SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 12-48841- -7 **FECHA**: 2014-05-08 10:52:26
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 35
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JERONIMO CASTILLO BONILLA

Asunto: Radicación: 12-48841- -7
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 5485
Folios: 35

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2010/049849				
Fecha de Presentación Internacional	22/09/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 12-048841-00000	22/Marzo/2012
Reivindicaciones:	Radicado No. 12-048841-00000	22/Marzo/2012
	Radicado No. 12-048841-00005	29/Agosto/2013
Dibujos:	Radicado No. 12-048841-00000	22/Marzo/2012
Secuencias:	Radicado No. 12-048841-00000	22/Marzo/2012
Prioridades:	US 61/245.273	23/Septiembre/2009
	US 61/289.886	23/Diciembre/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Para la presente solicitud se estudió el capítulo reivindicatorio completo (Radicado No. 12-048841-00005-0000).

Título de la solicitud: "Nuevas enzimas de hidrolasa de glucólisis y sus aplicaciones".

El problema planteado en esta solicitud consiste en la necesidad de convertir una planta u otros materiales celulósicos o hemicelulósicos en azucares fermentables con eficacia y producción mejoradas.

Para resolver este problema técnico la solicitud en estudio proporciona una composición de ocurrencia no natural que contiene un primer polipéptido o variante del mismo que tiene actividad beta-xilosidasa, actividad L-alfa-arabinofuranosidasa, o ambas, actividad beta-xilosidasa y actividad L-alfa-arabinofuranosidasa, seleccionado de un polipéptido Fv43D, Fv3A, Pf43A, Fv43E, Fv39A, Fv43A, Fv43B, Pa51A, Gz43A, Bxl1 de *Trichoderma reesei*, Fv51A, Af43A, Pf51A; y un segundo polipéptido o variante del mismo que tiene actividad xilanasa, seleccionado de una xilanasa de *Trichoderma* o xilanasa de *Aspergillus*; en donde la composición es capaz de convertir 60% o más de la xilana de la hemicelulosa de una biomasa en xilosa.

El objeto de la presente solicitud se relaciona con la clasificación internacional de patentes (CIP): C12N 9/24, C12P 19/00, C12P 7/04, C12P 7/08.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 42 - 44 no se considera una invención, de acuerdo con el Art citado por el hecho de que se refiere a material biológico tal como se encuentra en la naturaleza.

5. De la solicitud de patente

Titulo

El título no corresponde específicamente con el objeto de la solicitud por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones y presenta repeticiones de materia en diferentes reivindicaciones. Adicionalmente, carece de claridad de acuerdo a las siguientes consideraciones:

1.) Una composición debe caracterizarse a partir de sus ingredientes activos y excipientes y las proporciones de los mismos. En la reivindicación 1 únicamente se mencionan los ingredientes activos pero no se mencionan las cantidades o proporciones de cada uno de ellos. Además, se refiere a una composición que contiene dos polipéptidos, sin describir la forma en que se presentan dichos polipéptidos y sin caracterizarlos a partir de su estructura (secuencia de aminoácidos), tampoco se indica la estructura de las variantes de los mismos. Por otra parte, la expresión "*en donde la composición es capaz de convertir 60%...*" no corresponde a una característica técnica de la invención y por el contrario se refiere a un resultado a alcanzar que debe mencionarse en la memoria descriptiva, pero no en las reivindicaciones.

2.) Se recuerda a la solicitante que un polipéptido no debe caracterizarse a partir de porcentajes de identidad, ya que esto le resta claridad a la invención y no permite que se limite el alcance de la invención apropiadamente, ni a partir del ácido nucleico que lo codifica. Claramente definir por porcentaje de identidad frente a una SEQ no es admisible, porque tal y como se escribe no hay certeza de a qué secuencia se hace referencia con el código indicado, esto se evidencia de la lectura de la rv. 4, por ej: Fv43D puede ser 90% SEQ. 27 o SEQ. 28. Se sugiere caracterizar los polipéptidos a partir de su estructura completa, es decir, su secuencia de aminoácidos. Tampoco deben emplearse expresiones como "… *codifica por un ácido nucleico que es capaz de hibridar…*" pues esto corresponde a un resultado a alcanzar y no a una característica técnica. Asimismo, definir un polipéptido o una variante por la actividad no es admisible porque esto equivale a definirlo por el resultado a alcanzar. Por otra parte, los nombres y códigos no oficiales para identificar un polipéptido no dan cuenta de la estructura de los polipéptidos, tampoco el origen es suficiente y define de forma inequívoca la estructura del polipéptido que se combina en la composición. (reivindicaciones 4 - 7, 15).

3.) Las reivindicaciones 8 y 9 carecen de concisión puesto que indican que la composición tiene uno o más polipéptidos. Dicha expresión es restrictiva únicamente a lo mínimo pero no a lo máximo, por lo tanto no se conoce el alcance real de la invención, puesto que no específica cual es el máximo de polipéptidos con actividad celulasa que puede contener la reivindicación. Además, dentro de la caracterización de la composición deben mencionarse especificadamente cada uno de sus componentes y no recurrir a generalidades de ese tipo puesto que existen muchas enzimas con actividad celulasa y no se define cuál de ellas se selecciona para la presente invención.

4.) Las reivindicaciones 9 - 13 carecen de claridad, puesto que se refieren a componentes de una celulasa entera y su caracterización pero antes no se ha mencionado que la composición contenga dicha celulasa. Se sugiere hacer las aclaraciones necesarias.

5.) Es necesario aclarar el objeto reclamado en las reivindicaciones 23 – 24 pues se refieren a una biomasa de la composiciónd e a reinvidicación 1, cuando antes no se ha indicado que dicha compsicion contenga biomasa. Además, no es claro por qué la composición de peptidasas además se puede llegar a caracterizar por el sustrato que van a degradar.

6.) Los rangos empleados en las reivindicaciones 16, 17, 26 - 30, 46 - 50 son muy amplios y no definen claramente la invención, puesto que por ejemplo, una composición con una proporción del 2% en peso de beta-glicosidasa se entiende como una composición completamente diferente a una en la que dicha enzima constituya el 50%. Se sugiere restringir las reivindicaciones.

7.) Las reivindicaciones 18, 21, 22 están definidas en términos de un resultado a alcanzar, lo cual no permite tener claridad sobre el alcance de la invención puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado.

8.) La reivindicación 19 carece de sustento verdadero en el capítulo descriptivo. Se recuerda a la solicitante que toda la materia reclamada debe tener sustento en la descripción, demostrando actividad o efecto.

9.) En el caso de la reivindicación 31, no es suficiente con mencionar la composición, puesto que el ingrediente primordial de dicho caldo es el organismo fermentador.

Se sugiere incluir todas las características técnicas esenciales del objeto reclamado.

10.) El proceso para convertir biomasa en azucares descrito en las reivindicación 35 no menciona las condiciones específicas en que debiera realizarse, las características de la biomasa empleada ni la forma en que se recuperarán los azucares. Éste no se puede caracterizar solo por referencia a la composición de los polipéptidos. Se recuerda a la solicitante que un método es una secuencia lógica de etapas que incluye condiciones de reacción fisocoquímicas y biológicas, reactivos y sustrato.

11.) Similarmente, el proceso de sacarificación descrito en las reivindicaciones 36 – 39 no menciona las condiciones específicas en que debiera realizarse, entiéndase como condiciones de reacción fisocoquímicas y biológicas, reactivos y sustrato. Se sugiere caracterizar dicho proceso a partir de la secuencia lógica de etapas.

12.) La reivindicación 40 se define como resultado que se pretende alcanzar, la solicitante debe definir la cepa e indicar su número de depósito de material biológico por autoridad reconocida, o de lo contrario se considerará materia no patentable.

13.) Por último se recomienda agrupar las reivindicaciones por categorías, de modo que las reivindicaciones dependientes de una misma reivindicación aparezcan de forma consecutiva y no separada como ocurre con reivindicación 36 y sus dependientes 45 a 50.

Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa puesto que la composición reclamada en la reivindicación 19 no tiene sustento de realización ni demuestra su actividad o efecto.

Por otra parte, debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para las cepas reclamadas.

6. Unidad de invención (Art 25 D 486)

No se encuentra ningún elemento particular que permita concluir que se pueden reivindicar en la misma solicitud todas las modalidades de composición que resultan al seleccionar el primer polipéptido entre 14 opciones y el segundo polipéptido solo por la actividad xilanasa que desempeña o por el resultado que se pretende alcanzar.

Por consiguiente, la solicitud no cumple con el requisito de unidad de invención porque se refiere por lo menos 4 grupos inventivos, como se detalla a continuación:

- *Grupo inventivo 1:* Composiciones que combinan variantes con actividad beta-xilosidasa y actividad xilanasa (reivindicación 1 – 7, 21 – 50).

- *Grupo inventivo 2*: Composiciones que combinan variantes con actividad beta-xilosidasa, actividad L-alfa-arabinofuranosidasa y actividad xilanasa (reivindicación 1 – 7, 21 – 50).

- *Grupo inventivo 3:* Composiciones que combinan variantes con actividad beta-xilosidasa, actividad xilanasa y actividad celulasa (reivindicación 8 – 18).

- *Grupo inventivo 4:* Composiciones que combinan variantes con actividad beta-xilosidasa, actividad xilanasa y proteínas accesorias (reivindicación 1, y 19 – 50). Este

grupo inventivo carece de sustento verdadero en la memoria descriptiva.

Vale la pena aclarar que en estos grupos se ha dejado a un lado el tema de falta de certeza en cuanto a la cercanía estructural de las variantes a): Fv43D, Fv3A, Pf43A, Fv43E, Fv39A, Fv43A, Fv43B, Pa51A, Fo43A, Gz43A, Bx11 de Trichoderma reesei, Fv51A, Af43A o Pf51A y que por otra parte el componente b) se define por dos orígenes diferentes, Trichoderma y de Aspergillus. La falta de claridad en la definición de la materia, y el resultado de múltiples realizaciones sustentan la carencia de unidad de invención, como fue mencionado anteriormente.

De hecho, no hay unidad de invención entre los al menos cuatro grupos inventivos ya que el concepto común inventivo, composición que comprende variantes con actividad beta-xilosidasa y actividad xilanasa , no cumple con el requisito de nivel inventivo de acuerdo con los documentos D1 y D2 (Análisis de patentabilidad para la reivindicación 1).

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Octubre 01 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO 2007094852	"Cellulolytic enzymes, nucleic acids encoding them and methods for making and using them"	23/Agosto/2007
D2	Bhat 2000	"Cellulases and related enzymes in biotechnology"	2000
D3	Margolles-clark *et al.* 1997	"Expression patterns of ten hemicellulase genes of the filamentous fungus Trichoderma reesei on various carbon sources"	16/Septiembre/1997

El documento D1[1] revela polipéptidos con actividad celulolítica y su uso en una variaedad de procesos farmacéuticos, agronómicos y alimenticios, así como composiciones que los comprenden (resumen).

El documento D2[2] da a conocer una revisión del estado del arte de celulasas microbianas y enzimas relacionadas y su enorme potencial en biotecnología (resumen).

El documento D3[3] divulga los patrones de expresión de diez genes que codifican para hemicelulasas del hongo filamentoso *T. reesei* (resumen).

8. Examen de Patentabilidad (Art 14 D486)

1 http://www.google.com/patents/WO2007094852A2
2 M.K. Bhat. 2000. Cellulases and related enzymes in biotechnology. Biotechnology Advances 18: 355–383.
3 Emilio Margolles-clark1, Marja Ihnen, Merja Penttilä. 1997. Expression patterns of ten hemicellulase genes of the filamentous fungus Trichoderma reesei on various carbon sources. Journal of Biotechnology 57(1–3): 167–179.
Ver abstract en: http://www.sciencedirect.com/science/article/pii/S0168165697000977

Nivel inventivo (Art18 D486)

Grupo inventivo 1:

La reivindicación 1 consiste en "*Una composición de ocurrencia no natural que contiene: a) un polipéptido o variante del mismos…"*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Una composición de ocurrencia no natural que contiene	Una composición o producto de manufactura que comprende (rev. 155)	Preparación multienzimática para la hidrólisis de xilano que comprende (Pág. 370 tabla 5)
un primer polipéptido o variante del mismo que tiene actividad beta-xilosidasa, actividad L-alfa-arabinofuranosidasa, o ambas, actividad beta-xilosidasa y actividad L-alfa-arabinofuranosidasa, seleccionado de un polipéptido Fv43D, Fv3A, Pf43A, Fv43E, Fv39A, Fv43A, Fv43B, Pa51A, Gz43A, Bxl1 de *Trichoderma reesei*, Fv51A, Af43A, Pf51A;	una combinación de diferentes enzimas incluyendo celulasas y hemicelulasas como beta-xilosidasas y/o arabifuronidasas (rev. 137 y rev. 155).	Xilanasas, beta-xylosidasas y alfa-L-arabinofuranosidasas (Pág. 370 tabla 5)
y un segundo polipéptido o variante del mismo que tiene actividad xilanasa, seleccionado de una xilanasa de Trichoderma o xilanasa de Aspergillus	Y una xilanasa de Aspergillus (Pág. 306 líneas 16 - 27)	Siendo la xilanasa seleccionada de T. reesei (Pág. 371)

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención reclamada en la reivindicación 1 porque divulgan composiciones o preparaciones a partir de la mezcla de celulasas y enzimas relacionadas para el uso en biotecnología aplicada a la industria textil, farmacéutica, alimenticia, entro otras. El documento D1 divulga una composición o producto de manufactura que comprende una mezcla o cocktail de enzimas que comprende xilanasa, beta-xilosidasa y arabinofuranosidasa (rev. 155) o bien los polipéptidos que las expresan (rev. 137), cuyo polipéptido que tiene actividad xilanasa es seleccionado de Aspergillus (Pág. 306 líneas 16 - 27). El mismo documento D1 divulga un cultivo de fermentación con la mezcla de enzimas antes mencionada en donde el hongo filamentoso es Trichoderma reesei (Pág. 237 líneas 4 - 6), un método para convertir biomasa en azucares, que consiste en contacto de la biomasa con la composición multi-enzimática, por un proceso de sacarificación (resumen), y una célula recombinante que expresa un polipéptido con actividad beta-xilosidasa (Pág. 31). Por otra parte, el documento D2 enseña la función de las enzimas xilanasa, beta-xilosidasa y alfa-L-arabinofuranosidasa en la hidrólisis de xilano aplicado al bio-blanqueamiento de pulpas (Pág. 370 tabla 5), siendo la xilanasa purirficada de *T. reesei* (Pág. 371).

La diferencia entre la composición reclamada en la reivindicación 1 y la divulgada en los documentos D1 y D2 es la selección de polipéptidos de Fv43D, Fv3A, Pf43A, Fv43E, Fv39A, Fv43A, Fv43B, Pa51A, Gz43A, Bxl1 de *Trichoderma reesei*, Fv51A, Af43A, o Pf51A.

No existe un efecto inesperado o sorprendente que la selección de dichos polipéptidos específicos le confieran a la invención, puesto que la composición reclamada conlleva a la conversión de biomasa al igual que las anterioridades.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar las composiciones conocidas en los documentos D1 y el D2 para conseguir una composición alternativas a las ya existentes".

Sin embargo, el documento D2 ya sugería previamente la importancia de la selección de

organismos para la producción de una proteína o enzima específica (Pág. 376) debido a las relaciones de estructura y función de las celulasas desde bacterias hasta hongos (Pág. 377). Asimismo, proteínas con funciones celulolíticas y hemicelulolíticas son conocidas para diferentes microorganismos en el estado de la técnica. Ejemplo de ello es la enzima beta-xilosidasa de Trichoderma reesei (bxl1) divulgada en le documento D3. De este modo, una persona medianamente versada en la materia incluiría el polipéptido de *T. reesei* divulgado en el documento D3 en las composiciones conocidas en los documentos D1 y D2 para llegar a sí a la consecución de composiciones alternas a las ya existentes útiles en la conversión de biomasa en azucares. Por consiguiente, la solución aquí propuesta se considera obvia y sin altura inventiva.

Las reivindicaciones 2 - 50 no tienen características adicionales frente a las enseñanzas de los documentos D1 a D3, por lo tanto tampoco tienen nivel inventivo.

Por consiguiente, el grupo inventivo 1 carece de nivel inventivo, al igual que los grupos inventivos 2 – 4 debido a que este primer grupo corresponde el concepto común de la solicitud.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 2007094852	1 - 50	Nivel inventivo
D2	Bhat 2000	1 - 50	Nivel inventivo
D3	Margolles-clark *et al.* 1997	1 - 50	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-05-13

Elaboró: Edna Carolina Bonilla Leon
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ELSEVIER

Biotechnology Advances 18 (2000) 355–383

BIOTECHNOLOGY ADVANCES

Research review paper

Cellulases and related enzymes in biotechnology

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Abstract

Basic and applied research on microbial cellulases, hemicellulases and pectinases has not only generated significant scientific knowledge but has also revealed their enormous potential in biotechnology. At present, cellulases and related enzymes are used in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes. Indeed, the demand for these enzymes is growing more rapidly than ever before, and this demand has become the driving force for research on cellulases and related enzymes. The present article is an overview of the biotechnological state-of-the-art for cellulases and related enzymes. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Biotechnology; Cellulases; Hemicellulases; Pectinases

1. Introduction

Active research on cellulases and related polysaccharidases began in the early 1950s, owing to their enormous potential to convert lignocellulose, the most abundant and renewable source of energy on Earth, to glucose and soluble sugars (Coughlan, 1985a,b; Mandels, 1985; Reese, 1976; Reese and Mandels, 1984). Extensive basic and applied research during the 1970s and 1980s demonstrated that the enzyme-induced bio-conversion of lignocellulose to soluble sugars was rather difficult and uneconomical (Coughlan, 1985a; Ladisch et al., 1983; Mandels, 1985; Ryu and Mandels, 1980). Nevertheless, continued research on cellulases, hemicellulases and pectinases revealed their biotechnological potential in various industries, including food, brewery and wine, animal feed, textile and laundry, pulp and paper, agriculture, as well as in research and development (Bajpai, 1999; Bayer et al., 1994; Beguin and Aubert, 1994; Bhat and Bhat, 1997, 1998; Gilbert and Hazlewood, 1993; Godfrey and West, 1996b; Harman and Kubicek, 1998; Lamed and Bayer, 1988; Mandels, 1985; Poutanen, 1997; Saddler, 1993; Uhlig, 1998; Viikari et al., 1993; Visser et al., 1992; Visser and Voragen, 1996; Wong and Saddler, 1992, 1993).

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Today, the enzymes are commonly used in many industrial applications, and the demand for more stable, highly active and specific enzymes is growing rapidly. It was estimated that in 1995, the world sale of industrial enzymes would be >1.0 billion US dollars, while the world market for industrial enzymes is expected to be in the range between 1.7 and 2.0 billion US dollars by the year 2005 (Godfrey and West, 1996a). According to a recent publication, the industrial enzymes have already reached a market of 1.6 billion US dollars (Demain, 2000). Interestingly, 60% of the total world supply of industrial enzymes is produced in Europe, and the remaining 40% from USA and Japan. Also, approximately 75% of the industrial enzymes are hydrolases, with carbohydrolases being the second largest group.

Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications (Chesson, 1987; Thomke et al., 1980; Voragen, 1992; Voragen et al., 1980, 1986). Subsequently, these enzymes were used in the textile, laundry as well as in the pulp and paper industries (Godfrey 1996; Wong and Saddler, 1992, 1993). However, pectinases were used in the food industry as early as 1930 (Kertesz, 1930). During the last two decades, the use of cellulases, hemicellulases and pectinases has increased considerably, especially in textile, food, brewery and wine as well as in pulp and paper industries (Godfrey and West, 1996b; Harman and Kubicek, 1998; Saddler, 1993; Uhlig, 1998). Today, these enzymes account for approximately 20% of the world enzyme market (Mantyla et al., 1998), mostly from *Trichoderma* and *Aspergillus* (Godfrey and West, 1996b; Uhlig, 1998). Currently, several commercial enzyme producers are marketing tailor-made enzyme preparations suitable for biotechnology, and the updated details can be found in respective company web pages. The present review highlights the main uses of cellulases, hemicellulases and pectinases in biotechnology. Further background information on different applications can be found elsewhere (Godfrey and West, 1996b; Harman and Kubicek, 1998; Uhlig, 1998).

2. Cellulases, hemicellulases and pectinases in food biotechnology

Cellulases, hemicellulases and pectinases have a wide range of potential applications in food biotechnology. These are summarised in Table 1. Details of some of the most promising applications are given.

2.1. Extraction and clarification of fruit and vegetable juices

The production of fruit and vegetable juices is important both from the human health and commercial standpoints. The availability of nutritious components from fruits and vegetables to a wide range of consumers is thus facilitated throughout the year by the marketing of their juices. The production of fruit and vegetable juices requires methods for extraction, clarification and stabilization. During the early 1930s, when fruit industries began to produce juice, the yields were low, and many difficulties were encountered in filtering the juice to an acceptable clarity (Uhlig, 1998). Subsequently, research on industrially suitable pectinases, cellulases and hemicellulases from food-grade micro-organisms (*Aspergillus niger* and *Trichoderma* sp.), together with increased knowledge on fruit components, helped to overcome these difficulties (Grassin and Fauquembergue, 1996a). Currently, a combination of pectinases (pectin lyase, pectin methylesterase, endo and exo-polygalacturonases, pectin ace-

Table 1
Cellulases, hemicellulases and pectinases in food biotechnology

Enzyme	Function	Application	Reference
Macerating enzymes (pectinases, cellulases and hemicellulases)	Hydrolysis of soluble pectin and cell wall components; decreasing the viscosity and maintaining the texture of juice from fruits	Improvement in pressing and extraction of juice from fruits and oil from olives; releasing flavour, enzymes, proteins, polysaccharides, starch and agar	Galante et al., 1998b; Godfrey and West, 1996b; Uhlig, 1998
Acid and thermostable pectinase with polygalacturonase, pectin esterase and pectin transeliminase	Fast drop in the viscosity of berry and stoned fruits with the breakdown of fruit tissues	Improvement in pressing fruit mashes and high colour extraction	Grassin and Fauquembergue, 1996b; Uhlig, 1998
Polygalacturonase with high pro-pectinase and low cellulase	Partial hydrolysis of pro-pectin	Production of high viscosity fruit purees	Grassin and Fauquembergue, 1996b; Uhlig, 1998
Polygalacturonase and pectin transeliminase with low pectin esterase and hemicellulase	Partial hydrolysis of pro-pectin and hydrolysis of soluble pectin to medium sized fragments; formation and precipitation of acid moieties; removal of hydrocolloids from cellulose fibres	Production of cloudy vegetable juice of low viscosity	Grassin and Fauquembergue, 1996b; Uhlig, 1998
Polygalacturonase, pectin transeliminase and hemicellulase	Complete hydrolysis of pectin, branched polysaccharides and mucous substances	Clarification of fruit juices	Grassin and Fauquembergue, 1996b; Uhlig, 1998
Pectinase and β-glucosidase	Infusion of pectinase and glucosidase for easy peeling/ firming of fruits and vegetables	Alteration of the sensory properties of fruits and vegetables	Baker and Bruemmer, 1989; Baker and Wicker, 1996; Crocco, 1976; Gunata et al., 1990; Javeri et al., 1991; Krammer et al., 1991; Marlatt et al., 1992; Pabst et al., 1991
Arabinoxylan modifying enzymes (endoxylanases, xylan debranching enzymes)	Modification of cereal arabinoxylan and production of arabinoxylo-oligosaccharides	Improvement in the texture, quality and shelf life of bakery products	Hamer, 1991; Kulp, 1993; Maat et al., 1992; Poutanen, 1997

(*continued on next page*)

Table 1 (*continued*)

Enzyme	Function	Application	Reference
Cellulases and hemicellulases	Partial or complete hydrolysis of cell wall polysaccharides and substituted celluloses	Improvement in soaking efficiency; homogeneous water absorption by cereals; the nutritive quality of fermented foods; the rehydrability of dried vegetables and soups; the production of oligosaccharides as functional food ingredients and low-calorie food substitutents and biomass conversion	Beguin and Aubert, 1994; Bhat and Bhat, 1997; Mandels, 1985; Ryu and Mandels, 1980
β-Glucanases and mannanases	Solubilization of fungal and bacterial cell wall	Food safety and preservation	Fuglsang et al., 1995
Xylanases and endoglucanases	Hydrolysis of arabinoxylan and starch	Separation and isolation of starch and gluten from wheat flour	Heldt-Hansen, 1997
Pectin esterase with no polygalacturonase and pectin lyase activities	Fruit processing	Production of high quality tomato ketchup and fruit pulps	Heldt-Hansen, 1997
Rhamnogalacturonase Rhamnogalacturonan acetyl esterase and galactanase	Cloud stability	Production of cloud stable apple juice	Heldt-Hansen, 1997
Cellulase and pectinase	Release of antioxidants from fruit and vegetable pomace	Controlling coronary heart disease and atherosclerosis; reducing food spoilage	Meyer et al., 1998
Endo-mannanase	Modification of guar gum	Production of water-soluble dietary fibres to enrich the fibre content of foods	Bar and Lindley, 1994

tylesterase, rhamnogalacturonase, endo- and exo-arabinases), cellulases (endoglucanases, exoglucanases and cellobiases) and hemicellulases (endo- and exo-xylanases, galactanases, xyloglucanases and mannanases)—collectively called macerating enzymes—are used in the extraction and clarification of fruit and vegetable juices (Galante et al., 1998b; Grassin and Fauquembergue, 1996a). In addition, α-amylase and amyloglucosidase, active at acidic pH, were used to process starch-containing fruits, especially apples harvested during the early stages in order to prevent haze formation (Grassin and Fauquembergue, 1996a; Uhlig, 1998).

During the production of juice from fruits such as apples and pears, the whole fruits are crushed to pulp mash, which, after mechanical processing (pressing, centrifuging and filtering), yields a clear fruit juice and a solid phase called pomace (Galante et al., 1998b). Use of macerating enzymes increases both yield and process performance without additional capital investment. Macerating enzymes are generally used in two steps: (1) after crushing, to macerate the fruit pulp either to partial or complete liquifaction, which not only increases the juice yield and reduces the processing time, but also improves the extraction of valuable fruit components, and (2) after the juice extraction, whereby pectinases are used for its clarification, thereby lowering the viscosity of fruit juice prior to concentration and increasing the filtration rate and stability of the final product. Thus, the macerating enzymes play a key role in food biotechnology, and their demand will likely increase for extraction of juice from a wide range of fruits and vegetables.

2.2. Production of fruit nectars and purees

The production and preservation of fruit nectars and purees are of tremendous commercial importance to attract a wide range of consumers and particularly to use the fruits, which are easily perishable. Many tropical fruits are either not easily pressable, being too acidic or too strongly flavoured to be used to produce pleasant beverages without dilution, blending or both. Nevertheless, the juice from tropical fruits is delicious after dilution or blending with other fruit juices. Some good examples of these are apricot, peach, pear, plum, mango, guava, papaya and banana. Use of macerating enzymes not only improves the cloud stability, texture, and facilitates easy concentration of nectars and purees, but also decreases their viscosity rapidly (Grassin and Fauquembergue, 1996a). Hence, a suitable combination of macerating enzymes is expected to be ideal for the production of fruit nectars and purees.

2.3. Infusion of pectinases and β-glucosidases to alter the sensory properties of fruits and vegetables

Enzyme infusion has the potential to alter the texture, flavour and other sensory properties of foods. Vacuum infusion of pectinases to ease the peeling of citrus fruits has been commercialised (Baker and Bruemmer, 1989; Baker and Wicker, 1996). Several food companies in the UK, USA, Japan and South Africa are currently using this technique for the production of freshly peeled citrus fruits and salads. Other potential applications of pectinase infusion include: (1) reducing the excessive bitterness in citrus peels (Roe and Bruemmer, 1977); (2) restoring the flavour lost during drying (Crocco, 1976); and (3) improving the firmness of peaches and processed pickles (Baker and Wicker, 1996; Javeri et al., 1991). In addition, the infusion of pectinases and β-glucosidases increases the aroma and volatile characteristics of specific fruits and vegetables (Humpf and Schrier, 1991; Krammer et al., 1991; Marlatt et al.,

1992; Pabst et al., 1991). Thus, enzyme infusion to alter the sensory attributes of fruits, vegetables and other foods has enormous potential in food biotechnology.

2.4. Extraction of olive oil

In recent years, olive oil has attracted the world market because of its numerous health claims. Extraction of olive oil involves: (1) crushing and grinding of olives in a stone or hammer mill; (2) passing the minced olive paste through a series of malaxeurs and horizontal decanters; and (3) high-speed centrifugation to recover the oil (Galante et al., 1998b). Typically 100 kg of olives yield 16–20 kg of oil. Based on the presence of free fatty acid (FFA), olive oil is classified as either extra-virgin (<1% FFA), virgin (1–3% FFA) or ordinary (Galante et al., 1998b). To produce high quality olive oil, freshly picked, clean and slightly immature fruit have been used under cold pressing conditions (Galante et al., 1998b). However, high yields have been obtained with fully ripened fruit, when processed at higher than ambient temperatures, but this resulted in oil with high acidity, rancidity and poor aroma (Galante et al., 1998b). Hence, an improved method for the extraction of high quality olive oil was needed to meet the growing consumer demand.

The commercial enzyme preparation, Olivex (a pectinase preparation with low levels of cellulase and hemicellulase from *Aspergillus aculeatus*) was the first enzyme mixture used to improve the extraction of olive oil (Fantozzi et al., 1977). Systematic studies carried out in the 1980s, revealed that no single enzyme was adequate for the efficient maceration and extraction of oil from olives. Three types of enzymes viz. pectinases, cellulases and hemicellulases were found to be essential for this purpose (Galante et al., 1998b). Also, a combination of enzymes, consisting of pectinases (from *Aspergillus*), cellulases and hemicellulases (from *Trichoderma*), performed better than the enzymes from a single micro-organism (Galante et al., 1993). A commercial enzyme preparation Cytolase O was successfully used in Southern Italy, which increased the oil yield on an average by 1–2 kg per tonne of olives (Galante et al., 1998b). Furthermore, the use of macerating enzymes increased the anti-oxidants in extra-virgin olive oil and reduced the induction of rancidity (Galante et al., 1998b). The main advantages of using macerating enzymes during olive oil extraction are: (1) increased extraction (up to 2 kg oil per 100 kg olives) under cold processing conditions; (2) better centrifugal fractionation of the oily must; (3) oil with high levels of anti-oxidants and vitamin E; (4) slow induction of rancidity; (5) overall improvement in plant efficiency; and (6) low oil content in the waste water (Galante et al., 1998b). Likewise, the macerating enzymes could play a prominent role in the extraction of oils from other agricultural crops.

2.5. Improving the quality of bakery products

Exogenous microbial enzymes, namely amylases and proteases, have been used in industrial baking for many years (Hamer, 1991; Kulp, 1993; Linko and Linko, 1986; Poutanen, 1997). In recent years, hemicellulases, especially endo-xylanases have also been used to improve the quality of dough, bread, biscuits, cakes and other bakery products (Poutanen, 1997). Although the endo-xylanases are known to exhibit many beneficiary effects during dough handling and baking, their actual mechanism of action is not well understood. It has been hypothesised that the ability of endo-xylanases to hydrolyse arabinoxylan present in dough facilitates the redistribution of water in both dough and bread, and is responsible for the observed favourable efects on dough handling, bread volume, texture and stability (Maat

et al., 1992; Poutanen, 1997). Besides, the addition of endo-xylanases during dough processing is expected to increase the concentration of arabino xylo-oligosaccharides in bread, which have beneficiary effects on human health. Recently, arabinases, α-L-arabinofuranosidases, arabinoxylan α-L-arabinofuranohydrolases and esterases have been reported to play important roles in improving the texture, quality and sensory attributes of bakery products (Poutanen, 1997). However, a suitable combination of these enzymes is vital for achieving maximum benefit during dough processing and baking.

3. Cellulases, hemicellulases and pectinases in beer and wine biotechnology

Beer brewing and wine making are old technologies and have an ancient history. In simple terms, beer brewing involves malting the barley in a malt house followed by the preparation and fermentation of the wort in the brewery, while wine making requires the extraction of juice from grapes and subsequent fermentation of the juice by yeast. Enzyme technology plays a central role in both these processes. The addition of exogenous glucanases and related polysaccharidases are known to improve not only the beer and wine qualities, but also their overall production efficiency (Galante et al., 1998b). This section highlights the significance of cellulases, hemicellulases and pectinases in brewery and wine industries as well as summarises their applications (Table 2).

3.1. Beer brewing

This technology is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α- and β-amylases, carboxypeptidase and β-glucanase that hydrolyse the seed reserve. All these enzymes should act in synergy under optimal conditions to produce high quality malt. Nevertheless, many breweries end up using un-malted or poor quality barley, due to seasonal variations, different cultivars or poor harvest, which contains low levels of endogenous β-glucanase activity. The problem associated with the use of such un-malted or

Table 2
Cellulases, hemicellulases and pectinases in brewery and wine biotechnology

Enzyme/micro-organism	Function	Application	Reference
β-Glucanase/glucanolytic yeast	Hydrolysis of β-1,3, and β-1,4 glucan; reducing the viscosity and releasing reducing sugars during primary fermentation	Improvement in primary fermentation, filtration and quality of beer	Canales et al., 1988; Galante et al., 1998b; Oksanen et al., 1985; Pajunen, 1986
Pectin esterase	De-esterification and gelling of pectins	Improvement in the clarification of cider	Uhlig, 1998
Macerating enzymes (cellulases, hemicellulases and pectinases)	Hydrolysis of plant cell wall polysaccharides	Improvement in skin maceration and colour extraction of grapes; quality, stability, filtration and clarification of wines	Galante et al., 1998b; Grassin and Fauquembergue, 1996b; Uhlig, 1998
β-Glucosidase	Modification of aromatic residues	Improvement in the aroma of wines	Caldini et al., 1994; Gunata et al., 1990

poor quality barley and other cereals in combination with malt is the presence of 6–10% non-starch polysaccharide (NSP), mainly a soluble β-glucan. This forms gels during the brewing process and leads to poor filtration of the wort, slow run-off times, low extract yields and/or the development of haze in the final product. To overcome these problems, microbial β-glucanases, which hydrolyse β-glucan and reduce the viscosity of the wort are added either during mashing or primary fermentation. The commonly used β-glucanases are from *Penicillium emersonii, Aspergillus niger, Bacillus subtilis* and *Trichoderma reesei* (Galante et al., 1998b).

Based on the comparative study on the performance of different β-glucanases in beer wort production, Pajunen (1986) concluded that the enzyme preparation from *Trichoderma* was the best as judged by its cost/performance ratio. In an earlier study, Oksanen et al. (1985) observed that endoglucanase II and cellobiohydrolase II of the *Trichoderma* cellulase system were responsible for a maximum reduction in the degree of polymerisation and wort viscosity. It was also reported that the addition of 0.05–0.1 ml of a commercial *Trichoderma* cellulase preparation per kg of grists caused a 90% decrease in β-glucan content and reduced the filtration time by 30% (Oksanen et al., 1985). Furthermore, a marked improvement in filterability was reported with increasing doses of enzyme when tested in pilot scale (Oksanen et al., 1985).

The pilot and industrial scale brewing trials were performed using three commercially available β-glucanases from *Trichoderma, Bacillus subtilis* and *Aspergillus niger* on three different grist bills (65% malt/35% barley; 65% malt/35% rice and 50% malt/15% barley/35% rice) (Canales et al., 1988). In all three cases, the β-glucanase from *Trichoderma* performed better than the β-glucanase from the other two microbial sources. Besides, in all these and in previous trials, there was no difference in the quality of the final product when compared with normal products as judged by the taste panel (Canales et al., 1988; Galante et al., 1998b). Thus, β-glucanase, especially from *Trichoderma*, appears to be suitable for the production of high quality beer from poor quality barley.

3.2. Wine production

This is a biotechnological process in which both yeast cells and enzymes play a key role. In the last four decades, attempts have been made to improve the yeast strains used for fermentation of grape juice as well as to use exogenous microbial enzymes during wine making. Three main exogenous enzymes used in wine production are pectinases, β-glucanases and hemicellulases (Galante et al., 1998b). The main benefits of using these three enzymes during wine making include: (1) better skin maceration and improved colour extraction; (2) easy must clarification and filtration; and (3) improved wine quality and stability (Galante et al., 1998b). Recently, a fourth enzyme, β-glucosidase has attracted considerable attention in the wine industry because of its ability to improve the aroma of wines by modifying naturally present, glycosylated precursors (Caldini et al., 1994; Gunata et al., 1990).

The first microbial enzyme used in the wine industry was a commercial pectinase from *Aspergillus*, which contained varying amounts of pectin esterase, polygalacturonase, pectin lyase and small amounts of hemicellulase (Galante et al., 1998b). Addition of pectinase, while crushing grapes or to the wine must, improves juice extraction, reduces the clarification time and increases the terpene content of wine (Galante et al., 1998b). Also, pectinase preparations with high pectin

lyase and low pectin methyl esterase activities are preferred to minimise the methanol released from methylated polygalacturonic acid during wine production (Galante et al., 1998b).

In the early 1980s, it was suggested that *Trichoderma* β-glucanase could be successfully used for wine making from grapes infected with *Botrytis cinerea* (Dubordieu et al., 1981; Villetaz et al., 1984). This fungus generally attacks nearly ripe grapes under conditions of certain temperatures and humidity, and produces a high molecular mass soluble β-(1,3) glucan with short side chains linked through β-(1,6) glycosidic bonds, which causes severe problems during wine filtration. A β-glucanase from *Trichoderma harzianum*, which specifically hydrolyses this β-glucan was identified and patented (Galante et al., 1998b). This enzyme was also found to be useful for hydrolysis of glucans from yeast, that cause adverse effects during filtration and clarification of wine (Galante et al., 1998b).

Significant and reproducible improvements in grape pressability, settling rate and total juice yield were achieved using a combination of macerating enzymes compared to that using pectinase alone (Harbord et al., 1990). Nevertheless, such improvements were noticeable only with a correct balance of exogenous pectinolytic, cellulolytic and hemicellulolytic enzymes, when used to compensate the relatively low endogenous enzyme activities.

Using three varieties (Soave, Chardonnay and Sauvignon) of white grapes from Northern Italy, Galante et al. (1998b) assessed the performance of Cytolase 219 (a commercial enzyme preparation, derived from *Trichoderma* and *Aspergillus*, containing pectinase, cellulase and hemicellulase) in wine making. They reported a 10–35% increase in the extraction of the first wine must, a 70–180% increase in the must filtration rate, significant improvement in wine stability, 50–120 min decrease in pressing time, 30–70% decrease in must viscosity and 20–40% energy saving during cooling of fermenters. Currently, a number of commercial enzyme preparations are available specifically for improving the maceration of grapes, colour extraction, wine filtration and wine quality. In fact, the enzyme technology offers enormous benefits to wine industry, but new enzymes with better properties are expected to emerge and provide further benefits to both wine producers and consumers.

4. Cellulases and hemicellulases in animal feed biotechnology

The animal feed industry is an important sector of agro-business with an annual production of >600 million tonnes of feed, worth >50 billion US dollars. Of the total feed produced, the major share is taken by poultry, pigs and ruminants (up to 90%), while the pet foods and fish farming account for 10%. Cellulases and hemicellulases have a wide range of potential applications in the animal feed industry, as summarised in Table 3.

4.1. Role of cellulases and hemicellulases in monogastric feed

Hydrolases are the main class of enzymes used in monogastric feed. The use of hydrolases is either to (1) eliminate anti-nutritional factors (ANF) present in grains or vegetables; (2) degrade certain cereal components in order to improve the nutritional value of feed; or (3) to supplement animals' own digestive enzymes (e.g. proteases, amylases and glucanases), whenever these enzymes are inadequate during post-weaning period, as it is often the case with broilers and piglets (Galante et al., 1998b).

Table 3
Cellulases, hemicellulases and pectinases in animal feed biotechnology

Enzyme	Function	Application	References
Cellulases and hemicellulases	Partial hydrolysis of lignocellulosic materials; dehulling of cereal grains; hydrolysis of β-glucans; decrease in intestinal viscosity; better emulsification and flexibility of feed materials	Improvement in the nutritional quality of animal feed and thus the performance of ruminants and monogastrics	Beauchemin et al., 1995; Chesson, 1987; Cowan, 1996; Galante et al., 1998b; Graham and Balnave, 1995; Lewis et al., 1996
β-Glucanase and xylanase	Hydrolysis of cereal β-glucans and arabinoxylans, decrease in intestinal viscosity and release of nutrients from grains	Improvement in the feed digestion and absorption, weight gain by broiler chickens and hens	Bedford and Classen, 1992; Chesson, 1987; Galante et al., 1998b; Walsh et al., 1993
Hemicellulase with high xylanase actvity	Increase the nutritive quality of pig feeds	Reduction in the cost of pig feeds and the use of less expensive feeds for pigs	Chesson, 1987; Galante et al., 1998b; Graham et al., 1998; Thomke et al., 1980
Cellulases, hemicellulases and pectinases	Partial hydrolysis of plant cell wall during silage and fodder preservation; expression of preferred genes in ruminant and monogastric animals for high feed conversion efficiency	Production and preservation of high quality fodder for ruminants; improving the quality of grass silage; production of transgenic animals	Ali et al., 1995; Hall et al., 1993; Selmer-Olsen et al., 1993

β-Glucanases and xylanases have been successfully used in monogastric diets to hydrolyse non-starchy polysaccharides (NSP) such as barley β-glucans and arabinoxylans (Cowan, 1996; Hesselman et al., 1982; Rexen, 1981; Walsh et al., 1993). The presence of high levels of NSP in cereal-based diet results in poor feed conversion rate, slow weight gain, and sticky droppings by young animals, especially chicks (Bedford and Classen, 1992; Chesson, 1987; Galante et al., 1998b). Addition of β-glucanases and xylanases during feed production was found to degrade NSP and markedly improve the digestion and absorption of feed components as well as weight gain by broiler chickens and egg laying hens (Cowan, 1996; Hesselman et al., 1982; Rexen, 1981; Walsh et al., 1993).

The best xylanase/β-glucanase ratio was 6:1 to gain the maximum body weight by broiler chickens with a diet containing 60% wheat (Galante et al., 1998b). Some of the benefits claimed by the addition of a commercial enzyme preparation Econase™ to poultry feed include: (1) greater flexibility on diet formulation; (2) the use of inexpensive raw materials; (3) increased energy value of cereals; (4) improved digestibility, growth and feed conversion; (5) uniform animals; (6) clean eggs with increased yolk colour; (7) dry droppings; and (8) less environmental waste (Galante et al., 1998b). Similar claims were also made with other enzyme preparations high in xylanase and β-glucanase activities (Bedford and Classen, 1992; Cowan, 1996; Graham and Balnave, 1995).

The benefits of enzyme addition to poultry diet have also promoted the supplementation of these enzymes to pig diet. Initial studies have shown that the use of β-glucanases improved the performance of barley-fed pigs (Thomke et al., 1980). However, using cannulated pigs, it was demonstrated that the addition of enzyme preparations containing β-glucanase to a wheat mid-

dlings/barley diet improved the digestion of starch, lipids and proteins in the small intestine as observed in chickens, but had little effect on fibre digestion (Graham et al., 1988). These results suggested that pigs do not respond to β-glucanase in the same way as chickens, probably because the viscosity of the digesta in the intestine of piglets is relatively unaffected by dietary fibres. Nevertheless, subsequent studies with multi-enzyme preparations containing xylanase and β-glucanase showed potential benefits in the production of pigs (Bohme, 1990). Besides, the supplementation of xylanase-based multi-enzyme product reduced the overall cost of pig feed, and facilitated the use of inexpensive feed. Despite the above progress, further research is needed to recommend the right combination of hydrolytic enzymes and to include a wide range of diets for pigs.

4.2. Role of cellulases and hemicellulases in ruminant feed

Currently, there is a great deal of interest in using enzyme preparations containing high levels of cellulase and hemicellulase activities for improving the feed utilization, milk yield and body weight gain by ruminants. Nevertheless, the successful use of these enzymes in ruminant diet depends on: (1) their stability in the feed (during and after processing) and in the rumen; (2) the ability of enzyme components to hydrolyse plant cell wall polysaccharides; and (3) the ability of the animals to use the reaction products efficiently. Therefore, the enzyme preparations should be characterised by in vitro and in vivo experiments and should contain essential enzyme activities for different applications in order to guarantee success.

The forage diet of ruminants, which contains cellulose, hemicellulose, pectin and lignin, is more complex than the cereal-based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, hemicellulase and pectinase have been used to improve the nutritive quality of forages (Graham and Balnave, 1995; Kung et al., 199; Lewis et al., 1996). Nevertheless, the results with the addition of enzyme preparations containing cellulase, hemicellulase and pectinase to ruminant diet are somewhat inconsistent. Several studies have shown substantial improvements in feed digestibility and animal performance (Burroughs et al., 1960; Rust et al., 1965), while some researchers reported either negative effects or none at all (Perry et al., 1966; Theurer et al., 1963). Recently, Beauchemin et al. (1995) reported that the addition of commercial enzyme preparations containing cellulase and xylanase to hay diet increased the live weight gain of cattle by as much as 35%. Similarly, a 5–25% increase in milk yield has been reported in the case of dairy cows fed with forage treated with commercial fibrolytic enzymes (Lewis et al., 1996; Stokes and Zheng, 1995). In contrast, other studies no significant increase either in body weight or milk yield was observed (Lewis et al., 1996; Perry et al., 1966; Theurer et al., 1963). Thus, the overall success in improving the fibre digestion and ruminant performance may be limited. This could mainly be due to the presence of hydrophobic cuticle, lignin and its close association with cell wall polysaccharides and the nature of lignocellulose, which prevents the efficient utilization of fibre in the rumen. Hence, considerable basic and applied research effort, together with improved enzymes, will be needed to enhance fibre digestion by ruminants and thus, their performance.

Attempts have also been made to clone cellulase and xylanase genes in order to produce transgenic animals, which would secrete the required enzyme into the gastrointestinal tract of the animal to facilitate its feed digestion efficiency (Ali et al., 1995; Hall et al., 1993). In-

deed, this type of research should have considerable impact in understanding the role of cellulases and related enzymes in feed digestion and animal performance.

5. Cellulases in textile and laundry biotechnology

Cellulases have achieved their worldwide success in textile and laundry because of their ability to modify cellulosic fibres in a controlled and desired manner, so as to improve the quality of fabrics. Although, cellulases were introduced in textile and laundry only a decade ago, they have now become the third largest group of enzymes used in these applications. Bio-stoning and bio-polishing are the best-known current textile applications of cellulases (Table 4). Cellulases are also increasingly used in household washing powders, since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabric surfaces and improve the appearance and colour brightness (Table 4).

5.1. Bio-stoning of denim garments

Blue jeans and other denim garments have gained remarkable popularity in recent years. It is estimated that over 800 million pairs of blue jeans are produced worldwide every year, which represents a multi-billion dollar business. In denim fabrics, the indigo dye is mostly attached to the surface of the yarn and to the most exterior short cotton fibres. Repeated washings of denim fabric showed the wash down or aged effect, on which the entire denim industry has been built.

In textile mills, the indigo warp was heavily sized with starch, and the denim fabrics were woven into a very tight structure. This made them extremely sturdy and long lasting material, but rather stiff and uncomfortable to wear when it was new. Hence, the aged or faded jeans became very popular. In the late 1970s and early 1980s, industrial laundries developed methods for producing faded jeans by washing the garments with pumice stones, which partially removed the dye revealing the white interior of the yarn, which leads to the faded, worn and aged appearance. This was designated as ‘stone-washing.’ Although the use of 1–2 kg stones per kg of jeans for 1 h during stone-washing met the market requirements, it caused several problems including rapid wear

Table 4
Cellulases in textile and laundry biotechnology

Enzyme	Function	Application	Reference
Cellulase, preferably neutral and endoglucanase rich	Removal of excess dye from denim fabrics; soften the cotton fabrics without damaging the fibre	Bio-stoning of denim fabrics; production of high quality and environmentally friendly washing powders	Galante et al., 1998a; Godfrey, 1996; Uhlig, 1998
Cellulase, preferably acid and endoglucanase rich	Removal of excess microfibrils from the surface of cotton and non-denim fabrics	Bio-polishing of cotton and non-denim fabrics	Galante et al., 1998a; Godfrey, 1996; Kumar et al., 1994, 1996
Cellulase, preferably endoglucanase rich	Restoration of softness and colour brightness of cotton fabrics	Production of high quality fabrics	Galante et al., 1998a; Godfrey, 1996; Kumar et al., 1994

and tear of washing machines, large numbers of second class garments, unsafe working conditions, environmental pollution, and the need for manual removal of pumice from pockets and folds of garments. Therefore, there was an urgent need to overcome these problems in the denim industry. In the mid 1980s, biotechnology provided a perfect alternative for stone-washing using microbial cellulases, later known as 'bio-stoning.'

During the bio-stoning process, cellulases act on the cotton fabric and break off the small fibre ends on the yarn surface, thereby loosening the indigo, which is easily removed by mechanical abrasion in the wash cycle. The advantages in the replacement of pumice stones by a cellulase-based treatment include: (1) reduced wear and tear of washing machines and short treatment times; (2) increased productivity of the machines because of high loading; (3) substantial decrease of second quality garments; (4) less work-intensive and safer working conditions; (5) safe environment, since pumice powder is not produced; (6) flexibility to create and consistently reproduce new finished products; and (7) the possibility to automate the process with computer-controlled dosing devices when using liquid cellulase preparations (Galante et al., 1998a). However, a major drawback during bio-stoning is the strong tendency of the released dye to redeposit on the garments, which is known as 'back-staining.' Such a phenomenon masks the overall blue/white contrast of the finished product. Therefore, controlling the back-staining is important, especially when high levels of blue/white contrast are expected with no post-wash bleaching step.

Evaluation of abrasion and back-staining of denim garments by reflectance measurement using neutral (from *Humicola insolens*) and acidic (from *Trichoderma reesei*) cellulases revealed that the former caused higher abrasion and less back-staining than the latter (Galante et al., 1998a). The exact reason for the differential levels of back-staining by the acid and neutral cellulases is not known. Initially, the acidic pH during treatment was believed to be responsible, but this was found not to be the case. In fact, there are indications that some acid cellulases facilitate low levels of back-staining, while some neutral cellulases show high re-deposition of indigo (Galante et al., 1998a). Hence, these results cautioned that the pH profile alone should not be considered as the sole reason for its potential performance during bio-stoning.

It has been reported that the blue indigo re-deposition during bio-stoning with acid cellulase could be substantially prevented by either adding microbial protease (e.g. subtilisin) together with cellulase in the wash bath or by mixing the protease with the cellulase before adding to the washing machine (Galante et al., 1998a). The protease was believed to prevent the cellulase from binding the dye back to the surface of the denim, yet this did not affect the abraded look caused by the action of the cellulase. Nonetheless, an optimum ratio of cellulase to protease and the pH were critical in order to obtain maximum benefit. Besides, it has not yet been established whether an endo-rich or single endoglucanase from the *Trichoderma* cellulase preparation performs better during bio-stoning than the whole complex. Further experiments using two commercially available enzymes (an endo-rich cellulase, active at acidic pH and a recombinant endocellulase) are expected to reveal an ideal cellulase preparation for bio-stoning.

5.2. Bio-polishing of non-denim fabrics

Most of the natural materials used in fabric manufacturing contained cellulosic fibers, such as cotton, linen, ramie, viscose and lyocell, which had a tendency for 'fuzz' formation (short fibres protruding from the surface) as well as 'pilling' (fluffy/loosened fuzz attached to

the surface). These phenomena were considered as negative features of cellulosic fabrics. Hence, the prevention or permanent removal of fuzz formation and pilling was necessary to increase the commercial value of cellulosic fabrics. This was accomplished using cellulases in a process called 'bio-polishing' (Galante et al., 1998a).

Bio-polishing is usually carried out during the textile wet processing stage and includes desizing, scouring, bleaching, dyeing and finishing. During this process, the cellulases act on small fibre ends that protrude from the fabric surface, where the mechanical action removes these fibres and polishes the fabrics. The main advantages of using cellulases are: (1) removal of short fibres and surface fuzziness; (2) smooth and glossy appearance; (3) improved colour brightness and uniformity; (4) high hydrophilicity and moisture absorbance; (5) new and improved finishing and fashionable effects; and (6) environmentally friendly process. In fact, bio-polishing is currently a key step in the textile industry for producing high quality garments.

5.3. Defibrillation of lyocell

Lyocell is a pure cellulosic fibre from wood pulp obtained after the solvent spun with amino oxide. Although the lyocell possesses many positive attributes for the production of high quality fabrics, these fabrics often show tangles of primary fibrils on the surface commonly referred as 'fibrillation,' which is a negative aspect of lyocell. Indeed, the defibrillation of lyocell fabrics can be best controlled by cellulase treatment. Acid cellulases have proven to be most effective in treating 100% lyocell, while mixed lyocell garments can be processed successfully with neutral cellulases. The major benefits of cellulase treatment of lyocell based garments include: (1) significantly enhanced appearance and soft hand feel; (2) defiling and pill prevention; and (3) improved drapability and surface appearance even after repeated washings (Kumar et al., 1994).

Because lyocell is advantageous for textile companies, many enzyme producers decided to concentrate on developing new tailor-made cellulases specific for lyocell. These novel cellulases are expected to achieve the following goals: (1) wide range of finishing effects, particularly in blends with other fibres; (2) negligible loss of strength and weight; (3) better adaptability to high-speed jet machines; (4) possible combination with other chemical treatment steps; and (5) natural and pleasant hand feel. Two genetically engineered commercial endoglucanases are currently available and particularly suited for the treatment of lyocell fabrics (Galante et al., 1998a). Indeed, the great versatility of lyocell and other man-made fibres in textile industries will persuade the genetic and protein engineers to produce new and improved cellulases ideal for textile applications.

5.4. Are endo-enriched cellulase or whole cellulase preparations ideal for bio-finishing?

As in the case of bio-stoning, cellulase rich in endoglucanase activity appears to be better suited for bio-finishing. Nonetheless, it is still not clear which component of a cellulase complex should be either added or omitted, to achieve the best performance during bio-finishing. Using a cellulase preparation produced by *T. reesei* where the gene coding for endoglucanase II was deleted, Miettinen-oinonen et al. (1996) reported that a cellulase preparation low in endoglucanase activity showed up to 15% less strength loss in fabric than the complete cellu-

lase mixture at an equal abrasion level. Similarly, in bio-finishing experiments using cotton fabrics, the cellulase preparation with low endoglucanase activity gave less strength loss as a function of enzyme dosage. Thus, these authors concluded that a modified *T. reesei* cellulase preparation lacking endoglucanase II performed better during bio-stoning and bio-finishing of cotton and caused less strength loss and low fibre damage. In contrast, Kumar et al. (1996) who studied the performance of three different cellulase preparations from *T. reesei* using one complete cellulase mixture, while other two were enriched with different endoglucanase activities and no exoglucanase activity. These authors reported that one of the endo-enriched cellulase preparations gave the highest degree of defibrillation of both 100% lyocell and a 65/35 blend of lyocell/cotton with significantly less fabric strength loss in contrast with the complete cellulase mixture. In addition, the other endo-enriched cellulase was more effective when mild surface polishing was required and fabric strength loss was a major concern (Kumar et al., 1996). Based on these findings, the authors concluded that the performance of the whole cellulase preparations was quite different from the enzyme rich in endo-activity, and that the latter offered better performance in applications where losses in fabric strength and weight had to be minimised. Therefore, the immediate future objectives of textile industries are: (1) the selection of ideal cellulase preparations; (2) obtaining soft fabrics with no loss of strength properties; and (3) the use of cellulae in continuous fabric processes, in order to obtain the best results and to avoid undesirable side effects.

5.5. Use of cellulase in laundry

The cellulase preparations capable of modifying the structure of cellulose fibrils are added to laundry detergents to improve the colour brightness, hand feel and dirt removal from cotton and cotton blend garments. Most cotton or cotton blend garments, during repeated washings, tend to become fluffy and dull. This is mainly due to the presence of partially detached microfibrils on the surface of garments that can be removed by cellulases in order to restore a smooth surface and original colour to the garment. Also, the degradation of microfibrils by cellulase, softens the garment and removes dirt particles trapped in the microfibril network. This is currently accomplished by adding a commercial cellulase preparation from *H. insolens*, active under mild alkaline conditions (pH 8.5–9.0), and at temperatures over 50°C in washing powders (Uhlig, 1998). Although, the amount of cellulase added represents approximately 0.4% of the total detergent cost, it is considered rather expensive and hence, alternative cellulase preparations are required to attract the worldwide laundry market.

6. Cellulases and hemicellulases in pulp and paper biotechnology

Cellulases and hemicellulases have been used in the pulp and paper industry for different purposes. Commercial enzyme preparations contain various enzyme activities, where some may be vital, while others may be detrimental for a specific application. Therefore, enzyme mixtures or purified enzymes should be well characterised with respect to their substrate specificity and mode of action before using for a particular application in pulp and paper industry. Some of the main application of cellulases and hemicellulases in pulp and paper industry are summarised in Table 5 and described below.

Table 5
Cellulases and hemicellulases in pulp and paper biotechnology

Enzyme	Function	Application	Reference
Cellulases and hemicellulases	Modification of coarse mechanical pulp and hand-sheet strength properties; partial hydrolysis of carbohydrate molecules and the release of ink from fibre surfaces; hydrolysis of colloidal materials in paper mill drainage	Bio-mechanical pulping; modification of fibre properties; de-inking of recycled fibres; improving draining and runnability of paper mills	Akhtar, 1994; Buchert et al., 1998; Kantelinen et al., 1995; Leatham et al., 1990; Noe et al., 1986; Pere et al., 1996; Prasad et al., 1992, 1993; Rahkamo et al., 1996; Saddler, 1993; Viikari et al., 1993
Xylanases, mananases, β-xylosidase and α-L-arabinofuranosidase	Hydrolysis of re-precipitated xylan or removal of xylan from lignin-carbohydrate complexes; removal of glucomannan	Bio-bleaching of kraft pulps; reduction in chlorine requirement in subsequent bleaching and environmental pollution	Buchert et al., 1992; 1998, Suurnakki et al., 1996a; Tenkanen et al., 1992a; Tolan, 1992; Viikari et al., 1987
Purified cellulase and hemicellulase components	Partial or complete hydrolysis of pulp fibres	Bio-characterization of pulp fibres	Buchert et al., 1996b, 1997; Oksanen et al., 1997; Suurnakki et al., 1996c; Teleman et al., 1995

6.1. Bio-mechanical pulping

The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk and stiffness. Although these fibres are useful for producing different grades of papers, the main disadvantage of mechanical pulping is high-energy consumption. Bio-mechanical pulping using white-rot fungi resulted in substantial energy savings during refining, and improvements in hand-sheet strength properties (Akhtar, 1994; Leatham et al., 1990). Unfortunately, these encouraging laboratory results have not yet been commercialised.

Unrefined wood chips are generally less accessible to enzymatic modification, hence, the addition of an enzyme in mechanical pulping can be effective only after the initial refining. The effects of enzymatic modification of coarse mechanical pulp using cellulase and hemicellulase from *Trichoderma* prior to secondary refining were studied (Pere et al., 1996). Marginal modification of the above pulp, during secondary refining, led to an energy savings of 20% and 5% with cellobiohydrolase I and hemicellulase, respectively. Treatment with endoglucanase I from *Trichoderma*, slightly decreased the energy consumption at the expense of pulp quality, while no positive effect on energy consumption was observed with cellulase mixture. Energy consumption was reduced up to 30–40% with cellobiohydrolase I, when the refining was performed using low-intensity refiner. Use of cellobiohydrolase I also led to 10–15% energy savings during two-stage refining and resulted in increased tensile strength and high fibre qualities (Pere et al., 1996).

6.2. Bio-bleaching of kraft pulps

Use of hemicellulolytic enzymes was the first large-scale application of enzymes in the pulp and paper industry (Viikari et al., 1986, 1987). This was based on the observation, that limited hydrolysis of hemicellulose in pulps by hemicellulases (mainly xylanases) increased the extractability of lignin from the kraft pulps and reduced the chlorine required in subsequent bleaching. Although the exact mechanism of action of xylanase in bio-bleaching is not known, it has been proposed that the xylanase either hydrolysed the re-precipitated xylan partially or completely removed the xylan from the lignin-carbohydrate complexes. Both these processes were possible and allowed the enhanced leaching of entrapped lignin from the fibre cell wall and made the pulp more susceptible to the bleaching chemicals. The xylanase from *T. reesei* has been reported to act uniformly on all accessible surfaces of kraft pulp and to be effective during bio-bleaching (Saake et al., 1995; Suurnakki et al., 1996a).

Compared to xylanase, mannanase has attracted minimal attention in bio-bleaching because of its limited action on most pulps. Also, the mechanism of mannanase-aided bleaching appears to differ from the xylanase-aided bleaching, since the distribution of glucomannan is different from xylan in pulps (Buchert et al., 1992; Suurnakki et al., 1996b,c). In case of mannanase-aided bleaching the composition and configuration of the outer surface of pulp fibres appear to be important (Suurnakki et al., 1996c).

The role of xylanase in the de-lignification of kraft pulps has been extensively studied using two purified xylanases from *T. reesei* with different pI (5.5 and 9.0), pH optima and substrate specificities (Buchert et al., 1992; Tenkanen et al., 1992a,b). Interestingly, both xylanases performed almost in the same manner, in reducing kappa number and improving the brightness in subsequent chemical modifications (Buchert et al., 1992). Purified *T. reesei* hemicellulases have also been used in bleach boosting of different types of pulps. The xylanase from *T. reesei* was most effective when used in conventionally cooked pulps, and the effect was more pronounced with pulps produced from northern pine than radiata pine (Suurnakki et al., 1996a). Similar results have been reported with xylanases from other micro-organisms with respect to the origin of pulp and its production method (Allison et al., 1995; Nelson et al., 1995; Tolan, 1992).

It has been suggested that mannanase was most beneficial in pulp bleaching when used in combination with xylanase (Buchert et al., 1992; Suurnakki et al., 1996a), while the accessory enzymes such as β-xylosidase and α-L-arabinosidase played a minor role in xylanase-aided bleaching of pulps (Kantelinen et al., 1993; Luonteri et al., 1996). Interestingly, the endoglucanase I from *T. reesei* has been shown to increase the bleachability of pulps due to its xylanase activity (Buchert et al., 1994). Most of the commercial hemicellulase preparations currently used in bio-bleaching of different pulps originate from *T. reesei*.

6.3. Bio-modification of fibres

Cellulase and hemicellulase mixtures have been used for the modification of fibre properties with the aim of improving drainage, beatability and runnability of the paper mills (Noe et al., 1986; Pommier et al., 1989, 1990). In these applications, the enzymatic treatment was performed either before or after beating of the pulps. The aim of cellulase and hemicellulase treatment prior to the refining process is either to improve the beatability response or to modify the fibre properties. The addition of cellulase and hemicellulase after beating is to improve the

drainage properties of pulps, which determine the speed of paper mills. A commercial cellulase/hemicellulase preparation, named Pergalase-A40, from *Trichoderma* has been used by many paper mills around the world for the production of release papers and wood-containing printing papers (Freiermuth et al., 1994; Pommier et al., 1990).

In the late 1980s, the possibility of improving the drainage rates of recycled fibres by cellulase, especially by endoglucanase was identified (Pommier et al., 1989, 1990). This was subsequently confirmed by Kamaya (1996) using purified endoglucanases from *Trichoderma*. Both endoglucanases I and II from *Trichoderma* were equally effective in decreasing the Schoper-Riegler (SR) value of recycled soft wood craft pulp and indicated improved drainage, whilst cellobiohydrolase had no effect. Xylanase and mannanase treatment resulted in only a marginal improvement of the SR value. However, depending on the origin of the pulp, the efficiency of different enzymes might vary. For example, Pere et al. (1996) demonstrated the need of simultaneous solubilization of xylan and cellulose for the drainage improvement of reed cannary grass kraft pulp. By using endoglucanase I from *T. reesei*, which hydrolysed both cellulose and xylan, Pere et al. (1996) showed a drainage improvement by 30%, while endoglucanase II from the same fungus, which was specific for cellulose, showed only a limited effect on the drainage property.

A detailed understanding of the action of different cellulase and hemicellulase components on different types of pulps is vital for the development of enzymatic modification of fibres. Mansfield et al. (1996) studied the action of a commercial cellulase preparation (Novozyme SP from *Humicola insolens*) on different fractions of Douglas fir kraft pulp. They observed that the cellulase treatment decreased the defibrillation, which reduced the fibre coarseness. Also, with increasing dose of cellulase, the strength properties of fibre reduced. Pere et al. (1995) and Rahkamo et al. (1996) investigated the effect of major cellulase components from *T. reesei* on the fibre properties of unbleached soft wood kraft and dissolving pulps. They found that the cellobiohydrolases had moderate effect on fibre viscosity, while endoglucanases, especially endoglucanase II, dramatically decreased the pulp viscosity even at a low concentration. Nevertheless, cellobiohydrolase I treatment showed no effect on the hand-sheet properties even after PFI-refining, and suggested that this enzyme did not cause any structural damage to the fibres. On the other hand, endoglucanase II treatment damaged the strength properties, and indicated that this enzyme attacked cellulose fibres at sites where even low levels of hydrolysis resulted in large decrease in viscosity and led to a dramatic deterioration in the tensile index (Pere et al., 1996).

Oksanen et al. (1997) and Kamaya (1996) studied the effect of purified cellulase and hemicellulase components from *Trichoderma* on the beatability of pulp and technical properties of paper from bleached kraft pulps. Treatment of pulp with either cellobiohydrolase I and II had no effect on the development of pulp properties, whereas endoglucanase, especially endoglucanase II, improved the pulp beatability, sheet density and other properties of the paper. Xylanase and mannanase, however, did not modify the pulp properties significantly when less than 10% of the respective hemicellulose was hydrolysed (Oksanen et al., 1997).

6.4. Bio-de-inking

The application of enzymes in de-inking has been intensively studied in both laboratory and pilot scales, but the technique has not yet been commercialised (Buchert et al., 1998).

The two principal approaches in using enzymes for de-inking include the (1) hydrolysis of soy-based ink carriers by lipase, and (2) the release of ink from fibre surfaces by cellulases, xylanases and pectinases. Most applications proposed so far use cellulases and hemicellulases for the release of ink from the fibre surface by partial hydrolysis of carbohydrate molecules (Jeffries et al., 1994; Prasad et al., 1992, 1993). The main advantage of enzymatic de-inking is the avoidance of the use of alkali. De-inking, using enzymes at acidic pH, also prevents the alkaline yellowing, simplifies the de-inking process, changes the ink particle size distribution and reduces the environmental pollution. In addition, the enzymatic de-inking improves the fibre brightness, strength properties, pulp freeness and cleanliness as well as reduces fine particles in the pulp. Xylanase treatment has been reported to increase the strength properties, while cellulase treatment improved the brightness and freeness of the pulp (Prasad et al., 1993). In fact, the enzymatic de-inking has a great potential both from commercial and environmental standpoints and expected to be commercialised in the near future.

6.5. Bio-improvement of drainage properties and the performance of paper mills

During mechanical pulping, various wood components such as pitch, lignin and hemicellulose are dissolved and released into the drainage. These components are collectively called ‘dissolved and colloidal substances.’ During peroxide bleaching of mechanical pulps, other wood components including pectin are also released. All these components often cause severe problems in paper mills including pitch depositions, specks in the paper and decreased de-watering. Enzymes, especially carbohydrases, which act on the above-mentioned colloidal substances are expected to improve the overall performance of paper mills. Using a commercial enzyme preparation (Pergalase A 40) from *Trichoderma*, Kantelinen et al. (1995) demonstrated a remarkable decrease in the turbidity of thermo-mechanical pulping filtrates. Also, the enzymatic treatment, destabilised the lipophilic extractives in the filtrates and facilitated their attachment to thermo-mechanical pulping fibres. In addition, the same authors showed that the purified endoglucanase I from *T. reesei* was useful for disturbing the steric stability of colloidal pitch, while the xylanase from the same fungus was effective only at high concentrations.

6.6. Bio-characterisation of pulp fibres

Hydrolases acting on pulp fibres are useful tools for the characterisation of fibres. Purified xylanase and mannanase from *T. reesei* have been successfully used for selective solubilization of xylan and glucomannan from different pulps (Buchert et al., 1996a). In addition, either purified cellulase components or mixtures of cellulase and hemicellulase components have been used for partial or complete solubilization of pulp fibres and subsequent characterisation of hydrolysis products by either NMR or HPLC (Buchert et al., 1995; Teleman et al., 1995; Tenkanen et al., 1995). Selective enzymatic solubilization of xylan or glucomannan facilitates determination of the influence of the respective hemicellulosic components on fibre properties such as pore size distribution (Suurnakki et al., 1997), location of lignin (Buchert et al., 1996b), brightness reversion (Buchert et al., 1997) and hornification (Oksanen et al., 1997). Enzymatic solubilization of pulp carbohydrates under mild and non-destructive conditions is beneficial, especially in the analysis of acid-labile pulp components. The suitability

of this approach has been verified in the structural analysis of kraft xylan using *T. reesei* xylanase, which led to the identification of hexenuronic acid in kraft pulps (Buchert et al., 1995; Teleman et al., 1995).

7. Application of cellulases and related enzymes in research and development as well as in agriculture

Both mixture and isolated components of cellulases, hemicellulases and pectinases, have a wide range of potential applications in research as summarised in Table 6. Also, these enzymes and the fungi have potential applications in agriculture for controlling plant disease (Benitez et al., 1998; Chet et al., 1998) as well as in enhancing plant growth and development (Bailey and Lumsden, 1998). Some of the main applications of cellulases and related enzymes in research and development as well as in agriculture are described below.

Table 6
Applications of plant cell wall degrading enzymes and cellulolytic micro-organisms in research and development as well as in agriculture

Enzyme/microorganism	Function	Application	Reference
Mixture of cellulases, hemicellulases and pectinases	Solubilization of plant or fungal cell walls	Production of plant or fungal protoplasts, hybrid and mutant strains	Beguin and Aubert, 1994; Bhat and Bhat, 1997; Brown et al., 1986
Cellulases and related enzymes, preferably β-1,3 and 1,6 glucanases; *Trichoderma* sp. and *Geocladium*	Inhibition of spore germination, germ tube elongation and fungal growth	Bio-control of plant pathogenes and diseases	Benitez et al., 1998; Bruce et al., 1995; Chet et al., 1998; De La Cruz et al., 1995; Harman and Kubicek, 1998; Lorito et al., 1994
Trichoderma sp., *Geocladium* sp., *Chaetomium* sp., *Penicillium* sp., *Rhizopus nigricans*, *Fusarium roseum*	Enhancing seed germination, plant growth and flowering; improving root system; increasing the crop yields.	Agriculture	Bailey and Lumsden, 1998; Harman and Bjorkman, 1998; Harman and Kubicek, 1998
CBD of cellulases and cellulosomes; dockerins, cohesins and linkers of cellulosome	Affinity tag, affinity systems, conjugation and gene fusion	Affinity purification, immobilization and fusion of proteins, enzymes and antibodies; production of hybrid molecules for various applications	Bayer et al., 1994, 1995; Greenwood et al., 1989; Ong et al., 1989; Tomme et al., 1994
Cellobiohydrolase I promoter from *T. reesei* and glucoamylase promoter from *A. niger*	Expression of heterologous proteins and enzymes	Production of high levels of proteins, enzymes and antibodies	Dunn-Coleman et al., 1991; Harkki et al., 1989; Joutsjoki et al., 1993; Pentilla, 1998; Saloheimo et al., 1989; Saloheimo and Niku-Paavola, 1991; Ward et al., 1990
Native enzymes, subunits of cellulosome or recombinant enzymes	Improving the efficiency of a specific application	Production of designer cellulosomes	Bayer et al., 1994

7.1. Enzymatic production of plant and fungal protoplasts

Mixture of cellulases and other polysaccharidases produced by fungal strains of *Trichoderma* and *Penicillium* are used for the production of plant and fungal protoplasts (Table 6). These protoplasts can be fused to produce either hybrid or mutant strains with desired characteristics. Also, cellulases and related enzymes can be used as potential tools for generating new strains capable of producing high levels of enzymes of commercial interest.

Brown and co-workers (1986) evaluated a number of commercial and in-house cellulase preparations for the production of protoplasts from wild and mutant strains of *Penicillium*. They reported that the enzymes from *Trichoderma viride* Persoon (strain BIA), grown on solid-state culture using wheat bran, and *Penicillium pinophilum* 87160iii, grown as submerged culture on a mixed substrate (laminarin and *P. pinphilum* cell walls), were the best for the production of fungal protoplasts. Thus, a combination of enzyme preparations containing cellulase and hemicellulase activities can be successfully used for the production of plant and fungal protoplasts.

7.2. Enzymatic/microbial control of plant disease and enhanced plant growth

Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens an controlling the plant disease. It has been reported that β-1,3-glucanase and N-acetyl-glucosaminidase from *Trichoderma harzianum* strain P1 synergistically inhibited the spore germination and germ tube elongation of *B. cinerea* (Lorito et al., 1994). The β-1,3-glucanase from *T. harzianum* CECT 2413 induced morphological changes such as hyphal tip swelling, leakage of cytoplasm, and the formation of numerous septae, and inhibited the growth of *R. solani* and *Fusarium* sp. (Benitez et al., 1998). Also, the β-1,3- and β-1,6-glucanases from *T. harzianum* CECT 2413 hydrolysed filamentous fungal cell walls and inhibited the growth of fungi tested (Bruce et al., 1995; De La Cruz et al., 1995). Furthermore, the mutant strains of *T. harzianum*, which produced higher levels of β-1,3- and β-1,6-glucanases than the wild type strain, have also been shown to possess high anti-fungal activity. A hypercellulolytic mutant of *T. longibrachiatum*, which produced higher levels of β-1,4-endoglucanase than wild type, reduced the disease incidence by *Pythium* (a plant pathogen) on cucumber seedlings from 60 to 28% (Chet et al., 1998). Thus, a combination of fungal strains and their enzymes could be useful as bio-control agents to protect the seeds and plants from plant pathogens.

Many cellulolytic fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. are known to play a key role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system as well as increased crop yields (Bailey and Lumsden, 1998; Harman and Bjorkman, 1998; Harman and Kubicek, 1998). Although, these fungi have both direct (probably through growth-promoting diffusable factor) and indirect (by controlling the plant disease and pathogenes) effects on plants (Bailey and Lumsden, 1998; Harman and Bjorkman, 1998), it is not yet clear how these fungi facilitate the improved plant performance. Further research is vital to unravel the full potential of these micro-organisms in agriculture.

7.3. CBD-based affinity tag for the purification of enzymes, antibodies and immobilization of fusion proteins

Cellulose-binding domains (CBD) of fungal cellulases, which functions normally when fused to heterologous proteins, have been successfully used either as an affinity tag for the

purification of proteins or immobilisation of fusion proteins (Assouline et al., 1993; Greenwood et al., 1989, 1992; Ong et al., 1989; Tomme et al., 1994). Similarly, using the scaffoldin CBD of the *Clostridium thermocellum* cellulosome, a novel affinity column was prepared for the purification of antibodies. The biotinylated CBD bound to cellulose was attached to biotinylated protein A via avidin and used successfully for the purification of antibodies (Bayer et al., 1995). Thus, the CBD has a great potential in biotechnology.

7.4. Expression of heterologous proteins and enzymes

It has been reported that the mutant strains of *T. reesei* synthesized and secreted cellulases as high as 40 g per litre of culture medium and approximately 50% of cellulases accounted for cellobiohydrolase (Durand et al., 1988; Penttila, 1998). This implied that the cellobiohydrolase I gene possessed a strong promoter. Using this promotor, a number of heterologous proteins, enzymes and antibodies have been produced in mg and gram quantities (Penttila, 1998). The promotor of the *A. niger gal*A gene also triggered the synthesis and secretion of relatively high levels of glucoamylase (Penttila, 1998) and could be used for the production of heterologous proteins.

Calf chymosin was the first heterologous protein expressed in *T. reesei* and *Aspergillus* (Harkki et al., 1989; Ward et al., 1990). Also, the calf chymosin expressed in *Aspergillus*, was the first mammalian protein produced at the commercial level and used in the production of vegetarian cheese (Dunn-Coleman et al., 1991). The other heterologous proteins expressed in *T. reesei* using the cellobiohydrolase I promotor, include: (1) lignin peroxidase and laccase from *Phlebia radiata* (Saloheimo et al., 1989; Saloheimo and Niku-Paavola, 1991); (2) glucoamylase P from *Hormoconis resinae* (Joutsjoki et al., 1993); (3) phytase and acid phosphatase from *Aspergillus niger* (Paloheimo et al., 1993); (4) endochitinase from *Trichoderma harzianum* (Margolles-Clark et al., 1996); (5) antibody Fab fragments and single chain antibodies from murine (Nyyssonen et al., 1993); and (6) interleukin-6 from mammalian origin (Demolder et al., 1994). The production of high yields of human lysozyme as a fusion protein of either *T. reesei* cellobiohydrolase I, *A. niger* glucoamylase or the bacterial phleomycin resistance protein has been reported (Penttila, 1998). Thus, the cellobiohydrolase I and other strong promotors have great potential for the production of foreign proteins, and antibodies of commercial interest. Nonetheless, further research is essential for establishing the suitability of *T. reesei* cellobiohydrolase I and other promotors for the production of foreign proteins and antibodies. Also, the knowledge on protein folding and glycosylation are important while selecting an organism for the production of a specific protein or an enzyme.

8. Concluding remarks and future prospects

The progress in biotechnology of cellulases and related enzymes is truly remarkable and attracting worldwide attention. Currently, cellulases, hemicellulases and pectinases are widely used in food, brewery and wine, animal feed, textile and laundry, paper and pulp industries as well as in research and development. Some of these applications prefer one or two selected components of cellulase, hemicellulase or pectinase, while others require mixtures of cellulases, hemicellulases and pectinases for maximum benefit. Recent development on

the biochemistry, genetics and protein, as well as on the structure–function relationships of cellulases including cellulosomes and related enzymes from bacteria and fungi, has led to speculation and anticipation of their enormous commercial potential in biotechnology and research. In fact, the potential use of *C. thermocellum* cellulosome and its subunits in research, medicine and biotechnology has been elegantly described by Bayer and co-workers (Bayer et al., 1994). Also, the non-catalytic domains of cellulase system, such as the CBD, and the promoters of cellobiohydrolase I from *T. reesei* and related enzymes will undoubtedly play a key role in future biotechnology and research. Hence, to meet the growing demand for cellulases and related enzymes and to realise their full potential in biotechnology and research, continued multidisciplinary research on basic and applied aspects is vital. These developments together with improved scientific knowledge are expected to pave the way for a remarkable success in the biotechnology of cellulases and related enzymes in the 21st century.

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