

TRICHODERMA VIRIDE STRAIN FOR CELLULOLYTIC ENZYME PRODUCTION

FIELD OF THE INVENTION

5 The present invention relates to a novel strain of *Trichoderma viride*. More particularly, the present invention relates to a novel strain of *Trichoderma viride* useful for the production of cellulolytic enzyme complex, for degrading lignocellulosic materials. The invention also relates to a method of producing a supernatant comprising a cellulolytic enzyme complex derived from the strain, the application of the supernatant produced
10 thereto to degrade the lignocellulosic materials and its use in but not limited to bioethanol industry.

BACKGROUND OF THE INVENTION

15 Enzymes have been used for a long time for a variety of different industrial applications. For example, in the baking industry, in wine and fruit juice industries (where enzymes are used to break down pectins and beta-glucans), in textile industry (where cellulases are used to obtain soft and smooth cellulosic fabrics), in animal feed, and also, which is not the least application, in bioethanol industry (where cellulolytic enzymes are used to
20 hydrolyze lignocellulosic biomass to produce reducing sugars for fuel ethanol fermentation).

In recent years, conversion of biomass to biofuel has been the subject of intense research efforts. It has gained significant political and scientific momentum, owing to
25 concerns about the shortage of fossil fuels and the emission of green house gases (Antoni *et al.*, "Biofuels from Microbes"; *Appl Microbiol Biotechnol*, 77:23-35 (2007); Bayer *et al.*, "The Cellulosomes: Multienzyme Machines for Degradation of Plant Cell Wall Polysaccharides", *Annu Rev Microbiol*, 58: 521-554 (2004); Service RF, "Cellulosic Ethanol : Biofuel Researchers Prepare to Reap a New Harvest", *Science*, 315:1488-1491
30 (2007)). Global energy usage is projected to nearly double in the next two decades ([http://www.eia.doe.gov/oiaf/ieo/pdf/048.\(2007\).pdf](http://www.eia.doe.gov/oiaf/ieo/pdf/048.(2007).pdf).) and biological fuel production might serve as a sustainable, carbon-neutral energy source, compatible with the current engine technology.

35 Lignocellulosic biomass is by far the most abundant renewable source of sugars that can be fermented to high value products like fuel ethanol. These include hardwood, softwood,

grasses and agricultural residues. Other raw materials of potential interest include newsprint, office paper, municipal solid wastes, etc. Lignocellulose is comprised of three major components: cellulose, hemicellulose, and lignin.

- 5 Cellulose is a homopolysaccharide comprised of β -D-glucopyranose units, linked by β -(1-4)-glycosidic bonds. The smallest repetitive unit of cellulose is cellobiose, which can be converted into glucose residues.

Hemicelluloses are polymers of xylose, mannose, arabinose and galactose.

10

Lignin is a highly branched biopolymer of aromatic compounds filling the spaces in the cell wall between cellulose and hemicellulose. It has an important role in the physical characteristics of wood as well as in protecting lignocellulosic materials against biological attack, against cellulose-degrading microorganisms, etc. Various pretreatment methods
15 are applied to break down the intact structure of lignocellulosic materials. The aim of the pretreatment is to degrade biomass structure and to enhance enzymatic degradation. Hemicelluloses associate closely with lignin and cellulose. Therefore, most hemicelluloses are removed in many pretreatment methods. However, part of the remained hemicelluloses is still used as a substrate either for hydrolysis or for cellulolytic
20 enzyme production.

Lignocellulosic enzyme complex is comprised of cellulolytic enzymes (i.e. cellulase), a multicomponent enzyme complex containing three basic types of enzyme (i.e. endoglucanase, exoglucanase and β -glucosidase) and hemicellulolytic enzymes (i.e. xylanase and β -xylosidase, etc.). The synergistic actions of this enzyme complex bring
25 about the degradation of cellulose to glucose and the hemicellulose to xylose and other reducing sugars.

The production of cellulolytic enzyme complex is a major factor in lignocelluloses hydrolysis and biomass-to-ethanol process. It is important to make the process
30 economically viable because large-scale application in the degradation of lignocelluloses demands microorganisms with improved activity and productivity and cellulases with better resistance to product inhibition. A variety of microorganisms, including bacteria and fungi, have the ability to secrete cellulase to degrade the lignocelluloses to glucose monomers. Filamentous fungi, for example, *Trichoderma reesei* (See Mandels *et al.*,
35 "Measurement of Saccharifying Cellulose", *Biotechnol Bioeng Symp*, 6:21-33 (1976a)),

have excellent capacity for extracellular protein production. It is well known that *Trichoderma reesei* produces cellobiohydrolase (CBH) or exoglucanase and endoglucanase (EG) components of the cellulolytic enzyme complex in large quantities. However, the amount of β -glucosidase secreted by *Trichoderma reesei* is insufficient, causing the accumulation of cellobiose which produces repression and end product inhibition to the enzymes. In addition, cellulase from *Trichoderma reesei* shows relatively low specific activity, low thermal stability and high sensitivity to product inhibition (See Lynd *et al.*, "Microbial Cellulose Utilization: Fundamentals and Biotechnology", *Microbiol Mol boil Rev*, 66:506-577 (2002); Chan *et al.*, "Novel Mutation Method for Increased Cellulase Production", *J appl Microbiol*, 98:318-323 (2005); and Kavas *et al.*, "*Trichoderma Atroviride* Mutants with Enhanced Production of Cellulose and β -glucosidase on Pretreated Willow", *Enzyme Microb Tech*, 43:48-55 (2008)).

Other known cellulases producing fungi includes *Penicillium* (See Asdul *et al.*, "Strain Improvement of *Penicillium janthinellum* NCIM 1171 for Increased Cellulose Production", *Bioresour Technol*, 98:1467-1473 (2007); and Cheng *et al.*, "Genome Shuffling Improves Production of Cellulase by *Penicillium decumbens* JU-A10", *J Appl Microbiol*, 107:1837-1846 (2009)) and *Aspergillus* species (See Sridevi *et al.*, "Production of Cellulase by *Aspergillus niger* on Natural and Pretreated Lignocellulosic Wastes", *The Internet J Microbiol*, 7(1) (2009)). These species produce cellulolytic enzyme complexes having a higher β -glucosidase activity than *Trichoderma reesei*, but the total cellulase (FPase) activity is relatively lower than that from *Trichoderma reesei*. As a result, production of sugars from waste cellulose is still not commercially feasible.

Lignocellulosic enzymes can be prepared from a range of different microorganisms. In WO 95/26398, a modified cellulase is obtained by inclusion of foreign DNA sequence in a host cell which modifies the nature of the original strain selected from a list of microorganisms consisting of: *Bacillus*, *Streptomyces*, *Saccharomyces*, *Schizosaccharomyces* and *Aspergillus*.

In WO 96/05739, a mixture of enzymes comprising xylanase, protease, and beta-glucanase is obtained from different microorganisms.

In WO 2009/065199, a mixture of enzymes comprising endoglucanase, exoglucanase, beta-glucosidase and accessory enzymes such as xylanase is obtained from *Trichoderma reesei* and *Aspergillus awamori*.

In some other cases, the enzyme preparations are obtained from microorganisms that are subjected to genetic modification using recombinant DNA techniques.

5 SUMMARY OF INVENTION

The above and other problems are solved and an advance in the art is made by providing a novel strain of *Trichoderma viride* named NP13a useful for the production of cellulolytic enzyme complex in accordance with this invention. It is an advantage that the strain of
10 *Trichoderma viride* NP13a in accordance with this invention is useful for producing a cellulolytic enzyme complex having improved ability to degrade lignocellulosic materials. A second advantage of this invention is that the strain of *Trichoderma viride* NP13a is useful for producing cellulolytic enzyme complex without the need to subject the *Trichoderma viride* NP13a strain to genetic modification.

15

In accordance with an embodiment of this invention, there is provided an isolated culture of a strain of *Trichoderma viride* named NP13a on deposit with the ATCC as accession no. PTA-10765 and a mutant derived therefrom, having cellulolytic activity useful for production of cellulolytic enzyme complex.

20

In accordance with another embodiment of this invention, there is provided a supernatant obtained from the culture of the strain of *Trichoderma viride* NP13a. The supernatant comprises a cellulolytic enzyme complex which comprises cellulase, endoglucanase, exoglucanase, β -glucosidase, xylanase and β -xylosidase or mixtures thereof.

25

In accordance with another embodiment of this invention, a method for obtaining the supernatant is provided. The method comprises contacting the strain of *Trichoderma viride* NP13a with a medium to get a suspension, incubating the suspension, fermenting the suspension and followed by centrifuging the suspension to obtain the supernatant.

30

In accordance with other embodiments of this invention, a method for degrading lignocellulosic material to fermentable sugar is provided. The method comprises pretreating the lignocellulosic material, washing the pretreated lignocellulosic material with distilled water, drying the pretreated lignocellulosic material and contacting the
35 pretreated lignocellulosic material with the supernatant containing the cellulolytic enzyme complex derived from the strain of *Trichoderma viride* NP13a.

In accordance with other embodiment of this invention, a recombinant microbe comprising genes from the strain of *Trichoderma viride* NP13a is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

5

Figure 1 illustrates the enzyme activities of the *Trichoderma viride* strain NP13a and the commercially available hypercellulolytic *Trichoderma reesei* strain Rut-C30.

10 Figure 2 illustrates the time course of reducing sugar yield during enzymatic hydrolysis of waste newspaper using the enzyme complexes prepared from the *Trichoderma viride* strain NP13a and *Trichoderma reesei* strain Rut-C30, and using other commercial enzymes and their mixtures.

15 Figure 3 illustrates the time course of cellobiose yield during enzymatic hydrolysis of waste newspaper using the enzyme complexes prepared from the *Trichoderma viride* strain NP13a and *Trichoderma reesei* strain Rut-C30 and using other commercial enzymes and their mixtures.

DESCRIPTION OF THE INVENTION

20

The present invention relates to a new strain of *Trichoderma viride*, named NP13a. This strain is useful in producing a cellulolytic enzyme complex that can be used for the degradation of pretreated lignocellulosic materials. The *Trichoderma viride* strain NP13a was deposited with ATCC, 12301 Parklawn Drive, Rockville Md., United States of America on 31 March 2010, and bears the accession number of PTA-10765.

Strain isolation and identification

30 The *Trichoderma viride* strain NP13a of this invention was isolated from soil samples collected from the campus of Ngee Ann Polytechnic, Singapore. *Trichoderma viride* strain NP13a grows well on potato dextrose agar (PDA). On the PDA plate, colonies appear to be white when the *Trichoderma viride* strain NP13a first grows with fine mycelium. The colonies start to form dense mycelium from the centre of the colonies on day two or day three and spores begin to form. The colonies grow in a circular manner and their colour changes from white to green. After a longer period of growth, the colonies become dark green and powdery but are colourless when observed from the

35

back of the plate. The spores grow in a ring-like manner at the beginning. However, at the second stage of growth, the spores begin to grow singularly or in groups of 2 or 3 and are widely spaced apart. The spores' growth is curvy or wave-like. The size of the spores produced is 8 to 12 μm by 2.5 to 3 μm . After budding, surfaces of the spores turn
5 spiky and the size of the spores becomes in the range of 2.5 to 4.8 μm . The spores spread all over the plate by day seven or eight.

The above morphology of the strain NP13a on PDA plates indicates that the strain is a typical *Trichoderma* genus and *Trichoderma viride* species. It forms clear zones when
10 grown on swollen cellulose plates, indicating the secretion of cellulolytic enzymes.

NP13a mutant strains

Mutants of the *Trichoderma viride* strain NP13a can be made using techniques that are
15 well known in this field. For example, mutants can be produced by random mutagenesis using ethylmethane sulfonate (EMS) treatment. Mutants can also be created using ultraviolet light irradiation or nitrosoguanidine treatment using standard techniques. Whole genome engineering approaches such as protoplast fusion, genome shuffling, and global transcription machinery engineering (gTME) can be employed to create improved
20 mutants.

Recombinant microorganisms containing NP13a strain genes

Novel recombinant microbes containing genes from the *Trichoderma viride* strain NP13a,
25 and active mutants thereof, can be made by isolating gene(s) from the *Trichoderma viride* strain NP13a and active mutants thereof, and transforming a suitable host with the gene(s). In particular, genes that encode the lignocellulosic biomass degrading enzymes such as endoglucanase, exoglucanase, β -glucosidase, xylanase, β -xylosidase, and pectinase, etc. can be used to prepare recombinant microorganisms that produce these
30 specific cellulolytic enzymes, which are useful for lignocellulosic material hydrolysis. Such genes can also be optimized using standard techniques, such as enzyme directed evolution and then be transferred to a host cell or to the parent strain itself. Many ways are known for introducing a gene into a host microorganism under conditions that allow for stable maintenance and expression of the gene. One can provide DNA constructs
35 containing the transcriptional and translational signals for the expression of the gene, replication or integration sequences, promoter sequences or marker genes and then

transform them into the host microorganisms. The gene can be from a variety of organisms, such as the host organism, bacteria, bacteriophage, cyanobacteria, fungi, algae, or viruses, which are well known in the art. The construct can be a plasmid, or can be intended to be incorporated into the genome of the host. The host can be a bacterium,
5 fungus, algae, or any of the known organisms in the art. The transformants (transformed host cells) can be cultured in the most appropriate media for cell growth and for enzyme production. Enzymes produced can be extracted, isolated and purified using the standard techniques.

10 Enzyme production

It is well known that culture conditions affect significantly the production of cellulases and hemicellulases. In addition, the enzyme complex composition is sensitive to the composition of the substrate, and to the culturing conditions such as pH, temperature, etc.
15 Carbohydrates or their derivatives induce most of the cellulolytic enzymes.

Enzyme production of the hypercellulolytic fungus, for example, *Trichoderma reesei* Rut-C30 has been studied in most detail. It is generally accepted that cellobiose (See Fritscher *et al.*, "Cellobiose Metabolism and Cellobiohydrolase Biosynthesis by
20 *Trichoderma reesei*", *Exp Mycol*, 14:405-15 (1990)) and lactose (See Morikawa *et al.*, "Cellulase Induction by Lactose in *Trichoderma reesei* PC-3-7", *Appl Biochem Biotechnol*, 44:106-111 (1995)) enhance the production of endo- and exo-glucanase as well as β -glucosidase. However, the highest cellulase yields have been obtained on cellulose-containing carbon sources (See Bhat *et al.*, "Cellulose Degrading Enzymes and Their
25 Potential Industrial Applications", *Biotechnol Adv*, 15:583-620 (1997)). Secretion of β -glucosidase is highly dependent on the culture conditions. A relatively high pH can enhance β -glucosidase production.

In the present invention, cellulases and hemicellulases can be produced on various
30 carbon sources, including on an alpha cellulose substrate (Solka Floc); pretreated wood dust; pretreated wood chips; pretreated oil palm empty fruit bunch powders; and wheat bran or its mixture with the rest of the above mentioned lignocellulosic materials.

The lignocellulosic material or biomass used in the present invention is preferably
35 selected from the group consisting of herbaceous material, agricultural residue, forestry residue, waste paper, and pulp and paper mill residue.

In one embodiment of the invention, the lignocellulosic material or biomass include, but are not limited to, Empty Fruit Bunches (EFB) collected from Masai Palm Oil Mill / Refinery, Johore, Malaysia, wood dust collected from Wah & Hua Pte Ltd, wood chips
5 collected from Environmental Landscape Pte Ltd and/or wheat bran obtained from the market.

All the lignocellulosic materials or biomass collected were immediately autoclaved and dried. The dried biomass was cut, milled and then sieved to 500 μm powder. The
10 biomass then went through chemical pretreatment using a diluted acid such as phosphoric acid (H_3PO_4), hydrochloric acid (HCl), and sulphuric acid (H_2SO_4); or a diluted base such as sodium hydroxide (NaOH), calcium hydroxide ($\text{Ca}(\text{OH})_2$) and hydrogen peroxide (H_2O_2), and distilled water. The biomass was pretreated under room temperature or under pressurized steam for sometime. Thereafter, the pretreated
15 materials were collected, filtered, washed with distilled water until the pretreated materials had a pH of 7, and then dried. The pretreated biomass may then be used as a substrate to cultivate the cellulolytic *Trichoderma viride* NP13a and its mutants thereof, for producing cellulolytic enzyme complex.

20 The *Trichoderma viride* strain NP13a obtained from the soil samples collected from the campus of Ngee Ann Polytechnic, Singapore was grown on potato dextrose agar (PDA) at 30°C for development of spores. On day 7, the plates were washed with 0.5% Tween-80 to obtain a 10^8 spores/ml suspension.

25 Various organic and synthetic media which contain suitable sources of carbon and nitrogen may be used for fermenting the *Trichoderma viride* strain NP13a. Such media may, if desired, contain inorganic salts and other appropriate nutrients suitable for growth of the microorganisms. A suitable carbon source used in the present invention includes, but is not limited to, alpha cellulose substrate (Solka Floc). The effected biomass loading
30 is 1-5%. With regard to the sources of nitrogen, it is possible to use, for example, inorganic ammonium salts (e.g. ammonium sulfate) and organic ammonium salts (e.g. urea, amino acids, meat extract and peptone etc.). With regard to inorganic salts, it is possible to use, for example, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate (MgSO_4), calcium chloride (CaCl_2), iron(II) sulphate (FeSO_4), manganese
35 sulphate (MnSO_4) and zinc sulphate (ZnSO_4).

The inoculum size for the seed culture is preferably 0.1 to 5.0 % of the 10^8 /ml spores suspension prepared above. The seed culture was incubated for 1 to 3 days. The inoculum size of the seed culture to fermentation is preferably 5 to 20%. When the fermentation is effected at a temperature in the range from 20°C to 35°C and a pH of 4 to 8 with sufficient aeration for the strain to grow, the activities of the cellulolytic enzymes reaches a maximum within 4 to 14 days after the fermentation begins. After culturing of the strain is completed, the resultant supernatant solution and/or enzyme preparation originating from the culture liquor may be used in conventional manner for hydrolysis of lignocellulosic materials. The supernatant derived from the *Trichoderma viride* strain NP13a contains a cellulolytic enzyme complex comprised of cellulase, endoglucanase, exoglucanase, β -glucosidase, xylanase and β -xylosidase. Preferably, β -glucosidase and cellulase are present in a ratio of 2:1, and xylanase and cellulase are present in a ratio of 20:1.

Enzyme activity assay

Filter paper activity was assayed according to the procedure described by Ghose, T.K. (Ghose, T.K., *Pure Appl. Chem.* 59:257-268 (1987)) by incubating 0.5 ml of the suitably diluted enzyme samples with 1.0 ml, of 0.05 M citrate buffer (pH 4.8) containing one Whatman No.1 filter paper strip (50 mg, 1 x 6 cm). The reaction mixture was incubated at 50°C for 60 min.

Endoglucanase (CMCase) activity was carried out in a total reaction mixture of 1.5 ml containing 0.5 ml of suitably diluted enzyme samples and 1.0 ml, 2% (w/v) carboxymethylcellulose (CMC) solution in 0.05 M citrate buffer (pH 4.8). This mixture was incubated at 50°C for 30 min.

Xylanase activity was determined by catalyzing 1.0 ml of 1% xylan in 0.05 M phosphate buffer (pH 6.5) by 0.5 ml of suitably diluted enzymes at 40°C for 10 min (See Bailey, *et al.*, "Induction, Isolation and Testing of Stable *Trichoderma reesei* Mutants with Improved Production of Solubilizing Cellulase", *Enzyme Microb Technol*, 3:153-157 (1981)).

β -glucosidase activity was estimated using p-nitro phenyl β -D-glucopyranoside (pNPG) as the substrate (See Berghem and Pettersson, "Mechanism of Enzymatic Cellulose Degradation. Isolation and Some Properties of a Beta-glucosidase from *Trichoderma viride*", *Eur J Biochem*, 46:295-305 (1974)). The total assay mixture (1 ml) consisted of

0.9 ml of 1 mM pNPG and 0.1 ml of suitably diluted enzyme samples and was incubated at 50°C for 10 min. The reaction was terminated by the addition of 0.5 ml of 1 M sodium carbonate. The p-nitrophenol liberated was measured at 400 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan).

5

β -xylosidase activity was estimated in an analogous manner except that the p-nitro phenyl β -D-xylopyranoside (pNPX) was used as the substrate.

The reducing sugars were determined by using the dinitrosalicylic acid (DNS) method described by Ghose, T.K. (Ghose, T.K., *Pure Appl. Chem.* 59:257-268, (1987)). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of the product from their respective substrate per min under the assay conditions. The soluble protein, an indicator of the enzyme released, was measured by the Lowry Protein Assay using bovine serum albumin as the standard (Lowry et al., "Protein Measurement with Folin Phenol Reagent", *J Biol Chem*, 193:265-275, (1951)).

15

Enzymatic hydrolysis

The crude enzyme mixture of the *Trichoderma reesei* strain Rut-C30 and the *Trichoderma viride* strain NP13a and its mutants thereof, was each extracted from the fermentation broth after 7 days of growth. Waste newspaper was cut into approx 0.5 x 0.5 cm pieces after drying at 90°C for two days. Enzymatic hydrolysis of the waste newspaper was carried out at a biomass loading of 2.5% (w/v) in 20 ml of 0.05 M citrate buffer (pH 4.8) in 50-ml Falcon tubes. The experiments were performed in triplicates at 50 $\pm$ 1°C in a shaking water bath (Mettmert GmbH + Co. KG, Schwabach, Germany) with maximum strokes for 96 hours.

20

25

The commercial *Trichoderma reesei* cellulase (Celluclast 1.5 L), and β -glucosidase (Novozyme 188) were used as the controls. The enzyme activity for FPase was 10 IU/g newspaper and that for β -glucosidase was 30 IU/g newspaper in the mixture of Celluclast 1.5 L and Novozyme 188. Enzyme loading for the crude enzyme mixture from the fermentation broth was the same, i.e. 10 IU/g substrate of FPase. Samples were withdrawn every 24 hours. They were centrifuged at 4500 rpm, at 4°C for 5 min. The amount of reducing sugars released from the enzymatic hydrolysis was measured using the 3,5-dinitrosalicylic acid (DNS) method (Ghose, 1987). The concentration of cellobiose

30

35

was measured using High Performance Liquid Chromatography (HPLC) on an Aminex HPX-87H column (Bio- Rad, Richmond, CA, USA) at 75°C with 0.6 ml/min eluent of 5 mM sulfuric acid. Such analysis was conducted on a 1200 Series HPLC system (Agilent Technologies Inc.) equipped with a Refractive Index Detector. The glucose yields were calculated as: reducing sugar (g) x 0.9 x 100/initial cellulose (g) in biomass. Hydrolysis of polysaccharides involves water. For each mole of reducing sugar released, one mole of H₂O is required. A correction factor of 0.9 was therefore included in the calculation of the amount of polysaccharides hydrolyzed.

- 10 The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

15 Example 1

Enzyme production by the strain NP13a and its mutants thereof

- The strain of *Trichoderma viride* NP13a and its mutants thereof were cultivated in shake
20 flasks for enzyme production. The basic medium used for the seed culture and the enzyme production was Mandel's medium (Mandels and Sternberg, "Recent Advances in Cellulose Technology", *J. Ferment. Technol*, 54:267-286 (1976b)) containing 1.4 g of (NH₄)₂SO₄, 2.0 g of KH₂PO₄, 0.3 g of urea, 0.3 g of CaCl₂, 0.3 g of MgSO₄·7H₂O, 5.0 mg of FeSO₄·7H₂O, 1.6 mg of MnSO₄·H₂O, 1.4 mg of ZnSO₄·H₂O, and 2.0 mg of CoCl₂ as well
25 as 10 g of substrate such as lactose (for seed culture only), the delignified cellulose (Solka Eloc), in 1000 ml of distilled water. The initial pH of the medium was adjusted to 6 with 1 M HCl or 1 M NaOH before inoculation. A 250-ml Erlenmeyer flask containing 100 ml medium was inoculated with 1 ml of fungal spore suspension (10⁸ of spores) and incubated at 30°C on a rotary shaker (150 rpm) for 2 days. Shake flasks (250-ml
30 Erlenmeyer flasks containing 100 ml medium) were inoculated with 10 ml of the day-2 seed culture and cultivated on a rotary shaker (150 rpm) at 30°C. After 7 days, the cells were removed from the culture broth by centrifugation or filtration. The resulting supernatant solution was used as the crude enzyme preparation for enzyme activity assay and extracellular protein content measurement.

35

The *Trichoderma reesei* strain Rut-C30 was cultivated in the same manner as described above and the results are shown in Table 1.

Table 1 shows the enzyme activities and productivities of the crude enzyme mixtures prepared from the strains of *Trichoderma viride* NP13a and *Trichoderma reesei* Rut-C30 in shake flask fermentation. The results were compared with the data in the art (Cheng *et al.*, "Genome Shuffling Improves Production of Cellulase by *Penicillium decumbens* JU-A10", *J Appl Microbiol*, 107:1837-1846 (2007); and Asdul MG *et al.*, "Strain Improvement of *Penicillium janthinellum* NCIM 1171 for Increased Cellulase Production", *Bioresour Technol*, 98:1467-1473 (2007)). While the strain of *Trichoderma viride* NP13a (EU2-77) showed comparable FPase activity and productivity with the commercial strain *Trichoderma reesei* Rut-C30 and other strains reported in the art, the strain of *Trichoderma viride* NP13a exhibited higher specific β -glucosidase activity as compared to the rest of the strains listed in Table 1. This is a great advantage of the *Trichoderma viride* strain NP13a and the active mutants thereof.

Table 1. Comparison of the enzyme activities from the *Trichoderma viride* strain NP13a and some other strains in the art

Strains	FPase IU/ml	FPase Productivity U/l/hr	Specific Activity IU/mg		
			Endo- glucanase	FPase	Beta- glucosidase
Rut-C30	2.75 $\pm$ 0.18	16.37 $\pm$ 1.1	8.23 $\pm$ 0.3	1.02 $\pm$ 0.067	0.078 $\pm$ 0.011
JU-A10[*]	3.82 $\pm$ 0.28	42.44 $\pm$ 2.5	11.88 $\pm$ 0.45	3.41 $\pm$ 0.25	0.705 $\pm$ 0.008
EMS-UV-8[**]	3.20 $\pm$ 0.25	13.33 $\pm$ 1.0	8.33 $\pm$ 0.39	0.33 $\pm$ 0.03	0.625 $\pm$ 0.04
EU2-77	2.19 $\pm$ 0.05	13.04 $\pm$ 0.3	9.74 $\pm$ 0.15	1.30 $\pm$ 0.03	2.390 $\pm$ 0.05

*Cheng *et al.*, 2009; ** Asdul *et al.*, 2007

The enzyme activities of the *Trichoderma viride* strain NP13a (EU2-77) and the commercial strain, *Trichoderma reesei* Rut-C30 are compared in Fig. 1. Although the activities of the filter paper enzymes (FPase), endoglucanase (CMCase), β -xylosidase and protein concentration were higher for the *Trichoderma reesei* strain Rut-C30, the activities of β -glucosidase and xylanase for the *Trichoderma viride* strain NP13a were much higher than those of the *Trichoderma reesei* strain Rut-C30. In particular, β -glucosidase activity of the *Trichoderma viride* strain NP13a was almost 19 times of that of the *Trichoderma reesei* strain RUT-C30. This feature makes the *Trichoderma viride*

strain NP13a a potential microbial factory for cellulolytic enzyme complex production for use in the biomass-to-ethanol process.

Xylanase is an accessory enzyme for lignocellulosic material hydrolysis. It can help degrade hemicellulose, separating cellulose from lignin and consequently making cellulose more accessible to the cellulolytic enzymes. The higher activity of xylanase in the *Trichoderma viride* strain NP13a indicates that the enzyme complex prepared from the *Trichoderma viride* strain NP13a might be more efficient in lignocellulosic biomass hydrolysis.

The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible alternative for the conversion of lignocellulosic materials into fermentable sugars and fuel ethanol (Lin *et al.*, "Ethanol Fermentation from Biomass Resources : Current State and Prospects", *Appl Microbiol Biotechnol*, 69:627-642 (2006); Galbe *et al.*, "Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production", *Adv Biochem Eng Biotechnol*, 108:46-65 (2007); Merino *et al.*, "Progress and Challenges in Enzyme Development for Biomass Utilization", *Adv Biochem Eng Biotechnol*, 108:95-120 (2007)). However, the most widely studied enzyme system, namely *Trichoderma reesei* cellulases, has shown several disadvantages. Attempts to use these enzymes in the degradation of cellulosic wastes have not been successful for several reasons, such as low sugar yields, low specific activities, and end product inhibition.

Since the parent strain, *Trichoderma reesei* QM 6a, of the *Trichoderma reesei* strain RUT-C30 was isolated, there has been further developments on the chemical mutagenesis and UV irradiation to produce hypercellulolytic *Trichoderma reesei* mutants. *Trichoderma reesei* Rut-C30 is a mutant and produces approximately four times more cellulase than the original parent strain QM 6a (Reese ET, "History of the Cellulase Program at the U.S. Army Natick Development Center", *Biotechnol Bioeng Symp*, 6:9-20 (1976)). However, the enzymes prepared using *Trichoderma reesei* Rut-C30 is deficient in β -glucosidase. On the other hand, the *Trichoderma viride* strain NP13a of the present invention produces much more β -glucosidase than the *Trichoderma reesei* Rut-C30 strain. Such feature makes the *Trichoderma viride* strain NP13a of the present invention and the active mutants thereof, good candidates for the production of cellulases for lignocellulosic waste degradation.

Example 2

Hydrolysis of waste newspaper

Besides measuring the enzyme activities, it is also very important to determine the hydrolytic capacity of the produced enzymes on lignocellulosic materials. In many cases, there is no correlation between the initial hydrolysis of a Whatman No.1 filter paper strip (FPase assay) and the liberation of reducing sugars from the lignocelluloses. It may be that not the best mutant (according to FPase activity) was chosen for practical applications. In a previous study, hydrolysis efficiency of different enzymes at same filter paper activities per gram of cellulose in the waste newspaper was compared, and major differences among the sugar yields were obtained (Juhasz *et al.*, "Characterization of Cellulases and Hemicellulases Produced by *Trichoderma reesei* on Various Carbon Sources", *Process Biochem*, 40:3519-3525 (2005)). The enzyme complex giving the highest glucose yield was not the one having the highest FPase activity. Breuil *et al.* (see Breuil *et al.*, "Influence of β -glucosidase on the Filter Paper Activity and Hydrolysis of Lignocellulosic Substrates", *Bioresour Technol*, 39:139-142 (1992)) suggested to look at the profile of the individual sugars (especially cellobiose and glucose) released during the filter paper assay in order to be able to better predict the ability of a cellulase mixture to hydrolyze the cellulosic materials.

In order to investigate the hydrolytic potentials of the *Trichoderma viride* strain NP13a of the present invention, a dosage of FPase at 10 IU/g newspaper, 0.5 g of 0.5 cm x 0.5 cm non-pretreated waste newspaper strips were hydrolyzed by the crude enzyme mixture prepared from the shake flask fermentation and by the commercial enzymes and their mixtures in 20 ml of citrate buffer at pH 4.8 in 50-ml falcon tubes. After 96 hour incubation at 50°C in the shaking water bath, the final reducing sugar yields from the newspaper hydrolysates were obtained and the results are depicted in Figures 2 and 3. As shown in these figures, sugar yield was low for Celluclast 1.5 L and for the fermentation broth obtained from the *Trichoderma reesei* strain Rut-C30 (37.9% and 40.3%, respectively). In fact, Celluclast 1.5 L was also produced by *Trichoderma reesei* strain. Enzymes prepared from the *Trichoderma reesei* species are known to be deficient in extracellular β -glucosidase. It is only with the addition of Novozyme 188 (30 IU/g substrate) to Celluclast 1.5 L that resulted in an about 1.5-fold increase in the final sugar yield (55.1%).

Hydrolysis efficiencies of the enzymes produced by the *Trichoderma viride* strain NP13a and its mutant (EU2-77) were about 1.5 fold higher than that of the *Trichoderma reesei* enzymes (43.8% and 53.3%, respectively), but were slightly worse than that of the commercial enzyme mixture of Celluclast 1.5 L and Novozyme 188 (55.15%). The final
5 cellobiose concentrations shown in Figure 3 given by the *Trichoderma reesei* strain Rut-C30 (82.1 mg/g cellulose) was 4 times more than that from the *Trichoderma viride* strain NP13a (14.9 mg/g cellulose) and 10 times more than that from the commercial enzyme mixture (2.65 mg/g cellulose for Celluclast 1.5L and Novozyme 188 mixture). The accumulation of cellobiose may result in the inhibition of cellulolytic enzyme activities.

10 In this example, the culture broth from the *Trichoderma reesei* strain Rut-C30 and the commercial cellulase, Celluclast 1.5 L, produced high levels of cellobiose during the hydrolysis of waste newspaper, owing to the deficiency of extracellular β -glucosidase (0.21 IU/ml) (Sternberg *et al.*, "Beta-glucosidase-microbial Production and Effect on
15 Enzymatic Hydrolysis of Cellulose", *Can J Microbiol*, 23:139-147 (1977)). The reason for the low secretion of this enzyme into the fermentation broth of *Trichoderma reesei* is that the major part of the β -glucosidase is tightly bound to the cell walls of the fungus during cultivation and some part of the enzyme may be found inside the cells (Kubicek *et al.*, "Release of Carboxymethyl-cellulase and β -glucosidase from Cell Walls of *Trichoderma*
20 *Reesei*", *Eur J Appl Microbiol Biotechnol*, 13:226-231 (1981)).

Due to the higher amount of extracellular β -glucosidase produced, supernatants of the new *Trichoderma viride* strain NP13a of the present invention is able to hydrolyse the waste newspaper more efficiently than that of the *Trichoderma reesei* strain. For
25 *Trichoderma reesei* supernatants and Celluclast 1.5 L, β -glucosidase supplementation was necessary in order to achieve high sugar yields. Similar fermentation and hydrolysis results were also obtained with *Trichoderma harzianum* strain E58 (Saddler *et al.*, "A Comparison Between the Cellulase Systems of *Trichoderma harzianum* E58 and *Trichoderma reesei* Rut C30", *Appl Microbiol Biotechnol*, 22:139-145 (1985)). This strain
30 produces a higher level of extracellular β -glucosidase than the *Trichoderma reesei* strain Rut-C30, and is therefore more efficient in breaking down various lignocellulosic substrates into glucose molecules. Among the entire enzyme samples prepared, the mixture of the commercial enzymes, Celluclast 1.5 L and Novozyme 188, was the most efficient in reducing sugar production and cellobiose hydrolysis. Although the
35 *Trichoderma viride* strain NP13a could not completely hydrolyze the cellobiose, the end

product inhibition was not as significant, as its reducing sugar yield was comparable to that of the commercial enzyme mixture of Celluclast 1.5 L and Novozyme 188.

The hydrolysis results depicted in Figures 2 and 3 prove that the *Trichoderma viride* strain NP13a is an efficient factory for cellulolytic enzyme complex production with its potential applications in second generation biofuel production. In addition, the *Trichoderma viride* strain NP13a can also be further improved by random mutagenesis and genome shuffling for higher yield of secreted enzymes.

DEPOSIT

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of microorganisms for the Purpose of Patent Procedure, deposit of the *Trichoderma viride* strain NP13a was made with the American Type Culture Collection (ATCC) of 12301 Parklawn Drive, Rockville Md., USA, on 31 Mar 2010 and bears the accession number of PTA-10765. The deposit has been made under conditions that access to the cultures will be available during the pendency of the patent application disclosing them to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accordance with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposits, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures plus five years after the first request for a sample from the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

The above is a description of the subject matter the inventors regard as the invention and is believed that others can and will design alternative processes that include this invention based on the above disclosure.

5

CLAIMS

1. An isolated culture of a strain of *Trichoderma viride* named NP13a on deposit with the ATCC as accession no. PTA-10765, or a mutant derived therefrom, having cellulolytic activity useful for production of cellulolytic enzyme complex.

2. A supernatant obtained from the culture as defined in claim 1, comprising a cellulolytic enzyme complex capable of improving hydrolysis of lignocellulosic materials.

3. The supernatant of claim 2, wherein the cellulolytic enzyme complex comprises cellulase, endoglucanase, exoglucanase, β -glucosidase, xylanase and β -xylosidase.

4. The supernatant of claim 3, wherein the β -glucosidase and the cellulase are present in a ratio of 2:1.

5. The supernatant of claim 3, wherein the xylanases and the cellulase are present in a ratio of 20:1.

6. A method of obtaining a supernatant as defined in any of claims 2 to 5, comprising:

contacting a strain of *Trichoderma viride* named NP13a on deposit with the ATCC as accession no. PTA-10765 with a medium to get a suspension,

incubating the suspension;

fermenting the suspension; and

centrifuging the suspension to obtain the supernatant.

7. The method according to claim 6, wherein the medium comprises inorganic salts and other appropriate nutrients suitable for growth of the *Trichoderma viride* strain NP13a.

8. The method according to claim 7, wherein the inorganic salts is selected from the group consisting of potassium dihydrogen phosphate, magnesium sulphate, calcium chloride, iron(II) sulphate, manganese sulphate and zinc sulphate.

9. The method according to claim 7, wherein the medium further comprises inorganic ammonium salts and organic ammonium salts.

10. The method according to claim 6, wherein the medium is a substrate selected from the group consisting of cellulosic material, herbaceous material, agricultural residue, forestry residue, waste paper, and pulp and paper mill residue.
- 5 11. The method according to claim 10, wherein an effective substrate loading is in the range of 1 to 5%.
12. The method according to claim 6, wherein the suspension is incubated at a
10 temperature in the range of 20°C to 35°C.
13. The method according to claim 6, wherein the suspension is incubated for 1 to 3 days.
- 15 14. The method according to claim 6, wherein the suspension is fermented for a period between 4 to 14 days.
15. A method for degrading lignocellulosic material to fermentable sugar, comprising:
pretreating the lignocellulosic material;
20 washing the pretreated lignocellulosic material with distilled water;
drying the pretreated lignocellulosic material; and
contacting the pretreated lignocellulosic material with a supernatant containing a
cellulolytic enzyme complex derived from a strain of *Trichoderma viride* named NP13a
on deposit with the ATCC as accession no. PTA-10765.
- 25 16. The method according to claim 15, wherein the lignocellulosic material is pretreated with a diluted acid and distilled water under room temperature or under pressurized steam for a predetermined period of time.
- 30 17. The method according to claim 15, wherein the lignocellulosic material is pretreated with a diluted base and distilled water under room temperature or under pressurized steam for a predetermined period of time.
18. The method according to claim 15, wherein the pretreated lignocellulosic material
35 is washed until a pH of 7 is reached.

19. The method according to claim 15, further comprising recovering the degraded lignocellulosic material.

20. The method according to claim 19, wherein the degraded lignocellulosic material is a sugar.

21. The method according to claim 15, wherein the lignocellulosic material is selected from the group consisting of herbaceous material, agricultural residue, forestry residue, waste paper, and pulp and paper mill residue.

10