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ESTUDIOS SOBRE EL SISTEMA PECTINOLITICO DE *Aspergillus* sp. CH-Y-1043.

TESIS
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CAPITULO 1

Introducción General

RESUMEN

Aspergillus sp. CH-Y-1043 produce endo- y exo-pectinasas cuando crece sobre pectinas de diferentes grados de esterificación a valores de pH tan bajos como 2.5 y más aún a este valor de pH se alcanza la máxima producción de ambas enzimas a pesar de que el crecimiento a penas alcanza un 50% del obtenido a valores de pH mayores. La endo-pectinasa se produce a valores de pH de 2.5 a 3.5 cuando *Aspergillus* sp. CH-Y-1043 crece en pectina de bajo grado de esterificación mientras que con pectinas de alto grado de esterificación esta enzima se produce en todos los valores de pH analizados. La máxima producción de la exo-pectinasa se alcanza también a valores de pH de 2.5 a 3.5. Encontramos que esta enzima se encuentra asociada a la célula y puede ser liberada por incubación a diferentes valores de pH mientras que la endo-pectinasa no fue detectada en la fracción celular. Los resultados anteriores fueron confirmados por análisis electroforético (SDS-PAGE) acoplado a ensayos de actividad "in situ" con geles de agarosa-pectina, lo que permitió identificar una banda de proteína que corresponde a la endo-pectinasa y una banda más con actividad de pectin esterasa. También se evaluó la estabilidad de las pectinasas producidas por *Aspergillus* sp. CH-Y-1043 a diferente valores de pH.
Por otro lado, encontramos que la exo-pectinasa se produce en forma constitutiva cuando *Aspergillus* sp. crece en glucosa, sacarosa, fructosa, glicerol y ácido galacturónico. La actividad específica producida en estas fuentes de carbono está en el rango del 26% al 75% en relación a la producida en pectina o ácido poligalacturónico. La producción de esta enzima esta estrechamente relacionada con el crecimiento.
Finalmente, encontramos que las conidias intactas de *Aspergillus* sp. CH-Y-1043 son capases de degradar pectina "in vitro" aún cuando se inhibe la síntesis de proteína, indicando la presencia de pectinasa unidas a la célula de este microorganismo. Por lo menos, la exo-pectinasa fue encontrada en las conidias y en el micelio de *Aspergillus* sp. Su presencia no depende de la fuente de carbono utilizada para el crecimiento lo cual sugiere que es producida en forma constitutiva. Esta enzima puede ser liberada de las conidias y del micelio por incubación a diferentes valores de pH y la cantidad de enzima liberada puede ser aumentada por tratamiento con agentes químicos o enzimas líticas de pared celular. Resultados de análisis por "Western blot" de filtrados libres de células así como de extractos de conidias y micelio utilizando anticuerpos dirigidos contra conidias intactas de *Aspergillus* sp. CH-Y-1043 sugieren fuertemente que la exo- pectinasa detectada en las conidias y el micelio de este microorganismo es la misma que la exo-pectinasa constitutiva que se produce en ausencia de pectina.

SUMMARY

Aspergillus sp. CH-Y-1043 grown on pectins with various degrees of esterification produces endo- and exo-pectinases at pH values as low as 2.5. Maximal production was attained at this pH, although fungal growth only approximated 50% of that obtained at higher pH values. Endo-pectinase was produced at pH 2.5-3.5 when the fungus was grown on low degree esterified pectin. With higher degree esterified pectin this enzyme was produced at all pH values analyzed. Exo-pectinase production was less affected by pH values. Still, maximal production was also reached at pH 2.5-3.5. Exo-pectinase was found to be associated to the cell and could be released after incubation at different pH values, whereas endo-pectinase was not detected in the cellular fraction. Results confirmed by SDS-PAGE coupled to "in situ" activity assays in pectin-agarose gels allowed the identification of a protein band corresponding to endo-pectinase and a band with pectin esterase activity. Stability of Aspergillus sp. CH-Y-1043 pectinases at various pH values was also evaluated.

On the other hand, the exo-pectinase was found to be produced constitutively when *Aspergillus* sp. CH-Y-1043 grown on glucose, sucrose, fructose, glycerol and galacturonic acid. The specific activity was found to be in the range of 26% to 75% of that produced with pectin or polygalacturonic acid. The production of this exo-pectinase is strictly correlated to the exponential growth phase and it is highly sensitive to the pH of culture medium.

Finally, we found that intact conidia of *Aspergillus* sp. CH-Y 1043 were able to degrade pectin "in vitro" even when protein synthesis was inhibited, thus indicating the presence of cell bound pectinases. At least an exo-pectinase was found and this enzyme was also present in the mycelium of *Aspegillus* sp. Its presence was not dependent of the carbon source used for growth, suggesting its contitutive nature. This exo-pectinase could be released from conidia and mycelium by incubation at different pH values and the amount of enzyme released could be increased by treatments with chemical agents and hydrolytic enzymes. Results of western blot analyzes of culture filtrates and extract obtained from conidia and mycelium reacted with antibodies raised toward intact conidia of *Aspergillus* sp. CH-Y-1043 strongly suggest that the exo-pectinase detected in the conidia and mycelium of this fungus is the same that the constitutive exo-pectinase founded when the fungus grown in the absence of pectic substances.

INTRODUCCION GENERAL

Entre los polímeros naturales, los polisacáridos constituyen el material más abundante producido por la naturaleza, por lo cual aquellos procesos tendientes a su bioconversión enzimática y/o microbiana así como a la utilización más eficiente de estos materiales resultan de importancia en la investigación biotecnológica. La enorme variabilidad en composición y estructura de estos polisacáridos naturales hace que su bioconversión requiera no solo de una enzima sino de sistemas complejos de diferentes enzimas degradativas[1]. Muchos microorganismos, pero particularmente los hongos pueden crecer a partir de desechos de plantas y producir una amplia variedad de polisacarasas extracelulares con actividad celulolítica, pectinolítica y hemicelulolítica, entre otras [2-4].

La investigación sobre el aprovechamiento de subproductos de cultivos agrícolas ha estado orientada principalmente a la utilización de la celulosa y hemicelulosa presentes en estos materiales [1-2]. Sin embargo, existen subproductos tales como las pulpas de henequén, remolacha y cáscara de limón o naranja que además de la celulosa y hemicelulosa, también contienen apreciables cantidades de pectina y pueden ser utilizados en la producción de enzimas pectinolíticas extracelulares y biomasa microbiana [5-6], como actualmente se hace con la pulpa de remolacha y de manzana así como para la extracción de la pectina que contienen.

Las pectinasas son un grupo de por lo menos doce diferentes enzimas ampliamente distribuidas en la naturaleza, normalmente presentes en frutas y vegetales, así como en bacterias, levaduras y hongos, capases de degradar substancias pécticas [7-8]. Sin 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 estas 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 además, por su característica de excretar las enzimas al medio de cultivo [8-10].

Las pectinasas también juegan un papel importante en la degradación de la pared celular de plantas durante la patogénesis por microorganismos y están involucradas con el desarrollo, maduración y extensión de la pared celular de frutas y vegetales [1,11].

Casi todas las pectinasas extracelulares son sintetizadas en respuesta a la presencia de substancias pécticas, las cuales son generalmente insolubles o incapaces de ser transportadas al interior de la célula 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* [12].

Debido a la naturaleza inducible de la mayoría de las enzimas pectinolíticas, su producción está estrechamente ligada al substrato utilizado como inductor [13].

Aunque todos los inductores conocidos de las pectinasas son urónidos, productos derivados de la pectina o del ácido poligalacturónico, dependiendo de la concentración y grado de esterificación de la molécula presente como inductor, el microorganismo puede producir ciertas enzimas pectinolíticas preferencialmente sobre otras [3,10,12].

Substancias pécticas

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 [2]. Según la nomenclatura de la American Chemical Society [8], las substancias pécticas son carbohidratos complejos de propiedades coloidales formadas principalmente por unidades de ácido anhidrogalacturónico enlazadas en posición α-1,4, cuyos

grupos carboxilo pueden estar esterificados parcial o totalmente con grupos metilo, o neutralizados por una o más bases. Dentro de las substancias pécticas se encuentran: a) La protopectina, precursor insoluble de la pectina; b) los ácidos pectínicos, que son ácidos poligalacturónicos coloidales parcialmente esterificados; c) la pectina, que es ácido poligalacturónico soluble en agua con grado de esterificación y neutralización variables, pero mayores a los ácidos pectínicos, y d) los ácidos pécticos, que son ácidos poligalacturónicos coloidales prácticamente libres de ésteres [2-4]. 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 [4]. El tipo y cantidad de éstos así como el grado de esterificación dependen básicamente del origen de las substancias pécticas, las cuales se encuentran originalmente en forma de protopectina, cuya síntesis ocurre en las etapas tempranas de crecimiento, cuando el área de la pared celular aumenta[2]. 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 influida por el tipo y cantidad de pectina presente.

Enzimas pécticas

Las enzimas que actúan sobre las substancias pécticas pueden ser divididas en dos grandes grupos [4,9,10]; las enzimas despolimerizantes y las saponificantes (Fig. 1). Las enzimas despolimerizantes pueden dividirse a su vez en relación a su mecanismo de acción (hidrólisis o transeliminación), al substrato sobre el que prefencialmente actúan (pectina o ácido poligalacturónico) y de acuerdo al sitio de iniciación de la ruptura del polímero (tipo exo y endo).

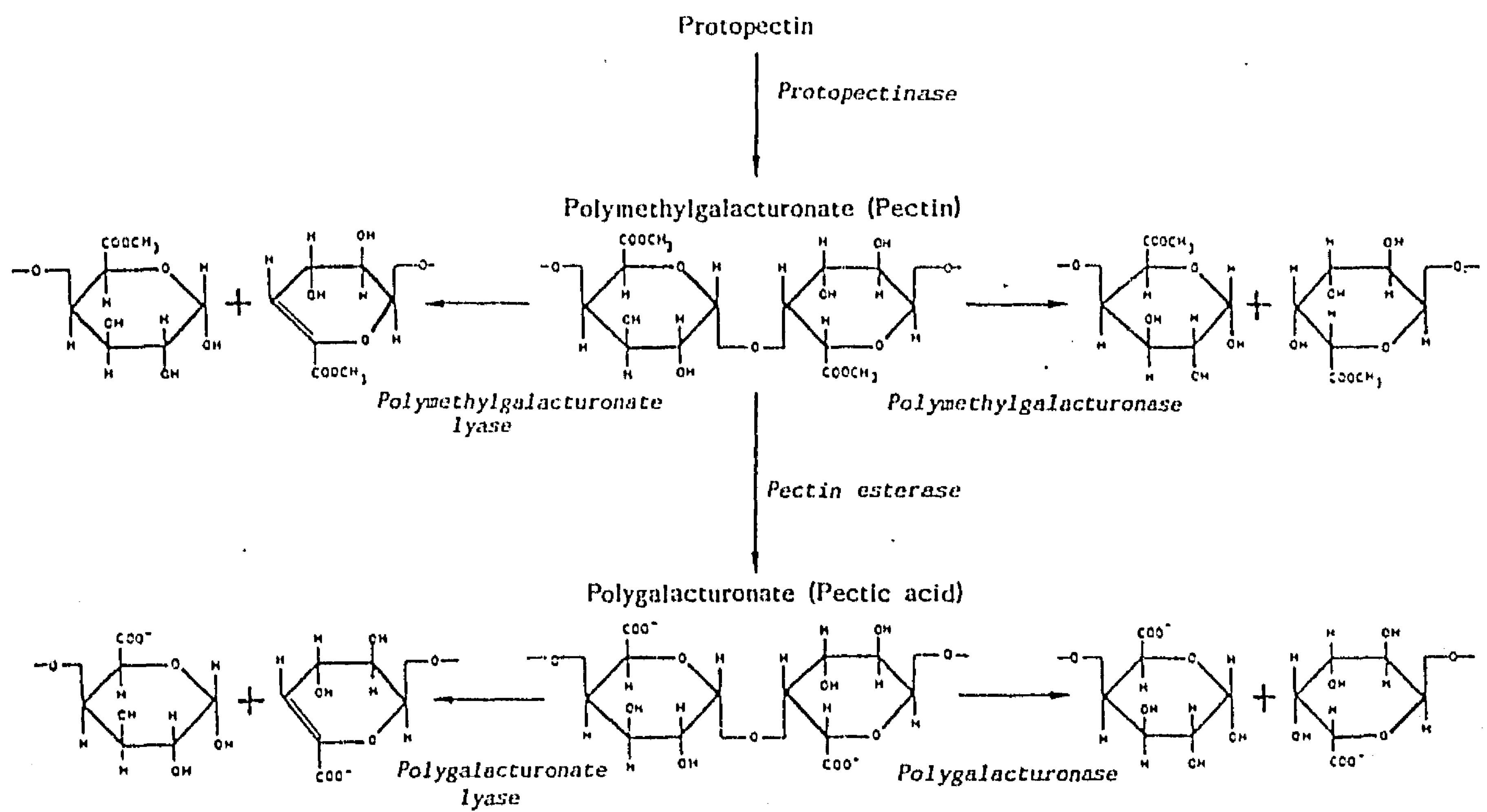

Fig.1. Mecanismo de acción de las enzimas pectinolíticas

Actualmente se han encontrado en diversos organismos, doce diferentes actividades pectinolíticas. 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 [9]. Las despolimerasas rompen los enlaces glucosídicos α-1,4 del substrato de preferencia por hidrólisis (hidrolasas) o por β-eliminación (liasas). El rompimiento hidrolítico puede iniciarse por el extremo de la cadena de substrato (enzimas de tipo exo), rindiendo productos de bajo peso molecular, principalmente monómeros pero conservando una proporción importante del polisacárido de alto peso molecular por lo que la viscosidad es poco afectada. 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 con una cosecuente y rápida disminución de la viscosidad [14]. El ataque por β-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 son producidas por hongos y no han sido encontradas en bacterias ni plantas superiores. Las pectato-liasas (endo y exo) son enzimas típicas 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 encuentran en plantas, hongos y algunas bacterias. Por último, la exo-pectin liasas no han sido encontradas en la naturaleza [7]. 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 substrato 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 [3,7].

Las pectinasas tienen un creciente número de aplicaciones en la industria de alimentos. Su utilización es esencial para el procesamiento industrial de frutas y vegetales, ya que al degradar las substancias pécticas no solo aumentan los rendimientos de extracción, sino también se reducen los tiempos de filtración de los jugos [15]. Asimismo, son de gran utilidad en la elaboración y clarificación de vinos y jugos y en la extracción de colorantes vegetales. Estas enzimas también son utilizadas para reducir la viscosidad en concentrados de frutas y para modificar y solubilizar las substancias pécticas que dificultan la sedimentación de partículas indeseables en productos derivados de frutas [3,7].

La producción de pectinasas en muchos microorganismos está limitada por mecanismos que regulan su síntesis y por factores ambientales. Debido a que la mayoría de estas enzimas son inducidas por la presencia de pectina y están sujetas a represión catabólica esta misma fuente de carbono [16-19], la utilización del cultivo continuo o del cultivo alimentado puede tener ventajas sobre el cultivo en lote ya que con ellos se puede eliminar o por lo menos reducir los problemas de represión catabólica ocasionados por altas concentraciones de la fuente de carbono o por la acumulación de los productos de su degradación. Por ejemplo, el cultivo alimentado ha sido exitosamente utilizado para incrementar los rendimientos de celulasas de *Myrothecium verrucaria* [20] y *Trichoderma viride* [21], así como para la producción de β-glucanasa de *Bacillus subtilis* [22]. Además existen otras posibilidades de aplicación, como es el caso de la producción de pectinasas de *Aspergillus* sp. CH-Y-1043 [23].

En virtud de que las substancias pécticas son polisacáridos de elevado peso molecular que difícilmente puede entrar a la célula, surge la pregunta de como se lleva a cabo la inducción

o de que manera los microorganismos pueden detectar la presencia de estos materiales en el medio ambiente externo. Se ha sugerido que algunos microorganismos producen niveles basales de pectinasas constitutivas que degradan el substrato polimérico y que los productos de bajo peso molecular de esta reacción sirven como inductores y fuentes de energía para promover el crecimiento e inducir la producción de otras pectinasas [11]. Esto es cierto para algunos microorganismos particularmente bacterias y un número reducido de hongos [11, 12, 24]. La síntesis de endo-poligalacturonato liasa es constitutiva en *E. carotovora* y *E. aroidae*, y debido a la velocidad diferencial de síntesis en glucosa, glicerol y pectato de sodio, se ha concluido que esta enzima está sujeta a represión catabólica. *E. carotovora, E. chrysanthemi* y *E. aroidae*, secretan una parte de la poligalacturonato-liasa que producen al medio de cultivo, y otra parte permanece asociada a la célula [19]. Así mismo, se ha descrito que la síntesis de endopoligalacturonato-liasa extracelular de *Aeromonas liquefaciens*, es constitutiva y sensible a la represión catabólica [19].

En hongos se sabe que *Pirenochaeta terrestris* produce pequeñas cantidades de endo poligalacturonasas constitutivas [25]. Asimismo, en *Aspergillus saitoi* [26] y *Botrytis cinerea* [11,24] se ha reportado la producción constitutiva de poligalacturonasas.

La regulación de la síntesis de estas enzimas ha sido más estudiada en bacterias que en hongos. Sin embargo, existen trabajos reportados realizados en hongos. Así, encontramos que la poligalacturonasa de *Aspergillus niger* está sujeta a represión catabólica por glucosa a nivel de transcripción y traducción [4,16]. Asimismo, se sabe que la síntesis de endopoligalacturonasa de *Pyrenochaeta terrestris* es reprimida por la adición de glucosa u otras hexosas a concentraciones de 0.05 M pero es estimulada a concentraciones de 0.005 M cuando el microorganismo es crecido en medios conteniendo pectina [17]. En la mayoría de los hongos se desconoce cual es el inductor directo de la síntesis de pectinasas pero se ha encontrado que

el ácido galacturónico induce la síntesis de endo-poligalacturonasa y endo-pectin-liasa en *Verticillium albo-atrium* [14] y que el D-galactarato (ácido múcico) parece ser el único compuesto estimulador de la síntesis de pectin esterasa y poligalacturonasa en *P. chrysogenum* [26].

La cepa de *Aspergillus* sp. CH-Y-1043 aislada y seleccionada, en el sureste de México, es un hongo blanco que fue identificado como *Aspergillus*, crece a 37 °C y puede utilizar como única fuente de carbono: pectina, pulpa de henequén y cáscara de limón no tratadas física ni químicamente [5-6,23,27-28]. La actividad pectinolítica extracelular que produce puede ser utilizada en la despectinización del jugo de manzana con una actividad específica comparable a la de las preparaciones comerciales de mayor demanda en México [5,6].

Hemos encontrado que el crecimiento y la producción de pectinasas de *Aspergillus* sp. CH-Y-1043 son dependientes de la concentración de pectina en el medio de cultivo [23]. El crecimiento se favorece por un aumento en la concentración de la pectina. Sin embargo, la actividad especifica producida es menor cuando se utilizan concentraciones crecientes de pectina. Este hecho indica que estas enzimas están sujetas a represión catabólica [23,27]. En este sentido, hemos demostrado que en cultivo alimentado, la velocidad relativa de síntesis de las pectinasas en esta cepa aumenta entre 3 y 4 veces cuando se utilizan concentraciones limitantes de la fuente de carbono en relación a cuando ésta se utiliza en exceso [23]. Estos resultados confirman que la síntesis de pectinasas de *Aspergillus* sp. CH-Y-1043, está sujeta a represión catabólica por la fuente de carbono.

Por otro lado, la gran versatilidad de *Aspergillus* sp. CH-Y-1043 para adaptarse a diferentes condiciones de cultivo, sus bajos requerimientos nutricionales y su capacidad para crecer a 37 °C la hace una cepa muy interesante como modelo de estudio de la producción, regulación,

secreción y caracterización de sistemas enzimáticos complejos como es el caso de las pectinasas que éste produce [23,27,28].

OBJETIVO

El objetivo del presente trabajo fue el de obtener mayor información sobre algunos de los factores que afectan la producción del sistema pectinolítico de *Aspergillus* sp. CH-Y-1043 en fermentación sumergida, así como la identificación de los componentes que forman este sistema enzimático.

En este trabajo se presentan los resultados de la identificación de las diferentes enzimas pectinolíticas producidas por *Aspergillus* sp. CH-Y-1043, así como el efecto del pH sobre la producción y estabilidad de estas enzimas, la producción de pectinasas en ausencia de materiales pécticos y la presencia de pectinasas unidas a las esporas y al micelio de este microorganismo. Estos resultados pueden contribuir a un mejor entendimiento del complejo proceso de degradación de pectina, así como a diseñar las formas para producir preparaciones pectinolíticas bien definidas que preferencialmente contengan enzimas puras en lugar de mezclas. Adicionalmente se pretende tener una visión más integrada del sistema pectinolítico producido por *Aspergillus* sp. CH-Y-1043.

CAPITULO 2

Influence of pH on endo- and exo-pectinase production by *Aspergillus* sp. CH-Y-1043

Canadian Journal of Microbiology. 1991, 37: 912-917.

Influence of pH on endo- and exo-pectinase production by *Aspergillus* sp. CH-Y-1043

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Aspergillus sp. CH-Y-1043 grown on pectins with various degrees of esterification produces endo- and exo-pectinases at pH values as low as 2.5. Maximal production was attained at this pH, although fungal growth only approximated 50% of that obtained at higher pH values. Endopectinase was produced at pH 2.5–3.5 when the fungus was grown on low degree esterified pectin. With higher degree esterified pectin this enzyme was produced at all pH values analyzed. Exopectinase production was less affected by pH values. Still, maximal production was also reached at pH 2.5–3.5. Exopectinase was found to be associated to the cell and could be released after incubation at different pH values, whereas endo pectinase was not detected in the cellular fraction. Results confirmed by SDS–PAGE coupled with *in situ* activity assays in pectin–agarose gels allowed the identification of a protein band corresponding to endopectinase and a band with pectin esterase activity. Stability of *Aspergillus* sp. CH-Y-1043 pectinases at various pH values was also evaluated.

Key words: *Aspergillus* sp. CH-Y-1043, extreme acidic pH pectinase production, *in situ* pectinase detection, cell-associated exopectinase.

AGUILAR, G., TREJO, B. A., GARCÍA, J. M., et HUITRÓN, C. 1991. Influence of pH on endo- and exo-pectinase production by *Aspergillus* sp. CH-Y-1043. Can. J. Microbiol. 37 : 912–917.

L'*Aspergillus* sp. CH-Y-1043, cultivé sur des pectines ayant divers degré d'estérification, produit des endo- et des exo-pectinases à des valeurs de pH aussi faibles que 2,5. La production maximale a été atteinte à ce pH, bien que la croissance fongique n'ait été que d'environ 50% des croissances observées aux valeurs de pH plus élevées. L'endopectinase a été produite aux pH 2,5–3,5, lorsque le champignon a été cultivé sur de la pectine faiblement estérifiée. À des dégrés d'estérification plus élevés de la pectine, cette enzyme a été produite à toutes les valeurs de pH analysées. La production d'exopectinase a été moins affectée par les valeurs de pH; toutefois, la production maximale a aussi été atteinte aux pH 2,5–3,5. L'exopectinase s'est révélée associée aux cellules et a pu être libérée après incubations à différentes valeurs de pH, tandis que l'endopectinase n'a pas été détectée dans la fraction cellulaire. Les résultats, confirmés par électrophorèses (SDS–PAGE) couplées à des essais *in situ* d'activités sur gels de pectine–agarose, ont permis d'identifier une bande protéique correspondant à l'endopectinase et une autre correspondant à l'activité de la pectine estérase. La stabilité des pectinases produites par l'*Aspergillus* sp. CH-Y-1043 à différentes valeurs de pH a aussi été évaluée.

Mots clés : *Aspergillus* sp. CH-Y-1043, production de pectinases à des pH acides extrêmes, détection *in situ* des pectinases, exopectinase associée aux cellules.

[Traduit par la rédaction]

Introduction

Microorganism enzyme production, as well as that of other metabolites, is affected by culture conditions, in particular by the pH of the medium and the carbon source. Evidence is ample and involves various kinds of microorganisms.

In *Neurospora crassa* (Nahas et al. 1982) the secretion of alkaline and acid phosphatase depends directly on the pH of the culture medium. Above pH 7.4, alkaline phosphatase secretion is stimulated, while acid phosphatase production is reduced. The opposite occurs at pH values below 5.7.

Pectinase production by fungi such as *Fusarium* (Perley and Page 1971), *Rhizoctonia* (Lisker et al. 1975), *Penicillium* (Phaff 1947), *Botrytis* (Leone and Van den Heuvel 1986; Hancock et al. 1964), *Aspergillus* (Dean and Timberlake 1989*a*, 1989*b*; Tuttobello and Mill 1961), and *Saccharomycopsis* (Fellows and Worgan 1984) correlates to pH variations.

Ueda et al. (1982) reported that the amount of pectinesterase I (PE I), pectinesterase II (PE II), polygalacturonase (PG), and pectin lyase (PI) in *Aspergillus oryzae* A-3 also varies according to the pH of the medium. The authors related the differences in production to the stability of the enzymes under various pH values. In *Rhizoctonia solani* optimal pH values for polygalacturonase and pectin lyase production have been found to correspond to 4.0 and 8.0, respectively (Lisker et al. 1975). Kimura and Mizushima (1974) noted in a strain of *Acrocylindrium* that the *in vitro* induction of endopolygalacturonase by galacturonic acid is greater at pH 2.5 than at 5.0 or 7.0. However, the authors do not specify this to be the most efficient pH for production with pectic material.

In previous work we studied the production of pectic enzymes by *Aspergillus* sp. CH-Y-1043 (Aguilar and Huitrón 1986, 1987), starting at pH 4.2. Whenever pH values increased, the production of endopectinase was reduced to the point of disappearing. The production of endopectinase at acidic pH values using lemon peel as carbon source has also been reported (Larios et al. 1989). We therefore studied the effects of pH on fungal growth and extracellular pectinase production and stability in *Aspergillus* sp. CH-Y-1043, using 47 and 60% esterified pectins. In addition, we analyzed whether the pectinases produced by this microorganism remain associated to the cell and whether their release is affected by pH.

Materials and methods

Microorganism

The strain *Aspergillus* sp. CH-Y-1043 was cultured as previously reported (Aguilar and Huitrón 1986).

Medium for fermentation and enzyme production

The basal medium, previously described (Aguilar and Huitrón 1986,

1987), was supplemented with either 60% esterified citrus pectin (Drogeria Cosmopolita, S.A., Mexico) or 47% esterified pectin (Sigma Chemical, U.S.A.), both at 1.0%. The pH of the carbon source was adjusted to 4.2–4.4 and it was sterilized separately. The pH of the basal medium was adjusted so that the final mixture gave the desired pH. Sterilization was carried out at 121°C and 15 psi (1 psi = 6.89 kPa) for 20 min.

Growth and production of pectinolytic activity were followed in 500-mL Erlenmeyer flasks. Each contained 200 mL of culture medium and was shaken at 200 rpm in an incubator shaker (New Brunswick Scientific Co., U.S.A.) kept at a constant temperature of 37°C. All fermentations were inoculated with a spore suspension of *Aspergillus* sp. CH-Y-1043 as described elsewhere (Anguilar and Huitrón 1990).

Assays for pectinolytic activity

Exo- and endo-pectinolytic activity were determined by measuring the formation of reducing groups and by the relative change in viscosity, respectively, as described elsewhere (Aguilar and Huitrón 1987). One unit (U) of exopectinolytic activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol galacturonic acid per hour at pH 5.0. One unit of endopectinolytic activity was defined as the amount of enzyme that reduced the viscosity of 10 mL pectin solution by 50% in 10 min at pH 4.2.

Analyses

Cell growth was measured as dry weight (DW). Reducing groups in culture media were determined directly in the cell-free filtrates by using the dinitrosalicylic acid (DNS) method (Miller 1959) and expressed as galacturonic acid. Protein was estimated in dialyzed cell-free samples by the method of Lowry et al. (1951), with crystalline bovine albumin as the standard.

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970), with a resolving gel containing 10% acrylamide, 2.7% bis-acrylamide, and 20 μg of bovine serum albumin (Sigma Chemical Co., U.S.A.) per millilitre to enhance renaturation of electrophoresed enzymes (Ried and Collmer 1985; Lacks and Springhorn 1980). The stacking gel contained 4% acrylamide and 2.7% bis-acrylamide. The SDS (Bio-Rad Laboratories) concentration was 0.1% in the gel and running buffer. Samples were maintained at 100°C for 60 s in sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 0.005% bromophenol blue).

Samples were electrophoresed at 30 mA for 2–3 h through 1.5 mm gel in a vertical slab gel unit SE-600 (Hoefer Scientific Instruments, U.S.A.). Acrylamide gels were incubated for 1–1.5 h, with shaking, in two or three changes of 0.01 M Tris-HCl, pH 7.6 (Ried and Collmer 1985; Lacks and Springhorn 1980). After this time the gels were placed onto agarose–pectin overlays and incubated at room temperature from 6 to 8 h. Subsequently the acrylamide gels were rinsed with distilled water and were protein stained overnight in a solution containing 0.125% (v/v) Coomassie Blue R-250 (Bio-Rad Laboratories), 50% (v/v) methanol, and 10% (v/v) acetic acid. The gels were destained in 10% (v/v) acetic acid with heating. The agarose–pectin gels were prepared and stained as described below.

Agarose–pectin overlays for in situ *detection of pectinolytic activity*

The agarose–pectin overlays (Ried and Collmer 1985; Bertheau et al. 1984) were prepared dissolving the agarose by heating in 0.17 M acetate buffer, pH 5.0, and then pectin was added and mixed. This mixture was cast on a plate sandwich with 0.75 spacers in a gel caster (Hoefer Scientific Instruments, U.S.A.) previously heated at 37°C. Final concentrations within the gels were 1% agarose and 0.1% pectin. After incubation with polyacrylamide gel the agarose overlays were stained with a solution of ruthenium red, 0.05%, for 30 min. These overlays were put onto a plastic sheet and dried at room temperature.

Endo- and exo-pectinase release

A suspension of *Aspergillus* sp. CH-Y-1043 spores was inoculated into a 2.8-L Fernbach flask containing 1.0 L basal medium to which pectin (47% esterified) had been added, and incubated at 37°C and 200 rpm. After 36 h growth (mid exponential phase) the mycelium was harvested by filtration through Whatman No. 1 filter paper and washed with NaCl isotonic solution containing 125 μg cycloheximide per millilitre until no absorbance ws detected at 280 nm in the water wash. The washed mycelium was suspended in distilled water to a final concentration of 20 mg/mL, and 10-mL samples of this suspension were transferred to 125-mL Erlenmeyer flasks containing 40 mL of basal medium with 125 μg/mL cycloheximide, adjusted to a pH values of 2.5, 3.5, 4.2, 5.2, and 6.2. The flasks were then incubated at 37°C and 200 rpm. Samples were collected at various times and centrifuged, and the resulting supernatant (SN) was filtered through a 5.0-μm Millipore membrane (Millipore Corp., U.S.A.) and used for the activity assays. The mycelium pellet was collected and washed repeatedly by centrifugation as above and resuspended in 2 mL distilled water. It was subsequently homogenized and centrifuged, and the resulting supernatant was used for the activity assays. This fraction is referred to as cell extract (CE).

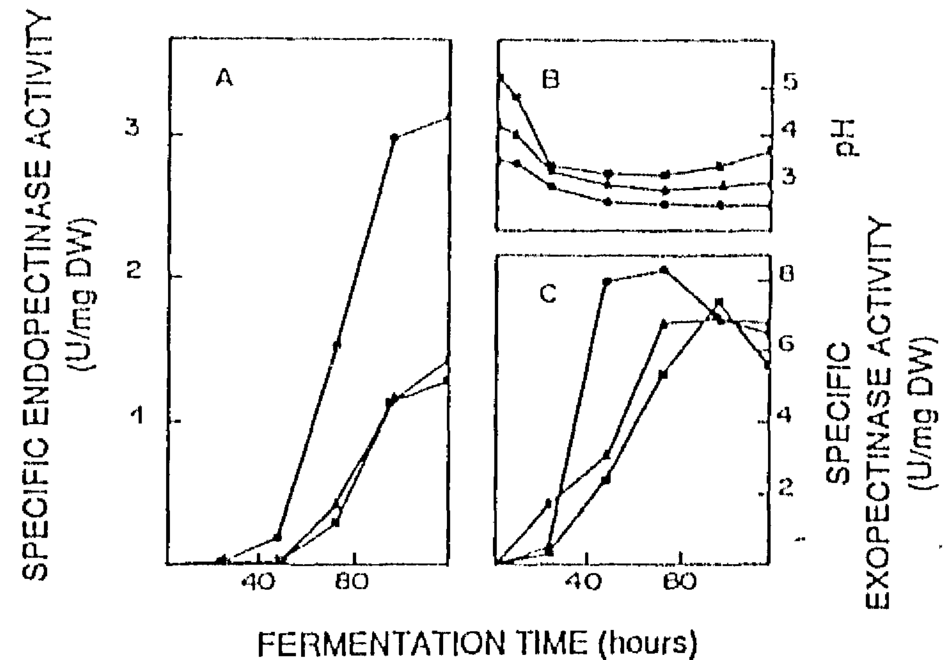

FIG. 1. Extracellular pectinolytic activity produced by *Aspergillus* sp. CH-Y-1043 growing on 1% pectin with a degree of esterification of 60% at different initial pH. (A) specific endopectinase activity; (B) pH profile; (C) specific exopectinase activity. pH_i 3.5 (●); 4.2 (▲), and 5.2 (■).

Endo- and exo-pectinase stability at different pH values

Ten millilitres of a cell-free filtrate was added to 10 mL of double-strength basal medium adjusted to pH values of 2.5, 3.5, 4.2, 5.2, and 6.2. Immediately after adding the filtrate, the flasks were incubated at 37°C and 200 rpm. Samples were taken at various times, and the residual activity was determined as mentioned above.

Results and discussion

Effect of the initial pH using 47% (P-47) and 60% (P-60) esterified pectins as carbon source on pectinase production

For these experiments the initial pH (pH_i) was set at 3.5, 4.2, and 5.2. Maximum endopectinase production using P-60 as carbon source was obtained at pH_i 3.5 (3.12 U/mg DW). At pH_i 4.2 and 5.2 activity was less than 50% (1.44 and 1.41 U/mg DW, respectively; Fig. 1A).

Under the same pH and carbon source conditions, exopectinase production at 120 h was 6.46, 6.79, and 5.61 U/mg DW, respectively (Fig. 1C). At pH_i 3.5 exopectinase production showed a rapid initial increase, which leveled off after 72 h. Note that at 48 h the activity was 2.5–3 times higher than for the other pH_i values.

In all cases, the initial pH value decreased and oscillated between 2.5 and 3.8, depending on pH_i (Fig. 1B). The effect of pH_i was clearly more noticeable for endo- than for exo-pectinase activity.

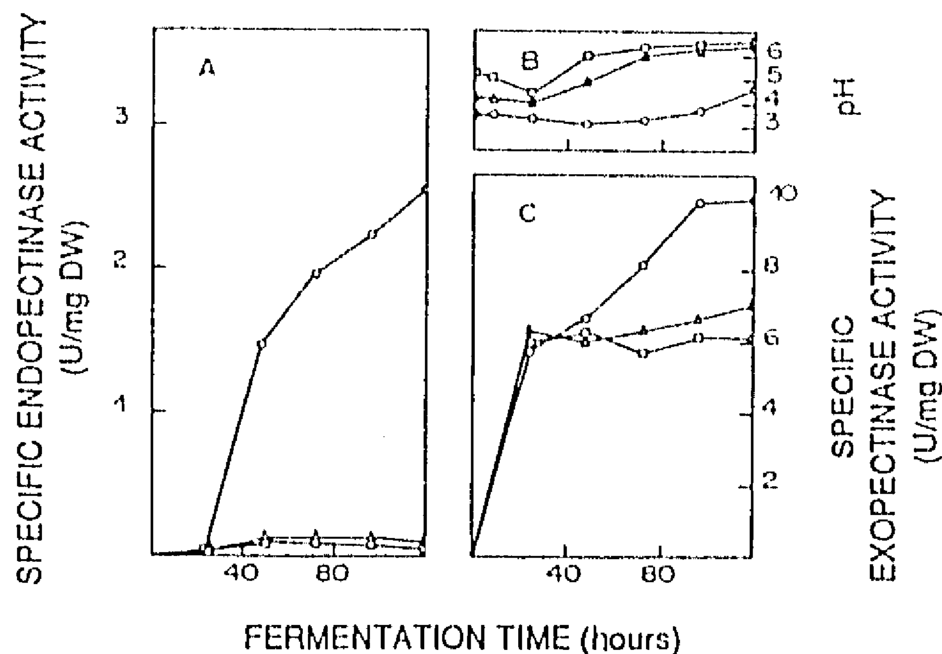

FIG. 2. Extracellular pectinolytic activity produced by *Aspergillus* sp. CH-Y-1043 growing on 1% pectin with a degree of esterification of 47% at different initial pH. (A) specific endopectinase activity; (B) pH profile; (C) specific exopectinase activity. pH_i 3.5 (○), 4.2 (△), and 5.2 (□).

Maximum endo- and exo-pectinase activity using P-47 as carbon source was obtained at pH_i 3.5 (Figs. 2A and 2C). At pH_i 4.2 and 5.2 endopectinase activity was less than 1% of maximum value, which correlates with a rapid increase in pH after 24 h of fermentation, reaching values above 6.0 (Figs. 2A and 2B).

Endopectinase production at pH_i 3.5 was higher with P-60 than with P-47 at 96 and 120 h, but it could be detected earlier with P-47. In fact, at 48 h, the activity with P-47 was 7.4 times higher than with P-60 (Figs. 1A and 2A). This delay in production with P-60 is due to a high concentration of pectin degradation products, which accumulate during the first hours of fermentation and exert catabolic repression on enzyme production in *Aspergillus* sp. CH-Y-1043 (Aguilar and Huitrón 1986, 1987). With P-60 at pH_i 3.5 the concentration of these products reached 3.4 mg/mL, whereas with P-47 it was 1.40 mg/mL (see Fig. 4D).

It is important to note that the initial degradation of pectin cannot be attributed to nonspecific reaction induced by pH or by the culture conditions. Uninoculated control cultures did not show an increase in these products (less than 0.3 mg/mL) after 120 h of incubation at 37°C (data not shown) at the pH values tested.

On the other hand, the lack of endopectinase production at pH 4.2 and 5.2 with P-47 cannot be attributed to catabolic repression. The maximal concentration of reducing groups in the culture medium at these initial pH values was similar (1.5 and 1.9 mg/mL) to that reached at pH_i 3.5. Moreover, with P-60 the concentration of pectin degradation products is higher (3.3 mg/mL at pH_i 4.2 and 2.9 mg/mL at pH_i 5.2) and does not stop enzyme production. This limited activity is probably due to a lack of induction at higher pH values. Perley and Page (1971) described a case of differential induction in *Fusarium roseum*, where endopolygalacturonase and pectinesterase are induced at pH 3.5 but not at pH 6.5 in pectin-containing media. This phenomenon is comparable with our results for endopectinase, which is strongly induced at pH 3.5 but virtually absent at pH values above 4.2, using low esterified pectin as carbon source.

Identification of endo- and exo-pectinase activity on polyacrylamide gels

Electrophoresis of the cell-free filtrates obtained using P-47 as carbon source revealed a major band migrating to a molecular mass of approximately 43 kDa when pH_i was 3.5 (Fig. 3A, lane 2). This protein band virtually disappeared at pH_i 4.2 and 5.2 (Fig. 3A, lanes 3 and 4). Electrophoresis of samples using P-60 displayed the 43-kDa band with the three pH_i values (Fig. 3A, lanes 5, 6, and 7). The *in situ* activity on agarose–pectin gels revealed a clear hydrolysis zone with the filtrate from P-47 at 3.5 and from P-60 at the three pH_i values (Fig. 3B, lanes *a*, *d*, *e*, and *f*), which corresponds to the 43-kDa band. The same analysis for P-47 at pH_i 4.2 and 5.2 revealed a very light hydrolysis zone (Fig. 3B, lanes *b* and *c*). This fact correlates very well with the quantitative endopectinase assay, indicating that the 43-kDa band corresponds to endopectinase.

We also observed zones stained darker than the unaltered pectin, characteristic of pectinesterase activity (Cruikshank and Wade 1980). These darker zones correspond to a 33-kDa protein band obtained with P-47 at pH_i 3.5 and with P-60 at pH_i 3.5, 4.2, and 5.2 (Fig. 3A, lanes 2, 5, 6, and 7; Fig. 3B, lanes *a*, *d*, *e*, and *f*) and indicate that *Aspergillus* sp. CH-Y-1043 also produces a pectinesterase. However, no quantitative assays were performed.

Growth and enzyme production at pH_i 2.5

In these experiments endopectinase production was highest whenever the pH values descended and remained between 2.5 and 3.0. Growth and pectinase production of *Aspergillus* sp. CH-Y-1043 were therefore evaluated at pH_i 2.5 with both types of pectin. Results are shown in Fig. 4. The production of endopectinase on P-47 reached 14 U/mg DW, which was the highest obtained for any combination of conditions, in fact, five times higher than that obtained at pH_i 3.5.

With P-60 the endopectinase production was virtually the same at pH_i 2.5 as that obtained with pH_i 4.2 and 5.2. Maximal activity appeared at pH_i 3.5 (see Figs. 1 and 4).

Practically all exopectinases appear to be produced during the first 24 h on P-47 and 48 h on P-60 (Fig. 4B).

Although the initial pH was very low, it continued to descend along the fermentation, reaching final values of 2.3 for P-47 and 2.0 for P-60 (Fig. 4C). Under these conditions, fungal growth was seriously affected and virtually ceased at 24 h (Fig. 4E). The maximal growth obtained was less than 50%, compared with growth at pH_i 3.5 (Fig. 4E), and with that obtained at the other pH_i values (4.2 and 5.2, data not shown).

The accumulation of pectin degradation products showed a different profile at pH_i 2.5 (Fig. 4D). The decrease of these products observed at the other pH_i values after 24 h was not visible here (Fig. 4D). Growth is arrested at 25 h; the carbon source remains virtually untouched and consequently accumulates. Moreover, the extracellular pectinase produced continued degrading unused residual pectin, which also favoured this accumulation.

The difference in concentration of accumulated reducing groups, always greater in P-60 than in P-47, could be due to the relative viscosity of the two pectins, which indicates differences in average molecular mass as well as in chain length. The relative viscosity of P-47 is approximately three times greater than that of P-60, which would make P-60 more susceptible to degradation. In parallel experiments, at pH_i 2.5, 60% esterified pectin with a relative viscosity simiar to P-47 showed comparable pectin degradation products concentration. Under these conditions, endopectinase activity after 72 h was 5.0 U/mg DW for P-47 and 6.4 U/mg DW for P-60. These results confirm that the highest production occurs at pH_i 2.5. It may also be concluded that the low enzyme production in P-60 of low viscosity

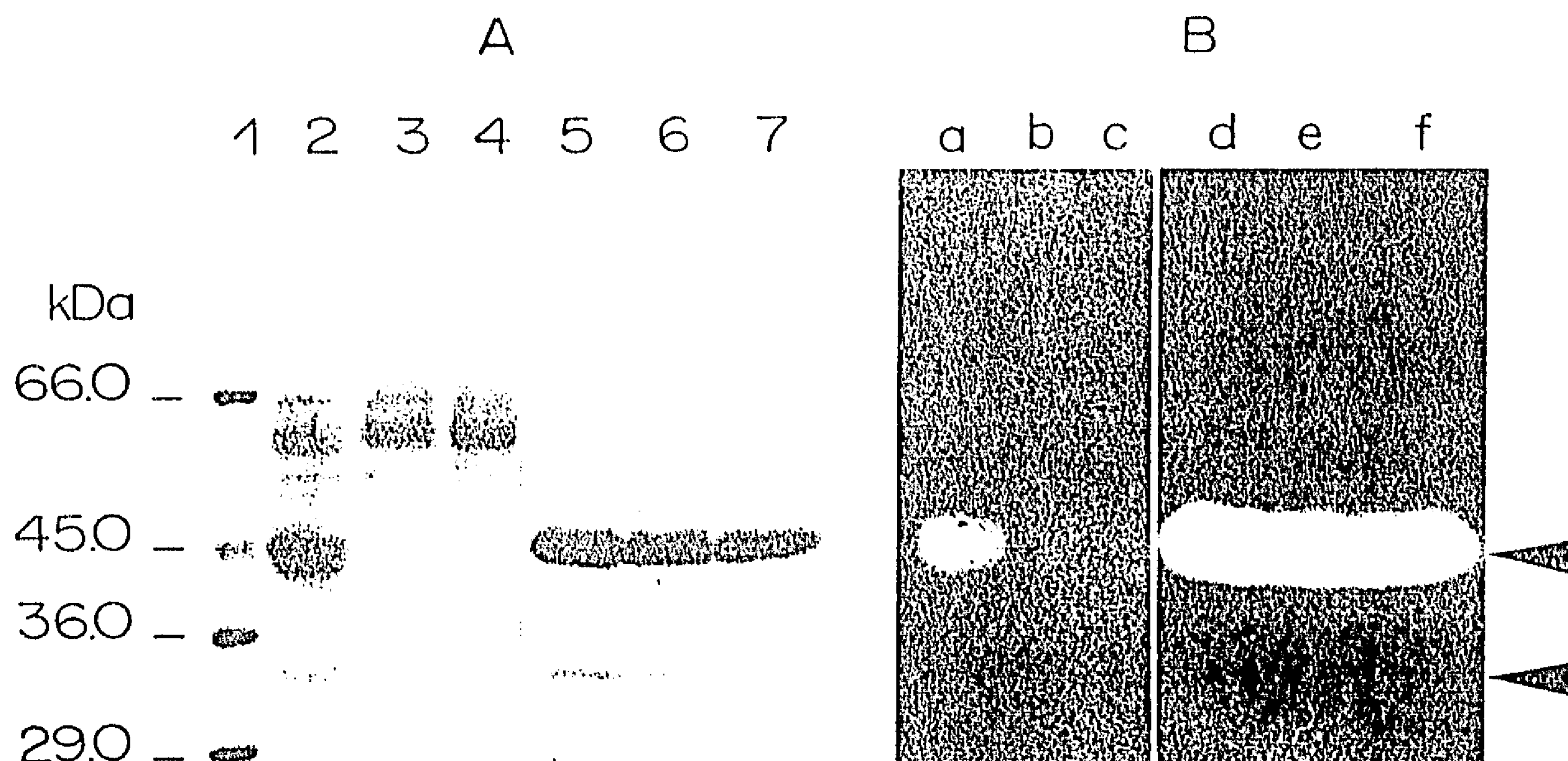

FIG. 3. (A) SDS–PAGE and (B) pectinolytic activity on pectin–agarose overlays of culture filtrates of *Aspergillus* sp. CH-Y-1043 at different initial pH. Electrophoresis on 10% acrylamide slab gel (14 × 16 cm) in the presence of SDS was according to Laemmli (1970), run at 30 mA constant current for 5 h. Crude cell-free samples were concentrated by lyophilization and dialyzed and applied to each well without further treatment. The polyacrylamide gel and the agarose–pectin overlay were incubated with 0.17 M acetate buffer for 6 h at room temperature. Lane 1, molecular mass markers (bovine albumin, 66.0 kDa; ovalbumin, 45.0 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa; carbonic anhydrase, 29.0 kDa; trypsinogen, 24.0 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.2 kDa). Fig. 3A, lanes 2–4, and Fig. 3B, lanes a–c. Filtrates from *Aspergillus* sp. CH-Y-1043 grown on 1% P-47 at pH_i 3.5, 4.2, and 5.2, respectively. Fig. 3A, lanes 5–7, and Fig. 3B, lanes d–f. Filtrates from *Aspergillus* sp. CH-Y-1043 grown on 1% P-60 at pH_i 3.5, 4.2, and 5.2, respectively.

at pH_i 2.5 is due to the higher concentration of pectin degradation products produced by a rapid accumulation in short periods of time.

The data obtained using 60% esterified pectin of similar viscosity to P-47 support the opinion that the production of endopectinase is favoured by highly methoxylated pectins. In addition, when galacturonic acid or polygalacturonic acid are used as carbon sources, endopectinase is not produced, independent of pH_i (Aguilar and Huitrón 1990). These facts lead us to think that endopectinase inducers could be methoxylated compounds originated by pectin degradation during fermentation.

Especially relevant is the fact that maximal pectinase production by *Aspergillus* sp. CH-Y-1043 is obtained at an extreme pH value, namely 2.5. A recent report states that *Aspergillus nidulans* possesses a very efficient homeostatic mechanism that allows it to grow at pH values ranging from very acidic (2.5) to very alkaline (10.5) (Rossi and Arst 1990). However, to our knowledge, no pectinolytic enzyme nor any other enzyme of industrial application has been demonstrated to show optimal production at pH 2.5, as occurs in this case.

Pectinase release and stability

To confirm whether the lack of endopectinase production at pH_i >4.2 was due to an effect on synthesis, rather than to other factors, such as enzyme retention at the cell membrane or poor enzymatic stability at higher pH, we performed the following experiments.

Aspergillus sp. CH-Y-1043 was grown on pectin for 36 h (mid exponential growth phase), and the cells were harvested and processed as described in the Material and methods section. The pectinolytic activity released from cells into the supernatant (SN), after incubation at different pH values, as well as that retained in the cellular extract (CE), were determined (see Material and methods).

The only activity detected both in the supernatant and in the cellular extract was exopectinase activity. Endopectinase activity was not registered in any of the two. The SN/EC ratio for exopectinase released at 6 h of incubation remained close to 1, and a maximum was obtained at pH 4.2 (SN/CE = 1.89). This indicates th[illegible] remains associated to the cell. It is probably synthesized and accumulated in the first phase of exponential growth and subsequently excreted into the medium. Endopectinase, in turn, seems to be synthesized and simultaneously excreted.

Samples of the supernatant obtained at 24 h of incubation were electrophoresed (Fig. 5). As expected, no pH value displays the 43-kDa band, confirming the absence of this enzyme and supporting the fact that this enzyme does not accumulate in the mycelium but is secreted simultaneously with its synthesis. A group of bands migrating to the 66-kDa molecular weight marker were observed as in previous experiments (see Fig. 3). These bands could correspond to exopectinase activity. However, it was not possible to obtain hydrolysis zones in the agarose–pectin gels, under these experimental conditions.

Tests for stability of the pectinases produced by *Aspergillus* sp. CH-Y-1043 at different pH values showed that endopectinase activity is very stable, decreasing only 11 and 19%, respectively, at the extreme pH values of 2.5 and 6.2 (Table 1). On the other hand, exopectinase activity was considerably more affected, diminishing up to 45% at pH 2.5 and between 20 and 28% at the other pH values (Table 1).

Because endopectinase is very stable with regard to pH variations (Table 1) and is not being accumulated in the cell, it

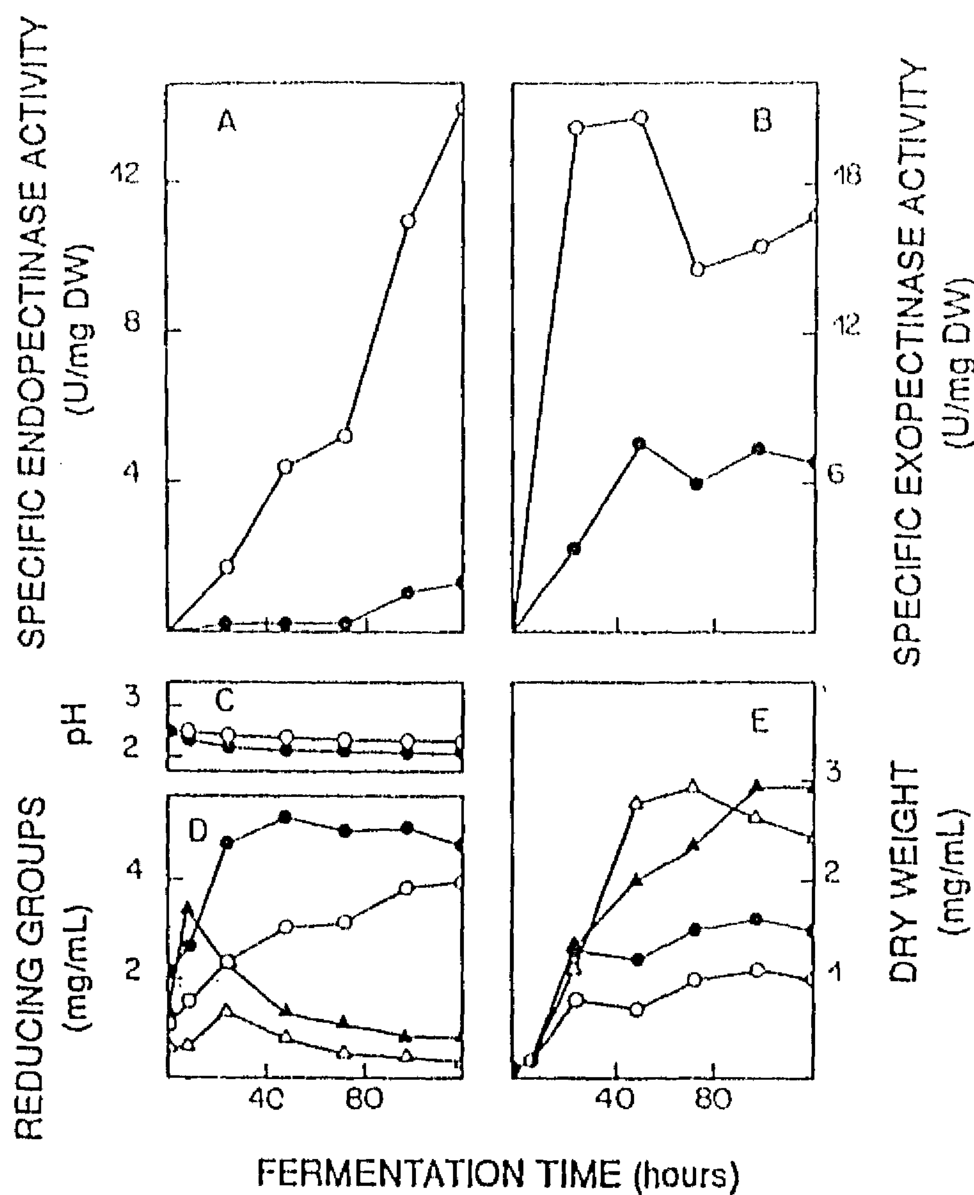

FIG. 4. Time course fermentation of *Aspergillus* sp. CH-Y-1043 at an initial pH of 2.5 with 1% pectin with a degree of esterification of 60% (●) and 47% (○). (A) Specific endopectinase activity; (B) specific exopectinase activity; (C) pH profile; (D) accumulation of degradation products from pectin as reducing groups; (E) dry weight. Also shown are (D) data of reducing groups and (E) dry weight with 1% pectin with a degree of esterification of 60% (▲) and 47% (△), both at pH$_i$ 3.5 for comparison.

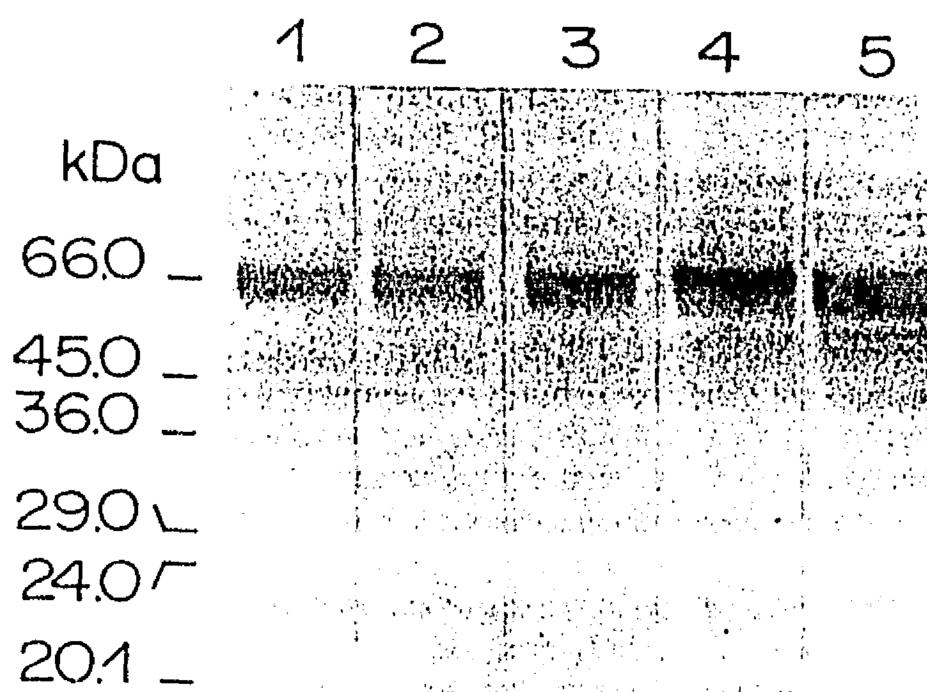

FIG. 5. SDS–PAGE of proteins released by *Aspergillus* sp. CH-Y-1043 after incubation for 24 h on basal medium with 125 μg/mL cycloheximide at different pH values. Samples were concentrated by lyophilization and dialyzed. Lanes 1–5, cell-free samples from *Aspergillus* sp. CH-Y-1043 incubated at pH 2.5, 3.5, 4.2, 5.2, and 6.2, respectively. Gel (14 × 8 cm) was run at 30 mA for 2 h. Molecular mass markers as for Fig. 3.

TABLE 1. pH stability of endo- and exo-pectinase activity[a]

	Residual activity (% of maximum)[b]	
pH	Endopectinase	Exopectinase
2.5	89	55
3.5	100	80
4.2	97	77
5.2	94	75
6.2	81	73

[a]Cell-free filtrate incubated at 37°C and 200 rpm for 120 h.
[b]The initial activity (2.0 and 23.0 U/mL for endo- and exo-pectinase, respectively) was taken as 100%.

seems that the lower activity at pH values above 3.5 is due to limited enzyme synthesis rather than to a loss of activity caused by poor stability or degradation during the fermentation. Additionally, the electrophoretic analysis of filtrates obtained at different pH values did not display variation in the profile of low molecular weight bands (data not shown).

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CAPITULO 3

Constitutive exo-pectinase produced by *Aspergillus* sp. CH-Y-1043 on different carbon sources

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CONSTITUTIVE EXO–PECTINASE PRODUCED BY *Aspergillus sp.* CH-Y-1043 ON DIFFERENT CARBON SOURCE

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SUMMARY
The production of a constitutive exo-pectinase by *Aspergillus sp.* CH-Y-1043 grown on glucose, sucrose, fructose, glycerol and galacturonic acid is reported. The specific activity was found to be in the range of 26% to 75% of that produced with pectin or polygalacturonic acid. The production of this exo-pectinase is strictly correlated to the exponential growth phase and it is highly sensitive to the pH of the culture medium.

INTRODUCTION

The pectic enzymes from fungal sources are industrially important in fruit juice technology since their capacity to degrade pectin and related substances not only results in greater yields of extracted juices but also reduced the time needed for filtration. Pectinases also play an important role in the degradation of cell wall material in plant pathogenesis and they have been associated to fruit development, ripening and cell wall extension (Ward and Moo-Young, 1989).

The production of many extracellular pectinases is known to be induced by the presence of pectic materials in the culture medium (Friederich et al, 1989, Leone and Van Den Heuvel, 1986, Fogarty and Kelly, 1983). However, since pectin is a high molecular weight polysaccharide, the question arises as to how induction takes place or how the cells can sense the substrate in the outer environment. It has been suggested that some microorganisms can produce low levels of basal constitutive activities that degrade the polymeric substrate, and that the low molecular products of the reaction serve as inducers or energy sources to promote cell growth and to induce the other pectinases (Leone and Van Den Heuvel, 1986). This is true for some microorganisms, particularly bacteria and a limited number of fungi. Constitutive levels of polygalacturonic acid trans-eliminase have been demonstrated in *Aeromonas liquefaciencs* (Hsu and Vaughn, 1969). *Bacillus sp.* and *B. subtilis* produce pectate-lyases constitutively on a wide variety of carbon and nitrogen sources (Kelly and Fogarty, 1978, Korowsky and Dunleavy, 1976). In fungi it is known that *Pyrenochaeta terrestris* produces small amounts of extracellular endo polygalacturonases constitutively (Keen and Horton, 1966). Also *Aspergillus saitoi* (Yamasaki et al, 1965) and *Botrytris cinerea* (Leone and Van Den Heuvel, 1986) are able to produce constitutive polygalacturonases.
The fungus *Aspergillus sp.* which we reported previously, is capable of producing variable amounts of inducible endo and exo pectinases on a wide variety of pectic materials and has potential for industrial production of these enzymes (Aguilar and Huitrón, 1987, Aguilar

and Huitrón, 1986, Saval and Huitrón, 1983, Saval et al 1982, Larios and Huitrón, 1989). This report contains our findings on the presence of a constitutive exo-type pectinolytic activity produced by *Aspergillus sp.* various carbon sources.

MATERIALS AND METHODS

Microorganism

The Microorganisms used in this work was the strain *Aspergillus sp.* CH-Y-1043, previously reported as *Aspergillus sp.*, cultured as reported (Aguilar and Huitrón, 1987).

Fermentation Medium

The basal medium contained (g/L) $(NH_4)_2SO_4$, 2.0, K_2HPO_4, 2.0, KH_2PO_4 2.0 and was supplemented with the chosen carbon source and 0.3°/o of yeast extract unless otherwise stated. Citrus pectin, polygalacturonic acid, galacturonic acid (Sigma Chemical Co., USA) glucose, sucrose, fructose or glycerol (J.T.Baker, S.A. de C.V. Mexico) all at 0.5°/o were used as carbon sources. All reagents were analytical grade. The carbon source and the yeast extract were sterilized separately from the basal medium. Sterilization was carried out at 121°C and 15 psi for 20 min. The initial pH in all experiments was 4.2-4.5.

Inoculum

Spores from 3 days old agar slants of *Aspergillus sp.* were collected by adding sterile distilled water to each slant. The spore suspension was adjusted to a final concentration in the culture medium of 1.0×10^6 spores/ml by counting in a counting chamber microscopic cell (American Optical Inc. USA).

Production of Pectinolytic Activity

For the production of pectinolytic activity, shake flask experiments were conducted in 500ml Erlenmeyer flasks, each one containing 200ml of culture medium and agitated at 200rpm in an incubator shaker (Newbruswick Sci. Co., USA) maintained at 37°C. To keep control of the pH, a 1.2 liter fermentor (Applikon, Dependable Ins., The Netherlands) filled with 1.0 L of culture medium was used. Sterile air was supplied at a rate of 1.0 vvm and the agitation speed was 200rpm. The pH was kept constant by addition of NaOH (0.4N) or H_2SO_4 (2°/o v/v) as required by means of a pH meter/controller (Mod. pH 22, Newbrunswick Scientific Co., USA). Temperature was maintained at 37°C.

Assays for Pectinolytic Activity

Samples were taken from flasks or fermentor at various times during fermentation and were inmediately filtered through Millipore membranes. Pectinolytic activity of these cell-free filtrates was determined: a) by quantifying, using the method of DNS (Miller, 1959), the number of reducing groups, expressed as galacturonic acid, which had been liberated after incubation with 0.9°/o pectin (45°C, 1h), one unit of exo activity was defined as the amount of enzyme which catalyses the formation of 1μmol of galacturonic acid/hour at pH 5.0 and (b) by measuring the relative change in viscosity of 1.0°/o pectin at 30°C in an Ostwuald viscosimeter. One unit of endo-activity was defined as the amount of enzyme which reduced the viscosity of 10ml of pectin solution by 50°/o in 10min at pH 4.2.

Cell Growth

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

Reducing Groups in Culture Medium

Reducing groups in culture media were directly determined in the cell-free filtrates using the method of DNS (Miller, 1959) and expressed as galacturonic acid. Whenever another carbon source was used, its concentration in the culture medium was expressed using the appropriate standard.

RESULTS AND DISCUSSION

When *Aspergillus sp.* CH-Y-1043 was grown on glucose as a sole carbon source pectinase levels remained low, suggesting the constitutive nature of this enzymes. However, since cell growth on glucose is very low we could not discern if the detected activity was produced during fermentation or if it was liberated from the spores during germination. There-

fore, cell growth and production of pectinases by *Aspergillus sp.* were evaluated in absence of pectic substances, but in the presence of yeast extract (YE). The growth and production on Pectin (P) and polygalacturonic acid (PGA) was also evaluated. The activity liberated by the spores during germination was evaluated through a control culture with basal medium alone.

The growth curves of *Aspergillus sp.* CH-Y-1043 on sucrose (S), fructose (F), glycerol (Gl), galacturonic acid (GA) or glucose (G) all enriched with YE are shown in Fig. 1. With these carbon sources more cell growth was obtained than with YE alone (doted line Fig. 1A). Maximum cell growth was the same for G, S, F and Gl. However with G it was around 2-2.5-fold higher during the first 24h compared to the other carbon sources with the exception of GA, where growth was slightly lower during the same period. Maxium cell growth attained with GA was 56°/o of other carbon sources (Fig. 1A). When no carbon source was added cell growth reached 27°/o of the maxium obtained with G, S, F and Gl and 48°/o of that obtained with GA. The addition of YE was important in order to promote cell growth because when G, S, F or GA are used as sole carbon sources growth is weak and with Gl it is practically absent (data not shown).

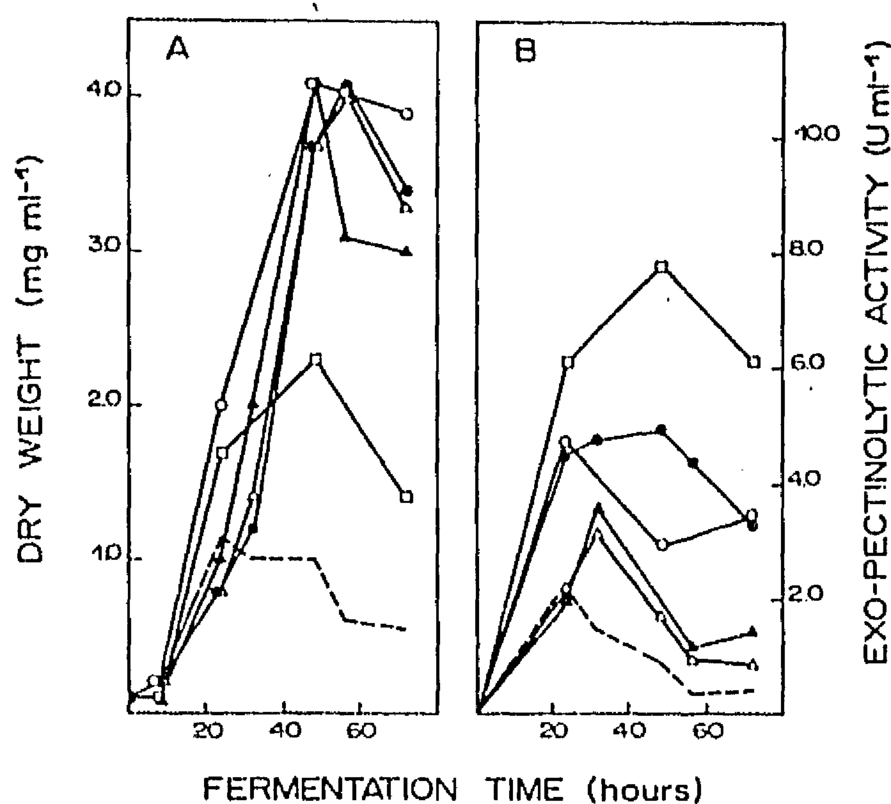

FIG. 1. Cell growth (A) and exo-pectinase activity (B) produced by Aspergillus sp. *growing on basal medium containing glucose (○), fructose (△), sucrose (▲), glycerol (●), galacturonic acid (□) all at 0.5°/o enriched 0.3°/o YE. (---) Basal medium, 0.3°/o YE without carbon source.*

With all these carbon sources exo-pectinolytic activity was produced (Fig. 1B). In contrast, endo type activity could not be detected in any case. Maxium activity was obtained when GA was used as carbon source (7.8 U/ml at 48h) despite the fact that cell growth was less than with other carbon sources. Activity was always higher than in media with no carbon source and in all cases it decreased after reaching the maxium.

The addition of YE to culture of *Aspergillus sp.* growing on P or PGA also stimulated cell growth (Fig. 2A). This stimulatory effect of YE as around 5-fold with PGA and only 2-fold with P. Maxium cell growth was obtained with either P or PGA both enriched with YE as carbon sources, slightly higher with P (Fig. 2A). Cell growth was lowest on PGA without YE (Fig. 2A).

It is interesting to note (Fig. 2B) that with P alone the production of exo-pectinolytic activity is maintained throughout fermentation even though cell growth ceases at 48h (Fig. 2A). Highest production was reached with either P or PGA enriched with YE. Endo activity was produced exclusively when P was used as a sole carbon source (0.28 U/ml). Endo-activity was not found with other carbon sources. Exo activity obtained in the control culture (basal medium only) was 0.032U/ml and no endo-activity could be detected.

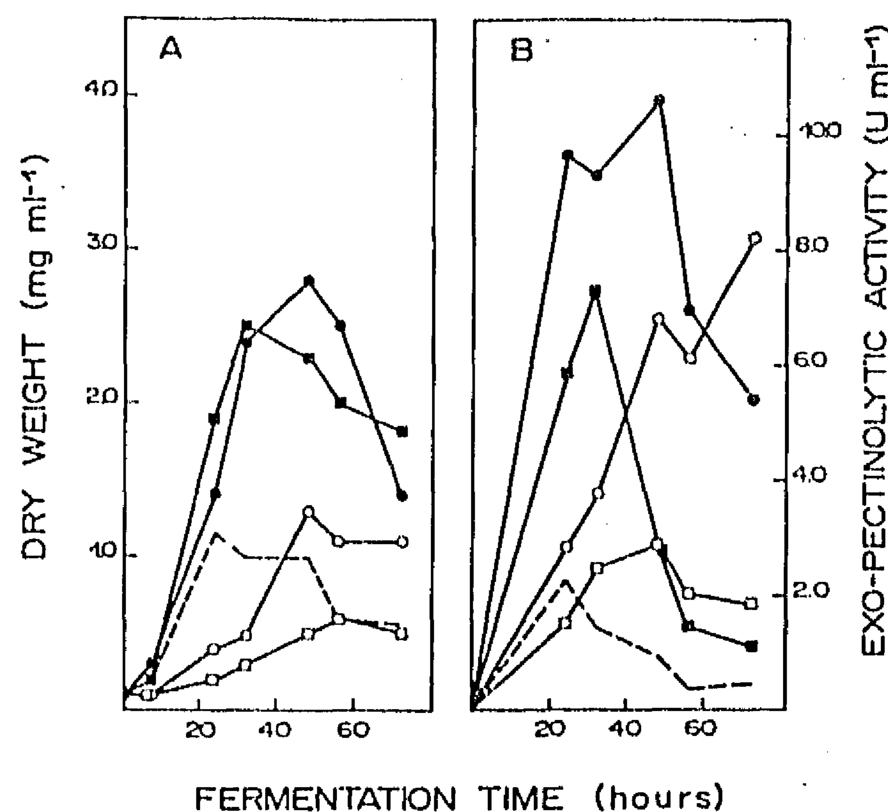

FIG. 2. Cell growth (A) and exo-pectinase activity (B) produced by Aspergillus sp. *growing on basal medium containing pectin (○, ●) or polygalacturonic acid (□, ■). YE (0.3°/o) without carbon source (---). Closed symbols YE, 0.3°/o added and open symbols without YE.*

Since cell growth varied from one carbon source to the next, the specific activity was calculated (Table I). Specific activity obtained with pectin was taken as 100°/o. The maximum specific activity was obtained with PGA and P without YE. In the absence of any carbon source but presence of YE the specific activity was 26°/o . With the exception of sucrose, all other carbon sources gave higher specific activities than that obtained with YE alone, but lower than the value for P. With GA, the final degradation product of P, the specific activity was 58°/o this value was higher than that obtained with PGA (41°/o). Specific activity of the control culture was only 4.0°/o indicating that the activity found with carbon sources was truly due to synthesis during cell growth.

TABLE I. SPECIFIC EXO-PECTINOLYTIC ACTIVITY (a)

	Maximum Exo-activity		
Carbon source (0.5°/o W/v) (b)	*U/mg D.W.*	*°/o*	*Final pH*
Basal medium	*0.32 (24)(c)*	*4*	*4.46*
None	*1.97 (24)*	*26*	*6.85*
Sucrose	*1.98 (24)*	*26*	*5.61*
Glucose	*2.35 (24)*	*31*	*6.00*
Fructose	*2.71 (24)*	*36*	*5.79*
Galacturonic acid	*4.34 (72)*	*58*	*7.96*
Glycerol	*5.66 (24)*	*75*	*5.74*
Polygalacturonic acid	*3.08 (24)*	*41*	*7.70*
Pectin	*6.83 (24)*	*91*	*6.95*
Polygalacturonic acid (without YE)	*8.16 (32)*	*108*	*6.21*
Pectin (without YE)	*7.54 (32)*	*100*	*5.70*

(a) Cell growth was carried out on basal medium with the chosen carbon source as indicated in Erlenmeyer flasks at 37°C, pHi 4.2-4 5
(b) In all cases 0.3°/o (w/v) yeast extract was added except where indicated.
(c) The number in brackets denotes the time at which maximum activity was attained

The final pH of all experiments tended to increase above 5.6 (Table I) partialy due to the presence of YE,. as may be observed with P or PGA when they were not enriched with YE. the final pH was lower to the corresponding experiment in the presence of YE. This in crease in the pH of the culture medium correlated with the great loss of activity observed in all previous fermentations.
Therefore, some experiments with controled pH were conducted. The initial pH was 4.2, as in the other fermentations. Normal evolution was allowed for the first 24h. At this time the pH was lowered by addition of a dilute acid solution to a pH value of 3.5 and it was kept thus until the end of fermentation. In these experiments, G or GA were used as carbon sources, both enriched with YE (0.3°/o) and the results were compared with those obtained with GA at 0.5°/o and 1.0°/o without YE. These results are shown in Fig. 3.
Cell growth with G or GA, enriched with YE (Fig. 3A), is similar to that obtained in the previous experiments with the same carbon sources (see Fig. 1A). When GA at 0.5°/o and 1.0°/o was used as a sole carbon source, growth was very limited (18°/o and 23°/o respectively, compared to the growth obtained with GA plus YE). Cell growth on G alone was lower than that on GA without YE (data not shown).

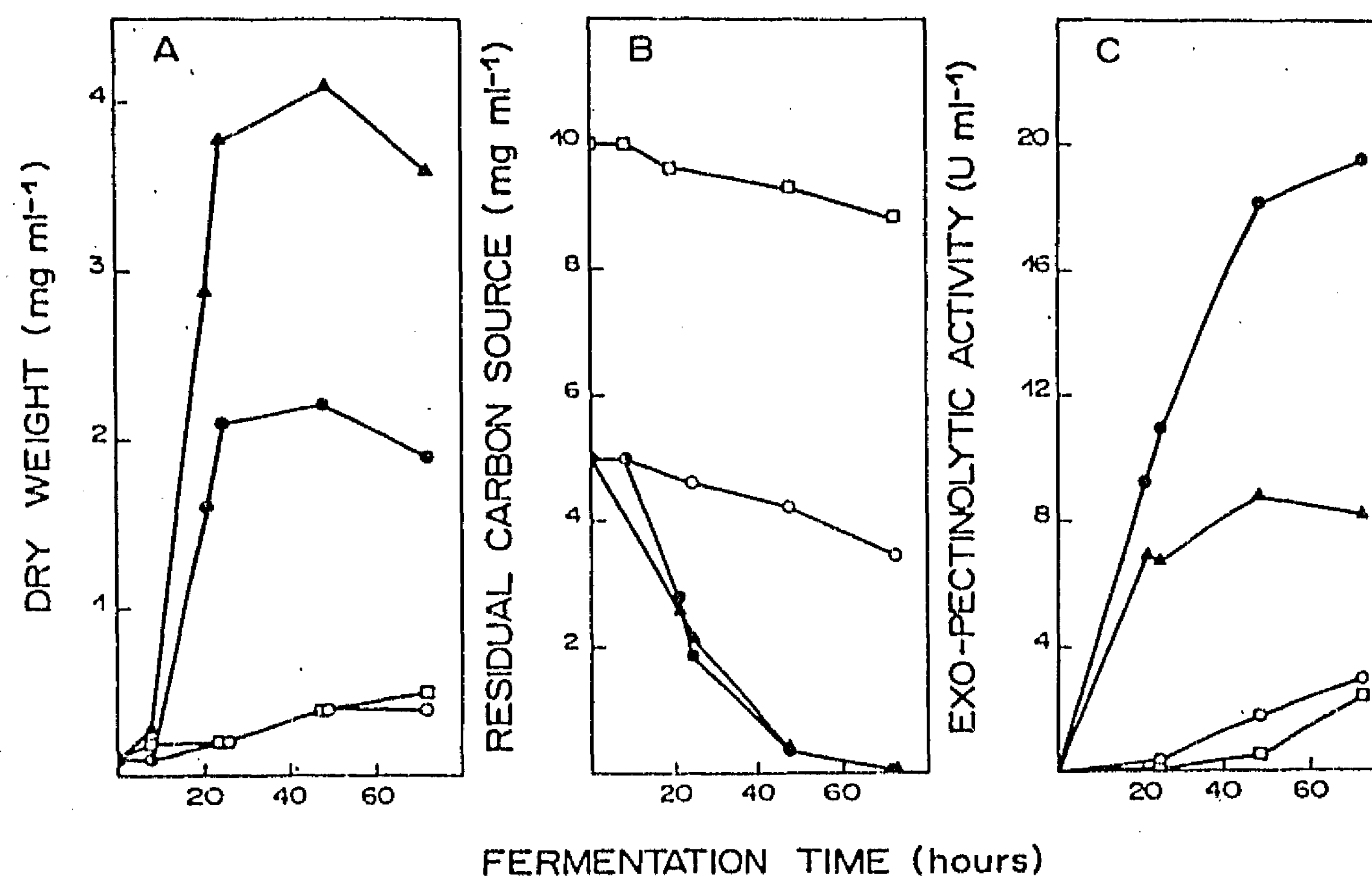

FIG. 3. Effect of pH control on cell growth (A), carbon source consumption (B) and exo-pectinolytic activity (C) produced by Aspergillus sp. *growing on basal medium containing 0.5°/o G + 0.3°/o YE (▲); 0.5°/o GA + 0.3°/o YE (●); 0.5°/o GA (○) and 1.0°/o GA (□).*

In the presence of YE the GA was practically exhausted during the first 48 h of fermentation, whereas in its absence the GA was consumed in lesser quantities (0.5°/o and 1.0°/o GA without YE: 31°/o and 11°/o respectively). Similar results were obtained with G plus YE (Fig. 3B).
In these experiments higher activity was obtained with GA plus YE (19.53U/ml) and this activity increased during fermentation even though cell growth ceased at 48h. With G plus YE the activity occurred during the first 24h, coincident with the exponential growth phase. Low increases were observed after this time (Fig. 3C). It is important to notice that the activity did not decrease as in the experiments where the pH was not controlled. Low but detectable activity was observed in medium containing GA without YE, when compared to the activity obtained with GA enriched with YE (Fig. 3C).

The specific activity for these ferementations was also calculated. As we expected the maxium was obtained when GA plus YE was used as carbon source (10.28U/mg DW). When 0.5°/o GA an 1°/o GA both without YE or G plus YE were used the specific ac-

tivities were 7.35U/mg DW 4.9U/mg DW and 2.4U/mg DW respectively. This latter value was similar to that obtained with G in the previous experiments where the pH was not controlled (see Table I). However, this result is not surprising since activity appeared during the first 24h and the pH was controlled after this time. From these experiments it was patent that whenever pH was not controlled the activity decreased very fast and when it was controlled the activity remained at the same level until fermentation ended indicating that high pH values affect the stability of the enzyme produced.

From the above results it is clear that a true synthesis of the exo-pectinase occurs in *Aspergillus sp.* growing in the absence of pectic substances, thus indicating the constitutive nature of this enzyme. The fact that the activity liberated from the spores is only 16°/o of the lowest values obtained supports this idea (see Table I). In all cases cell growth reaches a maxium, after which a decrease is observed. This fact correlates with the consumption of the carbon source (see Fig. 3A and B) and is probably due to cell lysis at high pH values. Cell growth and consequently the rate of substrate consumption is greatly stimulated by the addition of YE (see Fig. 3A and B). However, this stimulation is not the same for all the carbon sources tested. This effect could be associated to a different stimulation capacity of the assimilation pathways for these carbohydrates as in the case of *Aspergillus nidulans* growing in the presence of nitrate (Dijkema et al, 1986).

The production of this constitutive activity in the absence of pectic substances appeared to be strictly correlated to the active growth phase (Fig. 1). This correlation was also observed when the pH of the culture medium was maintained at 3.5 with G as carbon source (Fig. 3).

The constitutive exo-activity appeared to be highly sensitive to pH values near neutrality, since the activity of *Aspergillus sp.* growing on G with controlled pH remained constant, whereas the activity with normally evolving pH decreased considerably (see Fig. 1-3). A similar effect was observed with GA under pH control. However, increasingly higher GA concentration produced a decrease in exo-pectinolytic activity suggesting a repression-derepression effect. This fact is supported by the different levels of specific activity obtained depending on the carbon source (see Table I).

The higher specific activity obtained with GA, PGA and P also suggests an autocatalytic induction of this or other exo-pectinases due to the presence of these substrates as occurs with *Botrytis cinerea* (Leone and Van der Heuvel, 1987).

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CAPITULO 4

Conidial and mycelial bound exo-pectinase of *Aspergillus* sp.

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Conidial and mycelial-bound exo-pectinase of *Aspergillus* sp.

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Abstract: Intact conidia of *Aspergillus* sp. were able to degrade pectin 'in vitro' even when protein synthesis was inhibited, thus indicating the presence of cell bound pectinases. At least an exo-pectinase was found and this enzyme was also present in the mycelium of *Aspergillus* sp. Its presence was not dependent on the carbon source used for growth, suggesting its constitutive nature. This exo-pectinase could be released from conidia or mycelium by incubation at different pH values and the amount of enzyme released could be increased by treatments with chemical agents and hydrolytic enzymes.

Key words: Cell-bound exo-pectinase; Constitutive; *Aspergillus*; Enzyme release

Introduction

Pectinases have increased applications in the food industry; they also play an important role in the degradation of cell wall material in plant pathogenesis and have been associated with fruit development, ripening and cell wall extention [1,2]. In this context, the cellular localization of the different enzyme systems as well as their participation in the degradation of natural polysaccharides could help to understand the phenomena associated with microbial invasion to plant tissues or with the colonization of roots during symbiotic association of nitrogen fixing microorganisms [2–4].

Some enzymes and complex enzyme systems have been characterized and their cellular location established, as is the case of *Trichoderma reesei* cellulases [5–6]. The presence of a constitutive cell bound β-glucanase, which is present in conidia and mycelium of this fungus, has been reported [7,8]. This enzyme is responsible of initiating the 'in vitro' cellulose degradation and it is implicated in the induction of the other components *T. reseei*'s cellulolytic system [6]. On the other hand, Verhoeff [9] reported that the degradation of pectin by *Botrytis cinerea* is initiated by the action of pectinases located at the conidial surface. The presence of conidial bound pectinases has also been suggested by other authors [10–12].

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In previous works, we have reported the production of extracellular pectinases by the fungus *Aspergillus* sp. [11,13,14] as well as the production of a constitutive exo-pectinase [12]. We have found that when *Aspergillus* sp. is grown on pectin as carbon source pectin degradation products accumulate during the first hours of fermentation (lag phase). The decrease in the concentration of these products occurs at the begining of the exponential phase of growth. It has been suggested that the generation of these products is due to the presence of conidial pectinases [11,12]. Evidence on the presence of an exo-pectinase bound to the intact conidia and to the mycelium of *Aspergillus* sp. is shown in this work.

Material and Methods

Microorganism and culture conditions

The strain *Aspergillus* sp. [11–13] was used in this work. Conidia were harvested from a culture grown on potato dextrose agar (50 ml in 250 ml Erlenmeyer flasks) for 3 days a 37°C.

In order to evaluate the effect of the carbon source on the presence of the conidial pectinase *Aspergillus* sp. was grown on basal solid medium [13] to which the desired carbon source was added. The carbon sources were: citrus pectin (P), galacturonic acid (GA), glucose (G) or glycerol (Gl), all at a final concentration of 1% (w/v). After 3 days incubation, 10 ml of distilled water were added to each flask to make a conidial suspension. This suspension was filtered through 3 layers of miracloth filters, microscopically checked for the absence of mycelium and centrifuged at 500 rpm for 10 min. The packed conidia were repeatedly washed with isotonic sodium chloride solution containing cycloheximide (125 μg/ml) until no absorbance was detected at 280 nm. The washed conidia were resuspended with distilled water containing cycloheximide and used immediately for the determination of its pectinolytic activity or for chemical or enzymatic treatments.

Mycelium was obtained from liquid growing cultures of *Aspergillus* sp. in 500 ml Erlenmeyer flasks containing 200 ml of basal medium and 1% citrus pectin as carbon source. Flasks were inoculated with a conidial suspension (1×10^6 spores/ml, final concentration) and incubated at 37°C and 200 rpm for 36 h. After this time the mycelium was harvested by filtering the culture through Whatman 1 filter paper and washed using the same procedure as for the conidia. Mycelium was resuspended with distilled water containing cycloheximide and used immediately for the analyses.

Activity assays

Pectinolyitc activity was determined by measuring the increase in reducing groups from pectin by the DNS method [15] and expressed as galacturonic acid. The reaction mixture contained 1 ml of 1% pectin solution, and either 1 ml of conidial supension (1×10^8 conidia/ml), or 1 ml mycelial suspension (1.4–1.5 mg DW/ml) or 1 ml of supernatant or enzymatic cell-free filtrate. The reaction mixture was incubated at 45°C for 1 h unless otherwise stated. After incubation 3 ml of DNS solution were added to each sample and the samples were placed in a boiling water bath for 5 min, diluted properly and centrifuged at 5000 rpm for 10 min. The optical density of the supernatant of each sample was measured at 550 nm. In all cases boiled inactivated samples were used as blanks.

Chemical and enzymatic treatment of cells

One ml of either conidial or mycelial suspensions was centrifuged and resuspended in 1 ml 0.17 M acetate buffer, pH 5. To this suspension one ml of the releasing chemical agent or hydrolytic enzymes were added and the mixture was incubated at 37°C for 1 h unless otherwise stated. The chemical agents used were, sodium deoxycholate, sodium dodecyl sulphate (SDS), cetiltrimethyl-ammonium bromide (CTAB), Tween 80 and Triton X-100, all at a final concentration of 0.5%. The enzymatic treatments were carried out with an alkaline protease (Alcalase, Novo Industri A/C, Denmark) and with lytic enzymes from *Trichoderma harzianum* (Sigma, Chememical, USA) at a final concentration of 0.033 Andson U/ml and 5 mg/ml, respectively. After treatment samples were centrifuged and the supernatants collected. Cell packages were

resuspended by vortexing with 1 ml of the corresponding chemical agent and centrifuged again, the resulting supernatant was collected and mixed with those previously obtained and used so for the pectinolytic activity determination. The cell packages were washed twice by centrifugation with 0.17 M acetate buffer and resuspended on the same buffer and used immediately for the determination of the residual activity. Cycloheximide was used to inhibit protein synthesis during all treatment and enzymatic assays.

Other analytical methods

Protein concentration and reducing groups in the culture medium were determined by Lowry [16] and DNS methods [15], respectively.

Results and Discussion

As can be seen in Table 1 non-germinated conidia of *Aspergillus* sp. exhibit pectin degrading activity. The only activity detected was that of exo-pectinase; assays for endo-pectinase and pectin lyase were negative. Activity was present in conidia incubated in the presence and absence of cycloheximide, suggesting a pre-formed enzyme, probably synthezised during the sporulation stage. Since conidia were extensively washed it seems that the activity is bound to the surface of the conidial wall. Without cycloheximide, conidia exhibit slightly higher activity compared to the conidia incubated with cycloheximide, probably due to 'de novo' protein synthesis during germination.

Table 1

Exo-pectinolytic activity from conidia of *Aspergillus* sp. assayed in the presence or absence of cycloheximide at different times [a]

Condition [b]	Exo-Activity (U/10^8 conidia)		
	1 h	3 h	20 h
Control	0.722 ± 0.019	1.028 ± 0.055	1.040 ± 0.056
Control + Cycloheximide	0.687 ± 0.031	0.840 ± 0.040	0.850 ± 0.020

[a] Incubation was carried out at 37°C during 20 h using pectin as substrate. Results are the mean of four experiments (± SD); [b] Conidia were harvested and washed as described in the Materials and Methods section. Cycloheximide was added at a final concentration of 125 µg/ml in the reaction mixture.

Table 2

Exo-pectinolytic activity from conidia produced by *Aspergillus* sp. grown on different carbon sources [a]

Carbon source	Exo-Activity (U/10^8 conidia) [b]
Potato dextrose agar	0.703 ± 0.073
Pectin	0.474 ± 0.035
Galacturonic acid	0.496 ± 0.020
Glucose	0.450 ± 0.083
Glycerol	0.435 ± 0.047

[a] *Aspergillus* sp. was grown at 37°C during 72 h in Erlenmeyer flasks containing basal medium with the indicated carbon source added; [b] Activity was measured at 45°C for 1 h. Results are the mean of at least four experiments (± SD)

On the other hand, when incubated conidia were centrifuged after 20 h the activities in the supernatant fractions were 0.11 and 0.26 U/ml for the supernatant of conidia incubated in presence and absence of cycloheximide, respectively, thus supporting the 'de novo' synthesis and indicating that the enzyme was released, at last partially from conidia.

Since the exo-pectinase appeared to be formed during sporulation, the effect of different carbon sources on the levels of conidial exo-pectinase was evaluated. *Aspergillus* sp. was grown on media containing either P, GA, G, or Gl, as carbon sources (Table 2). In all cases the exo-pectinase was present irrespective of the carbon source used for growth, suggesting the constitutive nature of the enzyme.

Since conidial exo-pectinase is released during incubation, the effect of some chemical agents and hydrolytic enzymes was evaluated (Table 3). The enzyme was released from conidia with all the chemical agents evaluated. The use of SDS and CTAB results in a 23 and 26% inhibition of the enzyme activity. With sodium deoxycholate 60% of enzyme was released (0.566 U, compared to 0.098 U of the control). This amount of released enzyme represents more than 5 times higher activity than that released in the control.

The use of enzymes in the treatment of conidia results in a release of 70% with alkaline protease and 86% with cell wall lysing enzymes. Alkaline protease was used since its activity at pH of 5.0 is low and it would be possible to achieve a limited proteolysis which would not affect enzyme activity. The activity was lost with other proteolytic enzymes (microbial acid proteases). These results indicated that the enzyme is located at the external surface of the conidial wall.

Mycelium of *Aspergillus* sp. grown on pectin was treated in the same way as conidia (Table 3). Appoximately 39% of the enzyme was released by incubation of mycelium in buffer at pH 5, in agreement with our previous results [14]. As in the case of conidia, 63% of enzyme release is attained with sodium deoxycholate. Triton X-100 and Tween 80 had a similar effect on mycelium as that on conidia, a 66% and 65% release was obtained with these compounds, respectively. Inhibition by SDS and CTAB was also higher for mycelial exo-pectinase. With hydrolytic enzymes similar results to that of conidia were obtained (Table 3). When conidia or mycelium were treated with hydrolytic enzymes a higher activity was detected in the supernatant fraction. Since lytic enzymes contain pectin degrading activity, control tubes were assayed in parallel and the activity obtained in these tubes was subtracted to the corresponding test tubes. The pectinolyitc activity of the lytic enzymes, alone and mixed with culture filtrates of known activity was also evaluated. It was found that the activity obtained in the mixture was practically the same to that of the sum of each separate sample. No synergistic effect was found between the exo-pectinase produced by *Aspergillus* sp. and that of the lytic mixture. It is apparent then that the enzyme is more highly active in free form than when it is bound to the cell.

Table 3

Effect of various chemical and enzymatic treatments on exo-pectinase release from conidia and mycelium of *Aspergillus* sp.

Treatment [b]	Exo-pectinase activity [a]			
	Conidia		Mycelium	
	Cells	Supernatant	Cells	Supernatant
Control [c]	0.698	0.098 (12)	1.536	0.968 (39)
Sodium deoxycholate	0.377	0.566 (60)	0.866	1.448 (63)
SDS	0.330	0.283 (46)	0.565	0.539 (59)
CTAB	0.165	0.424 (72)	0.296	0.350 (54)
Triton X-100	0.472	0.424 (47)	0.781	1.536 (66)
Tween 80	0.594	0.147 (20)	0.701	1.296 (65)
Protease	0.472	1.085 (70)	0.928	1.680 (64)
Lytic enzymes	0.507	3.144 (86)	0.842	3.510 (81)

[a] The activity is expressed as $U/10^8$ conidia and U/mg DW for conidia and mycelium, respectively. The numbers in brackets indicate the percent of released activity in relation to the total for each case; [b] Conidia or mycelium were incubated for 1 h at 37°C in the presence of the releasing agent; [c] Control was either conidia or mycelium suspended in 0.17 M acetate buffer at pH 5.0. Data are from single experiments only, but repetitions yielded consistent results.

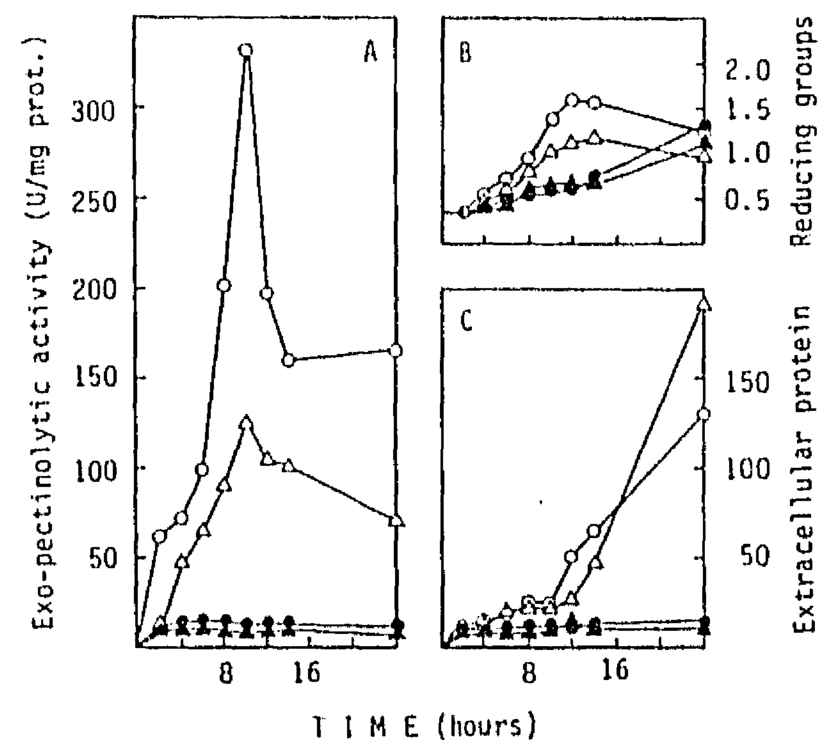

Fig. 1. Exo-pectinolytic activity (A), reducing groups (B) and extracellular protein (C) produced by *Aspergillus* sp. growing on 1% pectin at initial pH values of 3.5 (○, ●) or 4.2 (△, ▲). Cycloheximide (125 μg/ml) added (●, ▲); and without cycloheximide (○, △). Reducing groups and extracellular protein are expressed in mg/ml and μg/ml respectively.

The kinetics of production/release of the exo-pectinase is shown in Fig. 1. *Aspergillus* sp. was grown on pectin at pH values of 3.5 and 4.2, in the presence or absence of cycloheximide. The activity was detected from 2 h of culture in cell free filtrates and increased throughout the fermentation (Fig. 1A). Very low activity was de-

tected when cycloheximide was added and it remained constant. This activity found in the culture filtrate was due to the release of exo-pectinase from *Aspergillus* sp. conidia since no protein synthesis was occurring. Exo-pectinase specific activity reached a maximum at 10 h of fermentation at both pH values. Since the activity in the cell free filtrates from cultures containing cycloheximide was not due to the release of enzyme, the increased activity found in cultures without cycloheximide was due to a true synthesis of the enzyme. The increase in enzyme activity was consistent with an increase in extracellular protein (Fig. 1C) as well as with cell growth.

Cell growth and extracellular protein concentration were similar at both pH values. However the activity was higher at pH 3.5 (Fig. 1A).

As expected, no growth was obtained when cycloheximide was added to the cuture medium. However, an increase in reducing groups was observed (Fig. 1B). This increase in pectin degradation products was not due to a non-specific degradation of pectin by culture conditions, since non-inoculated cultures did not show an increase in these products (less than 0.3 mg/ml) after 120 h incubation at 37°C, as previously demonstrated [14].

When cycloheximide was not added, a higher increase in reducing groups was obtained which reached a maximum at 12 h fermentation. After this time a reduction in the concentration of these groups was observed since the rate of uptake of these compounds increased because the fungus had begun to grow at exponential rate.

From the above results it is clear that ungerminated conidia of *Aspergillus* sp. exhibit exo-pectinase activity even in the presence of cycloheximide, demonstrating a preformed conidial exo-pectinase. The fact that the enzyme could be released from conidia by treatment with ionic and non-ionic detergents indicates that the enzyme is partially bound by ionic interactions to the conidial wall. However, since longer treatments with these compounds did not release all the enzymes (data not shown) from conidia, it is believed that an amount of the enzyme is tightly bound or immersed in the cell wall matrix. This hypothesis is supported by the fact that higher amount of enzyme was released with wall-lytic enzymes. The enzyme was found not only in conidia but also in the mycelium of *Aspergillus* sp. and, as in conidia, it could be released by incubation with buffer or treatment with detergents or hydrolytic enzymes. The exo-pectinase appeared to be produced constitutively, irrespective of the carbon source used for conidiation.

On the other hand, it is not posible to state with the present data the nature of linkage of exo-pectinase to the cell wall. However, it is possible to conclude that the enzyme is bound to the external surface of cell wall, since it is sensitive to microbial acid proteases and it could be released with enzyme preparation containing β-glucanase activity. Similar results have been reported for CM-cellulase and β-glucosidase from *T. reseei* [5–7] and for invertase and trehalase from *N. crassa* [17]. It is possible that the exo-pectinase plays an important role in the generation of inducers for the other enzymes of *Aspergillus* sp. pectinolytic complex however this appreciation needs further research.

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CAPITULO 5

Discusión General

DISCUSION GENERAL

De los resultados anteriores es claro que el pH tiene un efecto muy marcado sobre la producción de las pectinasas extracelulares de *Aspergillus* sp. CH-Y-1043. En términos generales, la producción de la endo-pectinasa es la más afectada. Esta enzima se produce en un intervalo de pH de 2.5 a 4.2 obteniéndose un máximo a pH de 2.5.

Por otro lado, la exo-pectinasa es producida en un intervalo más amplio de pH y su producción no cambia tan drásticamente como la de la endo-pectinasa aunque tiende a disminuir conforme el pH aumenta. La máxima producción para esta enzima también se alcanza a pH de 2.5.

La producción de estas enzimas no solo depende del pH del medio, también se ve afectada por el tipo de pectina utilizada como fuente de carbono y particularmente, de su grado de esterificación. Utilizando pectinas de bajo grado de esterificación (P-47, Cap. 2) la endo pectinasa no se produce a valores de pH de 4.2 o mayores, mientras que con pectina de alto grado de esterificación (60%), la actividad se logra obtener hasta en pH de 5.2, aunque es menor a la obtenida a pH más ácidos.

La producción de exo-pectinasa, por el contrario, parece verse favorecida por el uso de pectinas de bajo grado de esterificación ya que su producción es ligeramente mayor con el tipo P-47 que con la P-60 (Cap. 2). Sin embargo, el efecto no es muy pronunciado.

Por otro lado, cuando se utiliza ácido galacturónico o poligalacturónico como fuentes de carbono, solo se produce la exo-pectinasa (Cap.3), independientemente del pH inicial del medio de cultivo. Estos hechos sugieren, por un lado, que la inducción de la endo-pectinasa está mediada por la presencia de derivados metoxilados generados por la degradación de la pectina durante la fermentación, y por el otro, que la exo-pectinasa se induce por la presencia de ácido

galacturónico que es uno de los productos finales de la degradación de la pectina.

A los diferentes valores de pH evaluados el crecimiento es similar con excepción de pH 2.5, condición en la cual, el crecimiento celular se reduce alrededor de un 50% en relación a lo obtenido a valores de pH más altos. Este hecho es particularmente importante ya que a 2.5 de pH se alcanza la máxima producción tanto de endo como de exo-pectinasas independientemente del tipo de pectina que se utilice como fuente de carbono.

En este sentido, el hecho de que el mejor pH de producción sea de 2.5, que es un valor muy ácido, tiene ventajas, ya que se reducen las posibilidades de contaminación, particularmente por bacterias, pues la mayoría de estas tienen serios problemas para crecer. *Aspergillus* sp. no solo produce actividad pectinolítica en estas condiciones, sino que produce la mayor actividad no solo en unidades volumetricas, sino en U/Unidad de biomasa lo que habla de una gran eficiencia de síntesis y/o secreción de enzimas a valores extremos de pH.

Encontramos que la ausencia de endo-pectinasa cuando el pH del medio es superior a 4.2 no se debe a pérdida de actividad de la enzima a estos valores de pH, que harían que la enzima no pudiera ser detectada en los ensayos de actividad, o a que la secreción de la enzima estuviera limitada a estos pH's, o que ésta se acumulara en el micelio de este microorganismo. La evaluación de la estabilidad de la enzima a diferentes valores de pH indicaron que su estabilidad es muy alta, reteniendo el 89 y el 81% de su actividad a pH de 2.5 y 6.2, respectivamente, después de 120 h de incubación a 37°C. Así mismo, encontramos que la endo-pectinasa tampoco es acumulada o retenida por el micelio de *Aspergillus* sp. ya que en experimentos con micelio lavado e incubado en medio basal no se logra liberar a la enzima y la actividad no se detecta ni en la fracción del sobrenadante ni en la célula completa u homogeneizada (Cap. 2).

Lo anterior indica el efecto del pH es sobre la biosíntesis de la enzima más que sobre

problemas de baja estabilidad o limitaciones en la secreción. Análisis, en geles de poliacrilamida, de filtrados libres de células provenientes de diferentes condiciones de pH, mostraron que no hay diferencias en los perfiles de bandas de bajo peso molecular entre estas muestras indicando que la endo-pectinasa tampoco es degradada en las condiciones que se evaluaron.

Por otro lado, la estabilidad de la exo-pectinasa es más reducida ya que ésta pierde hasta el 45% de su actividad a pH de 2.5. Esta enzima si es acumulada en el micelio y puede ser liberada cuando se incuba a diferentes valores de pH y se encuentra tanto en el micelio como en la fracción del sobrenadante. Indicando que al menos cierta cantidad de la enzima se acumula en el micelio para ser secretada posteriormente al medio de cultivo. Análisis electroforético de los sobrenadantes obtenidos de la incubación de células lavadas mostraron un grupo de bandas en la zona de 66 kDa sugiriendo que la exo-pectinasa se encuentra dentro de este grupo de bandas.

Por otro lado, los resultados también demuestran que *Aspergillus* sp. puede producir una exo-pectinasa en ausencia de materiales pécticos lo que sugiere la naturaleza constitutiva de esta enzima. Es decir, la exo-pectinasa detectada cuando *Aspergillus* sp. es crecido en glucosa, fructosa, sacarosa, glicerol o extracto de levadura, oscila entre el 26 y el 75%, en relación a la producida en pectina, indicando que la presencia de la enzima se debe a síntesis "de novo", ya que, además de que la producción aumenta durante la fermentación, la actividad liberada por las esporas solo corresponde a un 4% de la actividad que se obtiene cuando *Aspergillus* sp. es crecido sobre pectina (Cap. 3). La exo-pectinasa producida en ausencia de materiales pécticos también es susceptible al pH del medio de cultivo, lo que correlaciona con los resultados previos (Cap. 2) y sugiere que se trata de la misma enzima y que esta enzima es

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la primera que se produce y que pudiera estar involucrada en la generación de inductores para las producción, de por lo menos la endo-pectinasa.

Se encontró que tanto las esporas como el micelio de *Aspergillus* sp. presentan actividad degradativa sobre pectina (Cap. 4). Los ensayos de actividad de esporas no germinadas demuestran la presencia de actividad de tipo exo. Esta actividad no se debe a síntesis "de novo" de la enzima durante la incubación ya que la actividad está presente aún cuando se inhibe la síntesis de proteína (Cap. 4).

La exo-pectinasa de las esporas puede ser liberada por tratamiento con agentes químicos o enzimas hidrolíticas, sugiriendo que esta enzima se encuentra localizada en la superficie externa de las esporas. Esta enzima también se encontró en el micelio de *Aspergillus* sp. y su liberación es similar a lo observado con las esporas.

Su presencia en las esporas no depende de la fuente de carbono ya que la actividad esta presente independientemente de la fuente de carbono utilizada para el crecimiento, indicando que se trata de una enzima constitutiva. Hallazgos similares se han observado para otras enzimas como, CM-celulasa, β-glucosidasa, invertasa y trealasa [29-32].

La exo-pectinasa de las esporas participa en la degradación de la pectina durante la fase lag de crecimiento del microorganismo, lo que ocasiona una acumulación de los productos de degradación cuya disminución coincide con la fase exponencial de crecimiento (Cap. 4).

En relación a la identificación de los diferentes componentes del sistema pectinolítico de *Aspergillus* sp. se encontró que está formado por lo menos de, endo-pectinasa, exo-pectinasa, pectin esterasa y pectin liasa. La endo-pectinasa y la pectin esterasa fueron identificadas por electroforésis en geles de poliacrilamida acoplada a ensayos de actividad "in situ". La endo-pectinasa corresponde a una banda de proteína con una masa molecular de 43 kDa aproximadamente, mientras que la pectinesterasa corresponde a una proteína de 33 kDa

(Cap.2). Mediante este sistema de detección fue posible también identificar otras 2 bandas de proteína con actividad que migran en la zona de 66 kDa y que muy probablemente corresponden a la exo-pectinasa y a la pectin liasa (Trejo, resultados no publicados). Finalmente y en relación a la identidad de la exo-pectinasa presente en las esporas y el micelio, con aquella producida extracelularmente, las evidencias anteriores sugieren que se trata de la misma enzima. Por otro lado, mediante análisis por "Western Blot" se observo que anticuerpos dirigidos contra las esporas completas de *Aspergillus* sp. presentaban reactividad con una banda de proteína que migra en la zona de 66 kDa de filtrados libres de células provenientes de cultivos de *Aspergillus* sp. crecido tanto en pectina como en glucosa (Fig. 1). Estos resultados, junto con las evidencias antes presentadas sugieren fuertemente que la exo-pectinasa extracelular es la misma que la que está presente en las esporas y el micelio de este microorganismo y que es de naturaleza constitutiva. Es muy probable que esta enzima juegue un papel determinante en la generación de moléculas de bajo peso molecular que sirvan para promover el crecimiento y la inducción de los demás componentes del sistema pectinolítico de *Aspergillus* sp.

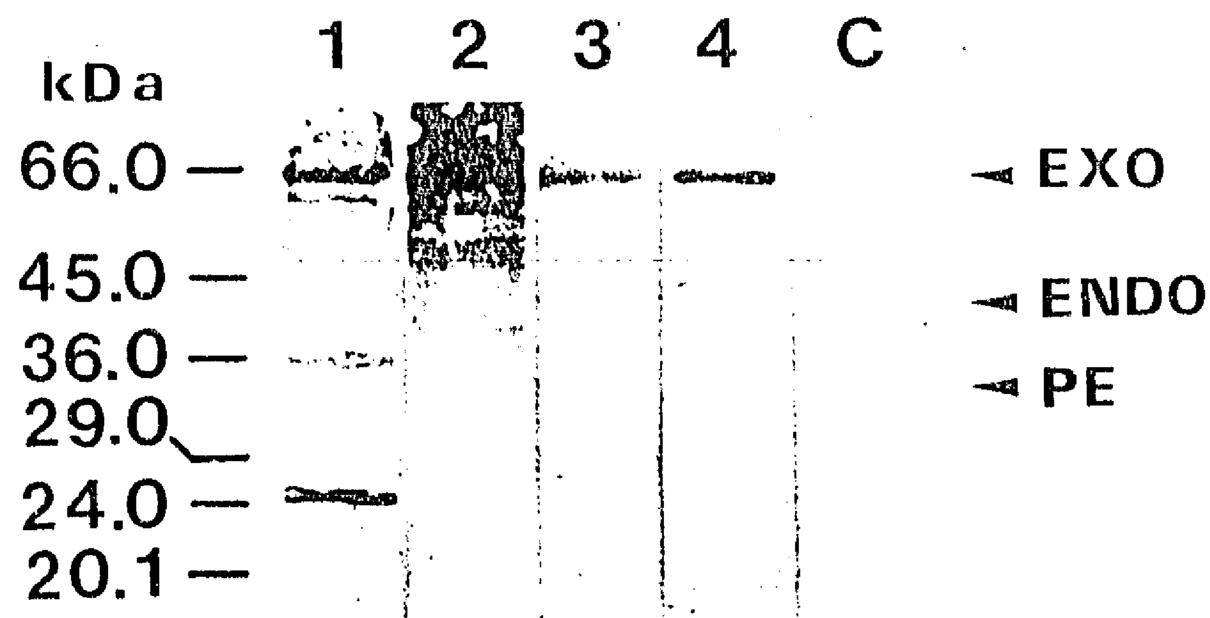

Fig. 1. Análisis por "Western blot" con anticuerpos dirigidos contra esporas intactas de *Aspergillus* sp. de extractos de esporas (Línea 1), micelio (Línea 2) y filtrados libres de células de *Aspergillus* sp creciendo en pectina (Línea 3) y glucosa (Línea 4), como fuentes de carbono. Los anticuerpos fueron obtenidos en conejos inmunizados con tres inyecciones intramusculares de esporas no germinadas y lavadas, en intervalos de una semana. Para cada inyección se utilizaron $1X10^{6}$ esporas suspendidas en 0.5 ml de solución salina isotónica y fueron mezcladas con 0.5 ml de adyuvante completo de Freund. La sangre fue colectada después de 7 días de la última inmunización y el suero fue separado y precipitado con sulfato de amonio. Las inmunoglobulinas precipitadas fueron resuspendidas en buffer fosfato y dializadas contra el mismo buffer durante una noche a 4°C, y conservadas en congelación. La electroforesis fue realizada por el método de Laemmli[33], en un gel de 10% de acrilamida. Después de la electroforesis, las proteínas separadas, fueron transferidas a membranas de nitrocelulosa de acuerdo al método de Towbin[34], para posteriormente hacerlas reaccionar con los anticuerpos anti-esporas. Como anticuerpo secundario se utilizó anticuerpo anti-conejo acoplado con peroxidasa. El extracto de esporas fue obtenido tratando esporas de *Aspergillus* sp. con enzimas líticas de *Trichoderma harzianum* durante dos horas a 37°C en buffer acetato 0.17 M a pH de 5, la mezcla de reacción fue filtrada y el filtrado fue dializado, concentrado por liofilización y utilizado así para la electroforesis. El extracto de micelio fue preparado de igual forma que el de esporas, utilizando micelio crecido durante 36 horas en medio basal conteniendo glucosa como fuente de carbono, adicionado de extracto de levadura. Las enzimas líticas de *T. harzianum* utilizadas en la preparación de los extractos fueron usadas como control (C). PE, pectin esterasa; ENDO, endo pectinasa y EXO, posición propuesta para la exo pectinasa.

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ANEXO 1

Pectin lyase from Aspergillus sp. CH-Y-1043.

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Como parte de las actividades realizadas dentro del programa de doctorado y, específicamente en lo que se refiere a la formación recursos humanos, se asesoró la tesis de un estudiante de licenciatura que dio lugar a la publicación de un trabajo en una revista de circulación internacional, la cual se presenta en este anexo.

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Pectin lyase from *Aspergillus* sp. CH-Y-1043

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Abstract. *Aspergillus* sp. CH-Y-1043 synthesizes pectin lyase when grown on citrus pectin at 37° C. Production is favoured by increased esterification degree of the pectin used as carbon source. This enzyme displays higher activity at pH values of 8.5–8.8 and temperatures of 40–45° C. The optimal substrate for the enzyme was highly esterified pectin and no enzymatic activity was registered on polygalacturonic acid. The activity is stimulated by, though not dependent on, divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+} and Co^{2+}) and inhibited by Zn^{2+}, and it is not sensitive to the addition of EDTA. The enzyme is very stable when exposed to pH variations: at 4° C it preserves more than 95% of its activity at pHs ranging from 2.0 to 10.0, and at 30° C stability is preserved at pHs ranging from 4.0 to 8.0. At a constant pH of 5.0, the enzyme conserves its stability at temperatures ranging from 4 to 50° C and at pH 8.0 sensitivity to temperature increased. The results on the endo-exo nature of the enzyme suggest that this is an exo-pectin lyase.

Introduction

Pectin lyases, capable of breaking the α-1,4 glycosidic bonds of pectin through trans-elimination, were discovered by Albersheim et al. (1960). Nowadays, these enzymes are classified according to the substrate of preference in two groups: pectate lyases, which are polygalacturonic acid specific, and pectin lyases, which act on pectin. Pectate lyases are mostly produced by bacteria and have alkaline optimal pH values, normally from 8.0 to 10.0 (Whitaker 1972; Fogarty and Kelly 1982). These kinds of enzymes are absolutely dependent on Ca^{2+} (Whitaker 1972; MacMillan and Vaughn 1964) and both the endo- (endo-pectate lyase: EC: 4.2.2.2) and the exo-type (exo-pectase lyase; EC: 4.2.2.9.) have been described.

In contrast to pectate lyases, pectin lyases are mostly synthesized by fungi. Optimal pH varies according to the genus. Thus, for *Aspergillus* and *Penicillium* it is between 5.2 and 6.0, whereas in *Sclerotia fructigena* and *Fusarium solani* it is 7.3 and 8.6, respectively (Fogarty and Kelly 1982). These enzymes do not require Ca^{2+} for activation. Nevertheless, Ca^{2+} and other ions may affect activity depending on the pH and on the degree of substrate esterification (Fogarty and Kelly 1982). All pectin lyases described to date are of the endo-type (endo-pectin lyase; EC 4.2.2.10). Exo-pectin lyase has not been identified.

Since pectin lyases are able to act on highly esterified pectins with no previous pectinase action, their use may be advantageous because, in the absence of methanol, the volatile esters responsible for the specific scent of fruits are not affected. Additionally, the use of pectin lyase may reduce the flocculating effects that appear when mixtures of pectinases containing polygalacturonases and pectin esterase, which in turn generate de-esterified pectin derivatives, come in contact with endogenous fruit Ca^{2+}, modifying juice quality (Fogarty and Kelly 1982; Alaña et al. 1989).

Our laboratory has been concerned with the pectinolytic system of *Aspergillus* sp. CH-Y-1043. This microorganism synthesizes hydrolytic endo- and exo-pectinase (Aguilar and Huitrón 1986, 1987, 1990; Larios et al. 1989; Solís et al. 1990) as well as a pectin esterase (Aguilar et al. 1991) when grown on pectin or agroindustrial by products. However, our strain is also capable of producing lyase activity, which increases its possible application. The present work describes our findings on some of the properties of pectin lyase produced by *Aspergillus* sp. CH-Y-1043, grown on citrus pectin.

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Materials and methods

Microorganism. *Aspergillus* sp. CH-Y-1043 was isolated in our laboratory. It was transferred and maintained on agar slants containing potato dextrose agar, as described previously (Aguilar and Huitrón 1987).

Pectin lyase production. As previously reported (Aguilar and Huitrón 1987), basal medium was employed, with added 47% or 60% esterified citrus pectin (Sigma, St. Louis, Mo., USA) to a concentration of 1.0% (w/v). Fermentation was performed in shake flasks at 37° C. The initial pH was 3.5 with constant shaking at 200 rpm in a rotary shaker (New Brunswick Scientific, USA). Samples were taken from flasks at various times during fermentation and were immediately filtered through a Millipore membrane. The clear cell-free filtrate was used for all the assays.

Analysis of lyase activity. Lyase activity was determined by monitoring the increase in absorbance at 235 nm (A_{235}) brought about by the appearance of the double bond between carbon atoms 4 and 5. The reaction mixture contained 1.0 ml of 1.0% substrate, 1.0 ml of 0.05 M TRIS-acetate buffer, pH 8.8, and 0.5 ml culture filtrate and was incubated at 40° C for a predetermined length of time. One 0.5-ml aliquot was taken from the reaction mixture and added to a test tube containing 4.5 ml of 0.01 M HCl to stop the reaction. Control tubes contained the enzymatic filtrate previously inactivated by boiling for 15 min. Substrates were pectins of different degrees of esterification and polygalacturonic acid. One unit (U) of lyase activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1 at 235 nm in the reaction mixture under the assay conditions.

Stability. The effect of pH on the stability of lyase activity was evaluated by incubation of the enzymatic filtrate at different pH values and at temperatures of 4° C and 30° C during 24 h. The buffers used were: 0.1 M Clark and Lubs for pH 1.0; 0.1 M HCl-sodium acetate for pH 2.0–3.0; 0.17 M Walpole's acetate for pH 4.0–6.0; 0.1 M McIlvaine for pH 3.0–8.0; 0.05 M TRIS-acetate for pH 7.0–9.0 and Sorensen glycine for pH 9.0–11.0 (Bates and Paabo 1976). To test stability at different temperatures the filtrate was incubated at 4, 30, 40, 50, 60 and 70° C for 1 h at pH 5.0 and 8.0. After this time, residual activity was determined in each sample.

Reduction in viscosity and percentage hydrolysis. The endo or exo nature with which the enzyme degrades the substrate was determined as described by MacMillan et al. (1964). A volume of 28 ml at a final concentration of 60% esterified 0.93% (w/v) pectin in 0.05 M TRIS-acetate buffer at pH 8.8 and 2.0 ml of enzymatic filtrate comprised the reaction mixture. The assay was carried out at 30° C.

Results and discussion

Pectin lyase production

Aspergillus sp. CH-Y-1043 produced pectin lyase activity when grown on pectin (Fig. 1). The activity was highest when 60% esterified pectin (P-60) was used (Fig. 1B) although cell growth on this carbon source was not as good as on 47% esterified pectin (P-47) (Fig. 1A). The specific activity at 72 h growth was 35% higher with P-60 than with P-47, indicating that production was favoured at an increased degree of pectin esterification. This was also true for the endo-pectinase produced by the same strain (Aguilar et al. 1991).

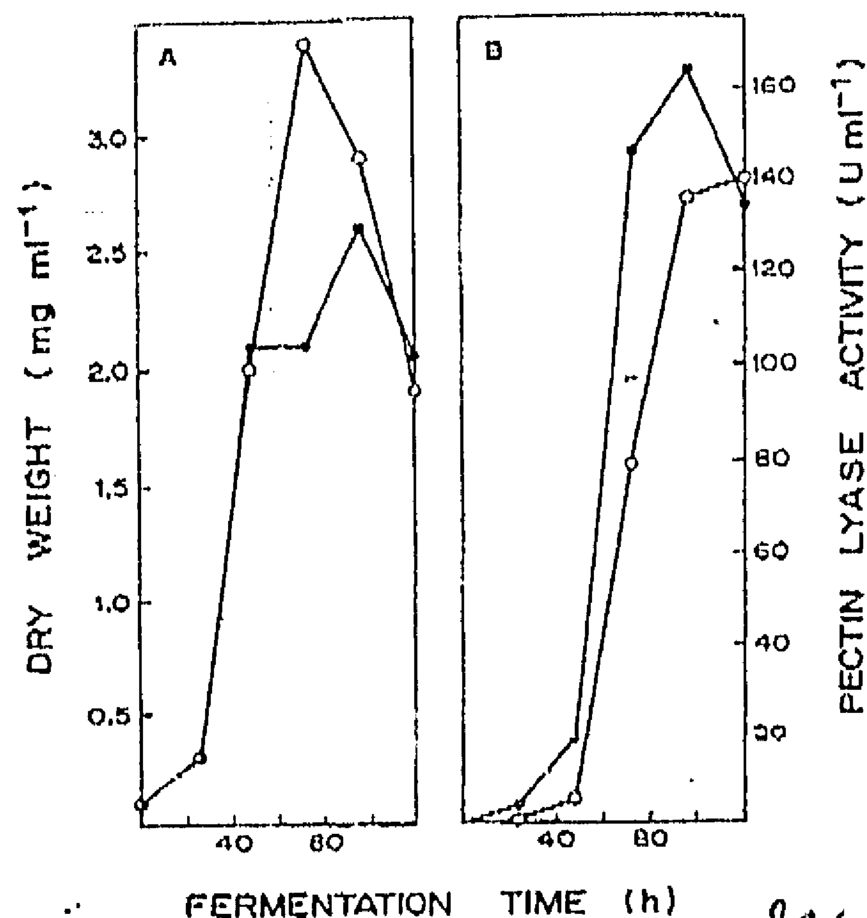

Fig. 1. Cell growth (A) and lyase activity production (B) by *Aspergillus* sp. CH-Y-1043 grown on 47% (○) or 60% (●) esterified pectin; U, units

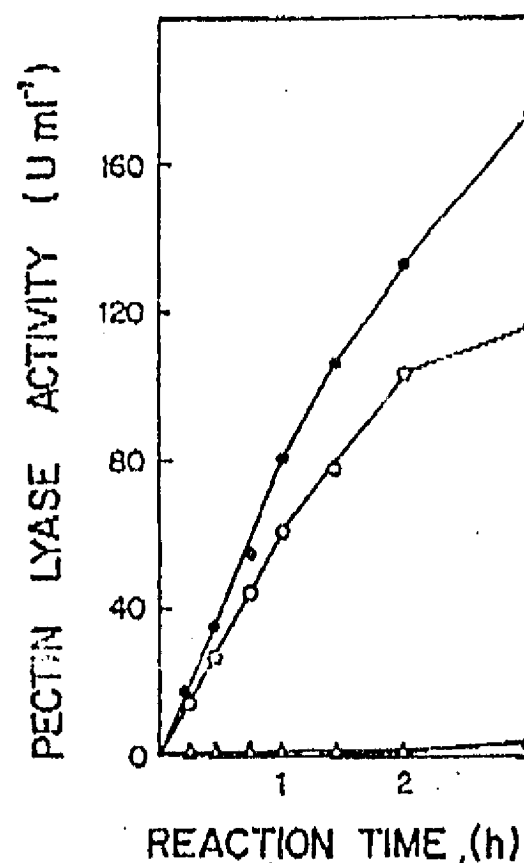

Fig. 2. Effect of substrate esterification degree on lyase activity using 60% (●) and 47% (○) esterified pectin and polygalacturonic acid (Δ). The reaction mixture was incubated in TRIS-acetate buffer, pH 8.8, at 40° C for 3 h

Effect of the esterification degree on lyase activity

Figure 2 shows that the highest activity at pH 8.8 occurred with highly esterified pectin (60%). Pectin of 47% esterification elicited 65% of that activity. With polygalacturonic acid, the activity was negligible. After the first 3 h, activity remained constant for the next 8 h

during which the reaction kinetics were followed (data not shown). Activity was also determined at pH 5.5 and the highest activity was registered with pectin of 60% esterification (data not shown). Similarly, no activity was observed with polygalacturonic acid. This indicates that we are dealing with a pectin lyase.

Effect of pH on lyase activity

The reaction mixture was incubated at pH values between 2.0 and 11.0, to determine the optimal pH for lyase activity. As shown on Fig. 3A, the optimal value lay between 8.5 and 8.8. Activity may be detected from pH 4.5. Above the optimal pH, the activity decreased rapidly; at pH 10.2 it was 90% lost and disappeared altogether at pH 10.8. The kind of buffer used did not influence the results.

The remarkably alkaline opitmal pH for *Aspergillus* sp. CH-Y-1043 lyase has not been previously reported for fungi of this genus. The highest optimal pH value for *Aspergillus* lyases was reported for *A. japonicus* (Ishii and Yokotsuka 1975), which changed from 6.0 to 7.0 on addition of Ca^{2+}.

Effect of temperature on lyase activity

The optimal temperature for lyase activity was determined by incubation of the reaction mixture at temperatures ranging from 30 to 60° C (Fig. 3B). The highest activity was observed between 40 and 45° C, and was in fact slightly higher at 45° C. Above this temperature, activity decreased rapidly, and more notably above 55° C.

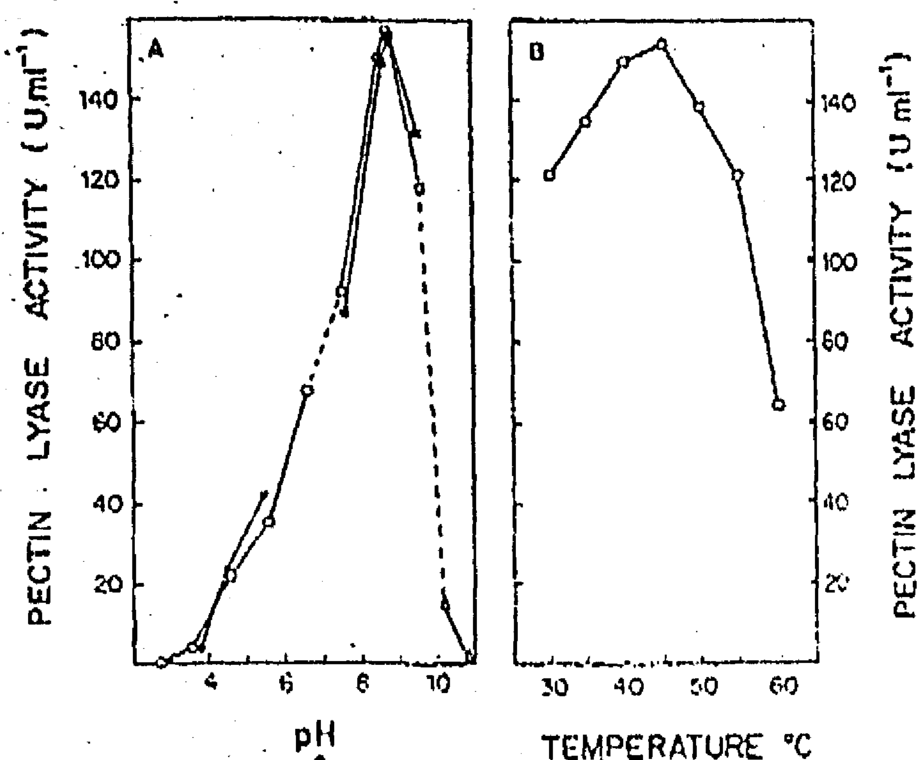

Fig. 3. Effect of pH (A) and temperature (B) on lyase activity. A The buffers used were: 0.1 M McIlvaine for pH 2.5-6.5 (O); 0.17 M acetate for pH 3.5-5.5 (●); 0.05 M TRIS-acetate for pH 7.5-9.5 (□); 0.05 M TRIS-HCl for pH 7.5-9.5 (■); 0.05 M, and 0.1 M Sorensen for pH 10.0 11.0 (Δ). The reaction mixture was incubated at 40°C for 3 h. B The activity was assayed at the indicated temperature during 3 h

At 60° C the residual activity was 40% of the maximum.

Even though activity was slightly higher at 45° C, the temperature chosen for the lyase assays was 40° C. This temperature combined with the high optimal pH value for pectin lyase activity, practically abolished the hydrolysing activity of other pectinases present in the culture filtrate. Under these conditions, substrate degradation was a product of lyase activity.

Stability

The effect of pH on stability is shown on Fig. 4A. After 24 h incubation, pectin lyase activity at 4° C was stable within the pH range 2.0–10.0, slightly decreasing at pH 11.0 where the residual activity was 85%. At 30° C the stability decreased above and below pH 4.0–8.0 with an optimal pH value at 7.0. At 30° C more that 60% activity was retained at pH 2.0, 3.0, and 11.0, and more that 75% at pH 9.0 and 10.0.

Figure 4B shows the effect of temperature on stability. At pH 5.0 pectin lyase activity was very stable within the temperature range 4–50° C; at 60° C the residual activity was 88% and at 70° C it disappeared altogether. On the other hand, at pH 8.0 activity started to decrease at 30° C; at 40° C the residual activity was 89% and at 50° C, 72%. At 60° C and 70° C the activity was not detectable.

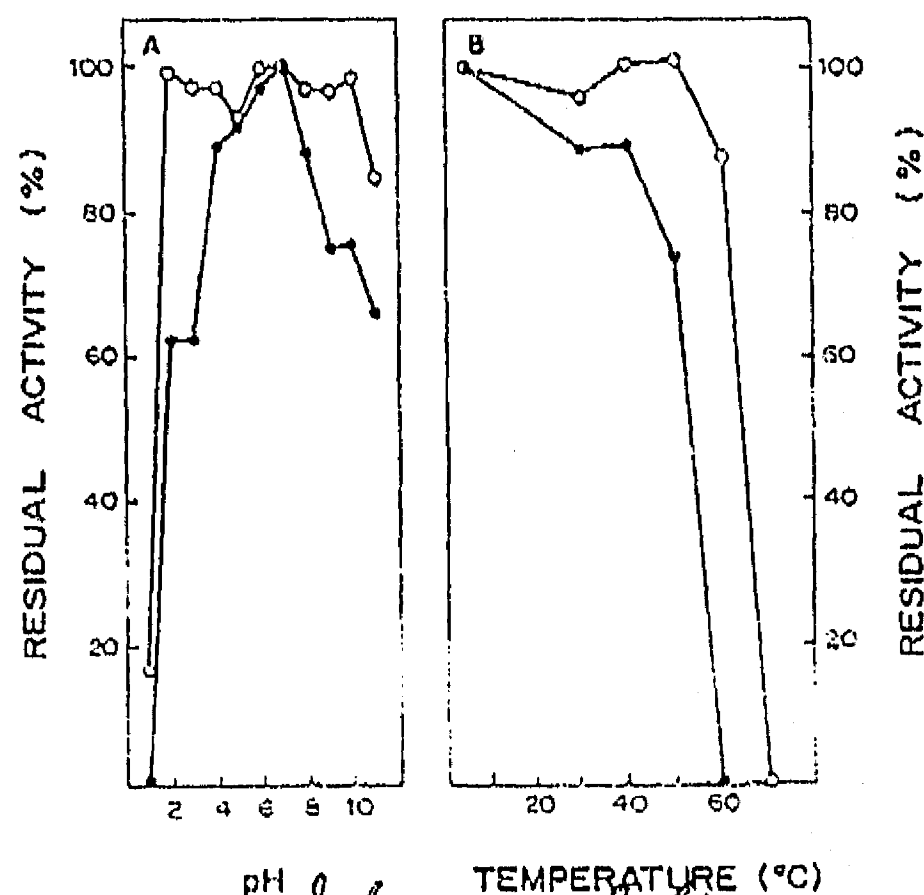

Fig. 4. Effect of pH (A) and temperature (B) on the stability of lyase activity. A For the buffers used, see Materials and methods. The filtrate was mixed with buffer and incubated at 4° C (O) and 30° C (●) during 24 h. Thereafter the activity was determined at pH 8.8 and 40° C for 3 h. B Incubation was carried out in 0.17 M acetate buffer at pH 5.0 (O) and in 0.05 M TRIS acetate buffer, pH 8.0, (●) at the indicated temperatures for 60 min. Thereafter, activity was determined at pH 8.8 at 40° C for 3 h

Effect of divalent cations on activity

The effect of Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, and Zn^{2+} on lyase activity was determined, since many lyases are Ca^{2+}-dependent (Whitaker 1972; Fogarty and Kelly 1982; Starr and Moran 1962). We also wished to test if the lack of activity of *Aspergillus* sp. CH-Y-1043 lyase on polygalacturonic acid was due to a deficiency of Ca^{2+} or other divalent cations. Thus, Ca^{2+} was added to the reaction mixture at the following concentrations: 0.04, 0.4, 4.0, 20.0 and 40.0 mM both at pH 5.5 and 8.8. The highest stimulation level was obtained at 20.0 mM Ca^{2+}, which increased activity 1.28 times at pH 5.5 and 1.78 times at pH 8.8 using pectin as the reaction substrate. No activity was detected with polygalacturonic acid at any Ca^{2+} concentration or pH value tested. In this case, the tested Ca^{2+} concentrations were 0.04 to 0.4 mM, since higher concentrations precipitated the substrate. The effects of Mg^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+} and Zn^{2+} were also tested (Table 1). Slight stimulation was obtained at pH 5.5 with Co^{2+} and Ba^{2+}; with Mg^{2+} and Mn^{2+} stimulation levels were similar to those reached with Ca^{2+}. Addition of Zn^{2+} inhibited activity by 39%. The inhibition effect of Zn^{2+} has also been reported for other lyases (Ward and Fogarty 1972). At pH 8.8 the only cations tested were Mg^{2+} and Ca^{2+}, since other cations caused substrate precipitation. Stimulation by Mg^{2+} under these conditions was 1.6 times. Stimulation was always higher at pH 8.8 than at pH 5.5, no matter if the added cation was Ca^{2+} or Mg^{2+} (Table 1) [illegible] not affect enzyme activity, but inhibited stimulation by Ca^{2+} (Table 1).

The fact that this lyase is not active on polygalacturonic acid even in the presence of Ca^{2+} or other cations confirms our previous findings and the identity of this pectin lyase, stimulated by Ca^{2+} and Mg^{2+}, but not absolutely dependent on these ions to degrade pectin.

When viscosity is reduced by about 50%, approximately 10% of the bonds are broken, as measured by the generated double bonds. According to existing literature (Fogarty and Kelly 1982; MacMillan et al. 1964; Tam 1983) this reveals an enzyme with terminal action.

The foregoing results indicate that the lyase produced by *Aspergillus* sp. CH-Y-1043 is extremely specific for highly esterified pectins, with an optimal pH unlike that of lyases produced by fungi of the same genus and more similar to bacterial pectin lyases. *Aspergillus* sp. CH-Y-1043 pectin lyase is not dependent on divalent cations, although the presence of Ca^{2+} and Mg^{2+} stimulated its activity, which is not always the case with hydrolytic pectinases (Whitaker 1972). The lyase is very stable at temperatures ranging from 4 to 50°C at pH 5.0 and therefore its use in clarification processes is quite possible, although, due to its elevated optimal pH value, its activity will not be maximal. However, the joint action of this lyase with hydrolytic pectinases also produced by this microorganism, could result in more efficient degradation of native substrates. Even though crude extract was used for the assays, the nature of the products derived from lyase activity and the elevated optimal pH value indicate that the substrate is degraded practically only by lyase, since no activity was detected in hydrolytic endo- and exo-pectinase assays under the same conditions. Although preliminary results suggest that we are dealing with an exo-pectin lyase, this will need to be verified with the purified enzyme. However assays with partially purified enzyme support the exo-pectin lyase hypothesis.

Table 1. Effect of divalent cations on lyase activity[a]

Cation[b] addition	Relative activity	
	pH 5.5	pH 8.8
Control (no addition)	1.00	1.00
Control + EDTA[c]	1.06	1.03
Control + EDTA + Ca^{2+}	0.99	0.98
Ca^{2+}	1.28	1.78
Ba^{2+}	1.13	nd
Co^{2+}	1.12	nd
Zn^{2+}	0.61	nd
Mn^{2+}	1.26	nd
Mg^{2+}	1.27	1.61

nd, not determined

[a] All assays were carried out with dialysed samples

[b] The final concentration in the reaction system was 20 mM

[c] EDTA was added at a final concentration of 20 mM

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