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Enzymatic saccharification of sugar beet pulp by novel mutants of Trichoderma reesei NRCAM 5 for bioethanol production

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***ABSTRACT*:** Fuel ethanol production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable production of renewable transportation fuels.Sugar beet pulp (SBP) represents a major source of lignocellulosic material (LCM) with considerable potential for use in biomass conversion to ethanol. In this study, Trichodermareesei and novel mutant of T. reeseiNRCAM5 were used for extracellular enzymes production from sugar beet pulp and subsequent fermentation of sugars to ethanol by Saccharomyces cerevisiae and kluyveromycesmarxianus. Endo-glucanase, exoglucanase, β-glucosidase, pectinase and invertase activity was investigated. Also, the purity and composition of enzyme-rich protein samples were evaluated under denaturing conditions by SDS-PAGE. Mutant T. reeseiNRCAM5 produces high levels of cellobiase, which can be further improved by controlled culture conditions. In addition, this mutant strain produces good levels of exoglucanase, endoglucanase and FPase, which together with the exceptionally high levels of cellobiase, makes it a good candidate for obtaining cellulases for saccharification of SBP. The T. reesei and T. reeseiNRCAM5 have both enzyme bonds of Cel6A (CBHII) and Cel7A (CBHI).The highest synergy was observed in FPase of T. reesei NRCAM5, that containing of large amount of CBH I and CBH II and minor amount of EG. The results indicated that the highest ethanol concentration can be obtained from the enzyme hydrolyzed sugar beet pulp byT. reesei NRCAM5. This can be explained to the fact that T. reeseiNRCAM5 can affect the cellulosic region differently and hence facilitates the enzyme treatment and thus the fermentation to produce the ethanol.

Keywords: Trichoderma reesei; γ-irradiation; SDS-Page; Sugar beet pulp;Cellulase.

INTRODUCTION

Ethanol produced by the direct fermentation of sucrose from sugar cane or that obtained from the starch of corn and other cereals (so-called first-generation biofuels) has been widely used in the past three decades as a blend to gasoline or as a neat fuel for vehicles in Brazil, the US and other countries0, 0.

Considerable research has also been carried out since the 1970 s on bioethanol production from lignocellulosics (second-generation biofuels) 0, 0, 0, 0. The enzymatic hydrolysis of cellulose to glucose catalyzed by cellulases is a key stage in the whole bioconversion process. Because of the recalcitrant crystalline nature of cellulosic fibers, significantly larger amounts of enzymes are needed during cellulose hydrolysis than in the process of starch hydrolysis where amylases were utilized in the production of first-generation bioethanol 0. Filamentous fungi are the major source of commercial cellulases. Cellulolytic fungi belonging to the genera Trichoderma (T. viride, T. longibrachiatum, T. reesei) have long been considered the most productive and powerful destroyers of crystalline cellulose 0, 0, 0, 0. Commercial cellulase preparations based on mutant strains of T. reesei (also known as Hypocreajecorina) are produced on an industrial scale by many companies worldwide 0. Thus, it is not surprising that most R&D projects on bioethanol production from lignocellulosics focus on using T. reeseicellulases0, 0, 0. As a result, many involved in bioethanol production projects believe that T. reesei is the only and indispensable choice for enzymatic cellulose saccharification. However, recent publications have increasingly demonstrated that alternatives to T. reesei enzymes in the production of second-generation biofuels exist 0, 0, 0, 0, 0, 0, 0, 0, 0, 0. Sugar beet pulp (SBP) represents a major source of

lignocellulosic material (LCM) with considerable potential for use in biomass conversion to ethanol. SBP is one of the most abundant industrial by products in the world, has a low commercial value and a large part is applied as cattle feed and the rest as waste one promising technology is to convert this abundant and renewable LCM to monomer sugar using enzymes that are to be applied after a pretreatment process and the microbes convert the sugars to ethanol. This cellulosic by product composed mainly of cellulose (21-27 %), araban (20-22 %), galactan (5.5-7 %), pectin (17-18 %), protein (9 %), sucrose (1-2 %) and ash (5 %) 0.Due to increase cost of fermentation process for bioethanol, cellulase production is one of the key steps for hydrolysis of the lignocellulosic materials. Strain improvement by mutations is an age-old and successful method 0, 0, 0, 0, 0. Strain improvement by mutations is an age-old as a successful method and it is a fundamental aspect of industrial microbiology, which is aimed at increasing enzyme yields, and reducing production costs 0. Therefore, several approaches including chemical mutations, UV or gamma irradiations and their combinations were applied to obtain enhanced cellulases producing strains 0. Nevertheless, strains that are genetically improved for high level of cellulases production have been successfully used in a number of applications including animal feed, pharmaceutical and textile industries 0. The objective of the present study was the utilization of an inexpensive and abundantly available lignocellulosic biomass of sugar beet pulp for cellulase production by γ-irradiatednovel mutants of TrichodermareeseiNRCAM 5 and the influence of fermentation time on saccharification of SBP and subsequent fermentation of sugars to ethanol were studied.

MATERIALS AND METHODS

SBP preparation

Sugar beet pulp was obtained as pressed pulp from an Iran sugar factory and was stored at -20 °C until the start of the experiments. It was suspended in water with magnetic stirring for 1h and then was filtered with a cheese cloth. This step was repeated until all reducing sugars were removed. Sugar beet pulp was ground in a hammer mill equipped with a 2 mm grid. The fraction with fine particle size was used in Trichoermafermentation media for enzyme production.

Analysis of SBP components

The proximate composition (dry matter, crude protein, crude fiber, crude lipid and ash) of sugar beet pulp was measured according to the methods described in AOAC0. Dry matter was determined by weight loss after drying (60 °C for 24 h). Crude proteins were determined indirectly from the amount of total nitrogen, measured by Kjeldhal's method, multiplied by factor 6.25. Crude lipid content was determined after Soxhlet extraction of lipids from dry samples using hexane as the solvent. Dry samples were refluxed at a boiling point of 60 °C during 5–6 h and then the dry crude lipid was gravimetrically determined. Ash content was determined gravimetrically after ashing at 550 °C for 6 h. Cell wall composition of defatted sugar beet pulp (cellulose, hemicellulose and lignin) was determined using the detergent fiber methods, developed by Van Soest and other0. This method is based in the sequential extraction and separation of three fractions: residue insoluble in solution of sodium lauryl sulfate buffered at pH 7 with EDTA-Borate (NDF); residue insoluble in solution of cetyl-trimethyl-ammonium-bromide ($C_{19}H_{42}BrN$) in 1N sulfuric acid(ADF); and residue insoluble in H_2SO_4 72% (w/w)(ADL). Then hemicelluloses is calculated as (NDF – ADF), cellulose as (ADF – ADL), and lignin is determined as the ADL fraction free of ash.

Culture conditions of Trichoderma reesei and T. reesei NRCAM 5

Trichodermareesei was obtained from the Persian Type Culture Collection (PTCC) strain number 1654. The fungi were maintained in the test tubes containing potato dextrose broth at 28 ˚C for 3 day. The T. reesei was subsequently maintained on potato dextrose agar (PDA) prepared according to manufacturer's instructions. The T. reesei NRCAM 5 (The γ-irradiated novel mutant obtained from T. reesei PTCC 1654) was obtained from Agricultural, Medical and Industrial Research School, Nuclear Science and Technology Research, Alborz, Iran. It was selected from a panel of γ-irradiated mutant strains of T. reesei previously screened for their capacity to improve the hydrolysis of sugar beet pulp.

Production of extracellular enzymes in shakeflasks

Trichodermareeseiand T. reeseiNrcam5 were maintained on agar media (MYG agar medium) containing ($g.l^{-1}$): malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 20. Washed spore suspensions were prepared from seven-day-old slant cultures in sterile saline solution and used as an inoculum of 1×10^7 spores/ml of medium. The spores were pelleted by centrifugation at 4500×g for 10 min, and washed twice in sterile saline solution. Seed cultures were produced in Trichoderma complete medium (TCM) which contained ($g.l^{-1}$): bactopeptone, 1.0; urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4$.$7H_2O$, 0.3; $CaCl_2$ .6H O, 0.3; $FeSO_4$.$7H_2O$, 0.005; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CoSO_4$.$7H_2O$, 0.002 and 2 $ml.l^{-1}$, Tween 80. The medium was adjusted to pH 4.8 and supplemented with 0.3% w/v of glucose. Cultures were produced in 50 ml volumes of TCM in 250ml Erlenmeyer flasks shaken at 180 rpm at 28 ˚C for 24 h. To induce production of extracellular enzymes washed mycelium was

transferred to 25ml of Trichoderma fermentation medium (TFM) which contained ($g.l^{-1}$): urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2$ .6H O, 0.3; $FeSO_4$.$7H_2O$, 0.005; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CoSO_4.7H_2O$, 0.002 and 2 $ml.l^{-1}$, Tween 80. This medium was adjusted to pH 4.8 and supplemented with 0.5% w/v of sugar beet pulp. Growth conditions were as described previously. Triplicate flasks were harvested after 48 h. Estimation of protein and extracellular β-glucanase, pectinase and cellulase activity was assayed in Trichoderma fermentation medium after centrifugation at 4500 ×g for 7 min at 4 ˚C 0.

Estimation of protein and Enzyme activity

The protein content in the TFM supernatant after 48 h fermentation were estimated by the dye binding method of Bradford 0. The amount of protein was calculated using bovine serum albumin (BSA) as a standard. A standard curve was prepared using 0, 2, 8, 12, 20 and 25 µg protein per ml in supernatant of TFM medium pH 4.8. The test was carried out using 150 µl of supernatant of TFM or standard and 3 ml of Bradford reagent. The standards and tests were replicate three fold. The absorbance was read at 595 nm on a spectrophotometer (Jenway, USA). β-glucanase, cellulase and pectinase activities were determined by measuring the amount of glucose and galacturonic acid released from substrates by the DNS method with glucose and galactronic acid as the standard. The reaction mixtures contained 0.5 ml of 50 $g.L^{-1}$Cellubiose, Avicel, CMC, Sucrose and pectin (as substrate) in 0.05M citrate buffer (pH 4.8) and 0.5ml of each supernatant of TFM medium. After incubation at 50 ˚C for 60min, the controls and samples were taken out of the 50 ˚C bath. The reactions were terminated by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS). The mixtures were also mixed well, then placed into a boiling-water bath for 5min, and cooled to room temperature. The absorbance of the reaction solutions was measured at 540 nm. The International unit (IU) of activity is defined as the amount of enzyme that liberates l µmol of glucose or galacturonic acid per minute in a standard assay. Also, for FPase assay, a filter paper strip of Whatman no. 1 (50 mg) was suspended in 1.0 ml of citrate buffer. After addition of the enzyme (0.5 ml), the reaction medium was incubated for 60 min at 50 ˚C. Finally, the reducing sugars were measured by the DNS method 0.

Electrophoresis and molecular size determination

Protein samples (5 ml) from TFM supernatants were precipitated with equal volume of acetone and resuspended in final volume of 100µl, frozen and kept at -70 ˚C until they were used. The molecular weight of the extracellular enzymes were determined by sodium dodecyl sulfate–poly-acryamide gel electrophoresis (SDS–PAGE) with a 4% (stacking) and 12.5% (separating) polyacrylamide gel by the method of Laemmli0. Before electrophoresis, equal volume of sample buffer (100 µl) that contained 65 mMTris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.2% (w/v) bromophenol blue was added to the protein sample (100 µl) and boiled for 5 min and applied to loading on the gels. The proteins were separated at constant Amperage of 20 A using the running buffer contained 25 mMTris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (5:1:4, v/v), and decolorized in methanol–acetic acid–water (1:1:8, v/v) 0.

Time of fermentation optimization

Time optimization was determined at pH 4.8 by assaying the extracellular protein and cellulase enzyme activity at several time of fermentation.

Saccharification of SBP and bioethanol production

In order to investigate whether the crude T. reesei and T. resseiNRCAM5 enzymes produced can be directly applied in SSF of SBP; the experiments were carried out in triplicate in 500-ml Erlenmeyer flasks with oven dried Sugar beet (SB) and sugar beet pulp (SBP) as a solid material for bioethanol production. The oven dried Sugar beet (SB) and sugar beet pulp (SBP) were rehydrated with Trichoderma complete medium (TCM, without tween 80, pH 4.8) to a final moisture content of 65 % and were then sterilized in an autoclave at 121°C for 30 min. To induce production of extracellular enzymes washed mycelia (T. reesei and T. resseiNRCAM5) were transferred to 30 of solid state medium (SSM) which contained SBP. Batch culture SSF was performed under static condition for 72 h at 28 °C. Then, the nutrients, $(NH_4)_2SO_4$, $MgSO_4.7H2O$ and KH_2PO_4, were sterilized separately and added to the SSM (non-fermented SB contained media and fermented SBP contained media) to final concentrations of 50 % (w/v) (Limtong et al., 2008). The medium was adjusted to pH 4.8. Saccharificationwas carried out for 24 h at 50°C, at 180 rpm. The flasks were then aseptically inoculated with Saccharomycscerevisiaeor kluyveromycesmarxianus suspension. The flasks were covered with parafilm in order to provide semi-anaerobic conditions for the yeast. Fermentation was performed on a shaker incubator at 35°C and 180 rpm for 48 h and all the cultures broth were centrifuged at 4500 rpm for 10 min, the supernatant were used for yield ethanol production assay.

Statistical analysis

The treatments were performed using a completely randomized design and all experiments were carried out at least in triplicate. The experimental data were subjected to analysis of variance (ANOVA) followed by a

Duncan's test. Significance was defined at P < 0.05. The SPSS (developer, 13) program was used for all statistical analysis

RESULTS AND DISCUSSIONS

Chemical composition of sugar beet pulp

The chemical constituents of sugar beet pulp are shown in Table1. The values show that hemicellulose content determined in sugar beet pulp is similar to other lignocellulosic materials 0, 0, 0. In terms of cellulose and lignin content, Martínez et al. 0 and Panagiotopoulos et al. 0reported similar values, with exception of the hemicellulose content which, in both cases, were lower than the one obtained in this work. The SBP used in this study contained approx 24.45% cellulose and 22.70% hemicellulose (Table 1). Lignin, one of the primary structural components of lignocellulosic biomass, has been found to be an obstacleto the biological conversion of lignocellulose to products 0. Because SBP has a low lignin content (<2%), pretreatment severity was expected to be lower than the severity required to pretreat other sources of lignocellulose.

Estimation of protein and enzymes activity

Enzyme production by Trichodermareeseiand T. reeseiNrcam5 were determined in shake flask cultures. These strains were further assessed for cellulases, pectinases and glucanase production in shake flasks containing 0.5 % (w/v) SBP. Enzyme production was attempted under two-step culture conditions.

Table1. Chemical composition of sugar beet pulp (Dry basis)

Composition	Value (%)
Moisture	95.20 ± 0.14
Protein	9.96 ± 0.11
Lipid	0.15 ± 0.07
Ash	4.80 ± 0.14
Total fiber (NDF)	48.65 ± 0.07
Cellulose	24.45 ± 0.78
Hemicellulose	22.70 ±0.99
Lignin (ADL)	1.50 ± 0.28
ADF	25.95 ± 1.06

In the first step, minimal medium containing 0.3 % glucose was used to grow the mycelia; in the second step, minimal medium containing sugar beet pulp as a carbon source (induction conditions) was used and the levels of extracellular enzyme activity were measured. The extracellular protein concentration of Trichodermareeseiand T. reeseiNrcam5 were determined by the dye binding method of Bradford. The amount of protein was calculated using bovine serum albumin (BSA) as a standard and results were showed in Table 2. Protein concentration was 350.26 and 180.76 $\mu g.ml^{-1}$ for T. reesei and T. reeseiNRCAM5, respectively. Determining the extracellular protein concentration is not always a simple task, since various factors may interfere with the final result 0. There are three main factors that influence these measurements: (a) each protein dosage method is based on a different identification and quantification principle; (b) the presence of non-protein components in the enzymatic solution and/or reaction medium can be a source of error if they interfere with the results of the quantitative method; and (c) other non-cellulase proteins present in the enzyme preparation may compromise the interpretation of the specific activity data. Such differences are also due to the fact that different enzyme isolates have different primary structures, besides different degrees of glycosilation. Therefore, these factors are reflected in the response of the proteins from different strain of Trichoderma. The cellulose enzymes activity of T. reesei and its mutants in TFM supernatant after 72 h incubation at 180 rpm and 28 ˚C on SBP as a substrate were investigated. Table 2 shows the production of β-glucosidase by T. reeseiNRCAM5 as compared to the wild type strain. The mutant strain of T.reeseiNRCAM5 secreted approximately 11 times more β-1, 4-glucosidase than the wild type. β-Glucosidase can accelerate cellulose degradation by reducing end product inhibition and thus plays an important role in this synergistic action. 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 0. Sucrose is the soluble carbon source that had the most significant negative effect on cellulase biosynthesis. Sugar beet pulp contains trace amounts (<0.5%) of sucrose. The mutant strain of T. reeseiNRCAM5 showed high levels of enzyme activity compared to the wild type. Table 2 shows the production of α-1,2-glucosidase by T. reeseiNRCAM5as compared to the wild type strain. The mutant strains of T. reeseiNRCAM5 secreted approximately 89.5 times more α-1, 2-glucosidase than the wild type. These suggested that this strain most of the sucrose has been used during fermentation. Overally, the highest enhancement of the efficiency of cellulose hydrolysis was observed in T. reeseiNRCAM5, which was several times higher than that of the parental strain T. reesei (Table 2). This strain showed an interesting ability of producing cellulase when grown in presents of sucrose, suggesting that it might be lessprone to carbohydrate repression than other Trichoderma strains. It was worthwhile noting that very high β-glucosidase activity does not increase the hydrolytic capacity and the overall FPase any further, as reported by

Kovács et al. 0. In addition, it also suggests that other accessory enzymes, such as xylanase may compensate the function of β-glucosidase, increase the biomass accessibility, and consequently contribute to the improvement in the sugar yield. To examine the influence of the enzymes over expression on sugar beet pulp hydrolysis, the activities of FPA, cellobiohydrolase and endoglucanase were investigated and the results shown in Table 2. Exoglucanases cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose. The T. reeseicellobiohydrolase (CBH) I and II act on the reducing and non-reducing cellulose chain ends, respectively 0.CBH (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1, 4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used for measuring exoglucanase activity because it has a low degree of polymerization of cellulose and it is relatively in accessible to attack by EGs despite some amorphous regions. Enzymes that show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases0. CMCase activities have been analyzed using CMC, and results are given in Table 2. The highest CMCase activity was obtained 7.11 U/ml in T. reeseiNRCAM5. EGs (CMCase) can randomly hydrolyze internal glycosidic bonds in cellulose chains. Avicelase activities have been analyzed using pure Avicel, and results are given in Table 2. The highest Avicelase activity was obtained 5.38 U/ml in T. reeseiNRCAM5. The most common total cellulase activity assay is the filter paper assay (FPA) using Whatman No. 1 filter paper as the substrate, which was established and published by the International Union of Pure and Applied Chemistry (IUPAC) 0. The results showed that the highest FPase was 5.71U/ml in T. reeseiNRCAM5. Besides measuring the enzyme activities, it was very important also 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 (FPA assay) and the liberation of reducing sugars from the lignocelluloses. It may happen that not the best mutant (according to FPA enzyme activity) will be the choice of practical applications. Therefore, the enzyme complex giving the highest glucose yield was not the one having the highest FPase activity. Filter paper activity (FPA) was measured. Data are represented as the mean of three independent experiments; error bars express the standard deviations.

Table2. Comparison of protein production (µg/ml) and enzyme activities (U/ml) of T. reesei and T. reeseiNRCAM5 in TFM supernatant after 72 h incubation at 180 rpm and 28 ˚C on SBP as a substrate.

Protein &Enzyme activities	Strains	
	T. reesei	T. reeseiNRCAM 5
Protein (µg/ml)	350.26±46.75	180.76 ±5.75
Avicelase (U/ml)	1.25±0.28	5.38 ±0.00
CMCase (U/ml)	3.05 ±0.28	7.11 ±1.07
β-glucosidase(U/ml)	0.57 ±0.07	6.61 ±0.80
FPase(U/ml)	1.36 ±0.17	5.71 ±0.44
Invertase(U/ml)	0.09 ±0.05	8.05 ±2.32
Pectinase (U/ml)	1.15 ±0.05	1.02 ±0.12

Breuil et al. 0 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. Table 2 shows the production of pectinase by T. reesei NRCAM5 as compared to the wild type strain. The mutant strain of T. reeseiNRCAM5 showed approximately same pectinase activity in compared the wild type. Among pectinase-producing fungi Aspergillusniger certainly is the most used 0, while among Trichoderma genus, only few species are recorded as good pectinase producers, like T. lignorum0 and T. reesei0. The polygalacturonase assay applied in this study did not discriminate between endo- and exo-polygalacturonase (pectinase) activities. Both an exo- and an endo-polygalacaturonase have been purified from T. reesei QM9414 when growth was performed on citrus pectin as carbon source 0. Furthermore, two endo-acting polygalacturonases from T. reesei grown on selective alkaline treated sugar beet pectin have been purified and characterized 0. Consequently, the varying level of polygalacturonase activity (Table 2) measured during growth on sugar beet pulp could indicate that T. reesei NRCAM5 produce several polygalacturonases; either different isoforms or with different mode of action.

Time of fermentation optimization

The results of extracellular protein production (µg/ml) and enzyme activities (U/ml) of T. reeseiNRCAM5 in TFM supernatant during 120 h incubation at 180 rpm and 28 ˚C on SBP as a substrate were showed in Table 3. The results of extracellular protein production showed a significant decrease during the time of fermentation. This decrease could be due to the consumption of remaining proteins in SBP that has been consumed by the T. reeseiNRCAM5. Pectinase activity increased during time of fermentation and showed a statistically significant difference. The highest endo-glucanase (CMCase), exo-glucanase (Avicelase), β-glucosidase (cellubiase) and total cellulase were showed in fermentation time of 72 h. The highest Invertase (or α-1,2-glucosidase) activity was observed in fermentation time of 120 h, due to fungi consumption of accumulated final

products in the environment of fermentation. The presence of cellulase and pectinase activities was attributed to the fact that cellulase and pectin could be used as nutrients for fungal growth. The cellulolytic fungus T. reeseiNRCAM5could degrade compounds present SBP and thereby decreases its inhibitory effect on the ethanolic fermentation byS. cerevisiae. Cellulases need to penetrate the polymer to access and hydrolyze it, unlike many common enzymes which take in their substrates to the active site pockets. Cellulases have specific domains for binding their substrate so that the enzyme sits on the polymer and effects a slow degradation. Above a given threshold concentration of cellulase defined by the biomass type and degree of polymerization, the concentration of enzyme might be having lesser effects on cellulose hydrolysis compared to the effect of reaction time. This is probably the reason for more efficient hydrolysis at an increased incubation time, even when used along with lesser enzyme loading.

Electrophoresis and molecular size determination of proteins

The electrophoresis patterns obtained by SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the extracellular proteins of TFM supernatants of T. reesei (lane 1) and T. reesei NRCAM5 (lane 2) are shown in Figure 1. There are observable differences in the protein banding pattern ranged from 11 to 245 KDa. Several molecular bands were observed in the protein profiles, while the TFM supernatant (not inoculated) was no specific protein binding.Cel5A is an EG that belongs to GH family5. The enzyme has an estimated molecular weight of 42 kilo Dalton (kDa), but has an apparent molecular weight of 48 kDa on a SDS-PAGE gel due to glycosylation. It has a pI of 5.5-5.6 0. This enzyme was only observed in T. reesei SDS-PAGE profiles in molecular weight of 41.63KDa (Fig.1). Cel5A hydrolyzes the β-1, 4-glycosidic bonds in cellulose using the retaining mechanism 0. The amount of expressed Cel5A has been estimated to be between 5-10 % of total expressed cellulase in T. reesei0,0. Cel6A is a GH family 6 CBH. The enzyme has an estimated molecular weight of 47 kDa, 53 kDa on a SDS-PAGE, and it has a pI of 5.9 0,0. Cel6A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the inverting mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the non-reducing end 0,0. There have been reports that Cel6A possesses some endoglucanaseactivity 0.

Table3. The extracellular protein production (µg/ml) and enzyme activities (U/ml) of T. reeseiNRCAM5 in TFM supernatant during 120 h incubation at 180 rpm and 28 ˚C on SBP as a substrate.

Protein & Enzyme activities	Time of fermentation (h) 0	48	72	96	120
Protein	329.66^{e}± 3.6	68.83^{d}± 4.6	46.50^{c}± 4.9	20.00^{b}± 3.5	7.33^{a}±3. 9
Avicelase	0.0^{a}±0.0	3.42^{c}±0. 6	4.49^{d}±0. 7	3.64^{c}±0. 4	2.40^{b}±0. 1
CMCase	0.0^{a}±0.0	5.42^{cd}±0 .4	5.71^{d}±0. 3	5.05^{bc}±0 .1	4.67^{b}±0. 1
β-glucosidase	0.0^{a}±0.0	9.70^{c}±0. 4	11.88^{d}± 0.8	5.40^{b}±0. 2	12.79^{b}±1 .6
FPase	0.0^{a}±0.0	3.47^{c}±0. 4	5.14^{d}±0. 6	2.25^{b}±0. 1	2.16^{b}±0. 1
Invertase	0.0^{a}±0.0	7.65^{c}±0. 1	8.12^{c}±0. 6	6.48^{b}±0. 4	14.73^{d}±1 .0
Pectinase	0.0^{a}±0.0	0.75^{b}±0. 0	0.91^{c}±0. 1	1.15^{d}±0. 1	1.23^{d}±0. 0

The amount of expressed Cel6A has been estimated to be between 17-20% of total expressed cellulase in T. reesei0,0. Cel7A is a GH family 7 CBH and it was the first T. reeseiGH family 7 cellulase that was discovered. Cel7A has an estimated molecular weight of 52 kDa, 66 kDa on a SDS-PAGE, and it has a pI of 4.3 0,0. These enzymes were observed in molecular weight of 71 for T. reeseiand T. reeseiNRCAM5SDS-PAGE profiles that were considered as a Cel7A (CBH I). Also, Cel6A (CBH II) was observed in T. reeseiand T. reeseiNRCAM5SDS-PAGE profiles in molecular weight of 59.09KDa (Figure1). Cel7A is the major cellulase produced by T. reesei, and it has been estimated that 50-60 % of total expressed cellulase in the fungus is Cel7A 0, 0. It is probably the key enzyme needed for hydrolysis of crystalline cellulose by the fungus. Cel7A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the reducing end 0, 0. Cel7B is a GH family 7 EG. Cel7B has an estimated molecular weight of 48 kDa, 50-55 kDa on a SDS-PAGE, and it has a pI of 4.5 0, 0. Cel7B is homologous to Cel7A, with about 45 % sequence identity. The main difference between the two GH family 7 structures is that the substrate-binding cleft is less covered by extended loops in the endoglucanase (Cel7B) than in the exoglucanase (Cel7A). Cel7B hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism. The amount of expressed Cel7B has been reported to be between 6-10% of total expressed cellulase in T. reesei0, 0.Cel7B enzyme bond was observed in SDS-PAGE profiles protein of T. reeseiand T. reeseiNRCAM5with molecular weight of 45.29KDa. Cel12A is a GH family 12 EG. The enzyme has

a molecular weight of 25 kDa, has a neutral pI of 7.5 0, 0, 0. Cel12A hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism. The two catalytic residues in Cel12A are the two carboxylates E116 and E200. The amount of expressed Cel12A has been reported to be less than 1% of total expressed cellulase in T. reesei0. The specific function for T. reesei Cel12A is not known. Some biochemical data on Cel12A can be found in the literature, including studies of activity on soluble substrates 0, and insoluble cellulase0. There have been reports that Cel12A, besides cellulose activity, has activity against β-glucan and xylan0, 0. It has been shown that Cel12A has an ability to induce extension of type I cell walls from cucumber and wheat 0.Cel12A enzyme bond was observed in SDS-PAGE profile protein of T. reesei and T. reeseiNRCAM5with molecular weight of 20.84KDa.The week bonds were observed in molecular weight of 19.11 KDa for T. reeseiand T. reeseiNRCAM5 that probably were related to xylanase II enzyme. Some biochemical data on EG can be found in the literature, including studies of activity on soluble substrates 0, and insoluble cellulase0. There have been reports that EG, besides cellulose activity has activity against β-glucan and xylan0, 0. Also, Cel61A was observed in molecular weight of 34.57KDa for T. reeseiand T. reeseiNRCAM5SDS-PAGE profiles. CEL 1A (BGL II) was observed in molecular weight of 115.90KDa for T. reeseiand T. reeseiNRCAM5SDS-PAGE profiles.

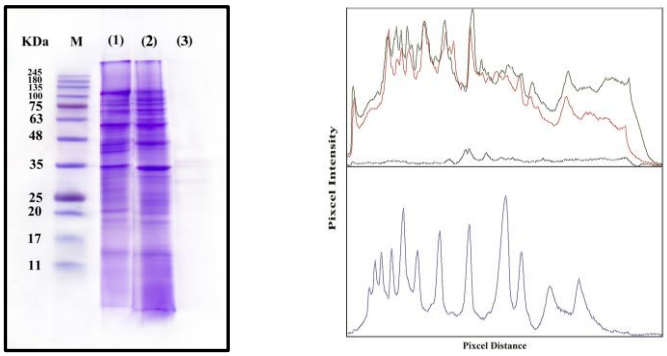

Figure1. Profiles of proteins secreted into the culture supernatants of (1) T. reesei, (2) T. reeseiNRCAM5 and (3) TFM supernatant (not inoculated) and densitometry analysis of them. “M” indicates a molecular weight marker.

β-Glucosidasehydrolyzes the soluble oligosaccharides, produced by cellulases, to glucose. The addition of β-glucosidases into the T. reeseicellulases system achieved better saccharification than the system without β-glucosidases0. β-Glucosidases hydrolyze the cellobiose which is an inhibitor of cellulase activity. Densitometry analysis showed that the protein concentration of the T. reeseiNRCAM5 is greater than the T. reesei.

A. Saccharification of SBP and bioethanol production

Saccharomyces cerevisiae is by far the most commonly used microbial species for industrial ethanol production from sugar- and starch-based raw materials. Also, strains of Kluyveromycesmarxianus have attracted much attention because they can grow at higher temperature and produced ethanol comparable to that by S. cerevisiae at industrial scale 0.

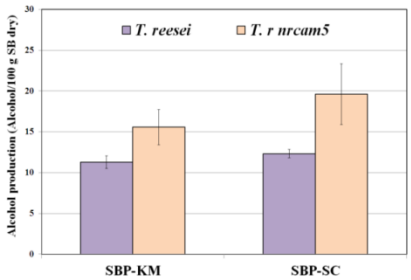

Figure2. Comparison percentage of alcohol production by Saccharomyces cerevisiae (SC) and kluyveromycesmarxianus (KM) from enzymatic hydrolyzed sugar beet pulp (SBP) as a substrate after 48 h incubation at 180 rpm and 35 ˚C.

The amount of alcohol production by S. cerevisiae (SC) and k.marxianus (KM) from enzymatic hydrolyzed sugar beet pulp (SBP) as a substrate inoculated with after 48 h incubation at 180 rpm and 35 ˚C were showed in Figure 2. The SBP hydrolyzed with T. reeseiNRCAM5 showed high levels of alcohol production compared to the SBP hydrolyzed with T. reesei. The results obtained demonstrate the effectiveness of enzymes in the saccharification of sugar beet pulp with extracellular enzymes of T. reeseiNRCAM5. The saccharification of SBP with T. reesei and subsequently fermentation of sugars to ethanoldid not observed a statistically significant difference between S. cerevisiae (SC) and k.marxianus (KM).

CONCLUSION

Significant progress has been made in the past several years in all aspects of lignocellulosic conversion to ethanol. The key to the establishment of a commercial process is a reduction in capital and operating costs of each of the unit operations. There is a much better understanding of the capital costs associated with pretreatment, suggesting ways to further reduce costs without compromising performance. Similarly enzyme costs have been reduced by a combination of protein engineering and process development. However, further cost reductions are required and will more than likely come from novel, tailored cocktails of enzymes with higher specific activities than current commercial enzymes. Finally, improvements have been made in microorganisms capable of converting multiple sugars into ethanol. A commercial organism must, however, be able to withstand the rigors of a full-scale process, including potential toxic compounds present in the sugar hydrolysate. Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Enzymatic hydrolysis of SBP to glucose is carried out by cellulase enzyme that is highly specific catalysts. The hydrolysis is performed under mild conditions (e.g. pH 4.5–5.0 and temperature 50 ◦C). Therefore, one may expect low corrosion problems, low utility consumption, and low toxicity of the hydrolyzates as the main advantages of this process compared to the acid hydrolysis by dilute acid at higher temperature. The results indicated that the highest ethanol concentration can be obtained from the enzyme hydrolyzed sugar beet pulp. This can be explained to the fact that T. reeseiNRCAM5can affect the cellulosic region differently and hence facilitates the enzyme treatment and thus the fermentation to produce the ethanol.

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