regulatorios de la producción de enzimas necesarias para el crecimiento celular, y puede conducir a la sobreproducción de enzimas.

METERIAL Y METODOS

Microorganismo

El microorganismo utilizado en este trabajo fué un hongo blanco del género **Aspergillus**, que produce pectinasas extracelulares a 37°C Este microorganismo fué aislado de suelo de una desfibradora de henequén en el estado de Yucatán, México [25,28]. **Aspergillus** sp. fué conservado y propagado en tubos conteniendo agar de papa-dextrosa (Merck-México).

Medio de Cultivo para la Fermentación

El medio de cultivo basal contenia: $(NH_4)_2SO_4$, 0.2%; KH_2PO_4, 0.2%; K_2HPO_4, 0.2% y pectina cítrica, 1.0% [26]. En todos los casos el pH del medio se ajustó con una solución diluida de ácido sulfúrico a 4.2-4.6. El medio fue esterilizado a 121°C y 15 psi durante 25 min.

Para el cultivo alimentado la fuente de carbono, nitrógeno o ambas fueron eliminados del medio basal y fueron adicionadas al fermentador como se indica más adelante.

En los casos en que el medio basal fue suplementado con D-glucosa, o ácido D-galacturónico, estos fueron adicionados

al medio de cultivo en condiciones asépticas a los tiempos indicados en las figuras. La adición se realizó agregando 10 ml de una solución de cada una de estas fuentes de carbono, previamente esterilizadas,a una concentración de 20 mg ml^{-1} (concentración final 1.0 mg ml^{-1}) o 100 mg ml^{-1} (concentración final,5.0 mg ml^{-1}) a cada 200 ml de medio de cultivo.Cuando se agregó cicloheximida, ésta fue esterilizada por filtración y se adicionó 1.0 ml de una solución de 25 mg ml^{-1}. Los experimentos en los cuales se hicieron adiciones se especifican en las figuras.

Alimentación de Nutrientes Limitantes

Los nutrientes utilizados como limitantes fueron eliminados del medio basal y alimentados al fermentador a una velocidad de flujo de 7.0 ml h^{-1} por medio de una bomba peristáltica (Master Flex, Cole Parmer, Inc. U.S.A., modelo 7550-40). Cuando los nutrientes limitantes fueron pectina o $(NH_4)_2SO_4$ estos se adicionaron a una concentración de 5.0 mg ml^{-1}(35 mg h^{-1}) y 0.19 mg ml^{-1}(1.33 mg h^{-1}), respectivamente. En el caso de doble limitación con pectina y $(NH_4)_2SO_4$ estos se adicionan simultaneamente a las mismas concentraciones anteriores. En todos los cultivos alimentados los valores de las determinaciones reportados en las figuras fueron corregidos por el efecto de dilución correspondiente al volumen agregado al tiempo que se tomo cada muestra.

Preparación del Inóculo

El inóculo fue preparado creciendo a **Aspergillus** sp. en tubos conteniendo agar de papa-dextrosa a 37°C durante 72 h. Después de este tiempo las esporas fueron cosechadas resuspendiendolas en solución isotónica de cloruro de sodio (0.15 M) y diluyendo con la misma solución hasta tener una absorbacia final de 5.0.

Fermentador Ciclónico

Como fermentador para los cultivos alimentados se utilizó un matraz Fernback (2.8 litros), adaptado con entradas y salidas para aire y medio de cultivo, puerta de inoculación y un dispositivo para recircular el medio de cultivo. Este dispositivo, localizado en la parte superior del matraz, consiste de dos salidas tangenciales que producen un flujo ciclónico que baña continuamente las paredes internas del matraz, impidiendo el crecimiento del hongo sobre esta superficie (Fig.2).

Producción de la Actividad Pectinolítica en Matraz

La producción de la actividad pectinolítica en matraz se llevó a cabo utilizando matraces Erlenmeyer de 500 ml conteniendo 200 ml de medio de cultivo, al cual se le adicionaron 2.0 ml de inóculo en condiciones asépticas. Los

cultivos fueron incubados a 37°C y agitados a 200 rpm en ur agitador rotatorio (Newbruswick Scientific, Co. U.S.A.).

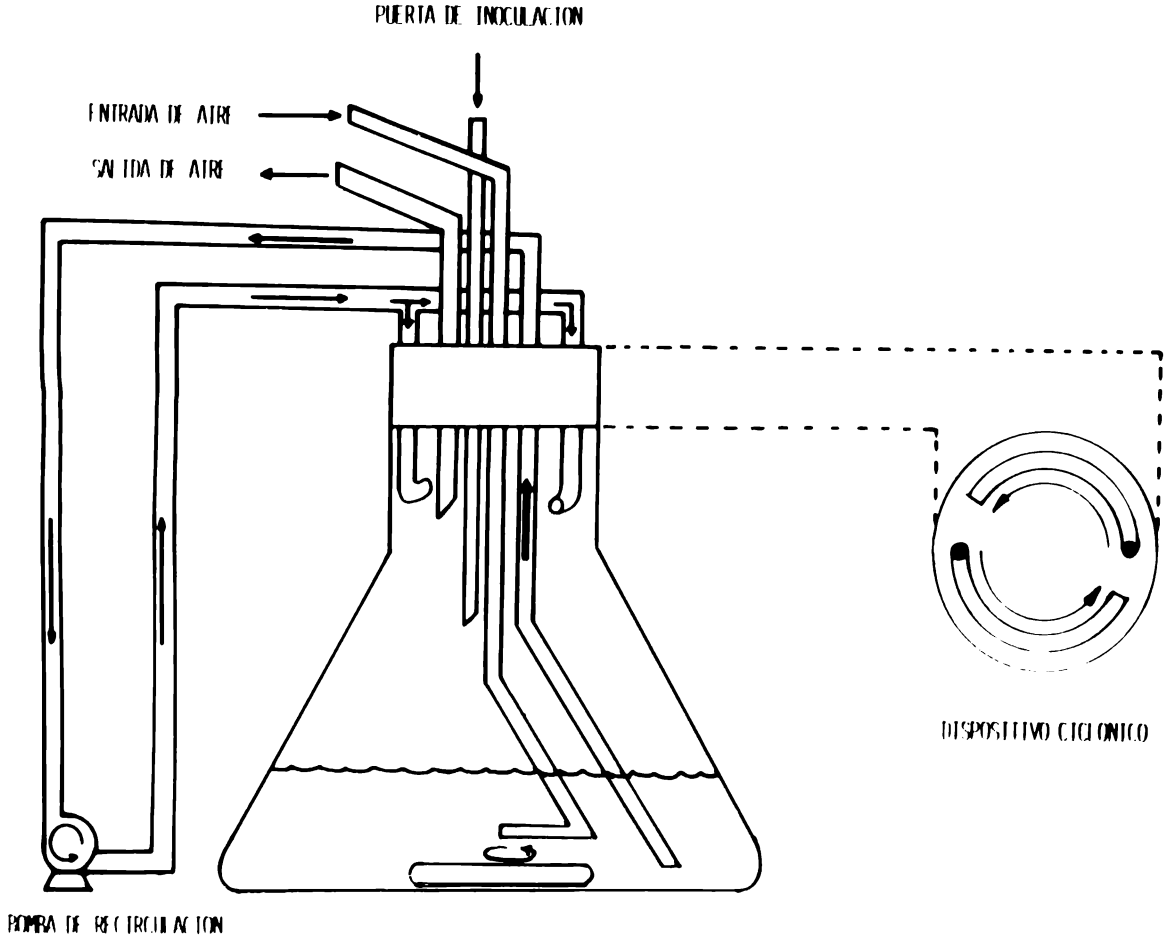


Fig.2. Diagrama simplificado del fermentador ciclónico usado para el cultivo alimentado.

Producción de la Actividad Pectinolítica en el Fermentador Ciclónico

La producción en este fermentador se llevó a cabo utilizando 1.0 l de medio de cultivo. El fermentador con el medio de cultivo fue puesto en un baño con agua a 37°C. El aire fue suministrado al fermentador,previamente esterilizado,a traves de un filtro fibroso y uno Millipore con una membrana de 0.45

µm, a una velocidad de 1.0 vvm. El medio de cultivo fue agitado por medio de un agitador magnético (Thermolyne, tipo 7200, Sybrom Co. U.S.A.). Una vez que las condiciones de cultivo se alcanzaron, se adicionaron 10 ml de inóculo, en condiciones asépticas. Inmediatamente después de la inoculación se inició la alimentación de los nutrientes a las velocidades antes especificadas y la recirculación del medio de cultivo a una velocidad de flujo de 1.0 l min^{-1}, por medio de una bomba peristaltica.

Determinación de la Actividad Pectinolítica.

Para la determinación de la actividad, se tomaron muestras a diferentes tiempos durante la fermentación las cuales fueron inmediatamente filtradas a través de un filtro Millipore con membrana de 0.45 µm. La actividad en el filtrado libre de células fue determinada: a) por cuantificación, por el método del ácido 2,3, dinitrosalicilico [42], del número de grupos reductores, expresados como acido galacturónico, liberados a partir de una solución de pectina al 0.9% incubado a 45°C durante 1 h, y b) por medio del cambio en la fluidez relativa de una solución de pectina al 1% a 30 °C en un viscosimetro de Ostwald [26].

Crecimiento celular

El crecimiento celular fue medido como peso seco (PS). Después de filtrar las muestras de fermentación a través de

una membrana Millipore de 0.45 µm, previamente llevada a peso constante, la masa celular retenida fue secada hasta peso constante a una temperatura de 70-75°C.

Determinación de Grupos Reductores en el Medio de Cultivo.

Los grupos reductores presentes en el medio de cultivo fueron determinados directamente del filtrado libre de células por el método del DNS [42]. Los productos de degradación de la pectina fueron expresados como ácido galacturónico.

RESULTADOS Y DISCUSION

Application of fed-batch cultures in the production of extracellular pectinases by *Aspergillus* sp.

G. Aguilar and C. Huitrón

Department of Biotechnology, Institute of Biomedical Research, National University of Mexico, AP 70228, 04510 Mexico, D. F.

(Received 2 November 1985; revised 18 March 1986)

The production of extracellular pectinases by Aspergillus *sp. in fed-batch cultures was studied in a fermenter with recirculation of medium. The continuous recirculation of medium resulted in a 40 and 60% increase in the extracellular pectinolytic activity as measured by reducing groups and viscometry, respectively, compared to the non-recirculated culture. The effect of dilution of the medium by addition of nutrients was evaluated. The results showed that the production of these activities was not affected with respect to the non-diluted culture. When nitrogen, pectin or both were added in limiting concentrations, we found that activities determined by reducing groups were 36, 249 and 455%, respectively, compared to the non-limited culture. The values for the pectinolytic activities measured by viscometry were only 5, 32 and 100%, respectively.*

Keywords: *Aspergillus* sp; extracellular enzymes; activity by reducing groups; activity by viscometry; fed-batch limited culture; induction; catabolic repression

duction

rch on the use of waste products from the agriculture ry has been principally oriented to the exploitation lulose and hemicellulose in the waste.[1-4] However, are byproducts such as the pulp of henequen, lemon, e and sugar beet which, in addition to the cellulose emicellulose, also contain appreciable quantities of . This pectin may be utilized to induce extracellular olytic enzymes as is being done with the pulp of both beet and apple. The enzymes so produced are used in dustrial processing of fruits and vegetables since their ty to degrade pectin and related substances not only in greater yields of extracted juices but also reduces ne needed for filtration.[5]

Mexico, abundant quantities of pulp from henequen mon are produced each year. We have reported that uen pulp can be successfully used at the fermenter res) level for the production of bacterial protein[6,7] f extracellular pectinases by *Aspergillus* sp.;[8,9] this fungus was isolated from the soil near a henequen sing plant.[8] This fungus grows at 37°C and, when on henequen pulp as carbon source, has a pectinoctivity which can be used in the depectinizing of juice. When equal concentrations of protein are used, mparable to that of several commercial pectinase preons.

e regulatory phenomena associated with the produc- f pectinases by fungi have been little studied in spite importance of such information at the industrial The synthesis of pectinases is induced by substances which contain pectin[11] and is subject to catabolic repression as reported for *Aspergillus niger*[12] and *Pyrenochaeta terrestris*.[13] For this reason, the use of a fed-batch culture would have advantages over a batch culture particularly when the presence of high concentrations of a nutrient negatively affects the yield or the productivity of the desired product. Such an effect has been reported for the polygalacturonate trans-eliminase of *Aeromonas liquefaciens* in which production was stimulated by limiting the concentration of the sustrate.[14] With fed-batch culture it is possible to eliminate or at least reduce such problems as substrate inhibition and catabolic repression and to increase the operating time of the fermentation. Fed-batch cultures have been successfully used to increase the yields of cellulases from *Myrothecium verrucaria*[15] and from *Trichoderma viride*[16], and the production of β-glucanase from *Bacillus subtilis*.[17] It has also been used in studies on the regulation of production of cellulases from *M. verrucaria*,[18] of pectinases and xylanases from *Verticillium albo-atrum*,[19] of cephalosporin from *Cephalosporium acremonium*[20] and of glutamine synthetase from *Neurospora crassa*.[21]

Given that *Aspergillus* sp. can produce pectinases from henequen pulp[8] and lemon pulp,[10] our principal interest was centred on the use of these byproducts of the agricultural industry as a source of enzymes. We felt it important to study some of the variables which affect the production of pectinases. Therefore, we used citrus pectin as model substrate to evaluate, first, the feasibility of increasing the production of these enzymes by *Apergillus* sp. in the fed-batch culture and second, the use of more complex substrates such as agroindustrial wastes. Using fed-batch cul-

0229/86/090541–05 $03.00
Butterworth & Co. (Publishers) Ltd

ture, we also analysed some of the factors involved in the regulation of biosynthesis of pectinases by *Aspergillus* sp.

In this report we present the results of the application of fed-batch culture to the production of extracellular pectinases by *Aspergillus* sp. under conditions in which the carbon source (citrus pectin) or nitrogen source (ammonium sulphate) or both were limited.

Materials and methods

Microorganisms

The microorganism used in this work was a white fungus of the genus *Aspergillus* which produces extracellular pectinolytic activity at 37°C.[9] The microorganism was propagated and stored on potato–dextrose–agar slants (Merck-Mexico).

Culture medium of fermentation

The culture medium contained 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.2% K_2HPO_4 and 1.0% citrus pectin. In the fed-batch culture, pectin or $(NH_4)_2SO_4$, or both, were eliminated. These reagents were added to the fermenter as indicated below. In all cases, the pH was adjusted to 4.2–4.6 with a dilute sulphuric acid solution. The medium and fermenter were sterilized for 25 min at 121°C and 15 lb in^{-2}.

Feeding of limiting nutrients

Those nutrients eliminated from the basal medium were added at a flow rate of 7.0 ml h^{-1} by means of a peristaltic pump (Master-Flex, Cole Parmer, Inc. USA, model 7550-40). When the limiting nutrient was either pectin or $(NH_4)_2SO_4$, the corresponding nutrient was fed in at a concentration of 5.0 mg ml^{-1} (35 mg h^{-1}) or of 0.19 mg ml^{-1} (1.33 mg h^{-1}), respectively. In the case of double limitation, pectin and $(NH_4)_2SO_4$ were fed in at the same concentrations. In both the fed-batch cultures, the values from the determination reported in the graphs were corrected by the dilution factor corresponding to the volume added at the time of sampling.

Preparation of inoculum

The inoculum was prepared by growing the microorganism in potato–dextrose–agar slants for 72 h at 37°C. After this time the spores were harvested by addition of 5 ml of isotonic sodium chloride solution and were diluted with the same solution to give a final absorbance value of 5.0.

Cyclonic fermenter

As fermentation reactor, we used a Fernback flask (2.8 litres) which was fitted with inlets and outlets for air and for culture medium, an inoculation port, and a device to recirculate the culture medium. This device, located in the upper part of the flask, had two tangential outlets so that a cyclonic flow was produced which continually bathed the walls of the flask, thereby avoiding growth of the fungus on the interior surface of the flask. We will refer this system as cyclonic fermenter.

Production of pectinolytic activity

For the production of pectinolytic activity, 1 litre of culture medium was added to the cyclonic fermenter described above and the flask was placed in a 37°C water bath. Sterile air was supplied to the fermenter throu fibrous filter and 0.45 μm membrane (Millipore Co., at a rate of 1.0 vvm. The mixture was agitated on a mag stirrer (Thermolyne, type 7200, Sybrom Co., USA). the culture conditions were reached, 10 ml inoculun added, under aseptic conditions, to the fermenter. Im ately thereafter, recirculation of culture medium was st at a flow rate of 1.0 l min^{-1} by means of a peristaltic p

Assays for pectinolytic activity

Samples (10 ml) were taken from the fermenter at di times during the fermentation and were immediately fil through 0.45 μm Millipore membranes. The pectin activity in the cell-free filtrate was determined: (a quantifying, using the method of DNS,[22] the numb reducing groups, expressed as galacturonic acid, whic been liberated after incubation with 0.9% pectin (45°C and (b) by measuring the relative change in the vis of 1.0% pectin at 30°C in an Ostwald viscosimeter.[9]

Cell growth

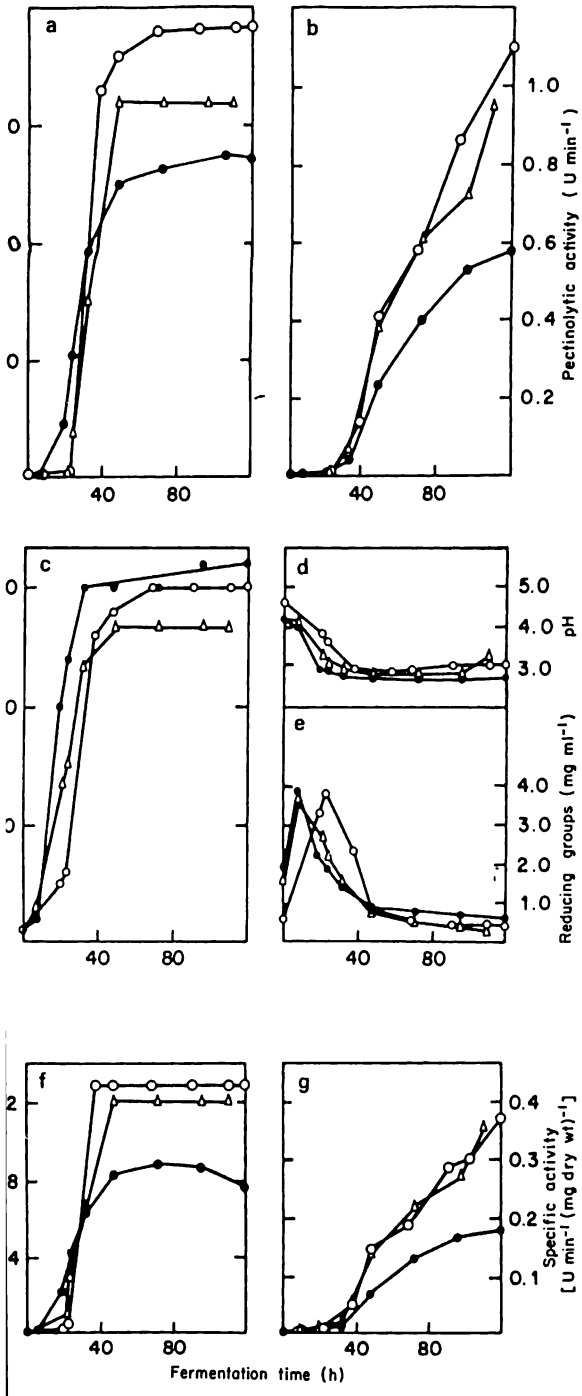
The cell growth was measured as dry weight (DW). filtering through a Millipore membrane, previously dri constant weight, the retained cell mass was dried to cor weight at 70 to 75°C.

Results and discussion

A fed-batch culture may be of constant or variable vo We selected the system with variable volume because p cannot be added at the concentrations required to ma a constant volume since, at high concentrations, p considerably increases the viscosity and more easily gels.

In the fed-batch cultures, in order to measure on effect of the concentration of limiting substrate an that of dilution during addition, a volume of water to that used in fed-batch culture was added to contr tures. The effect of recirculation of culture medium fermentation was also tested by using the cyclonic s described above.

The maximum pectinolytic activities in the dilute non-diluted fermentations, each with recirculatio medium, and that in the non-diluted, non-recirc fermentation were reached at the same time as gr growth (*Figure 1*). When the activity at 96 h of th diluted, non-recirculated fermentation was taken as the value for the diluted, recirculated fermentation was and that for the undiluted, recirculated fermentation as measured by reducing groups (*Figure 1a*). Whe pectinolytic activity was measured by relative fluid 96 h (*Figure 1b*), the values obtained for the three fe tations were 100, 136 and 161% respectively. The g curves from the three fermentations were similar (*Figu* but a greater increase was observed when the least pe lytic activity was produced. In the recirculated fermen lower growth but increased activity were detected result was probably due to a mechanical effect on th celia since it was observed by microscopy that, in the culated fermentations, the mycelia were fragm whereas in the non-recirculated fermentations, the m were long and unbroken. Oxygen did not appear responsible for the augmented activity since an incre growth was not observed in the recirculated ferment The pH was decreased during the first 36 h of fermen



1 Production of extracellular pectinolytic activity by *illus* sp. under conditions of dilution and recirculation of medium. △, Fermentation diluted with sterile water at a flow 7.0 ml h^{-1} and with recirculation rate of 1000 ml h^{-1}; ○, ted fermentation with recirculation at same flow rate; ●, tation without dilution and without recirculation. Extra- pectinolytic activity measured by: a, reducing groups; and he change in the relative fluidity. c, Cell growth; d, pH profile; mulation of reducing groups. Specific activity expressed as: educing groups (mg DW)$^{-1}$; and g, U min^{-1} (mg DW)$^{-1}$

alue of 2.8–3.0; the pH curve was similar for all three ntations.

ring the first hours of each fermentation, reducing s which were produced during the degradation of the accumulated (*Figure 1e*). In each fermentation, the t concentration of reducing groups was obtained at rt of the phase of greatest growth, after which time ncentration was reduced. The pectinolytic activities d maximum levels when the concentrations of degrada- roducts were almost used up (start of stationary

In both the recirculated, diluted and non-diluted fermentations, the specific activities as measured by reducing groups (*Figure 1f*), or by viscometry (*Figure 1g*) were similar and were greater than in the non-diluted, non-recirculated fermentation.

These results demonstrated that the detected enzymatic activity was not affected by the dilution of culture medium (*Figure 1c*), thereby implying that in the fed-batch cultures, the results obtained were due to the addition conditions with respect to the concentration and type of substrates added. The pectinolytic activity was less in the fermentation without recirculation of culture medium than that in the recirculated fermentations, whether diluted or not. The accumulation and utilization of the degradation products of pectin were similar in all three fermentations. According to these preliminary results, we decided to use recirculated fed-batch culture for our study with diluted, recirculated fermentation as control.

Our objectives were to evaluate the possibility of improving production of pectinases through use of recirculated fed-batch culture and to test if, under these conditions of limiting nutrients, the effect of the pectin degradation products on the production of these enzymes could be determined. Therefore, experiments were carried out in which pectin (35 mg h^{-1}), $(NH_4)_2SO_4$ (1.33 mg h^{-1}), or both (at these same concentrations) were added at these low concentrations in order to avoid the accumulation of these substrates in the culture medium. The results of the extracellular pectinolytic activity obtained under these conditions are shown in *Figures 2a* and *2b*.

In comparison with the control, when *Aspergillus* sp. was grown in limited nitrogen source, the production of the activity was delayed almost 80 h and was less as measured both by reducing groups and by viscometry. When pectin was the limiting nutrient, a greater increase in activity with respect to the control measured by reducing groups was observed at 96 and 120 h fermentation. In the culture grown under doubly limiting conditions (pectin and nitrogen), the activity was detected at 24 h and the maximum increase in activity was almost double that of the control (*Figure 2a*). A reduction was observed in the activity measured by viscometry with respect to the control (*Figure 2b*), since when either nitrogen or pectin was the limiting nutrient, the activity measured by viscometry was detected until just before 80 h and the maximum activity was approximately fourfold less than that of the control. Under doubly limited conditions, the production of this activity, although detected at the same time (36 h) as in the control culture, never reached the levels obtained in the control; however, it was greater than that found under the other conditions of limiting nutrients (*Figure 2b*).

Under conditions of limiting pectin or $(NH_4)_2SO_4$, a linear growth curve was obtained (*Figure 2c*) which was a function of the velocity of feeding and was preceded by a lag phase that may be attributed to the period of enzymatic induction since pectinases are inducible enzymes. However, under doubly limiting conditions, a non-linear growth curve was obtained which is probably due to the difference in the rate of utilization of the limiting nutrients and to the fact that the growth of *Aspergillus* sp. when tested under limiting pectin and nitrogen was not the same. These results were compared with the growth obtained in diluted, recirculated culture which was taken as control. Likewise, for the pectin degradation products in the limiting nitrogen culture, measured as reducing groups (*Figure 2e*) the maximum concentration of the accumulated products, similar to that

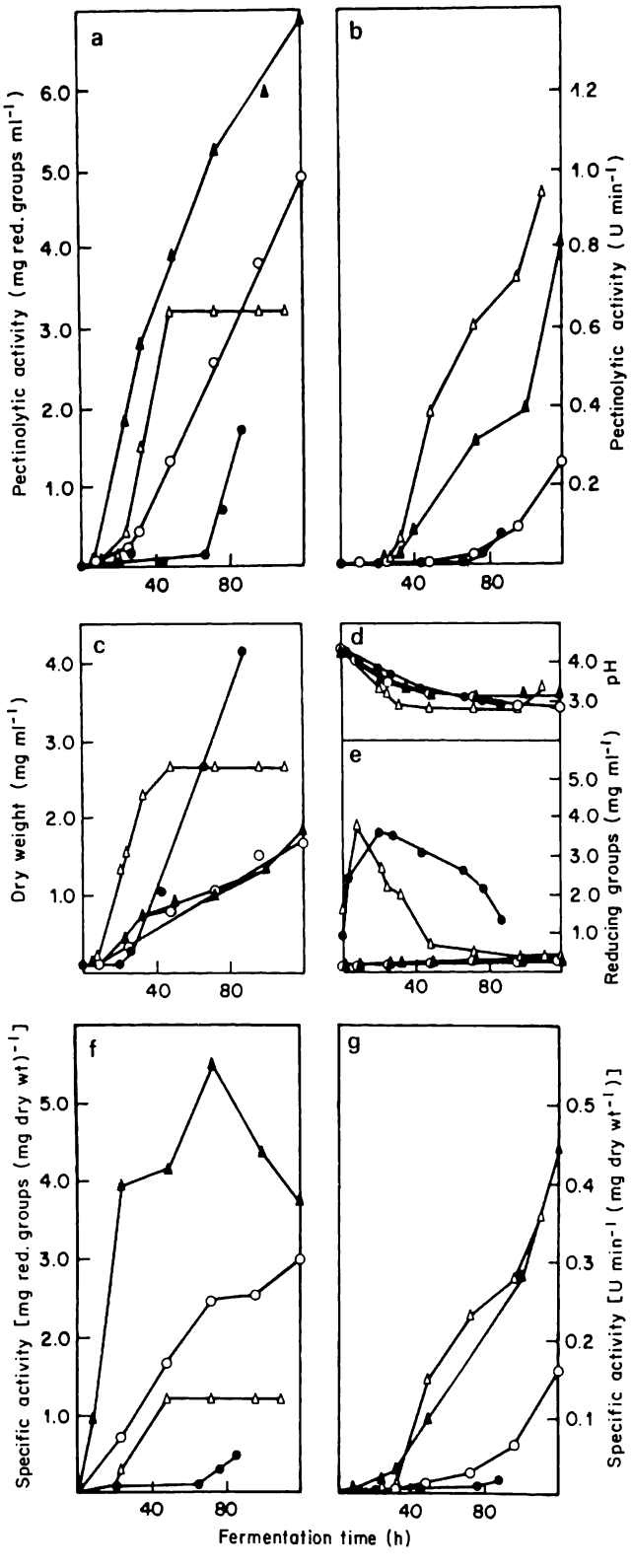


Figure 2 Time course of fed-batch fermentation of *Aspergillus* sp. under different limiting conditions. a, b, Extracellular pectinolytic activity; c, cell growth; d, pH profile; e, accumulation of degradation product from pectin as reducing groups. f, g, Specific activity. •, Ammonium sulphate limited at a feed rate of 1.33 mg h^{-1}; ○, pectin limited at feed rate of 35 mg h^{-1}; ▲, doubly limited at a feed rate of 1.33 mg h^{-1} and 35 mg h^{-1} for ammonium sulphate and pectin respectively; △, diluted batch culture as control for comparison (as *Figure 1*)

of the control, was obtained. However, since the rate of utilization of these products was less as a consequence of the lower growth due to limiting nitrogen source, their concentration in the culture medium remained elevated for a longer period of time in comparison to the control. When pectin was the limiting nutrient, the concentration of these products was maintained at a very low level which di increase during the fermentation; therefore, there w accumulation of these products. The same behaviou found in the doubly limited fed-batch cultures. The sults may be correlated with the production of enzy activity. It appears that, when the concentration c ducing groups was elevated, the production of activit delayed in the nitrogen limited culture and that, when concentration remained very low (as in the doubly li fed-batch culture), the production was begun at an e time or at least simultaneously with that of the p limited, control culture. Although this is applicable t activity measured by reducing groups, the activit measured by viscometry did not show such a direct co tion.

To compare adequately the production of the enzy activities obtained under the different limiting condi given that the growth was distinct for each conditio specific activities for each experiment were calcu (*Figure 2f, g*). When only the nitrogen source was lin 36% of the pectinolytic activity measured by red groups was produced and only 5% of that measured b cometry with respect to the control (*Table 1*). Hov when pectin was the limiting nutrient, there was a increase in the activity measured by reducing groups respect to the control. This was increased to 455% both pectin and nitrogen were limiting nutrients. Whe pectinolytic activity as measured by viscometry, pectin as limiting nutrient, 32% activity was obtained respect to the control; when both pectin and nitrogen limited, 100% (*Table 1*). These results strongly sugges the production of the activity determined by reducing g is subject to catabolic repression by the reducing g producing during fermentation. The results obtained f activity measured by viscometry showed a corre with the high concentration of degradation products, in ing catabolic repression in the nitrogen-limited cu However, in the pectin-limited and doubly limited cu where there was no accumulation of degradation pro the pectinolytic activity did not reach the level of th trol (*Figure 2b*). This may be due not just to the cat repression but to some other phenomenon such as concentration of inducer(s) caused by limitation of pe

The relation between the extracellular pectinolytic ity produced by *Aspergillus* sp. and the cell grow control, nitrogen-limited, pectin-limited and doubly li fermentations are shown in *Figure 3*, in order to co the relative rate of synthesis in these cultures. The syn of pectinases, as measured by reducing groups, was under pectin-limited or doubly limited conditions. nitrogen-limited conditions and in the control, a de the production of the same activity was observed whic correlated with the high accumulation of reducing g during fermentation (*Figure 2e*). It would seem tha activity was repressed at high concentration of the de tion products of pectin and was derepressed at low c tration of these products (*Figure 3a*).

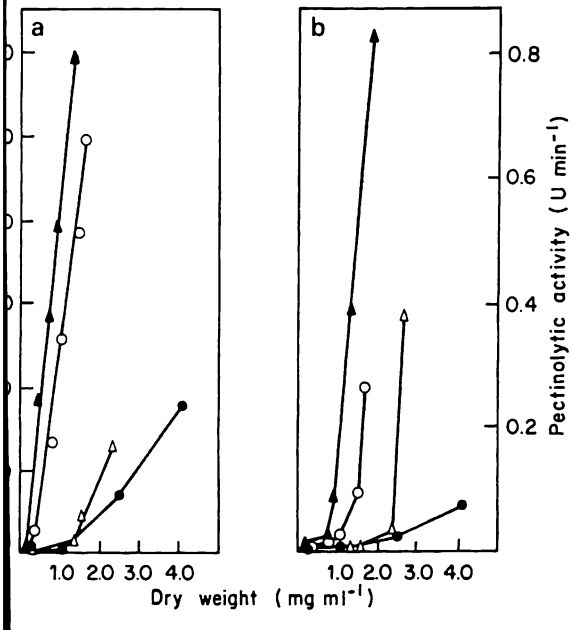
In the case of pectinolytic activity determined b cometric assay, a delay in the synthesis of activit observed (*Figure 3b*). This delay period although no pletely eliminated, was considerably shortened in the p limited and doubly limited cultures. This may be due fact that in these conditions there was no accumulat degradation products (*Figure 2e*). When nitrogen was ing, greater delay of the same activity was observed (*3b*), suggesting a major sensitivity to catabolic repr

1 Comparison of the maximum specific activity in batch and fed-batch cultures of *Aspergillus* sp.

re	Activity			
	Reducing groups [mg (mg DW)$^{-1}$]	(%)	Fluidity change [U min^{-1} (mg DW)$^{-1}$]	(%)
ol[a]	1.21	100	0.356	100
with recirculation	1.28	106	0.340	96
without recirculation	0.88	73	0.170	48
;en-limited	0.43	36	0.018	5
ı-limited	3.01	249	0.115	32
ı/nitrogen-limited	5.51	455	0.356	100

control culture was a batch culture with recirculation and feeding with sterile water at a flow rate of 7.0 ml h^{-1}

d by the accumulation of degradation products.
; demonstrated above, the production of pectinolytic
ty by *Aspergillus* sp. was sensitive to catabolic repres-
by the degradation products of pectin. However, given
liversity of intermediates that were formed (mono-,
ri-, tetragalacturonic acid etc., with or without a
oxyl group), we were unable to determine which of
these was (were) the one(s) directly responsible for
cing the repression, or if a product(s) of metabolism
se compounds was responsible.
e results presented in this study show that when the
ty was measured by reducing groups, the pectinases
d a repression–derepression response depending on
ctual accumulation of the degradation products of
. When the activity was measured by viscometry a
sensitivity was observed to catabolic repression,
ally under nitrogen limitation. In the pectin-limited
oubly limited cultures this activity did not reach the



3 Relation between extracellular pectinolytic activity and
wth of limited cultures of *Aspergillus* sp. from the data of
2. •, Ammonium sulphate limited; ○, pectin limited; ▲, doubly
(pectin and ammonium sulphate); △, diluted batch culture
ol for comparison. Other details as *Figure 2*

same levels as the control, conditions in which catabolic repression is not expected, because there was no accumulation of degradation products. This activity may be explained by a lower concentration of the inducer(s) due to pectin limitation.

Acknowledgement

The authors wish to thank Drs Ma. Elena Flores and Susana Saval for useful discussions and Ma. Angeles López for secretarial assistance.

References

1 Dekker, R.F.H. *Biotechnol. Bioeng.* 1983, **25**, 1127–1146
2 Tangnu, S.K. *Process Biochem.* 1982, **17** (3) 36–45
3 Linko, M. and Nybergh, P. *Proceedings of Symposium on Bioconversion in Food Technology* (P. Linko, ed.) University of Technology, Espoo, Finland, 1978, pp. 64–67
4 Cunningham, R.L. and Carr, M.E. *Biotechnol. Bioeng. Symp.* 1984, **14** 95–103
5 Kilara, A. *Process Biochem.* 1982, **17** (4) 35–41
6 Blancas, A., Alpizar, L., Larios, G., Saval, S. and Huitrón, C. *Biotechnol. Bioeng. Symp.* 1982, **12**, 171–175
7 Blancas, A., Aguilar, G. and Huitrón, C. *Biotechnol. Bioeng. Symp.* 1984, **14**, 479–484
8 Saval, S., Solórzano, R., Alpizar, L., Cea, A. and Huitrón, C. *Proc. Int. Symp. on the Use of Enzymes in Food Technology*, Versailles, France, 1982, pp. 531–535
9 Saval, S. and Huitrón, C. *Dev. Ind. Microbiol.* 1983, **24**, 547–551
10 Saval, S. MSc Thesis, National University of México, 1985
11 Perley, A.F. and Page, O.T. *Can. J. Microbiol.* 1971, **17**, 415–420
12 Shinmyo, A., Davis, I.K., Nomoto, F., Tahara, T. and Enatsu, T. *Eur. J. Appl. Microbiol.* 1978, **5**, 59–68
13 Keen, N.T. and Horton, J.C. *Can. J. Microbiol.* 1966, **12**, 443–453
14 Hsu, E.J. and Vaughn, R.H. *J. Bacteriol*, 1969, **98**, 172–181
15 Hulme, M.A. and Stranks, D.W. *Nature* 1970, **226**, 469–470
16 Gottvaldova, M., Kucera, J. and Podrazky, V. *Biotechnol. Lett.* 1982, **4**, 229–232
17 Markkanen, P., Reinwall, A. and Linko, M. *J. Appl. Chem. Biotechnol.* 1976, **26**, 41–46
18 Hulme, M.A. and Stranks, D.W. *J. Gen. Microbiol.* 1971, **69**, 145–155
19 Cooper, R.M. and Wood, R.K.S. *Nature* 1973, **246**, 309–311
20 Matsumura, M., Imanaka, T., Yoshida, T. and Taguchi, H. *J. Ferment. Technol.* 1981, **59**, 115–123
21 Limón-Lason, J., Lara, M., Resendiz, B. and Mora, J. *Biochem. Biophys. Res. Commun.* 1977, **78**, 1234–1240
22 Miller, L. *Anal. Chem.* 1959, **31**, 426–428

Studies for the large scale isolation of membrane-associated progesterone 11α-hydroxylase: enzyme stability in *Rhizopus nigricans* ATCC 6277b cells

D. F. Broad, S. Pontin and P. Dunnill

Department of Chemical and Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK

(Received 10 January 1986; revised 8 May 1986)

Factors influencing the stability of the membrane-associated enzyme progesterone 11α-hydroxylase of Rhizopus nigricans *are reported. Stability of the enzyme activity was enhanced over 18 h by glucose (2.5% w/v) and more so by aeration and by operations performed below 20°C. The results are related to the requirements of large-scale enzyme isolation.*

Keywords: Progesterone 11α-hydroxylase; stability; *Rhizopus nigricans*

Introduction

Membrane-associated enzymes catalyse several reactions of industrial interest, including steroid hydroxylation. Therefore, it is of interest to examine how to isolate membrane-associated enzymes on a large scale with particular emphasis on understanding why recoveries are generally so poor.

We have adopted the progesterone 11α-hydroxylase system of *Rhizopus nigricans* ATCC 6227b as a model and have examined its production[1] and in a preliminary manner its extraction.[2] The progesterone 11α-hydroxylase of *R. nigricans* ATCC 6227b is a member of the cytochrome *P*-450 monooxygenase group of enzymes and functions with an NADPH–cytochrome *P*-450 reductase.[3] The enzyme complex is associated with the endoplasmic reticulum of the mycelial cells.[3]

We are now embarked on an examination of the unit operations of extraction and purification on a pilot-scale. Such detailed studies are difficult to accomplish unless it is possible to subdivide the process by storage stages. This is because substantial amounts of data, both engineering and analytical, must be collected at each stage and on a large scale this extends batch operation over several hours. For this reason we have examined the conditions under which whole cells can be held with minimum loss of enzyme activity.

Material and methods

Materials

Potato dextrose agar and Sabouraud dextrose agar were obtained from Oxoid Ltd (Basingstoke, Hants, UK), yeast extract (Yeatex) from Bovril Food Ingredients Ltd (Burton on Trent, Staffs, UK) and dextrose monohydrate (Cerelose) from F. Allen Ltd, London, UK. Progesterone was a ous gift of Dr J. P. R. Herrmann (Glaxo Researcl Greenford, UK). D-Glucose was obtained from Fisor (Loughborough, Leics, UK) and Tween 80 from Koch Ltd (Colnbrook, Bucks, UK). 11α-Hydroxyproges was obtained from Sigma Ltd (Poole, Dorset, UK).

Growth, induction and assay of proges 11α-hydroxylase activity in R. nigricans *in l culture*

A spore suspension containing 5×10^8 spores was fuged at 4000*g* for 5 min. The spore pellet was resusp with vigorous agitation in 5 ml sterile growth mediun to inoculation in 250 ml growth medium (25 g l^{-1} de monohydrate, 20 g l^{-1} yeast extract, 1 ml l^{-1} trace min pH 4.5 adjusted with orthophosphoric acid) contai 'siliconized' 2 litre Ehrlenmeyer flasks fitted with bungs. Cultures were grown for 24 h at 28°C on a reci shaking incubator (New Brunswick, Edison NJ, USA a 5 cm throw set at 100 strokes min^{-1}.

Progesterone 11α-hydroxylase activity was induc 3 h in the cells by adding 0.5 g progesterone (final c tration 2 g l^{-1}) in 1 ml 0.01% (w/v) Tween 80 t flask after 21 h growth. Enzyme activity was deter by measuring the amount of 11α-hydroxyproges formed during 18 h at 4°C.[5]

Storage of R. nigricans *cells*

After growth in liquid culture, *R. nigricans* cell harvested by filtration through nylon mesh and with cold 0.5% (w/v) NaCl. Washed *R. nigricans* cell resuspended at a concentration of 0.067 g wet cells r the requisite storage medium. Aliquots of 30 m

0141–0229/86/090546–03 $03.00

STIMULATION OF THE PRODUCTION OF EXTRACELLULAR PECTINOLYTIC ACTIVITIES OF *Aspergillus* sp. BY GALACTURONIC ACID AND GLUCOSE ADDITION

ABBREVIATED TITLE:

STIMULATION OF THE PRODUCTION OF PECTINASES

SOMETIDO PARA SU PUBLICACION EN
"ENZYME AND MICROBIAL TECHNOLOGY".

Guillermo Aguilar and Carlos Huitrón

Department of Biotechnology, Institute of Biomedical Research, UNAM.

National University of México.

A.P. 70 228, 04510 México, D.F.

STIMULATION OF THE PRODUCTION OF EXTRACELLULAR PECTINOLYTIC ACTIVITIES OF *Aspergillus* sp. BY GALACTURONIC ACID AND GLUCOSE ADDITION

Guillermo Aguilar and Carlos Huitrón
Departament of Biotechnology, Institute of Biomedical Research, UNAM.
National University of Mexico. A.P. 70228, 04510 Mexico, D.F.

We studied the effect of the addition of galacturonic acid and of glucose on the production of the pectinolytic activities by *Aspergillus* sp. The results showed that a differential response in the production of the activities was produced. The production of the pectinolytic activity as measured by reducing groups was no negatively affected when low concentration of either glucose or galacturonic acid were added to growing pectin containing cultures of *Aspergillus* sp. in spite of the time of addition. The production of the activity measured by viscosimetry was stimulated by addition of galacturonic acid at 24 h suggesting that galacturonic acid participates in the induction of pectinolytic activities of *Aspergillus* sp. When the addition was made at t = 0 the increase in pH seems to affect the production of this activity. The addition of glucose at 24 h had no effect on the production of the viscosimetric activity; however catabolic repression was observed upon the addition at t = 0. The production of both pectinolytic activities was strongly repressed when glucose was added at high concentration and was affected less when galacturonic acid was added at the same high concentration.

Keywords: Aspergillus sp., catabolic repression, reducing groups accumulation, activity by reducing groups, activity by viscosimetry.

INTRODUCTION

The pectinases are a groups of enzymes that degrade pectin-containing substances and are widely used in the industrial processing of fruits and vegetables. These enzymes are produced by plants and microorganisms such as bacteria of the genera Bacillus, Clostridium, Pseudomonas, Xantomonas and Erwinia and the fungi Aspergillus and Penicillium (1,2). At present the majority of the commercial preparations of pectinases are obtained starting from fungi, especially species of the genus Aspergillus such as A. niger, A. oryzae and A. wentii since the fungi produce different extracellular enzymes with pectinolytic activity. For the production of pectinases, apple bagase, citrus fruit rind and sugar beet pulp, supplemented with glucose, maltose, starches or ground cereals have been used as substrates. (3-5). Our research group has worked on the production of pectinases from the fungus Aspergillus sp. grown on henequen pulp (6,7) or on lemon rind (8,9). This microorganism was isolated in the Yucatán peninsula, in the southeast of México (10) and was selected because of the high levels of extracellular pectinases, which it produces when grown at 37°C in simple medium containing either pectin, henequen pulp, polygalacturonic acid, tragacant gum, or other agroindustrial waste products (8).

Regulation of the syntheses of pectinases has been better studied in bacteria than in fungi; however, it is known that the syntheses of pectinases in fungi of the genera Aspergillus, Fusarium and Verticillium, among others, are induced by pectin or pectin-containing substances (6-13). The polygalacturonase of Aspergillus niger is subject to catabolic repression by glucose at the level of transcription as wells as translation (14,15). It has likewise been reported that the synthesis of an endopolygalacturonase in Pyrenochaeta terrestris is repressed when a pectin medium was supplemented with glucose or other hexoses at concentrations greater than 0.05 M but is stimulated at concentrations of 0.005 M (16).

At present, it is not known whether the direct inducer(s) of the pectinolytic activity is one or more of the various compounds involved in the induction of the enzymes which compose the pectinolytic system in fungi. Galacturonic acid induces the syntheses of endopolygalacturonase and endopectintranseliminase in Verticillium albo-atrum and D-galactarate (mucic acid) appears to be the only stimulating compound (i.e. direct inducer) of polygalacturonase and pectinesterase in Penicillium chrysogenum (19).

Our groups is working on some aspects of the regulation of the production of pectinases by Aspergillus sp. In a previous paper using fed-batch cultures in which pectin was used as the carbon source under various conditions of limiting carbon or nitrogen we found that the production of extracellular pectinolytic activity by Aspergillus sp. is affected by the

concentration of the pectin degradation products which accumulate in the culture medium during the first hours of fermentation (11). Syntheses of enzymes with pectinolytic activities are repressed at high concentrations of these degradation products and are derepressed at low concentration of these products. These same products have been shown to produce a differential response in the activity as measured by the amount of reducing groups in relation to the activity measured by viscosimetry (11). In this line we are interested in the evaluation of the effect of the addition of exogenous carbon sources on the production of the pectinolytic activity of Aspergillus sp.

Herein, we report our results on the effect of galacturonic acid and glucose on the production of pectinases when they are added at different times to cultures of Aspergillus sp. grown on pectin, and on the identification of some factors involved in the differential response of these pectinolytic activities.

MATERIALS AND METHODS

Microorganism

The microorganism used in this work was a white fungus of the genus Aspergillus which produces extracellular pectinolytic activity at 37°C (7,10). The microorganism was propagated and stored on potato-dextrose-agar slants (Merck-Mexico).

Culture Medium for Fermentations

The culture medium contained 0.2% $(NH_4)_2SO_4$; 0.2% KH_2PO_4; 0.2% K_2HPO_4 and 1.0% citrus pectin. In all cases, the pH was adjusted to 4.2-4.6, with a dilute sulfuric acid solution. The medium was sterilized 25 min at 121°C and 15 psi. In the cases where D-glucose, D-galacturonic acid, cycloheximide or mixtures of these compounds were added to the culture medium, the addition was made in asceptic conditions at the times stated in the graphs. After autoclaving at the conditions as above 10 ml of each solution of either D-glucose or D-galacturonic acid at a concentration of 20 mg ml^{-1} or 100 mg ml^{-1} were added to 200 ml of culture medium to give a final concentration of 1.0 mg ml^{-1} or 5.0 mg ml^{-1}, respectively. One ml of a solution of cicloheximide (25 mg ml^{-1}), sterilized by filtration, were added to the culture medium to give a final concentration of 125 $\mu g\ ml^{-1}$. The experiments where addition was made are specified in the Figures.

Preparation of Inoculum

The inoculum was prepared by growing the microorganism in potato-dextrose agar slants for 72h at 37°C. After this time the spores were harvested by addition of 5 ml of isotonic sodium chloride solution and were diluted with the same solution to give a final optical density value of 5.0.

Production of Pectinolytic Activity

For the production of pectinolytic activity, shake flask experiments were conducted in 500 ml Erlenmeyer flasks, each containing 200 ml of culture medium, 2.0 ml of inoculum were added, under asceptic conditions, to each flask. Cultures were incubated at 37°C and 200 rpm in an incubator shaker (Newbruswick Scientific, Co. U.S.A.)

Assays for Pectinolytic Activity

Samples were taken from the flasks at distinct times during the fermentation and were immediately filtered through 0.45 μm Millipore membranes.The pectinolytic activity in the cell-free filtrate was determined: (a) by quantifying, using the method of DNS (20), the number of reducing groups, expressed as galacturonic acid which had been liberated after incubation with 0.9% pectin (45°C, 1 h), and (b) by measuring the relative change in the viscosity of 1.0% pectin at 30°C in an Ostwald viscosimeter as we reported previously (8).

Cell Growth

The cell growth was measured as dry weight (DW). After filtering through a Millipore membrane, previously dried to constante weight, the retained cell mass was dried to constant weight at 70 to 75°C.

Reducing Groups in Culture Media

Reducing groups present in culture media were determined directly in the cell-free filtrates using the method of DNS (20). The degradation products from pectin were expressed as galacturonic acid. When other carbon source was used it concentration in the culture medium was determined as reducing groups and expressed using the appropiate standard (i.e. glucose).

RESULTS AND DISCUSSION

Effect of Addition of Low Concentrations of Galacturonic Acid or Glucose on the Production of Pectinolytic Activities.

Because galacturonic acid (GA) one of the degradation products from pectin and glucose (G) a known catabolite represor we decide to evaluate how this compounds affect the production of pectinolytic activities by Aspergillus sp., the effects on such production by the addition of low concentration of GA or G at different times to cultures of Aspergillus sp. which had been grown on pectin, were evaluated.

GA was added to cultures of Aspergillus sp. grown on pectin in three distinct manners: In the first, 10 ml of a solution of GA was added only at time zero, to a flask containing culture to give a final concentration of 1.0 mg GA ml^{-1}; this same quantity of GA (final concentration 1.0 mg ml^{-1}) was added to the second culture-containing flask only at 24 h and, to the third flask at 24, 48, 72, and 96 h of

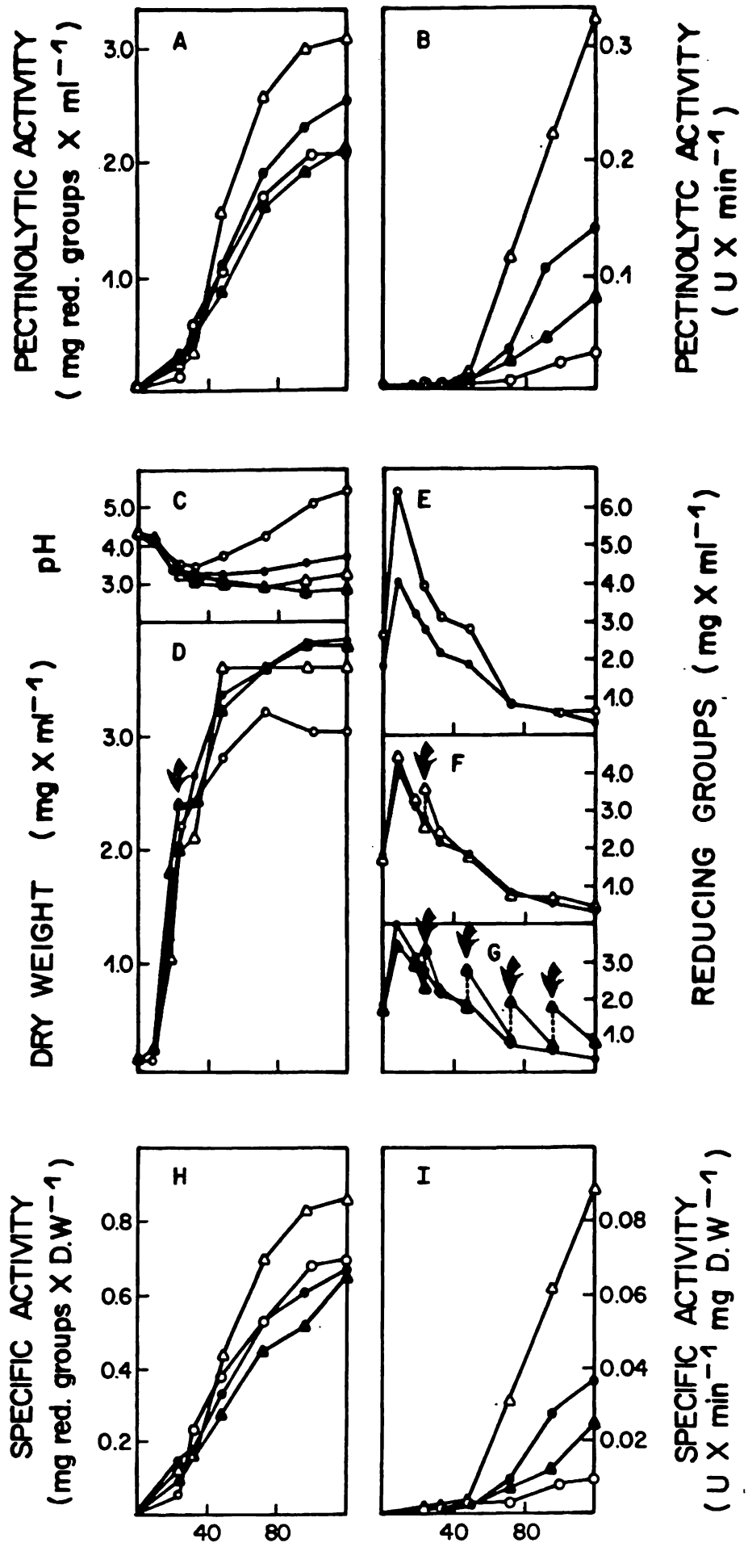

PECTINOLYTIC ACTIVITY (mg red. groups X ml⁻¹)
PECTINOLYTC ACTIVITY (U X min⁻¹)
A
B
C
D
E
F
G
H
I
pH
DRY WEIGHT (mg X ml⁻¹)
REDUCING GROUPS (mg X ml⁻¹)
SPECIFIC ACTIVITY (mg red. groups X D.W⁻¹)
SPECIFIC ACTIVITY (U X min⁻¹ mg D.W⁻¹)
FERMENTATION TIME (hours)

FIGURE 1

Fig.1. Effect of the addition of galacturonic acid (1.0 mg x ml^{-1}, final concentration) to cultures of Aspergillus sp. growing on pectin. Extracellular pectinolytic activity (A,B), pH profile (C), cell growth (D), degradation products from pectin as reducing groups (E,F,G), and specific activity (H,I). Pectin,1.0% without addition (●); pectin 1.0% plus a sole addition of GA at 0 h (○) or 24 h (△), and pectin 1.0% plus GA added at 24, 48, 72 and 96 h (▲). The arrows (⇓) shows the time of addition.

fermentation.

The resulting extracellular pectinolytic activities produced under these three culture conditions were measured by reducing groups (Fig. 1A) and by viscosimetry (Fig. 1B). When the GA had been added at t = 0 h or at 24 h intervals, the activities as measured by reducing groups were 82.0% and 83.0%, respectively, that produced in the control culture (Fig. 1A). However, when this carbon source had been added at 24 h, the pectinolytic activity was 123% that of the control culture (Fig. 1A).

As measured by viscosimetry the activity in the culture to which GA had been added at t = 0 was only 21% that of the control culture (Fig. 1B). This negative effect on the activity was also observed in the culture to which GA has been added at 24 h intervals; the activity so produced was 58% that of the control (Fig. 1B). However, when GA had been added to the culture at 24 h, the activity was 232% that of the control (Fig. 1B). This finding was not due to a stimulation of the activity per se because results of assays in vitro (data not shown) demonstrated that the levels of activities with or without GA were the same. Therefore, it appeared that GA acted at the level of synthesis of the enzymes involved in these activities, probably as an inducer.

The pH profiles were very similar for the control culture and for those with additions of GA at 24 h or at 24 hours intervals (Fig. 1C); whereas, the pH profile of the culture with GA addition at t = 0 increased from 32 h to 120 h, at which time pH 5.4 was reached. The growth curves for

these fermentations were similar, with the exception of that obtained with the addition of GA at t = 0 in which lower growth was observed (Fig. 1D). This lower growth may correlate with the increase in pH of this culture.

During the first hours of fermentation, the pectin degradation products accumulated in the culture media. (Fig. 1E-G). This was true even in that culture to which GA had been added at t = 0, thereby presenting the microorganism with a more easily assimilable carbon source than pectin(P). Contrary to the excepted use of GA first, followed by the degradation of P, in this culture, the accumulation of reducing groups reached 6.4 mg R.G. ml^{-1}, a value 2.4 mg R.G. ml^{-1} greater than that of the control (Fig. 1E). When the GA had been added at 24 h, the pattern of consumption of the reducing groups was almost the same as that of the control (Fig. 1F). When the GA had been added at 24 hours intervals, after each addition, the concentration of reducing groups reached control levels (Fig. 1G). This indicated that the microorganism in this culture consumed a greater quantity of its carbon source than did the control; however, this consumption was not reflected in its growth.

The specific activity, measured as reducing groups was similar to that of the control when the GA had been added either at t = 0 (105%) or at 24 h intervals (99%), (Fig. 1H , Table I). However as measured by viscosimetry, the specific activity was 27% and 69% that of the control when

Effect of the addition of galacturonic acid and glucose on the production of pectinolytic activities of Aspergillus sp. growing on pectin [a].

Compound added [b]	REDUCING GROUPS ACTIVITY [c]		VISCOSIMETRIC ACTIVITY [c]	
	mg R.G. x ml^{-1}	mg R.G. x mg D.W.(%)	U x min^{-1}	U x min^{-1} x mg D.W.$^{-1}$(%)
None	2.52	0.660 (100.0)	0.139	0.0360 (100.0)
Galacturonic acid (1.0 mg/ml; 0h)	2.07	0.690 (105.0)	0.029	0.0096 (27.0)
Galacturonic acid (1.0 mg/ml; 24h)	3.09	0.860 (130.0)	0.322	0.0890 (247.0)
Galacturonic acid (1.0 mg/ml;every 24h)	2.09	0.653 (99.0)	0.080	0.0250 (69.0)
Glucose (1.0 mg/ml;0h)	2.34	0.688 (104.2)	0.077	0.0230 (63.8)
Glucose (1.0 mg/ml; 24h)	2.71	0.900 (136.3)	0.153	0.0510 (141.6)
Galacturonic acid (5.0 mg/ml;24h)	1.60	0.400 (60.6)	0.102	0.0250 (69.4)
Glucose (5.0 mg/ml;24h)	1.32	0.200 (30.3)	0.042	0.0063 (17.5)
Cycloheximide (125µg/ml; 0h)	1.31	0.500 (76.0)	0.039	0.0150 (42.0)
Cycloheximide (125 µg/ml;24h)	1.48	0.672 (102.0)	0.040	0.0180 (50.0)
Galacturonic acid (1.0 mg/ml;24h) + Cycloheximide (125 µg/ml;24h)	0.65	0.290 (44.0)	0.024	0.0110 (31.0)

[a] All cultures were grown in shake flasks containing 1.0% of pectin incubated at 37°C and agitated at 200 rpm for five days.

[b] The numbers in bracket denotes the final concentration and time of addition, respectively of the compound indicated in table.

[c] Values obtained at 120 hours.

the GA was added at t = 0 or at 24 hour intervals respectively, (Fig. 1I, Table I). When GA had been added only at 24 h, increases of 30%, as measured by reducing groups, and of 147%, as measured by viscosimetry, in the specific activity were observed (Fig. 1G-H; Table I). The differential responses in the specific activity, detected by both methods but especially by viscosimetry, upon the addition of GA at different times and at low concentrations suggests that GA participates in some way in the induction of pectinases in Aspergillus sp. Such induction by GA may depend on the concentration and physiological state of the cells at the time of addition because, when GA was added at t = 0, the production of activity measured by viscosimetry appeared to be repressed. However, we could not discard the possibility that the increase in pH in this case might have negatively affected the activity. The addition of GA at 24 h when the cells were in the phase of most rapid growth, notably stimulated the production of activity. When a total of 4.0 mg GA had been added in sucessive additions, the activity was reduced, particularily that measured by viscosity, probably due to catabolic repression by GA at this concentration.

To separate flasks, glucose was added only at t = 0 or at t = 24 h to give a final concentration of 1.0 mg ml^{-1}. When G, had been added at t = 0, the activity measured by reducing groups was 93% that of the control and, when added at 24 h, 107% of control (Fig. 2A).

As measured by viscosimetry, the activities were 55% and 110% that of control when G had been added at t = 0 or at 24 h, respectively (Fig. 2B). The pH profiles obtained when G had been added at t = 0 or at 24 h were

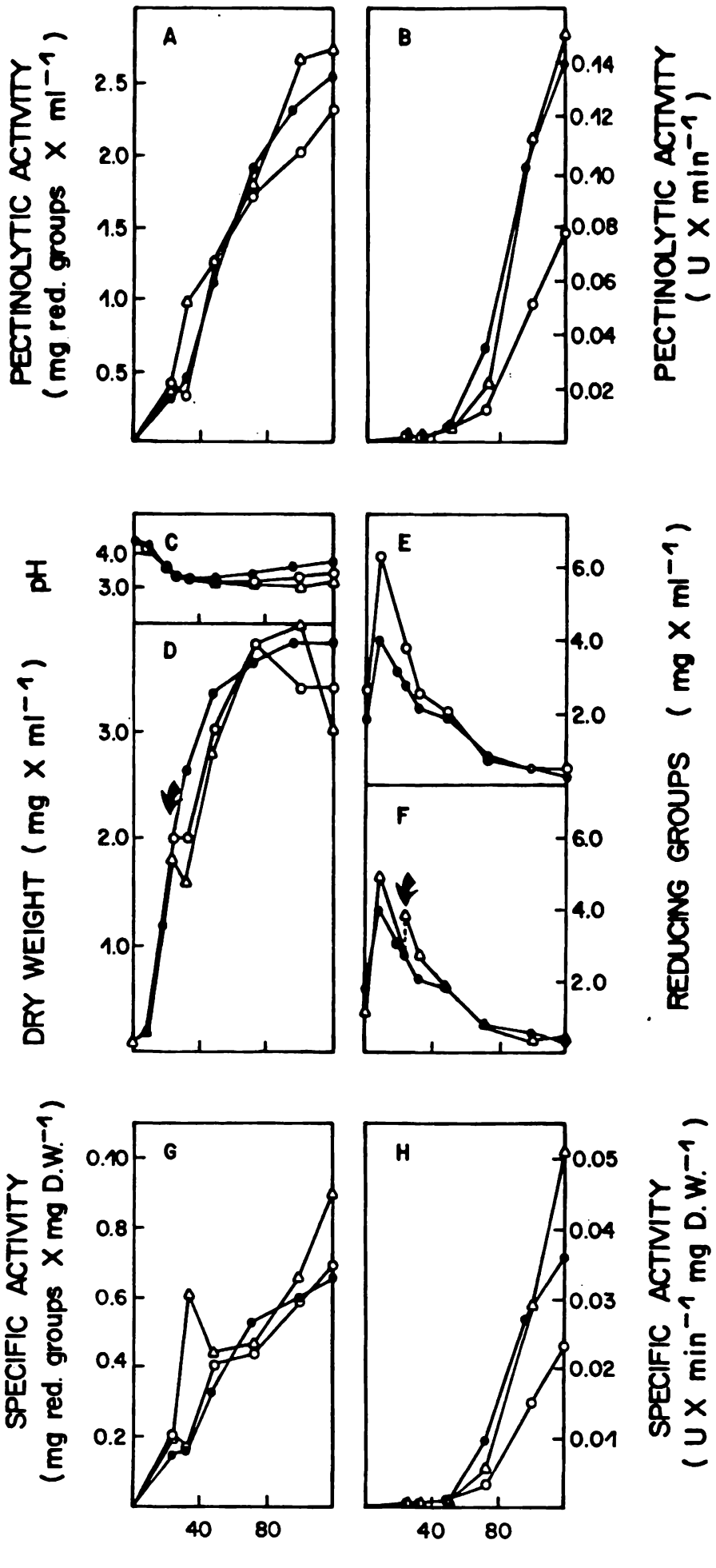

A
B
C
D
E
F
G
H
PECTINOLYTIC ACTIVITY (mg red. groups X ml⁻¹)
PECTINOLYTIC ACTIVITY (U X min⁻¹)
pH
DRY WEIGHT (mg X ml⁻¹)
REDUCING GROUPS (mg X ml⁻¹)
SPECIFIC ACTIVITY (mg red. groups X mg D.W.⁻¹)
SPECIFIC ACTIVITY (U X min⁻¹ mg D.W.⁻¹)
FERMENTATION TIME (hours)

FIGURE 2

Fig.2. Effect of the addition of glucose (1.0 mg ml^{-1}) to cultures of Aspergillus sp. growing on pectin. Extracellular pectinolytic activity (A,B), pH profile (C), cell growth (D), degradation products from pectin as reducing groups (E,F), and specific activity (G,H). Pectin, 1.0% without addition, as control (●); pectin, 1.0% plus a sole addition of G at 0 h (○) or 24 h (△). The arrows (⬍) shows the time of addition.

similar to that of the control, showing a decrease during the first 30 h of fermentation with the pH maintained at 3.0 to 3.3 (Fig. 2C).

During the first 24 h of fermentation, the growth of the cultures to which G had been added was similar to that of the control culture (Fig. 2D). Thereafter in the cultures with G similar to what occurred with the cultures containing GA a slight plateau was reached, however, at 72 h, the growth approached control levels.

The acumulation of reducing groups and their utilization during fermentation was essentially equal to that of the culture when GA had been added (Fig. 2E and 2F). When G had been added at t = 0, the accumulation of reducing groups was 6.4 mg ml^{-1} which was 2.4 mg ml^{-1} greater than the control value and almost equal to that obtained when GA had been added at t = 0.

The specific activity, measured by reducing groups was practically the same or slightly grater than that of the control when glucose had been added at t = 0 or at 24 h, respectively (Fig. 2C). However, this activity was less affected since when measured by viscosimetry, the activity was only 62.4% that of the control, when G had been added at t = 0 (Fig. 2H), whereas this activity was 141% that of control when the G had been added at 24 h (Fig. 2H). This apparent stimulatory effect of glucose on the production of pectinolytic activities by Aspergillus sp. especially as measured by viscosity, was slightly less than that observed upon the addition of GA at 24 h (Table I).

Effect of the Addition of High Concentration of Galacturonic Acid or Glucose on the Production of Pectinases.

To evaluate the effect of high concentration of **GA** or **G** on the production of pectinolytic activity by Aspergillus sp., experiments were carried out in which 10 ml of solution containing 100 mg ml^{-1} of either **GA** or **G** per millimeter were added to pectin culture containing (200 ml) to give a final concentration of 5.0 mg ml^{-1}. These carbon sources were added at 24 h to determine the effect of **GA** or **G** on cultures alrealy induced and to eliminate the increase in pH that results when such an addition is made at the start of fermentation.

With respect to the control,the activities measured by reducing groups was 63% for the addition of **GA** and 52% for that of **G** (Fig. 3A). The activity measured by viscosimetry were 73 and 30% for the addition of **GA** and **G**, respectively (Fig. 3B). While the pH profiles of the cultures to which **GA** or **G** had been added were similar and slightly lower than the pectin control (Fig. 3C).

Higher growth was obtained when **G** had been added; for that with **GA**, the growth was essentially equal to the control level (Fig 3D). It should be noted that, in this case although **GA** was added in high concentration, the growth obtained was equal to that of the control contrary to what happened when **G** had been (Fig. 3D).

The accumulation of reducing groups was similar for both cultures during

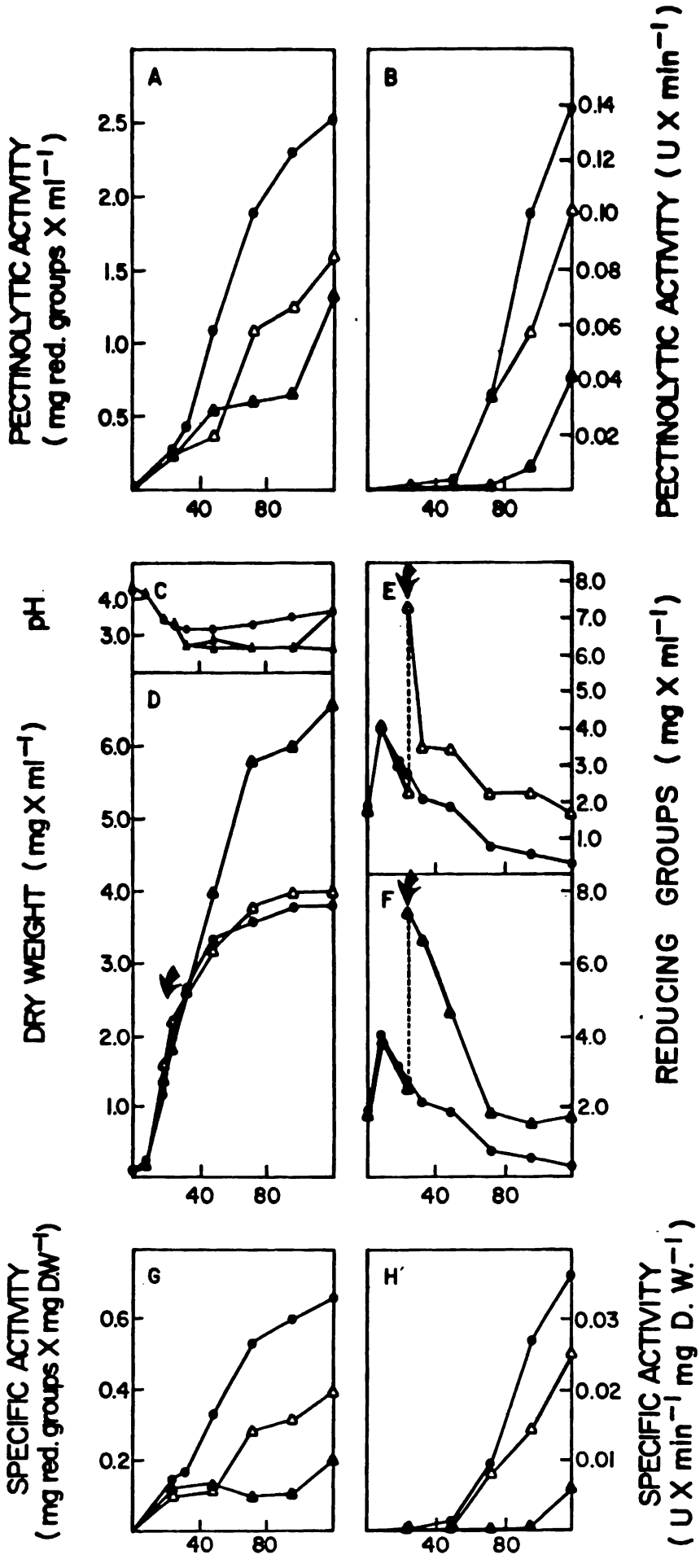

A
B
C
D
E
F
G
H'
PECTINOLYTIC ACTIVITY (mg red. groups X ml⁻¹)
PECTINOLYTIC ACTIVITY (U X min⁻¹)
pH
DRY WEIGHT (mg X ml⁻¹)
REDUCING GROUPS (mg X ml⁻¹)
SPECIFIC ACTIVITY (mg red. groups X mg DW⁻¹)
SPECIFIC ACTIVITY (U X min⁻¹ mg D. W.⁻¹)
FERMENTATION TIME (hours)

FIGURE 3

Fig.3. Effect of the addition of galacturonic acid or glucose at high concentration to cultures of Aspergillus sp. growing on pectin. Pectin 1.0% without addition as control (●), pectin, 1.0% plus a sole addition at 24 h, of GA, 5.0 mg x ml^{-1} (△) or G,5.0 mg x ml^{-1} (▲). The arrows (⇕) shows the time of addition. Other details as in Fig.2.

the first 24 h, at which time the respective additions were made. Thereafter the concentration of reducing groups decreased to a level of 1.6 mg ml^{-1} in these cultures; however the concentrations always remained greater than that in the control (Fig. 3E and 3H).

When the specific activities were calculated, it was found that, after the additions of **G**, the activity measured by reducing groups remaind constant until 96 h and then slightly increased at 120 h, at which time, the concentration of reducing groups was less than 2.0 mg ml^{-1}. The specific activity for reducing groups, in this case was 30% that of the control (Fig. 3G and Table I). When **GA** had been added the specific activity measured by reducing groups remained constant for 24 h after the addition; thereafter the activity increased at 120 h to 60% of the control value (Fig. 3G and Table I).

When **G** had been added, the specific activity measured by viscosimetry was detected at 96 h and at 120 h it reached 17.5% of that of the control. With **GA**, the specific activity was very similar to the control value during the first 72 h and reached a maximum level of 69% of the control activity at 120 h (Fig. 3H and Table I).

These results demonstrate that the pectinolytic activity produced by Aspergillus sp. was sensitive to catabolic repression by high concentrations (5 mg ml^{-1}), the activity measured by viscosimetry was more sensitive to this catabolic repression than was that measured by reducing groups (Fig. 3G and H, Table I).

When GA was added at this high concentration the effect of the repression was more evident for the activity measured by reducing groups than that viscosimetry. It is therefore possible that GA may be the inducer, in which case, the production of this activity that is sensitive to catabolic repression would depend on a delicate balance between induction and repression by this compound.

Effect of the Addition of Galacturonic Acid and Cycloheximide on the Production of Pectinases by Aspergillus sp.

Finally galacturonic acid and cycloheximide were added simultaneously in order to evaluate the effect on the synthesis of pectinolytic activities in Aspergillus sp. When these compounds were added simultaneously at 24 h, the activity which had been produced before their addition disappeared but reestablished itself after 96 h . The final activity reached in this culture was 28% of the control value (Fig. 4A).

When cycloheximide alone was added at t = 0, the production of activity measured by reducing groups, began after 72 h and reached a final value which was 52% that of the control; when added at 24 h, the activity produced during the first 24 h diminished but reestablished itself 72 h, the final activity was 59% of the control value (Fig. 4A).

When GA and cycloheximide had been added activity as measured by viscosimetry was detected at 96 h and was only 17% that of the control (Fig. 4B).

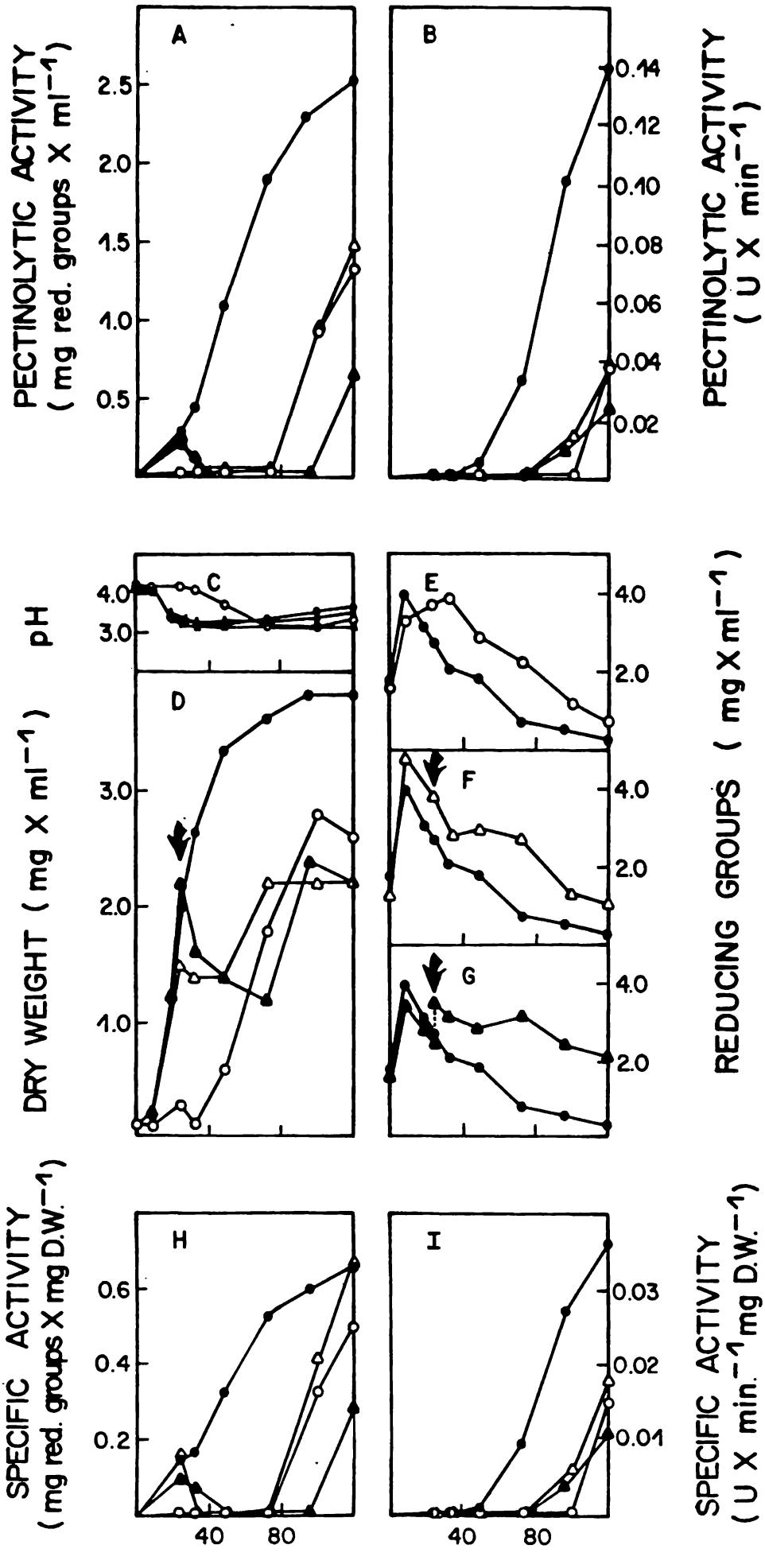

A
B
C
D
E
F
G
H
I
PECTINOLYTIC ACTIVITY (mg red. groups X ml^{-1})
PECTINOLYTIC ACTIVITY (U X min^{-1})
pH
DRY WEIGHT (mg X ml^{-1})
REDUCING GROUPS (mg X ml^{-1})
SPECIFIC ACTIVITY (mg red. groups X mg D.W.$^{-1}$)
SPECIFIC ACTIVITY (U X min.$^{-1}$ mg D.W.$^{-1}$)
FERMENTATION TIME (hours)

FIGURE 4

Fig.4. Effect of the addition of galacturonic acid and cycloheximide to cultures of Aspergillus sp. growing on pectin. Pectin,1.0% without addition as control (●), pectin, 1.0% plus a sole addition of cycloheximide at 0 h (○) or at 24 h (△) and pectin 1.0%, plus a sole addition of GA with cycloheximide at 24 h (▲). The final concentration of GA and cycloheximide were 1.0 mg x ml^{-1} and 125 µg x ml^{-1}, respectively. The arrows (↓) shows the time of addition. Other details as in Fig. 1.

This behaviour was very similar to that obtained when cycloheximide alone had been added at 24 h but the final activity was greater (29%), (Fig. 4B). When cycloheximide alone had been added at t = 0, the activity measured by viscosimetry was 28% and was produced only at 120 h of fermentation (Fig. 4B).

The pH profiles of these cultures were very similar to the control, with the exception of the culture in which cycloheximide had been added at t = 0. In that culture, the pH remained constant during the time in which the microorganism did not grow due to the effect of the cycloheximide and after 32 h, decreased to values similar to those of the control (Fig. 4C).

When cycloheximide had been added at t = 0, the growth of the culture was delayed 32 h but started to grow again after this time (Fig. 4D). When cycloheximide either with or without galacturonic acid, had been added at 24 h, the growth stopped upon addition and was reinitiated 48 h and 24 h later, respectively (Fig. 4D).

With respect to the reducing groups produced by degradation of pectin, it is interesting to note that when cycloheximide had been added at t = 0, the initial degradation of the pectin was not affected, suggesting the presence of preformed pectinolytic activity very probably associated with the cell (Fig. 4E). In the other two cases, the addition of cycloheximide stopped the consumption of the reducing groups; consumption was reinitiated when growth was reestablished (Fig. 4F and 4G).

The specific activity measured by reducing groups, when cycloheximide had been added at 24 h was practically equal to the control value (Fig. 4H; Table I). When the addition was done at t = 0, the specific activity was 76% that of the control; when cycloheximide and galacturonic acid had been added, the specific activity was only 44% of the control value (Fig. 4H; Table I). In no case did the activities measured viscosimetry reach control levels; the specific activities obtained were 42% when cycloheximide had been added at t = 0, 50% when added at 24 h, and 31% when cycloheximide and galacturonic acid had been added simultaneously (Fig. 4I; Table I).

CONCLUSIONS

The results presented in this article showed that the pectinolytic activity, measured by reducing groups and by viscosimetry, which was produced by Aspergillus sp. was sensitive to catabolic repression by pectin degradation products and by high concentrations of exogenous glucose and galacturonic acid. However, the effect of the catabolic repression was more pronounced for the activity measured by viscosimetry than for that measured by reducing groups.

In other hand, the proposed presence of a preformed enzymatic activity which was associated with the cells be responsible of accumulation of reducing groups was supported be the fact that these reducing groups accumulated in all the cases in which pectin was used as carbon source, even in the case in which cycloheximide was added at t = 0. The accumulation

of these products was almost equal to that of the control culture to which cycloheximide had not been added. Our results also suggested that this activity was not inducible. Data from preliminary experiments in which activity in whole cells was measured provided further support (data not shown).

In a previous study it was not possible to identify the product which specifically responsible for producing the catabolite repression (11). However the results presented here suggested that the catabolic repression produced by the pectin degradation products depended on the concentration of galacturonic acid liberated by the degradation of pectin. Since when galacturonic acid had been added successively, the production of activity was reduced, confirming its role as represor. Catabolic repression was also observed when glucose had been added at t = 0 but only in that activity measured by viscosimetry. The apparent increase in activity when glucose was added at 24 h was probably due to increased use of the glucose as energy source than of either pectin or galacturonic acid. The results also suggested that galacturonic acid very probably acts as an inducer of these enzymes and that its role as repressor and or inducer was directly related to its concentration in the culture medium because, when galacturonic acid, was added at 24 h at low concentrations the production of pectinases increased 30% and 147%, the activity being measured by reducing groups or by viscosimetry respectively. Because addition of high concentrations (5.0 mg ml^{-1}) of glucose or galacturonic acid repressed the production of pectinases, we think that the production of these enzymes depended on a delicate balance between induction and

repression as a function of the concentration of galacturonic acid. We also observed. That the galacturonic acid affected the de novo synthesis of pectinases. When galacturonic acid and cycloheximide had been added simultaneously to the culture, the synthesis of the activity measured by reducing groups was delayed 24 h in relation to that obtained in the culture to which cycloheximide alone had been added at 24 h, thereby demonstrating the catabolic repression by galacturonic acid. In contrast, the production of activity measured by viscosimetry was initiated at the same time independent of whether the cycloheximide had been added with or without galacturonic acid which supported the idea that the galacturonic acid may be involved in the induction of the pectinases.

The authors wish to thank the Organization of American States (OAS) for its partial support of these studies, and Drs. Susana Saval and Ma. Elena Flores for useful discussions and Aurora Hernandez for secretarial assistance.

REFERENCES

1. Rombouts,F.M., and Pilnik,W., in Economic Microbiology, Microbial Enzymes and Bioconversions, A.H. Rose, Ed., Academic Press, New York, 1980, Vol. 5, pp. 227-282.
2. Fogarty,W.M. and Kelly, C.T., in Microbial Enzymes and Biotechnology, W.M. Fogarty, Ed., Applied Science Publishers, London and New York, 1983, pp. 131-182.
3. Kilara, A., Process Biochem. 1982, 17 (4), 35-41.
4. Nyiri,L., Process Biochem. 1968, 3. 27-30.
5. Beckhorn, E.J., Labbee, M.D. and Underkofler, L.A., J. of Agr. Food Chem. 1965, 13,30-36.
6. Saval,S., Solorzano, R., Alpizar, L., Cea, A., and Huitrón, C., in Use of Enzymes in Food Technology, P. Dupuy, Ed. Technique et Documentation Lavoisier, France, 1982, pp. 531-535.
7. Saval, S. and Huitrón, C. Developments in Industrial Microbiology. 1983, 24, 547-551.
8. Saval, S., M. Sc. Thesis 1985, National University of Mexico,Mexico.
9. Huitrón, C., Saval, S., and Acuña, M.E., Annals of the New York, Academy of Sciences, Ed. A.I. Laskin, G.T. TSAO and L.B. WINGARD,JR. 1984, 434,110-114.
10. Saval, S., Solorzano, R., Alpizar, L., Cea, A. and Huitrón, C., in Biotecnologia de Enzimas, C. Huitrón, Ed., UNAM-OEA-CONACYT, Mexico, 1985, pp. 203-215.
11. Aguilar, G., and Huitrón, C., Enz. Microb. Technol, 1986,(8),541-545.
12. Perley, A.F., and Page, O.T., Can J. of Microbiol. 1971, 17,415-420.
13. Cooper, R.M., and Wood, R.K.S., Nature. 1973, 246, 309-311.

14. Shinmyo,A.,Davies,I.K.,Nomoto,F.,Tahara,T.,and Enatsu,T., Eur. J. of Appl. Microbiol. 1978, 5, 59-68.

15. Tahara,T., Kotani,H., Shinmyo,A., and Enatsu,T.,J. of Ferment. Technol. 1975, 53 (6), 409-412.

16. Keen,N.T., and Horton,J.C., Can. J. of Microbiol. 1966. 12,443-453.

17. Ward,O.P., and Fogarty,W.M., Appl. Microbiol. 1974, 17,346-354.

18. Lobanok,A.G., Mikhailova, R.V., and Sapunova, L.I., Mikrobiologiya. 1977, 46, 747.

19. Phaff,H.J., Arch. Biochem. 1974, 13,67-81.

20. Miller, L. Anal. Chem. 1959, 31, 426-428.

DISCUSION GENERAL

Los resultados presentados en este trabajo demuestran que la utilización del cultivo alimentado tiene ventajas en relación al cultivo en lote, ya que, con el adecuado manejo de las condiciones de limitación, se logró incrementar la producción de las pectinasas producidas por **Aspergillus** sp. Otra ventaja de este cultivo es que se logra reducir de manera muy importante la cantidad de sustrato utilizado, con lo que se optimiza substancialmente su consumo. Cuando se limitó con pectina, se agregó, después de 120 horas de fermentación, un total de 4.2 g de este sustrato que representa un 42.0% en relación al cultivo en lote al cual se agregaron 10 g totales, de pectina. En el caso de la limitación con sulfato de amonio se agregaron 0.16 g totales después de 120 horas, apenas un 8.0% de lo que se agrega en lote (2.0 g).

Los incrementos logrados en la producción de pectinasas, medidas por grupos reductores, fueron del 249% cuando se limito con pectina y del 455% cuando se limito con pectina y sulfato de amonio simultaneamente. Para la actividad medida por viscosimetría solo se obtuvo un 32%, en relacion a control y con la doble limitación solo se logra restituir los niveles obtenidos en el control. Estos resultados

sugieren que, si bien la actividad medida por grupos reductores se reprime por altas concentraciones de grupos reductores que se acumulan, en condiciones de limitación de nitrógeno y en el cultivo en lote, y se desreprime a bajas concentraciones de estos productos, como en el cultivo limitado de pectina y en el doble limitado, la actividad medida por viscosimetría, aunque responde a un patrón similar, parece estar relacionada a una limitación de inductor causada por la limitación de la pectina.

Como se demuestra en este trabajo la, producción de pectinasas de **Aspergillus** sp. está sujeta a represión catabólica por los productos de degradación de la pectina. Sin embargo, la actividad medida por viscosimetría parece más sensible a la represión que la medida por grupos reductores, lo que sugiere que su producción depende de un delicado balance entre inducción-represión, estrechamente relacionada a la concentración de estos grupos, y muy probablemente de ácido galacturónico. Esto se desprende de la observación de que en condiciones donde la pectina es limitante,condición en que se reduce o elimina la represión catabólica, tambien se limita la concentración de inductor con la consecuente disminución en la producción de la actividad medida por viscosimetría.

Esto se ve apoyado por el hecho de que cuando el ácido galacturónico es adicionado a bajas concentraciones (1.0

mg/ml) a cultivos en lote de **Aspergillus** sp., creciendo en pectina, la producción de las actividades se estimula en un 30% y en un 147% para la actividad medida por grupos reductores y viscosimetría, respectivamente. Con la adición de ácido galacturónico en altas concentraciones (5.0 mg/ml), se presenta represión catabólica para ambas actividades.

Estos resultados apuntan a que el ácido galacturónico tiene un doble papel como inductor y represor de las pectinasas producidas por **Aspergillus** sp., dependiendo de su concentración en el medio de cultivo y de la velocidad con que éste sea liberado durante la degradación de la pectina.

Por otro lado, con la adición de glucosa se observa represión catabólica tanto en bajas como en altas concentraciones, con excepción de la adición de glucosa a las 24 h, a baja concentración, donde se observa una aparente estimulación, probablemente debida a un mejor aprovechamiento de este sustrato como fuente de energía, aunque también existen la posibilidad de que parte de la actividad pectinolítica de este microorganismos pudiera ser constitutiva.

Finalmente, nuestros resultados sugieren que la acumulación de grupos reductores durante las primeras horas de fermentación, parece ser debida a una actividad

pectinolítica asociada a la célula más que a una actividad pectinolítica extracelular. Esto sugiere que la actividad responsable de la degradación inicial de la pectina sea una actividad preformada, que está presente en la superficie externa de las esporas, ya que su efecto se manifiesta aún en presencia de cicloheximida desde el inicio de la fermentación, y que esta actividad sea la responsable de proporcionar los inductores necesarios para la producción de las actividades pectinolíticas extracelulares en **Aspergillus** sp.

CONCLUSIONES

Con este trabajo se ha podido demostrar que las pectinasas producidas por **Aspergillus** sp.,son sensibles a la represión catabólica ejercida por los productos de la degradación de la pectina,y por altas concentraciones de glucosa y ácido galacturónico. Asímismo, se ha demostrado la utilidad del cultivo alimentado no solo para aumentar la productividad de este microorganismo, sino también para el estudio de algunos de los factores que afectan la síntesis de pectinasas en **Aspergillus** sp., además se logró un mejor aprovechamiento de los nutrientes en el medio de cultivo.

Por otro lado, se ha demostrado que la recirculación del medio de cultivo favorece la producción de pectinasas y que la dilución causada por la adición continua de nutrientes no tiene efecto ya que se obtienen prácticamente los mismos resultados que en cultivo en lote, sin dilución.

Asimismo, se ha determinado el papel del ácido galacturónico como represor de la actividad pectinolítica de **Aspergillus** sp., y aunque los resultados presentados aquí apoyan que este compuesto también participa como inductor de las pectinasas en **Aspergillus** sp., no son concluyentes en este sentido. Sin embargo, otros

experimentos realizados creciendo al microorganismo en condiciones de limitación de ácido galacturónico como única fuente de carbono, apuntan en esa dirección (datos no mostrados).

Por otro lado, se ha demostrado que **Aspergillus** sp. tiene grandes posibilidades de ser utilizado en la producción industrial de pectinasas, tanto en fermentación en lote como en cultivo alimentado. Este último, además de ser susceptible de implementarse a nivel industrial, puede facilitar las operaciones de recuperación, ya que al controlarse la velocidad de crecimiento, se puede disminuir y mantener en el nivel deseado la masa celular sin afectar la producción, con lo que se logra reducir algunos de los problemas como el taponamiento de filtros, ocasionados por el abundante crecimiento microbiano normalmente obtenido en las fermentaciones en lote. Asi mismo , se puede reducir la viscosidad de los caldos de fermentación lo que representa menor gasto de energía, y mejores coeficientes de transferencia de masa.

Si bien este trabajo ha permitido alcanzar un mayor conocimiento de algunos de los factores que afectan la síntesis de pectinasas en **Aspergillus** sp., así como aumentar su productividad con el cultivo alimentado, conviene destacar que este trabajo forma parte

de una linea de investigación más amplia en la que se han abordado diversos aspectos de la producción de estas enzimas, con diferentes estrategias y puntos de vista y que mediante la integración de todos los conocimientos acumulados se podrá lograr su aplicación práctica.

REFERENCIAS

1) Kilara, A. **Proc. Biochem.**1982,**17**(4), 35-41.

2) Whitaker, J. R. **Enzyme Microb. Technol.**1984,**6**,341-349.

3) Fogarty, W.M. y Ward, O.P. **Progress in Industrial Microbiology.**1974,**13**,59-119.

4) Fogarty. W.M. y Kelly, C.T., en: **Microbial Enzymes and Biotechnology**,W.M. Fogarty, Ed. Applied Science Publishers, London and New York, 1983, pp. 131-182.

5) Grampp, E.G. **Proc. Biochem.**1981, April/May: 24-27.

6) Pilnik, W. y Rombouts, F.M., en: **Enzymes and Food Processing.** Applied Science Publishers, LTD. 1981, Cap.6, pp. 105-128.

7) Nyiri,L. **Proc.Biochem.**1968,(8), 27-30.

8) Rombouts, F.M. y Pilnik, W. **Proc.Biochem**, 1978, **13**(8), 9-13.

9) Beckhorn, E.J., Labbee,M.D. y Underkofler, L.A. **J. Agr. Food Chem.** 1965,**13**, 30.

10) Tsuyumu, S. **Nature.** 1977,**269**, 237-238.

11) Biryucov, V.V., Gadzhieva, L.A. y Shersonova, M.V. **Prikladnaya Biokhimiya i Mickobiologiya.**1980,**16** (6), 876-882.

12) Rexová-Bencová,L. y Marcovik,D.Adv. en: **Carbohydrate Chemistry and Biochemistry.**1976,**33**, 323-385.

13) Rombouts, F.M. y Pilnik, W. en: **Economic Microbiology, Microbial Enzymes and Bioconversions**, A.H. Rose, Ed., Academic Press, New York. 1980, Vol. 5. pp. 227-282.

14) Kertezs, I. **Methods in Enzymol**. 1955,1,158-166.

15) Kurowski,W. H. y Dunleavy, J.A. **Eur. J. of Appl. Microbiol**. 1976,2:103-112.

16) Moran, F., Nasuno, S. y Starr, M.P. **Arch. Biochem. Biophys**. 1968,123:298.

17) Hsu, E.J. y Vaughn, R.H. **J. Bacteriol**, 1969,98, 172-181.

18) Perley, A.F. y Page, O.T. **Can. J. Microbiol**.1971,17, 415-420.

19) Shinmyo, A., Davis, I. K., Nomoto, F., Tahara, T. y Enatsu, T. **Eur. J. Appl. Microbiol**.1978,5, 59-68.

20) Keen, N. T. y Horton, J. **C. Can. J. Microbiol**.1966, **12**, 443-453.

21) Tahara,T.,Kotani,H. Shinmyo,A., y Enatsu, T. **J. Ferment. Technol**.1975,53(6), 409-412.

22) Ward, O.P. y Fogarty, W.M. **Appl. Microbiol**.1974,17, 346-354.

23) Lobanok, A.G., Mikhailova, R.V. y Sapunova, L.I. **Mikrobiologiya**.1977,46, 747.

24) Phaff, H. J. **Arch. Biochem**.1947,13, 67-81.

25) Saval,S.,Solórzano,R., Alpizar,L., Cea,A. y Huitrón,C. en: **Biotecnología de Enzimas**, C. Huitrón, Ed. UNAM-OEA-CONACYT,México,1985, pp. 203-215.

26) Saval,S. **Tesis de Maestria en Investigación Biomédica Basica**, UACPyP del CCH, Universidad Nacional Autónoma de México, 1985.

27) Saval,S.,Solórzano,R., Alpizar,L.,Cea,A. y Huitrón,C. **Proc. Int. Symp. on the Use of Enzymes in Food Technology**, Versailles, France, 1982, pp. 531-535.

28) Saval,S. y Huitrón, C. **Dev. Ind. Microbiol**.1983, **24**, 547-551.

29) Huitrón,C.,Saval,S.y Acuña,M.E. **Annals of the New York Academy of Sciences**, A.I. Laskin,G.T. Tsao y L.B. Wingard Jr. Eds. 1984,**434**,110-114.

30) Pirt,S.J. **Annals of the New York Academy of Sciences**,1979,**326**,119-125.

31) Hulme,M.A. and Stranks,D.W. **Nature**.1970,**226**, 469-470.

32) Gottvaldova,M.,Kucera,J. and Podrazky,V. **Biotechnol.Lett**.1982,**4**,229-232.

33) Markkanen,P.,Reinwall,A. and Linko,M.J. **Appl. Chem. Biotechnol**.1976,**26**,41-46.

34) Hulme,M.A. and Stranks,D.W. **J. Gen.Microbiol**.1971, **69**, 145-155.

35) Cooper,R.M. and Wood,R.K.S. **Nature**1973,**246**,309-311.

36) Matsumura,M.,Imanaka,T.,Yoshida,T. and Taguchi,H. **J.Ferment.Technol**.1981,**59**,115-123.

37) Limón-Lason, J.,Lara,M., Resendiz,B. and Mora,J. **Biochem.Biophys.Res.Commun**.1977,**78**,1234-1240.

38) Ellwood,D.C. y Tempest,D.W. **Adv. in Microb. Physiol.**1972,7,83-117.

39) Wang,D.C.I.,Cooney,C.L.,Demain,A.L.,Dunnill,P.,Humprhey, A.E. y Lilly,M.D. en: **Fermentation and Enzyme Technology.** John Willey and Sons.1979.

40) Bull,A.T. en: **Companion to Biochemistry**,A.T.Bull, J.R. Lagnado, K.F. Tripton y J.O. Thomas,Eds. Logman-London 1974.

41) Yamane,T. y Shimizu,S. **Advances in Biochem. Eng./Biotech.**1984,**30**,147-194.

42) Miller,L. **Annal.Chem.** 1959,**31**,426-428.