Cellulolytic Fungi and the Bioconversion of Fiber from *Agave sisalana*

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ABSTRACT *Agave sisalana*, a plant thriving in semi-arid regions of Thailand, is an important source of fiber for the sisal and handicraft industries of the country. Isolations of 94 soil and plant samples from agave plantations in 3 provinces revealed 52 fungal isolates with cellulolytic activities on both Czapex' dox with a filter paper and CMC agar media. These isolates were propagated in a liquid medium at 30, 37 and 45°C. The fungal isolate with the highest cellulolytic activity (FP = 0.274 U/ml at 37°C) was identified as *Acrophialophora* sp. At 45°C, the fungus also grew and produced detectable cellulolytic activity, while *Trichoderma reesei* did not. Bioconversion using the simultaneous saccharification and fermentation (SSF) involving *T. reesei* and *Saccharomyces cerevisiae* gave the highest ethanol yield of 0.30 g/g. Preliminary tests on SSF using *Acrophialophora* sp. and *S. cerevisiae* gave the maximum ethanol yields of 0.11 g/g and 0.17 g/g with agave fiber and filter paper, respectively.

KEYWORDS: bioconversion, *Agave sisalana*, *Acrophialophora* sp.

INTRODUCTION

Agave sisalana is a desert plant found in certain arid and semi-arid regions of the world including Brazil, China and India. In Thailand, the plant grows well in poor-quality soil in the northeastern part of the country and along the south central coast where the land is semi-arid. The annual leaf production of the cultivated plants is above 45 tons per acre. It is an important economic plant providing strong fiber for rope manufacture and the production of handicrafts. In general, the plant contains 62% cellulose available for bioconversion into ethanol and certain fungi living in its growing areas are likely to be adapted for its degradation. We have isolated fungi from agave plantations in Thailand and screened them for cellulolytic activity. The isolate with highest activity was used for bioconversion of agave fiber into ethanol.

MATERIALS AND METHODS

Sample collection, isolation, selection and screening

Ninety four soil and plant samples including dry leaves and dead stumps of *A. sisalana* were collected from agave plantations in Nakorn Ratchasima, Prachuab Kirikhan and Petchaburi provinces of Thailand. The isolation technique for cellulolytic fungi of Mandels and Sternberg[1] was used. To begin, 10 g (or 10 ml of fluid) of sample was suspended and mixed for 2 minutes in 100 ml distilled water. One ml of the suspension was added to 100 ml of Czapex' dox medium containing 2.0 x 10.0 cm Whatman no 1 filter paper as the sole carbon source in a 250 ml-Erlenmeyer flask. The flasks were incubated at 32°C for 7 days. Fungi growing on the filter paper were isolated in pure culture on potato dextrose agar (PDA). They were then transferred to carboxymethyl cellulose (CMC) agar for further selection as described by Hankin and Anagnostakis.[2] The plates were incubated at 32°C for 3 days and followed by the addition of 0.01% Congo red and rinsing with 1M NaCl. Fungi that produced clear zones were further tested for cellulolytic enzyme activity as described by Punnapayak and Emert[3] at 30, 37 and 45°C for 7 days.

Substrate and enzyme preparations

The biomass of agave fiber was alkali pretreated with 2.5 M NaOH as described by Punnapayak and Hoffmann.[4] Crude cellulases were prepared from *Trichoderma reesei* QM 9414 and the new isolate *Acrophialophora* sp. Briefly, each fungus was cultivated on a PDA plate for 7 days at 32°C for *T. reesei* and 37°C for *Acrophialophora* sp. Five square blocks (0.5 x 0.5 cm2) of the mycelial mat were inoculated into 100 ml of liquid medium containing 0.1% $MgSO_4$, 0.5% $CaHPO_4$, 0.4%$(NH_4)_2$ SO_4, 0.7% (w/v) cornsteep liquor, 3% microcrystalline cellulose (Avicel), 0.2% Tween 80, 0.0005% $FeSO_4$, 0.00014% $ZnSO_4$, 0.00016% $MnSO_4$, 0.00036% $CoCl_2$ in each 250-Erlenmeyer flask. The flasks were then shaken at 120 rev/min. They were incubated at 32°C for *T. reesei* and 40°C for *Acrophialophora* sp. for 15 days after which the culture broth was centrifuged at

1,465 x g for 15 minutes and the clear filtrate was used as a crude enzyme preparation.

Bioconversion experiment

A simultaneous saccharification and fermentation (SSF) technique was used for the bioconversion of fiber to ethanol.[5] Each pretreated material (5% dry wt) was supplemented (10% v/v) with a mixture of 3% $(NH_4)_2SO_4$, 0.22% $MgSO_4$. $7H_2O$ and 0.12% $CaCl_2$ and autoclaved at 121°C for 20 minutes. Crude cellulase produced from *T. reesei* (0.398 µ/ml filter paper activity, and 23.003 µ/ml CMCase) or from the new isolate *Acrophialophora* sp.(0.390 µ/ml filter paper activity, and 22.429 µ/ml CMCase) were added at 80% (v/v) with an inoculum of 2.6 x 10^9 cells of *Saccharomyces cerevisiae*. Flasks containing the mixtures were tightly plugged, sealed with aluminum closures and were incubated at 40°C in a rotary shaker (120 rev/min) under anaerobic condition for 6 days.

Sample analysis

Fungi were identified using microscopic examinations and guidelines described by Barnett and Hunter,[6] Domsch and Gams.[7] Cellulase activities in liquid media were determined using a filter paper (FP) activity assay as described by Mandels and Sternberg[1] and a Carboxymethyl cellulase actvity assay (CMCase), as described, by Acebal, *et al*.[8] Cellulase activities on CMC agar were recorded as clear zone ratios = clear zone diameter / colony diameter.[9] Glucose was measured by a glucose analyzer, (YSI 27; Yellow springs, USA). Total reducing sugars were measured using dinitrosalicylic acid[10] and ethanol was detected by gas chromatography using a Porapak Q Column. The conversion efficiency of the bioconversion reaction was calculated[11] as a percentage yield of product (ethanol) from the substrate (dry plant biomass). All experiments were performed in quadruplet and repeated twice. The data are means of the results of the experiments.

Results and Discussion

Isolation of 94 soil and plant samples from agave plantations in 3 provinces of Thailand yielded 52 fungal isolates with cellulolytic activity on both Czapex'dox and CMC agar media. The results are summarized in Table 1. Most came from soil samples, followed by dead stumps and dry leaves. Fresh leaves and leaf fluid byproduct from decorticating leaves for fiber gave very few fungi. Thus, soil was probably the major arena for agave degradation. One isolate ranked at the top, with activities comparable to *T. reesei* at 30°C (0.246 versus 0.284 filter paper activity, µ/ml). However, this fungus was capable of producing cellulase at 45°C while *T. reesei* was not. Identification revealed that it was *Acrophialophora* sp. with typical 1-celled, hyaline or sub-hyaline, ovoid to broadly fusoid and cutenulate conidia[6] (Fig 1).

The conversion of agave fiber into ethanol is being studied as a possible adjunct process in the sisal rope manufacturing industry. The SSF process was selected because of its advantage over a regular two-step hydrolysis and fermentation process.[4] Data on SSF using agave fiber with *S. cerevisiae* and the enzyme from *T. reesei* are summarized in Table 2. Whatman filter paper was used as a comparative substrate. Having gone through pulping, it is likely that the cellulose fibrils in the filter paper are much less crystalline than natural cellulose. The maximum ethanol yields obtained were 0.30 g/g (59% conversion) from agave and 0.40 g/g (78% conversion) from filter paper.

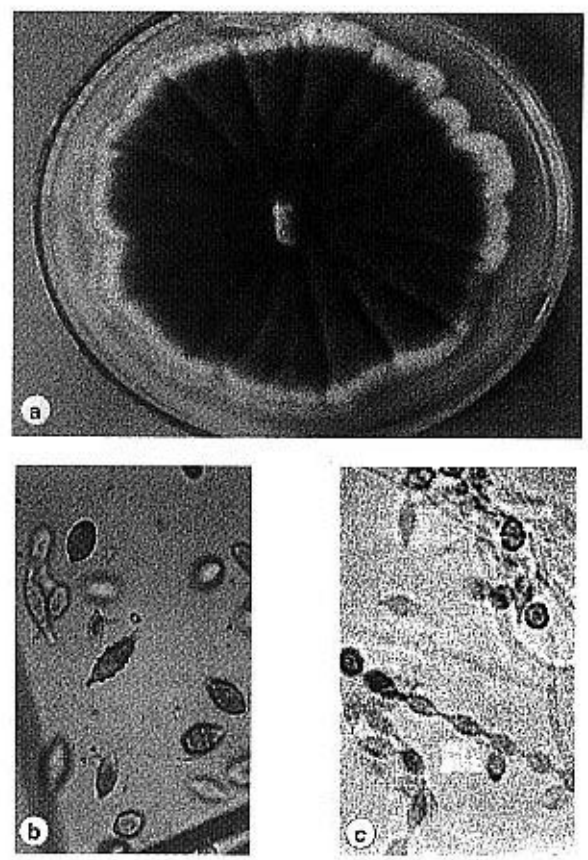

Fig 1. *Acrophialophora* sp. (a) colony morphology (b) released mature conidia (1,200 X) (c) young cutenulate conidia (1,400 X).

Table 1 Fungi with cellulolytic activity isolated from agave plantations.

Sample description	Fungus colony color	Clear zone ratios	Filter paper activity μ/ml 30°C	37°C	45°C
Soil	Brown	2.43	0.246	0.274	0.048
	Brown	2.10	0.194	0.170	0.010
	Dark brown	2.07	0.182	0.172	0.009
	Purple	1.73	0.160	0.175	0.000
	Grayish brown	1.65	0.154	0.159	0.000
	Green	1.54	0.148	0.199	0.003
	Pink	1.54	0.134	0.163	0.000
	Purple	1.53	0.134	0.117	0.000
	Black	1.48	0.134	0.103	0.000
	Yellowish brown	1.45	0.133	0.117	0.000
	Pale orange	1.41	0.129	0.081	0.000
	Black	1.42	0.127	0.117	0.000
	Dark orange	1.38	0.127	0.077	0.000
	Black	1.33	0.120	0.072	0.000
	Green	1.34	0.119	0.132	0.000
	Brown	1.30	0.117	0.119	0.000
	Yellowish brown	1.29	0.117	0.051	0.000
	Green	1.28	0.104	0.044	0.000
	Green	1.28	0.100	0.104	0.000
	Green	1.28	0.096	0.100	0.000
	Yellowish brown	1.27	0.096	0.100	0.000
	Brown	1.27	0.095	0.106	0.000
	Grayish brown	1.26	0.091	0.034	0.000
	Black	1.27	0.081	0.100	0.000
	Grey	1.27	0.078	0.081	0.000
	Dark brown	1.25	0.077	0.023	0.000
	Grey	1.26	0.068	0.099	0.000
	Grayish brown	1.25	0.067	0.017	0.000
	Green	1.24	0.066	0.010	0.000
	Grayish brown	1.25	0.064	0.081	0.000
	Purple	1.25	0.059	0.058	0.000
	Grey	1.24	0.049	0.065	0.000
	Green	1.24	0.046	0.008	0.000
	Black	1.23	0.045	0.011	0.000
	Green	1.23	0.035	0.060	0.000
	Black	1.21	0.031	0.016	0.000
	Green	1.21	0.031	0.001	0.000
	Black	1.21	0.023	0.005	0.000
	Black	1.20	0.023	0.022	0.000
	Grey	1.19	0.017	0.044	0.000
	Grayish brown	1.17	0.012	0.022	0.000
Dead stump	Dark brown	2.15	0.201	0.194	0.023
	Brown	1.48	0.133	0.137	0.000
	Brown	1.30	0.119	0.120	0.000
	Pale pink	1.25	0.064	0.072	0.000
	Grayish white	1.23	0.033	0.005	0.000
	Grey	1.14	0.009	0.011	0.000
Dry leaf	Brown	1.73	0.154	0.157	0.002
	Dark brown	1.36	0.121	0.122	0.000
	Green	1.26	0.072	0.102	0.000
Leaf	Green	1.85	0.177	0.188	0.004
Liquid from Sisal factory	Black	1.22	0.032	0.043	0.000
	T. reesei QM9414	-	0.284	0.166	0.000

Using *S. cerevisiae* and the enzyme from *Acrophialophora* sp., in a similar SSF, the maximum ethanol yields obtained were 0.11 g/g (22% conversion) and 0.17 g/g (33% conversion) from agave and paper substrate, respectively (Table 3).

Glucose and total reducing sugars were monitored during all SSF experiments to be certain that the hydrolysis took place and that the glucose level remained low throughout the process. This indicated a well functioning SSF where glucose was quickly taken up by the yeast and simultaneously fermented into ethanol and, therefore, resulted in low glucose accumulation.

Our investigation has indicated the possibility of using fungi for the biomass conversion of agave fiber. Ethanol is an important commodity with many

Table 2. Simultaneous saccharification and fermentation of agave using *T. reesei* and *S. cerevisiae*.

Fermentation period (days)	Ethanol (g/g dry material)		Glucose (g/l)		Total reducing sugar (g/l)		Conversion (%)	
	P	A	P	A	P	A	P	A
1	0.09 ± 0.02	0.22 ± 0.05	1.74 ± 0.14	1.30 ± 0.14	8.02 ± 0.23	5.02 ± 0.09	18	43
2	0.20 ± 0.06	0.25 ± 0.01	1.99 ± 0.14	1.44 ± 0.08	9.40 ± 0.54	5.90 ± 0.10	39	49
3	0.25 ± 0.07	0.30 ± 0.01	1.30 ± 0.10	1.15 ± 0.05	6.55 ± 0.14	4.67 ± 0.09	49	59
4	0.36 ± 0.11	0.29 ± 0.03	1.20 ± 0.04	0.81 ± 0.10	5.87 ± 0.13	4.04 ± 0.19	71	57
5	0.40 ± 0.14	0.27 ± 0.03	0.79 ± 0.10	0.67 ± 0.08	4.99 ± 0.19	2.71 ± 0.41	78	53
6	0.39 ± 0.14	0.24 ± 0.04	0.55 ± 0.06	0.45 ± 0.06	3.50 ± 0.12	2.22 ± 0.05	77	47

P = filter paper substrate A = agave substrate

Table 3. Simultaneous saccharification and fermentation of agave using *Acrophialophora* sp. and *S. cerevisiae*.

Fermentation period (days)	Ethanol (g/g dry material)		Glucose (g/l)		Total reducing sugar (g/l)		Conversion (%)	
	P	A	P	A	P	A	P	A
1	0.14 ± 0.03	0.10 ± 0.05	0.88 ± 0.05	0.62 ± 0.05	3.53 ± 0.09	2.10 ± 0.05	28	20
2	0.17 ± 0.11	0.11 ± 0.07	1.01 ± 0.09	0.84 ± 0.05	4.28 ± 0.10	2.55 ± 0.07	33	22
3	0.17 ± 0.16	0.11 ± 0.14	0.74 ± 0.05	0.59 ± 0.06	3.69 ± 0.13	1.79 ± 0.07	33	22
4	0.16 ± 0.20	0.08 ± 0.09	0.48 ± 0.06	0.33 ± 0.06	2.62 ± 0.17	1.44 ± 0.06	31	16
5	0.13 ± 0.18	0.07 ± 0.10	0.35 ± 0.07	0.28 ± 0.06	2.24 ± 0.10	1.15 ± 0.10	26	14
6	0.10 ± 0.21	0.06 ± 0.06	0.20 ± 0.08	0.10 ± 0.03	1.32 ± 0.14	0.68 ± 0.09	20	12

P = filter paper substrate A = agave substrate

uses. The bioconversion of fiber residue biomass from rope manufacturing may be an added benefit to the process. Ethanol yields from the SSF of *T. reesei* and *S. cerevisiae* are considered satisfactory at the level of 59% conversion from agave fiber. Without process optimization, SSF with *Acrophialophora* sp. enzyme was still preliminary. The superiority of both ethanol yields and percent conversion for the filter paper substrate compared to those for the fiber substrate suggested that yields could be improved with proper fiber pretreatment. Although this new fungal isolate could also produce cellulase at 30°C like *T. reesei*, it was relatively thermotolerant. Our model of bioconversion process using SSF might mimic the degradation process in nature where the agave residues and fungi are present. Further investigation and optimization of the bioconversion process are recommended.

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