



TRICHODERMA VIRIDE STRAIN FOR CELLULOLYTIC ENZYME PRODUCTION

BACKGROUND OF THE INVENTION

Enzymes have been used for a long time for a variety of different industrial applications. Examples are known in the baking industry, in the wine and fruit juices industry (where enzymes are used to break down pectins and beta-glucans), in the 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 industries (where lignocellulolytic enzymes are used to hydrolyze lignocellulosic biomass to produce reducing sugars for fuel ethanol fermentation).

Recently, conversion of biomass to bio-fuels has been the subject of intense research efforts and gained significant political and scientific momentum, owing to concerns about the shortage of fossil fuels and the emission of green house gases (Antoni et al., 2007; Bayer et al., 2004; Service, 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 current engine technology. 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. The additional raw materials of potential interest are newsprint, office paper, municipal solid wastes, etc.

Lignocellulose is comprised of three major components: cellulose, hemicellulose, and lignin. Cellulose is a homopolysaccharide composed of β -D-glucopyranose units, linked by β -(1-4)-glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The cellulose-hydrolyzing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases (CMCases), cellobiohydrolases (exoglucanases), and β -glucosidases. The CMCases catalyze random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. β -glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose.

As the production of cellulolytic enzymes is a major factor in lignocelluloses hydrolysis and biomass-to-ethanol process, it is important to make the process economically viable, because large-scale application in the degradation 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, typically *Trichoderma reesei* (Mandels et al., 1976a), have excellent capacity for extracellular protein production. It is well known that *T. reesei* produces the cellobiohydrolase (CBH) and endoglucanase (EG) components of cellulolytic enzyme complex in large quantities. However, the amount of β -glucosidase secreted by *T. reesei* is insufficient causing the accumulation of cellobiose, which produces repression and end product inhibition to the enzymes. In addition, cellulase from *T. reesei* shows relatively low specific activity, low thermal stability and high sensitivity to product inhibition (Lynd et al., 2002; Chan et al., 2005; Kavas et al., 2008). Other important cellulase producing



fungi such as *Penicillium* (Asdul et al., 2007; Cheng et al., 2009) and *Aspergillus* species (Sridevi et al., 2009) produce cellulolytic enzyme complex showing higher β -glucosidase activity than *T. reesei*, but the total cellulase (FPase) activity is relatively low. As a result, production of sugars from waste cellulose is still not commercially feasible.

Prior arts:

Lignocellulosic enzymes can be prepared from a range of different micro-organisms. In WO 95/26398 patent application, a modified cellulase is obtained by inclusion of foreign DNA sequence in a host cell which modifies the nature of the original strain which is chosen in the following list of micro-organisms: *Bacillus*, *Streptomyces*, *Saccharomyces*, *Schizosaccharomyces*, *Aspergillus*. In the present invention our main aim was to avoid foreign gene inclusion in the micro-organism which is the producer of the enzyme.

In the WO 96/05739 patent application, a mixture of enzymes (xylanase, protease, and beta-glucanase) is obtained from different micro-organisms.

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

In a number of cases the enzyme preparations have been obtained from microorganisms subjected to genetic modification using recombinant DNA techniques.

We hereby discovered and developed a new microorganism belonging to the genus of *Trichoderma* that contains a mixture of enzyme activities which can be used successfully to hydrolyze lignocellulosic biomass.

BRIEF DESCRIPTION OF THE DRAWING

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

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

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

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Lignocellulose is one of the most common biopolymers in nature. Degradation of lignocellulosic biomass to monomeric reducing sugars through the concerted action of cellulolytic enzymes has great importance, since sugars can serve as raw materials in a number of biotechnological production processes, e.g. the bioethanol production. Over the past decades, cellulolytic enzyme based research has attracted worldwide attention. Recently, continuous efforts have been made in the search for novel enzyme producing microorganisms to make the biomass-to-ethanol process more economically viable. Production of cellulolytic enzymes in-situ using such microorganisms instead of using commercial enzymes can improve the economy of the process.

The main components of lignocelluloses are cellulose, hemicelluloses and lignin. Hemicelluloses are polymers of xylose, mannose, arabinose and galactose. 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 preventing lignocellulosic materials against biological attack, against cellulose degrading microorganisms etc. Various pretreatment methods are applied to break down the intact structure of lignocelluloses. The aim of 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 remained hemicelluloses is still used as substrate either for hydrolysis or for cellulolytic enzyme production.

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

This invention is related to the discovery of a new strain of *Trichoderma viride* NP13a which can produce a cellulolytic enzyme complex that can be potentially used for the degradation of the pretreated lignocellulosic materials.

Strain isolation and identification

Strain NP13a was isolated from the campus of Ngee Ann Polytechnic in Singapore. It grows well on potato dextrose agar (PDA). On the PDA plate, the colonies appear to be white when it first grows with fine mycelium. Colonies start to form dense mycelium from the centre of the colonies on day two or day three and spore formation is observed. Colonies grow in a circular manner and their colour change to green from white. After a longer period of growth, colonies become dark green and powdery. Colonies are colourless observed from the back of the plate. Spores are growing in a ring-like manner at the beginning. However, at the second stage of growth, spores are growing singularly or in groups of two or three and are widely

apart. Moreover, spore 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 the spores turn spiky and the size of the spores are in the range of 2.5 to 4.8 μm . Spores spread all over the plate on day 7 or 8. The above morphology of strain NP13a on PDA plates indicates that it is a typical of the *Trichoderma* genus and *T. viride* species. It forms clear zones when grown on swollen cellulose plates, indicating the secretion of cellulolytic enzymes.

NP13a mutant strains

Mutants of strain NP13a can be made using techniques that are 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 mutants.

Recombinant microorganisms containing NP13a strain genes

Novel recombinant microbes containing genes from the NP13a strain, and active mutants thereof, can be made by isolating gene(s) from 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, α -glucosidase, xylanase, α -xylosidase, and pectinase etc. can be used to prepare recombinant microorganisms that produce these 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 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, 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.

Enzyme production

It is a 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. Carbohydrates or their derivatives induce most of the cellulolytic enzymes. Enzyme production of the hypercellulolytic fungus, e.g. *Trichoderma reesei* Rut-C30 has been studied in most detail. It is generally accepted that cellobiose (Fritscher et al., 1990) and lactose (Morikawa et al., 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 (Bhat et al., 1997). Secretion of β -glucosidase is highly dependent on the culture conditions. Thus, a relatively high pH can enhance β -glucosidase production. In the present invention, the production of cellulases and hemicellulases can be produced on various carbon sources: on alpha cellulose substrate (Solka Floc), on pretreated wood dust, on pretreated wood chips and on pretreated oil palm empty fruit bunch powders, and on wheat bran or its mixture with the rest of the above mentioned lignocellulosic materials.

Biomass collection and substrate pretreatment

Empty fruit bunches (EFB) were collected from Masai Palm Oil Mill / Refinery, Johore, Malaysia. Wood dust was collected from Wah & Hua Pte Ltd and wood chips were collected from Environmental Landscape Pte Ltd. Wheat bran was obtained from the market. All the lignocellulosic materials collected were immediately autoclaved and dried. They were then stored in sealed plastic bags for future use. All the biomass was cut, milled and then sieved to 500 μ m powder. They then went through chemical pretreatment, using dilute acid such as H_3PO_4 , HCl and H_2SO_4 , or dilute base such as NaOH, $Ca(OH)_2$, H_2O_2 , and distilled water under room temperature or under pressurized steam for sometime. The pretreated materials were collected, filtered, washed with distilled water until pH neutral, and then dried. After that, the pretreated biomasses were used as the substrates to cultivate the cellulolytic fungus *T. viride* NP13a and its mutants thereof, for cellulolytic enzyme complex production.

Microorganism cultivation and culturing media

Trichoderma reesei rut-C30 was obtained from American Type Culture Collection with a designated no. of ATCC 56765. The fungus *T. viride* NP13a was obtained from soil samples collected from the campus of Ngee Ann Polytechnic. Fungi such as *T. reesei* Rut-C30, the strain *T. viride* NP13a and its mutants thereof, were grown on potato dextrose agar (PDA) at 30°C for the development of spores. On day 7, the plates were washed with 0.5% Tween-80 and make 10^8 spores/ml suspensions.

Various organic and synthetic media which contain suitable sources of carbon and nitrogen may be used. Such media may, if desired, contain inorganic salts and other appropriate nutrients suitable for growth of the microorganisms. Suitable carbon source in this invention specifically refers to alpha cellulose substrate (Solka Floc). The effected biomass loading 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, KH_2PO_4 , $MgSO_4$, $CaCl_2$, $FeSO_4$, $MnSO_4$ and $ZnSO_4$.

The inoculum size for the seed culture is preferably 0.1-5% of the 10^8 /ml spore suspension prepared above. The seed culture was incubated for 1- 3 days. The inoculum size of the seed culture to fermentation is preferably 5 – 20%. When the fermentation is effected at a temperature of from 20 to 35°C and a pH of 4 – 8 with sufficient aeration for growth of the fungal strains, the activities of the lignocellulolytic enzymes reaches a maximum 4 – 14 days after the beginning of fermentation. After completion of the culturing, the resultant liquor and/or enzyme preparation originating from the culture liquor may be used in conventional manner for hydrolysis of lignocellulose.

Enzyme activity assay

Filter paper activity was assayed as described by Ghose (1987) by incubating 0.5 ml of the suitably diluted enzyme samples with 1.0 ml 0.05 M citrate buffer (pH 4.8) containing one Whatman No.1 filter paper strip (50 mg, 1 × 6 cm). The reaction mixture was incubated at 50 °C for 60 min.

Endoglucanase (CMCase) activity was carried out in the 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, while xylanase activity was determined by catalyzing of 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 (Bailey, 1991).

β-glucosidase activity was estimated using *p*-nitro phenyl β-D- glucopyranoside (pNPG) as the substrate (Berghem and Pettersson, 1974). The total of assay mixture (1 ml) consisted of 0.9 ml of 1 mM pNPG and 0.1 ml of suitably diluted enzyme samples and it was incubated at 50 °C for 10 min. The reaction was terminated by the addition of 0.5 ml 1 M sodium carbonate. The *p*-nitrophenol liberated was measured at 400 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). β -xylosidase activity was estimated in an analogous manner except that *p*-nitro phenyl β-D- xylopyranoside (pNPX) was used as the substrate.

The reducing sugars were determined by dinitrosalicylic acid (DNS) method (Ghose, 1987). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol 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., 1951).

Enzymatic hydrolysis

The crude enzyme mixtures of *T. reesei* Rut-C30, *T. viride* NP13a, and its mutants thereof, were extracted from the fermentation broth after 7 days of growth. Waste newspaper was cut into approx 0.5×0.5 cm pieces after drying at 90°C for two days. Enzymatic hydrolysis of 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 ±1°C in a shaking water bath

(Memmert GmbH + Co. KG, Schwabach, Germany) with maximum strokes for 96 h. The commercial *T. reesei* cellulase (Celluclast 1.5 L), and β -glucosidase (Novozyme 188) were used as the controls. Celluclast 1.5 L, Novozyme 188 and Cellic Ctec were kindly donated by Novozymes Malaysia sdn bhd. 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 h. They were centrifuged at 4500 rpm, 4°C for 5 min. The amount of reducing sugars released from enzymatic hydrolysis was measured using the 3,5-dinitrosalicylic acid (DNS) method (Ghose, 1987). Concentration of cellobiose 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) $\times$ 0.9 $\times$ 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.

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

EXAMPLES

Example 1

Enzyme production by the strain NP13a and its mutants thereof

Fungal strains such as *T. reesei* Rut-C30, the strain *T. viride* NP13a and its mutants thereof, were cultivated in shake flasks for enzyme production. The basic medium used for seed culture and enzyme production was Mandel's medium (Mandels and Sternberg, 1976b) containing 1.4 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.3 g urea, 0.3 g CaCl₂, 0.3 g MgSO₄·7H₂O, 5.0 mg FeSO₄·7H₂O, 1.6 mg MnSO₄·H₂O, 1.4 mg ZnSO₄·H₂O, and 2.0 mg CoCl₂ as well as 10 g substrate such as lactose (for seed culture only), the delignified cellulose (Solka Floc), in 1000 ml distilled water. The initial pH of the medium was adjusted to the 6.0 with 1M HCl or 1M 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 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.

Table 1 shows enzyme activities and productivities of the crude enzyme mixtures prepared from the strain *T. viride* NP13a and *T. reesei* Rut-C30 in shake flask

fermentation. Results were compared with the data in the art. While the strain NP13a showed comparable FPase activity and productivity with the commercial strain *T. reesei* Rut-C30 and other strains reported in the art, the strain NP13a exhibited higher specific of α -glucosidase activity compared to the rest of the strains listed in Table 2. This is a great advantage of the strain NP13a and the active mutants thereof.

Table 1. Comparison of enzyme activities from the strain NP13a and some 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±0.18	16.37±1.1	8.23±0.3	1.02±0.067	0.078±0.011
JU-A10[*]	3.82±0.28	42.44±2.5	11.88±0.45	3.41±0.25	0.705±0.008
EMS-UV- 8[**]	3.20±0.25	13.33±1.0	8.33±0.39	0.33±0.03	0.625±0.04
EU2-77	2.19±0.05	13.04±0.3	9.74±0.15	1.30±0.03	2.390±0.05

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

Enzyme activities of the strain NP13a and the commercial strain *T. reesei* are compared in Fig. 1. Although activities of filter paper enzymes (FPase), endoglucanase (CMCase), α -xylosidase and protein concentration were higher for *T. reesei* Rut-C30, activities of α -glucosidase and xylanase for the strain NP13a were much higher than those for *T. reesei* Rut-C30. In particular, α -glucosidase activity of the strain NP13a was almost 19 times of that for RUT-C30. This feature makes the strain NP13a a potential microbial factory for cellulolytic enzyme complex production for their 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 strain NP13a indicates that the enzyme complex prepared from 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 lignocellulosics into fermentable sugars and fuel ethanol (Lin and Tanaka, 2006; Galbe and Zacchi, 2007; Merino and Cherry, 2007). However, the most widely studied enzyme system, namely *T. 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 *T. reesei* QM 6a was isolated, it was further developed chemical mutagenesis and UV irradiation to produce hypercellulolytic *T. reesei* mutants. *T. reesei* Rut-C30 is a mutant and produces approximately four times more

cellulase than the original parent strain QM 6a (Reese, 1976). However enzymes prepared using Rut – C30 is deficient in α -glucosidase. On the other hand, *T. viride* NP13a produced much more β -glucosidase than *T. reesei* Rut-C30. Such feature makes the strain NP13a 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 was also very important to determine the hydrolytic capacity of the produced enzymes on the 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 happen that not the best mutant (according to FPase activity) will be the choice of 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., 2005). The enzyme complex giving the highest glucose yield was not the one having the highest FPase activity. Breuil et al. (Breuil et al., 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 cellulosic materials.

In order to investigate the hydrolytic potentials, with a dosage of FPase at 10 IU/g newspaper, 0.5 g 0.5 cm×0.5 cm non-pretreated waste newspaper strips were hydrolyzed by the crude enzyme mixtures prepared from 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 h incubation at 50°C in the shaking water bath, the final reducing sugar yields from the newspaper hydrolysates were depicted in Figs. 2 and 3. Sugar yield was low for celluclast 1.5 L and for the fermentation broth from *T. reesei* Rut-C30 (37.9% and 40.3%, respectively) as demonstrated. In fact, celluclast 1.5 L was also produced by a *T. reesei* strain. Enzymes prepared from *T. reesei* species is known to be deficient in extracellular β – glucosidase. The addition of Novozyme 188 (30 IU/g substrate) to celluclast 1.5 L resulted in an about 1.5-fold increase in the final sugar yield (55.1%). Hydrolysis efficiencies of the enzymes produced by the *T. viride* wild strain NP13a and its mutant EU2-77 were about 1.5 fold higher than that of *T. reesei* enzymes (43.8% and 53.3%, respectively), but slightly worse than that of the commercial enzyme mixture of Celluclast 1.5 L and Novozyme 188 (55.15%). The final cellobiose concentrations shown in Fig. 3 given by *T. reesei* Rut – C30 (82.1 mg/g cellulose) was 4 times more than that from *T. viride* (14.9 mg/g cellulose) strains 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.

In this example, the culture broth from *T. reesei* 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., 1977). The reason for the low secretion of this enzyme into the fermentation broth of *T. 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., 1981). Regardless of this fact, the majority of researchers and industrial companies prefer this particular species for cellulase production. Due to the higher amount of extracellular β -glucosidase produced, supernatants of our new *T. viride* strain NP13a hydrolysed the waste newspaper more efficiently than that of *T. reesei*. For *T. reesei* supernatants and for Celluclast 1.5 L, β -glucosidase supplementation was necessary in order to achieve high sugar yields. Similar fermentation and hydrolysis results were obtained with *T. harzianum* E58 (Saddler et al., 1985). This strain produced higher levels of extracellular β -glucosidase than *T. reesei* Rut-C30, and therefore proved to be more efficient to break down various lignocellulosic substrates into glucose molecules. Among the entire enzyme samples prepared, the mixture of commercial enzymes Celluclast 1.5 L and Novozyme 188 was the most efficient in reducing sugar production and cellobiose hydrolysis. Although the strain NP13a could not completely hydrolyze the cellobiose, end product inhibition was not significant as its reducing sugar yield was comparable to that from the commercial enzyme mixture of Celluclast 1.5 L and Novozyme 188.

The hydrolysis results prove that the strain NP13a might be an efficient factory for cellulolytic enzyme complex production with its potential applications in second generation biofuel production. In addition, the strain NP13a can 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 ... 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 accord 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 last 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.

TRICHODERMA VIRIDE STRAIN FOR CELLULOLYTIC ENZYME PRODUCTION

ABSTRACT

Purpose: This invention relates to a new mycelial fungus strain *Trichoderma viride* NP13a PTA-10756 characterized by high lignocellulolytic activity of enzyme complex containing cellulase, endo-glucanase, α -glucosidase, xylanase, and α -xylosidase. The enzyme complex can be successfully used for the saccharification of lignocellulosic materials.

Constitution: Fungus strain *Trichoderma viride* NP13a is prepared by the multistep isolation and screening. It is submerged cultured on liquid nutrient medium containing pretreated lignocellulosic biomass powder or cellulose as the source of carbon. After seven-day culturing cultural fluid is separated centrifugation. Invention can be used for preparing fermented sugars designated for processing to ethanol, and as fodder supplements also.

Creativity: This invention proposed a highly lignocellulolytic *Trichoderma* fungus strain, which can produce an enzyme complex that can be efficiently used for lignocellulosic materials saccharification. This cocktail of the enzyme complex is more efficient in lignocellulosic material saccharification than that produced from the commercial hypercellulolytic strain *Trichoderma reesei* Rut-C30. Its biomass hydrolysis capability is comparable to that from the commercial enzyme blend from Novozymes. It would be a good candidate to be used for the preparation of fermented sugars designated for bioethanol production.

FIELD OF THE INVENTION

This invention provides a new fungal strain that can be used for cellulolytic enzyme complex production. It presents the compositions of the enhanced cellulolytic enzyme complex for degrading of lignocellulosic materials. It also relates to the method of cellulolytic enzyme mixture production using such strain, and in particular to the application of these enzymes to the hydrolysis of lignocellulosic materials.

Figure 1

Figure 2

Figure 3

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