Influence of Enzymatic Cocktails on Conversion of Agricultural Lignocellulose to Fermentable Sugars

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Agricultural lignocellulosic biomass is regarded as an important source of biofuels, especially bioethanol and biohydrogen. The following aspects have been studied: the effect of type of substrate used in production of cellulolytic enzymes, the activity of several enzymatic cocktails used to hydrolyse three types of agricultural biomass and the influence of provenience of enzymatic cocktails on sugars yields in the hydrolysis process. Fungi investigated in this study (T. longibrachiatum DSM 769) release higher titter of enzymes when raw, unpretreated agriculture residual biomass is used as substrate and inducer for biosynthesis of cellulolytic enzymes. Cellulolytic enzymes produced in culture media containing a certain type of agricultural lignocellulosic biomass as substrate, can be used in hydrolysis of other types of agricultural lignocellulosic biomass with similar sugar yields. Cellulases produced in culture media containing purified crystalline cellulose as substrate does not contain all necessary types of enzymes to hydrolyze lignocellulosic complex from agricultural biomass to produce high yields of sugars. On-site production of cellulases can be an effective approach biorefinery of lignocellulose to produce biofuels or other biochemicals by fermentation.

Keywords: lignocellulose, cellulase, Trichoderma, sugars, biofuels, pretreatment

Lignocellulosic biomass derived from agricultural residues or fast-growing energy crops represent one of the most promising energy carriers for the future. We consider biomass as the most environmentally friendly battery, collecting sun energy by photosynthesis and applying appropriate conversion technologies, this energy can be transferred into solid, liquid or gaseous fuels (such as pellets, ethanol, hydrogen or methane). While conversion of lignocellulose into ethanol have been extensively studied[1,2], the technology is mature and applied in large scale [3], conversion of sugars obtained from lignocellulose into alternative biofuels such as hydrogen did not passed the laboratory scale [4-6].

Biohydrogen is a promising fuel: it is carbon-free and its combustion produces only water [7]. Although hydrogen constitutes a clean fuel, currently available methods leading to its production, such as methane reforming and partial oil and coal oxidation, demand fossil fuels and a high amount of energy [8]. Biological approaches that produce hydrogen offer several advantages over current physicochemical methods: they occur at ambient temperature and pressure, and they use renewable raw materials as substrates [9]. Truus et al., [10] demonstrated important yields of hydrogen from lignocellulosic crops by extreme thermophilic bacteria *Caldicellulosiruptorsaccharolyticus* and *Thermotoganeapolitana*. Hydrogen yields of 2.9 to 3.4 mol H_2 per mol of hexose, corresponding to 74 to 85% of the theoretical yield, were obtained in these batch fermentations.

Although aspects regarding hydrolysis of lignocellulose to obtain readily fermentable sugars and conversion of monosaccharides to bio-H_2 have been previously studied, preliminary aspects connected to provenience of enzymes used in hydrolysis phase have to be considered and studied. On-site production of cellulolytic enzymes is a promising approach in industrial applications [11], however enzymatic cocktails obtained in liquid or solid state fermentations differs depending on several factors.

In this work we have studied the effect of type of substrate used in production of cellulolytic enzymes, the activity of several enzymatic cocktails used to hydrolyse three types of agricultural biomass and the influence of provenience of enzymatic cocktails on sugars yields in the hydrolysis process.

Experimental part

In the first part of the study we have evaluated the influence of pretreatment of lignocellulosic complex from agricultural residues on the capacity to produce and release cellulolytic enzymes in *Trichoderma.*The microorganism used in this experiment is *Trichodermalongibrachiatum* CMIT36, obtained from D.S.M.Z. Germany, where is stored as *T. Longibrachiatum* DSM 769 (other name: *T. reesei* ATCC26921, initially named *T. reesei* Simons), is a mutant of *T. Viride* Persoon, or QM 9123 (ATCC24449), also referred as QM 9124. In one of the biosynthesis batches we applied a mixt culture (co-culture) of *T. longibrachiatum* and *Aspegillus niger* (local strain). All fungi are preserved in this collection by freezing the spore suspension at –70°C in glycerol 16% as cryoprotective agent. Pretreatment of lignocellulosic biomass was made by grinding biomass, dispensing in Erlenmeyer flasks where was treated with NaOH 2% solution, and autoclaved at 1 bar for 30 min. The pretreated biomass was washed several times with sulfuric acid solution and water until *p*H 5,5.Cellulase production by submerged liquid cultures (***SLC***)was carried out in 300 mL flasks containing 50 mL Mandels medium added with lignocellulosic biomass to obtain with 2% cellulose in culture medium (KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.14%, M_gSO_4 x $7H_2O$ 0.03%, $CaCl_2$ x $2H_2O$ 0.04%, urea 0.03%, peptone 0.03%, tween 80 0.05%, $FeSO_4$ x 7 H_2O sol. 5 mg % 1 mL, $ZnSO_4$ x 7 H_2O sol. 1.4 mg % 1mL, $MnSO_4$ x 7 H_2O sol 1.56 mg % 1 mL, $CoCl_2$ sol 2 mg % 1 mL, distillated water ad. 100 mL, *p*H 5.5 sterilization 20 min at 121°C). The flasks containing sterile medium were inoculated with 10% spores suspension. Wheat straw, corn stover and miscanthus

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biomass milled with a 2 mm mesh where used as carbon source and substrate for cellulase production. As control, Mandels medium with 2% cellulose Avicel PH101 was used. The inoculated media were incubated in an incubator shaker. During fermentation, samples were harvested in the last part of the fungal growth (days 7-9) when cellulosic activity have been previously proved as maximal [12]and FPU and CMC-ase activity were analysed.

Enzyme assays

Two assays[13, 14], have been applied:

- one using as substrate CMC for endoglucanase; the reaction was carried out at 50°C for 10 min. One International Unit (***IU***) of enzyme was defined as the amount of enzyme that released 1 µmol of reducing sugar per min under standard conditions;

- and one using as substrate filter paper for saccharifying cellulase; the reaction was carried out at 50°C for 60 min. One Filter Paper Unit (***FPU***) was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute under standard conditions.

In both assays the amount of reducing sugar was determined by DNS method.

In the second part of the study, we have investigated the influence of the type of substrate used to produce cellulolytic enzymes on the activity of these enzymes crossing applied on the same substrate and on other types of substrates. Portions consisting of 3 g of biomass were distributesin 100 mL Erlenmayer flasks and pretreated as previously described using NaOH combined with steam treatment. The pretreated biomass was hydrolysed using fresh culture liquids obtained in the first part of the study and other types of commercial cellulases and enzyme cocktails obtained in laboratory by authors applying the same culture conditions described in the first part of the study. The enzyme load was 15 U of cellulase/gram cellulose.

The commercial enzymes used in this study where purchased from *Novozyme*™. The following products where used, part of a *Novozymes* cellulosic ethanol enzyme kit:

-NS22086 cellulase complex, contains cellulase, cellobiaseand xylanase (endo -1,4-) and catalyzes the breakdown of cellulose material into glucose, cellobiose, and glucose polymers.

-NS22119 enzyme complex contains a wide range of carbohydrases, including arabinase, β-glucanase, cellulase, hemicellulase, pectinase, and xylanase.

-NS22118 β-glucosidase, also known as cellobiase; hydrolyzescellobiose to glucose.

-NS22083 xylanase, purified endoxylanase with a high specificity toward soluble pentosans, able to liberate pentose sugars from biomass hemicellulose fractions.

Citrate buffer *p*H 5 was used as hydrolysis medium for commercial enzymes and for laboratory fresh cultures. The hydrolysis of the 3 grams portions of biomass distributed flasks containing 60 mL hydrolysis liquid were carried out in sterile conditions at 50°C and *p*H 4.8 for 48 h. Two probes (at 24 and 48 h) were harvested in aseptic conditions, centrifuged and stored in the freezer. After hydrolysis the concentration of reducing sugars in harvested probes was determined.

To determine the total sugars released during hydrolysis, the 2 mL liquid samples harvested from hydrolysis and fermentation media were centrifuged 10 min at 9.000 rpm and supernatant was analyzed by DNS method (T.K. GHOSE [14]). The concentration of reducing sugars released during the enzymatic hydrolysis was indirectly determined measuring the absorbance at 540 nm of the color reaction.

Results and discussions

Regarding the question whether a pretreatment of lignocellulosic complex will increase enzymes yields in cellulolytic fungi cultivated on lignocellulosic biomass in Mandelsmendium, the results in table 1 indicate the contrary. Although overall enzymatic activities are lower comparing to other authors [15,16], our results demonstrate that the necessity of a pretreatment of lignocellulosic biomass to be used as substrate for cellulose production is not justified.

Fungi investigated in this study (*T. Longibrachiatum* DSM 769) release higher titter of enzymes when raw, unpretreated agriculture residual biomass is used as substrate and inducer for biosynthesis of cellulolytic enzymes. These findings confirm general consideration that the enzymatic activity of the microorganisms is higher in media containing complex polymeric substrates, difficult to be degraded than in media containing readily usable substrates.

The cultures obtained in this phase of the study were prepared by centrifugation to separate fungi biomass and insoluble components from culture medium. The obtained liquids represent the cellulosic preparatesto be used in the second part of the study.

In the second part of the study we applied different types of enzymes to hydrolyse each of the three types of biomass

Biomass/pretreatement	Saccharifyingcellulase (FPU/mL)		Endoglucanase (IU/mL)	
	day 7	day 9	day 7	day 9
corn stover	1.01	1.21	0.90	1.21
pretreated corn stover	0,.95	1.01	0.75	0.90
miscanthus	0.97	0.88	0.97	1.06
pretreatedmiscanthus	0.35	0.64	0.24	0.90
wheat straw	0.70	0.70	0.95	1.01
pretreated wheat straw	0.31	0.46	0.20	0.84
celluloseAvicel (control)	0.35	0.53	0.24	0.46

Table 1
ENZYMATIC ACTIVITY OF *T. LONGIBRACHIATUM* IN SLC USING PRETREATED AND UNPRETREATED LIGNOCELLULOSE AS SUBSTRATE

used in this study and abbreviated in tables 2-4 as follow: corn stover (CoSt), wheat straw (WhStr), miscanthus (Misc). Enzymes used for hydrolysis of cellulosic biomass are abbreviated *enz* in tables 2-4 followed by the type of substrate used in the biosynthesis phase (first phase of the study): corn stover (CoSt), wheat straw (WhStr), miscanthus (Misc),crystalline cellulose Avicel (A), co-culture of *Trichoderma longibrachiatum* and *Aspergillus niger* (B) and commercial enzymes described in the material and methods paragraph (22086, 22119, 22118, 22083).

The results in tables 2-4 indicates that the highest reducing sugars yields in all three types of biomass has been obtained using the cocktail formed of commercial enzymes Novozymes22119 and 22083 (complex contains a wide range of carbohydrases, including arabinase, β-glucanase, cellulase, hemicellulase, pectinase, xylanase and purified endoxylanase). This study proves the synergic action of endo-hydrolases (22083contains endoxylanase) in combination with exo-hydrolases (22119 contains xylanase). When applied separately (lines 7 in tables 2-4), without the help of endoxylanase to open action sites for xylanase, the reducing sugars yields are very low (under 20% of the yields obtained in combination with endoxylanase, as in lines 9 in tables 2-4. The second best yields have been obtained with commercial enzymes Novozymes22086, which is a cocktail of endo-, exo-glucanase and cellobiase (lines 6 7 in tables 2-4). As in the case of applying only cellobiase (line 87 in tables 2-4), as expected, the yields are very low as there are no endo-glucanases to open action sites for cellobiases. Still, the surprising results have been obtained in the case of applying the whole crude cellulolytic enzyme pool produced by *T. longibrachiatum* in culture media containing agricultural cellulosic biomass as substrate (lines 1-3 in tables 2-4). The yields are very close to those obtained with commercial enzymes, especially in the case of wheat straw, but in this case the yields obtained with commercial

	Type of biomass+ type of enzyme	Time of hydrolysis (hours), released reducing sugars mg%			
		12	24	48	60
1	CoSt+enzCoSt	520	1085	1189	1220
2	CoSt+enzWhStr	508	1033	1241	1241
3	CoSt+enzMisc	589	825	1033	1050
4	CoSt+enz A	510	840	987	998
5	CoSt+enz B	360	450	846	896
6	CoSt+22086	682	1470	1709	1709
7	CoSt+22119	251	299	371	371
8	CoSt+22118	323	323	371	371
9	CoSt+22119+22083	1064	1088	1566	1805

Table 2
REDUCING SUGARS YIELD IN THE PROCESS OF ENZYMATIC HYDROLYSIS OF CORN STOVER

	Type of biomass+ type of enzyme	Time of hydrolysis (hours), released reducing sugars mg%			
		12	24	48	60
1	Misc+enzMisc	127	1346	1450	1465
2	Misc+ enzCoSt	121	1346	1450	1460
3	Misc+enzWhStr	133	1137	1450	1455
4	Misc+enz A	538	777	992	1016
5	Misc+enz B	371	682	610	610
6	Misc+22086	849	1566	1709	1709
7	Misc+22119	228	275	371	371
8	Misc+22118	228	275	323	347
9	Misc+22119+22083	889	1578	1725	1730

Table 3
REDUCING SUGARS YIELD IN THE PROCESS OF ENZYMATIC HYDROLYSIS OF MISCANTHUS

Table 4
REDUCING SUGARS YIELD IN THE PROCESS OF ENZYMATIC HYDROLYSIS OF WHEAT STRAW

	Type of biomass+ type of enzyme	Time of hydrolysis (hours), released reducing sugars mg%			
		12	24	48	60
1	WhStr+enzWhStr	91	1189	1554	1554
2	WhStr+enzCoSt	104	1085	1346	1346
3	WhStr+enzMisc	50	1137	1241	1241
4	WhStr+enz A	443	729	897	897
5	WhStr+enz B	371	490	849	849
6	WhStr+22086	299	849	1518	1518
7	WhStr+22119	610	228	275	275
8	WhStr+22118	275	275	323	371
9	WhStr+22119+22083	777	1542	1566	1566

enzymes are lower than in the case of miscanthus and corn stover. The other finding addresses the cross-application of enzymes on the same substrate used for biosynthesis and on the other types of substrates (lines 1-3 in tables 2-4). Concerning this matter, in the case of corn stover and miscanthus our results indicates no important differences when the hydrolysis is carried out using the enzyme synthesized on the same substrate (lines 1), or with enzymes synthesized on different substrates (lines 2 and 3). In the case of wheat straw, the yield obtained by application of the whole crude cellulolytic enzyme pool produced by *T. longibrachiatum* in culture media containing wheat straw as substrate (line 1 in table 4) is higher as in the case of hydrolysis with enzymes synthesized on different substrates (lines 2 and 3 in table 4) and comparable with the yield obtained by application of commercial enzymes (lines 6 and 9 in table 4). In the case of the whole crude cellulolytic enzyme pool produced by *T. longibrachiatum* in culture media containing crystalline cellulose as substrate (lines 4) and co-cultures of *T. longibrachiatum* combined with *Aspergillus niger* (lines 5), the low yields obtained in our study demonstrate that composition of the enzyme pool is influenced by the composition of the substrate used in the biosynthesis process. In other words, fungi synthesize the type of enzyme needed to gain soluble sugars for growth and function of metabolism. When the substrate is complex (the case of agricultural biomass), the synthesized enzyme pool is complex enough to brake lingo-cellulosic structure of the biomass. When the substrate is a purified structure composed of a reduced range of biochemical bonds (β-1,4 glycosidic, β-1,6 glycosidic as in crystalline cellulose), fungi will release exclusively the enzymes needed to brake these types of structures. Our results demonstrate that the substrate used in microbial growth play an additional role in microbial metabolism, connected to gene expression and protein synthesis – the role of inductor. The activity of each gene is activated by an inductor when the protein encoded by that gene is needed in the metabolism. The presence of complex molecules, organic polymers in culture media of fungi induces the activity of genes encoding the enzymes needed to hydrolyze the structure of those polymers.

Sugars obtained after hydrolysis of lignocellulose can be used as substrate in fermentations to produce biofuels such as bio-hydrogen, biogas or other biochemicals by fermentation.

Conclusions

Cellulolytic enzymes are synthesized by fungi of *Trichoderma longibrachiatum* in submerged liquid cultures with lignocellulosic biomass as carbon source and substrate for enzyme synthesis.

Pretreatment of lignocellulosic biomass used as substrate in production of cellulolytic enzymes does not increase enzyme yields, by contrary, unpretreated biomass in culture medium of cellulolytic fungi induce production of highly active enzyme pool.

Cellulolytic enzymes produced in culture media containing a certain type of agricultural lignocellulosic biomass as substrate, can be used in hydrolysis of other types of agricultural lignocellulosic biomass with similar sugar yields.

Cellulases produced in culture media containing purified crystalline cellulose as substrate does not contain all necessary types of enzymes to hydrolyze lignocellulosic complex from agricultural biomass to produce high yields of sugars.

Commercial products conditioned in liquid form release higher yields of sugars in case of hydrolysis of agricultural biomass comparing with fresh enzymatic preparations obtained from laboratory cultures of cellulolytic fungi.

On-site production of cellulases can be an effective approach biorefinery of lignocellulose to produce biofuels or other biochemicals by fermentation.

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