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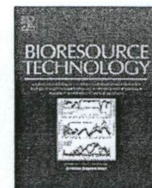
Bioresource Technology 107 (2012) 200–204

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Use of *Trichoderma reesei* RT-P1 crude enzyme powder for ethanol fermentation of sweet sorghum fresh stalks

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ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form 4 November 2011
Accepted 4 December 2011
Available online 21 December 2011

Keywords:
Ethanol
Sweet sorghum
Trichoderma reesei
Saccharomyces cerevisiae

ABSTRACT

Use of *Trichoderma reesei* RT-P1 crude enzyme powder and of this powder with 10% v/v *Saccharomyces cerevisiae* for ethanol fermentation of sweet sorghum fresh stalks were investigated. The optimal conditions were determined by orthogonal experiment method. With *T. reesei* crude enzyme powder, the optimal condition for the Keller cultivar was at 25 g with 4 g enzyme loading and for the Cowley cultivar at 30 g with 5 g enzyme loading, both with 8 days fermentation at pH 5 and 30 °C. At the optimal conditions above, ethanol concentration, productivity and yield of the Cowley cultivar (35.00 g/L, 0.18 g/L h and 0.38 g ethanol/g substrate, respectively) were higher than those of the Keller cultivar (20.46 g/L, 0.11 g/L h and 0.28 g ethanol/g substrate). The addition of 10% v/v *S. cerevisiae* to fermentation at the optimal conditions showed no significant variations in ethanol concentration, productivity and yield for both cultivars.

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1. Introduction

Renewable energy for sustainable development is utilized nowadays to reduce global warming and lessen dependence on diminishing fossil fuel reserves, and bioethanol is one type of renewable energy. Sweet sorghum (genus *Sorghum bicolor* L. Moench) has potential as a substrate for ethanol production due to its high tolerance to a wide ranges of climatic and soil conditions (Nimbkar et al., 2006; Ratnavathi et al., 2010).

Most prior studies of ethanol fermentation used sweet sorghum fresh juice containing sugar concentrations of 15–22 Brix with *Saccharomyces cerevisiae* yeast strain. Variations of this yeast strain, such as commercial dry yeast (*S. cerevisiae*), Fermax yeast, Superstart yeast, *S. cerevisiae* TISTR 5048, *S. cerevisiae* CFTR 01, *S. cerevisiae* SG, and Na-alginate powder immobilized yeast *S. cerevisiae* CICC 1308, were also used in the study of ethanol fermentation. It was found that the effects on ethanol concentration and sugar conversion efficiency depended on different yeast strains and processes used under the same fermentation conditions (Kundiyana et al., 2010; Laopaiboon et al., 2007; Ratnavathi et al., 2010; Liu and Shen, 2008).

Laopaiboon et al. (2009) studied the use *S. cerevisiae* NP01 mutant strain in high sugar concentration sweet sorghum juice and found that the strain could be used in the fermentation of sweet sorghum juice with very high gravity (VHG). The strain was a suitable microorganism for ethanol fermentation under VHG level up to 34 Brix because it could survive due to the presence of nitrogen and retain metabolism under very high ethanol concentrations from 120 g/L up to 135 g/L with high viable cells remaining in the fermentation broth. Yue et al. (2010) investigated the influence of nitrogen source on ethanol production from concentrated sweet sorghum juice using *S. cerevisiae* 3013 mutant strain under anaerobic condition. The researchers found that urea promoted the specific growth rate and had high ethanol tolerance, thereby increasing the ethanol yield while lowering the formation of by-products.

There were studies on ethanol production using sweet sorghum grain (carbohydrate), sweet sorghum residue (bagasse), and dried sweet sorghum stalks as substrates. The substrates were fermented in liquid media using various microorganisms under their respective optimal conditions of solid state fermentation to maximize yields of ethanol. The microorganisms were mixed cultures of *Fusarium oxysporum* F3 and *S. cerevisiae* 2541 with carbohydrate (Mamma et al., 1996), mutant strain of baker yeast 3013 with sorghum bagasse (Yu et al., 2007), *Neurospora crassa* with sorghum bagasse (Dogaris et al., 2009), *Mucor hiemalis* with sweet sorghum bagasse (Goshadrou et al., 2011), mutant strain of baker yeast AF37X (thermotolerant yeast strain) with dried sweet sorghum stalks (Yu et al., 2008), and angel active dry yeast (Shen et al., 2011) with sun-dried sweet sorghum stalks.

The authors of this paper have found no previous studies that investigated ethanol production from sweet sorghum fresh stalks

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doi:10.1016/j.biortech.2011.12.009

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using *Trichoderma. reesei*. This work thus was undertaken to study the possible use of *T. reesei* crude enzyme powder for ethanol fermentation of sweet sorghum fresh stalks of Keller and Cowley cultivars. The crude enzyme powder was obtained from fresh crude enzyme of *T. reesei* RT-P1, the latter of which was produced under solid state fermentation using *T. reesei* RT-P1 with initial cell concentration of 10^9 cell/mL mixed with pH 5 liquid media and cassava waste as substrate in the ratio of 1:1 (w:v) at 26 °C and 6 days cultivation. The fresh crude enzyme was then dried at 50–60 °C until the water content was less than 15% w/w to produce *T. reesei* RT-P1 crude enzyme powder. The application of *T. reesei* RT-P1 crude enzyme powder to ethanol fermentation was investigated by loading 100 mL pH 5 liquid media with 6% w/w crude enzyme powder and 8% w/w un-pretreated pineapple peel waste for 3 days cultivation at room temperature of 30 °C. Ethanol concentration obtained was 40 g/L (Siwarasak et al., 2011).

The objectives of this study are to find the optimal conditions for ethanol fermentation from sweet sorghum fresh stalks with *T. reesei* crude enzyme powder using orthogonal experiment method and to determine the effects of 10% v/v *S. cerevisiae* starter mixed with *T. reesei* crude enzyme powder on ethanol production from sweet sorghum fresh stalks under simultaneous saccharification and fermentation.

2. Methods

The optimal conditions of ethanol submerged state fermentation were determined using two kinds of sweet sorghum fresh stalk cultivars, i.e. Keller and Cowley, with *T. reesei* crude enzyme powder. The $L_{25}(5^3)$ orthogonal experiment design was applied to find the optimal conditions and four different batches of ethanol fermentation were investigated at the optimal conditions.

2.1. Experimental design

The orthogonal experiment method $L_{25}(5^3)$ was used by varying three control parameters: concentration of sweet sorghum fresh stalks, enzyme loading, and incubation time. The parameters were investigated at 5 different levels as shown in Table 1. The experiments were tested in duplicate to produce 25 different fermentation samples per one sweet sorghum stalk cultivar.

2.2. Sweet sorghum source

Two kinds of sweet sorghum cultivars, i.e. Keller and Cowley, were used in this work as carbon source. Fresh sweet sorghum plants used as raw materials, which were chopped into unspecified different lengths by a hammer mill at the plantation and stored in the freezer, were obtained from Suphun Buri Agricultural Research and Development Center, Thailand. Since no specific particle sizes of sweet sorghum fresh stalks were favored in the experiment, the stalks passing through the sieve were selected. Hence, the lengths of most of the stalks were within 5–15 mm range. Prior to use, the frozen stalks were thawed at room temperature.

Table 1
Levels and control parameters of orthogonal experiment method, $L_{25}(5^3)$.

Level	Control parameters		
	A	B	C
1	10	1	1
2	15	2	3
3	20	3	5
4	25	4	7
5	30	5	9

A = substrate, %w, B = enzyme loading, %w and C = incubation time (days) (SD between duplicates = 3%).

Table 2
Culture media composition.

Composition	Media	
	LM-1	LM-2
Sucrose (g)	–	200
$CaHPO_4$ (g)	1	–
$MgSO_4{\cdot}7H_2O$ (g)	1	0.50
Urea (46%$(NH_4)_2SO_4$) (g)	8	0.22
Phosphate (NPK-0-52-34) (g)	15	0.10
Pure water (mL)	1000	1000
pH	5	5

2.3. Microorganisms and media

T. reesei produced cellulases enzyme during its growth. *T. reesei* RT-P1 crude enzyme powder or cellulases was produced under solid state fermentation using pH 5 liquid media without sucrose added because sweet sorghum fresh stalks had sugar and cellulose as carbon source. *T. reesei* RT-P1 from potato dextrose agar (PDA) plates with initial cell concentration of 10^9 cell/mL was inoculated into 200 mL sterilized pH 5 liquid media which was used as nutrients (Table 2, LM-1). The mixture was stirred for 30 min and blended with 200 g sterilized cassava waste. The blended substrate was transferred to a glass bowl, covered with perforated wrap film, and incubated at 26 °C for 6 days. At day 6, green fresh crude starter matured and to derive crude enzyme powder, the fresh crude was then dried in an oven at 50–60 °C until the water content was less than 15% w/w. *T. reesei* RT-P1 crude enzyme powder showed the cellulase activity of 8 FPU.

S. cerevisiae RT-P2 from yeast malt extract agar (YMA) plate was used for preparation of yeast starter. As seen in Table 2, LM-2 liquid media for the yeast starter contained a high concentration of 200 g/L sucrose while low on the other nutrients relative to LM-1. As previously discovered by the authors of the paper, LM-2 with a high sugar concentration promoted the growth of *S. cerevisiae* RT-P2 as witnessed in the preparation of yeast starter in which only a few colonies of *S. cerevisiae* RT-P2 from YMA were needed to inoculate into 100 mL liquid media LM-2 and incubated merely 24 h before use.

2.4. Submerged state fermentation

The $L_{25}(5^3)$ orthogonal experiment was performed by inoculating *T. reesei* crude enzyme powder into 100 mL sterilized LM-1, stirring for 30 min before adding sterilized sweet sorghum fresh stalks, covering the mixture with cotton wool, and shaking at 120 rpm and 30 °C.

Based on the optimal fermentation conditions obtained from analysis results of orthogonal experiment, the verification of ethanol submerged state batch fermentation was performed in 250 mL shaking flasks with 12 days fermentation at 30 °C. The method of the ethanol batch fermentation was the same as that of the orthogonal experiment.

The effects of 10% v/v *S. cerevisiae* RT-P2 starter mixed with *T. reesei* crude enzyme powder on ethanol production at the optimal conditions in simultaneous saccharification and fermentation (SSF) were determined using 250 mL shaking flasks with 100 mL LM-1, initial pH 5, *T. reesei* crude enzyme powder, and sweet sorghum fresh stalks. The mixture was inoculated with 10% (v/v) *S. cerevisiae* RT-P2 starter, covered with cotton wool, and shaken at 120 rpm and 30 °C.

Samples of both batch fermentation and SSF were collected daily for analysis of spore, ethanol, cellulase activity, and reducing sugar concentration. The experiments were conducted in duplicate.

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2.5. Analytical methods

The spore concentration was prepared in pure water suspension and determined by a hemacytometer (Boeco, Germany) with a 40× microscope. Cell mass of *T. reesei* RT-P1 was measured by gravity method and cellulase activity was determined by filter paper (Ghose, 1987). Reducing sugar concentrations were estimated with 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) while ethanol concentrations were estimated by the dichromate colorimetric method, which was based on the complete oxidation of ethanol by potassium dichromate in the presence of sulfuric acid to form acetic acid (Williams and Reese, 1950).

2.6. Calculations

Ethanol yield ($Y_{p/s}$) was calculated as the final ethanol produced and expressed as g ethanol per g sugar utilized. The volumetric ethanol productivity, Q_p and the percentage of conversion efficiency or yield efficiency (E_y) were calculated by the following equations:

$$Q_p = \frac{P}{t} \quad (1)$$

Table 3
Orthogonal experiment results of cell concentration for Keller and Cowley cultivars.

No.	Sweet sorghum, g	Crude enzyme, g	Timeday	Cell concentration, g/L	
				Keller cultivar	Cowley cultivar
1	10	1	1	1.65	1.69
2	15	5	1	5.04	4.65
3	20	4	1	4.67	5.06
4	25	3	1	3.94	3.94
5	30	2	1	3.15	3.21
6	10	2	3	4.92	4.42
7	15	1	3	1.75	2.60
8	20	5	3	6.33	6.90
9	25	4	3	8.17	6.80
10	30	3	3	4.10	6.21
11	10	3	5	5.27	4.90
12	15	2	5	4.29	4.94
13	20	1	5	1.82	4.10
14	25	5	5	9.33	10.06
15	30	4	5	10.31	7.27
16	10	4	7	8.56	8.02
17	15	3	7	5.33	5.90
18	20	2	7	3.81	5.08
19	25	1	7	8.25	5.69
20	30	5	7	7.48	11.73
21	10	5	9	10.33	9.08
22	15	4	9	10.54	10.54
23	20	3	9	5.06	8.04
24	25	2	9	4.15	4.04
25	30	1	9	6.83	8.17

Table 4
Orthogonal experiments of cell mass range analysis for Keller and Cowley cultivars.

Level	Control parameters					
	Keller cultivar			Cowley cultivar		
	A	B	C	A	B	C
k_1	6.15	4.06	3.69	5.62	4.45	3.71
k_2	5.39	4.06	5.05	5.73	4.34	5.40
k_3	4.34	4.74	6.21	5.84	5.80	6.25
k_4	6.77	8.45	6.69	6.12	7.55	7.28
k_5	6.38	7.70	7.38	7.32	8.48	7.98
R	2.43	4.39	3.70	1.70	4.15	4.27
SD	0.96	2.11	1.46	0.69	1.85	1.67
Q	A_4	B_4	C_5	A_5	B_5	C_5

A, B, and C are substrates; %w, enzyme loading; %w and incubation time; day.

Table 5
Kinetic parameters of Keller and Cowley cultivars in batch fermentation (Batch) and simultaneous saccharification and fermentation (SSF).

Parameters	Keller cultivar		Cowley cultivar	
	Batch	SSF	Batch	SSF
Ethanol, g/L	20.46	22.58	35.30	35.00
Reducing sugar, g/L	71.87	82.56	93.62	95.62
E_y, %	55.82	53.63	73.94	71.77
t_d of 1st exponential phase, h	22.27	20.23	20.50	20.65
R^2	0.99	0.95	0.93	0.99
t_d of 2nd exponential phase, day	2.19	3.30	2.66	3.29
R^2	0.99	0.99	0.99	0.98
Q_p, g/L h	0.11	0.12	0.18	0.18
Q_x, g/L h	0.05	0.05	0.05	0.05
$Y_{p/s}$	0.28	0.27	0.38	0.37
$Y_{p/x}$	2.23	2.38	3.53	3.60
$Y_{x/s}$	0.13	0.11	0.11	0.10

$$E_y = \frac{Y_{p/s} \times 100}{0.51} \quad (2)$$

where P is the final ethanol concentration produced (g/L), t is the incubation time (h) giving the highest ethanol concentration for batch fermentation and 0.51 is the maximum theoretical ethanol yield of glucose consumption (Laopaiboon et al., 2007).

Reducing sugar concentration in Table 5 was displayed in g/L, which was extrapolated from the weight of fresh stalks and volume of liquid media based on the data that whole sweet sorghums contained approximately 58.2% w/w dry basis of combined sucrose and glucose (Billa et al., 1997) and fresh stalks 80% w/w initial moisture.

3. Results and discussion

T. reesei cell concentration in 25 fermented samples of a sweet sorghum cultivar was measured as response values for optimization of ethanol fermentation. The highest growth of *T. reesei* corresponded to the highest cellulase activity converting sweet sorghum fresh stalk to ethanol (Lever et al., 2010). Thus, cellulose of sweet sorghum fresh stalks was converted into reducing sugar by cellulase from *T. reesei* growth during ethanol conversion as shown in Figs. 1 and 2.

The effects of orthogonal experiment on cell concentration of each batch are presented in Table 3.

Cell concentration (g/L) range analyses of $L_{25}(5^3)$ orthogonal experiments of Keller and Cowley sweet sorghum fresh stalks are presented in Table 4. It was found that the optimal conditions are $A_4B_4C_5$, i.e. 25% w/w substrate, 4% w/w *T. reesei* crude enzyme powder and 9 days incubation for the Keller cultivar; and $A_5B_5C_5$,

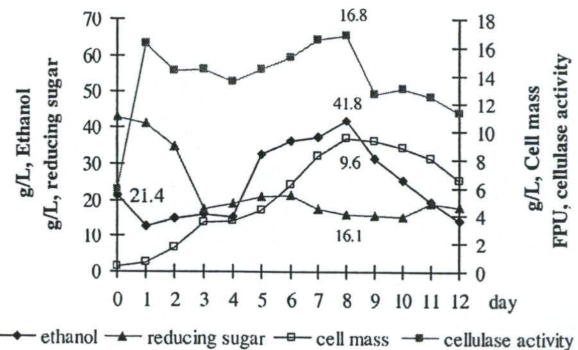

Fig. 1. Ethanol, reducing sugar, cellulase activity and cell concentration profiles of Keller cultivar in batch fermentation (SD between duplicates = 2%).

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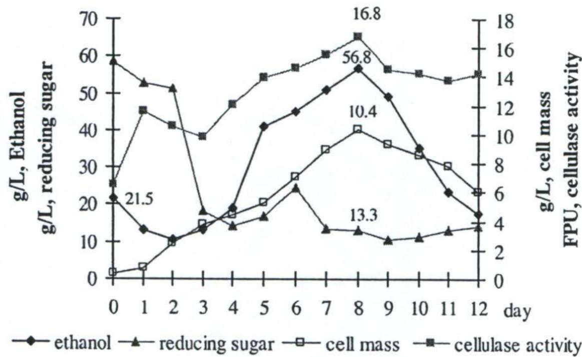

Fig. 2. Ethanol, reducing sugar, cellulase activity and cell concentration profiles of Cowley cultivar in batch fermentation (SD between duplicates = 1%).

i.e. 30% w/w substrate, 5% w/w *T. reesei* crude enzyme powder and 9 days incubation for the Cowley cultivar.

The ranking, from high to low, for the Keller cultivar of influential affecting factors to cell concentration was *T. reesei* crude enzyme powder > incubation time > substrate. However, the ranking of the Cowley cultivar was incubation time > *T. reesei* crude enzyme powder > substrate.

Ethanol batch fermentation from sweet sorghum fresh stalks with *T. reesei* crude enzyme powder was carried out in 250 mL shaking flasks with 100 mL LM-1 and the corresponding parameters of the optimal conditions A_4B_4 for the Keller cultivar and A_5B_5 for the Cowley cultivar and 12 days fermentation. The results of batch fermentation are shown in Figs. 1 and 2 for the Keller and Cowley cultivars, respectively. Shen et al. (2011) stated that microorganisms during harvest naturally fermented sweet sorghum; therefore, ethanol concentrations for the Keller and Cowley cultivars were initially around 20–25 g/L for batch fermentation and SSF. Ethanol values increased to 41.8 g/L for the Keller cultivar and 56.8 g/L for the Cowley cultivar at day 8 of cultivation. Ethanol concentration then decreased to about 20 g/L from days 9 to 12 of cultivation due to the fact that the fermentation was performed in separate flasks covered with cotton wool on a shaker at 120 rpm for 12 days, thereby causing the evaporation of ethanol when it became highly concentrated. However, cell concentration profile of *T. reesei* declined owing to inadequate carbon and nitrogen sources at 9–12 days fermentation and the occurrence of product inhibition (Nadir et al., 2009).

In addition, ethanol concentration during fermentation increased because of increasing cellulose hydrolysis by cellulase as *T. reesei* grew. At the maximum ethanol concentration, the reducing sugar concentration was consumed by approximately 62.5% w/w for the Keller cultivar and 77% w/w for the Cowley cultivar. It was found that ethanol yield efficiency and productivity of the Cowley cultivar were higher than those of the Keller cultivar. The results indicated that initial sugar concentration had significant effects on these parameters. The higher ethanol concentration and ethanol yield efficiency were the results of higher substrate concentration (Laopaiboon et al., 2007).

As for the variation of batch fermentation at the optimal conditions, it was found that the profiles of ethanol, reducing sugar and cell concentration of the Keller cultivar were similar to those of the Cowley cultivar. Sugar was reduced in the initial stage of 3–4 days fermentation for cell growth, implying that sugar was consumed for cultivation. *T. reesei* RT-P1 crude enzyme provided the ethanol profile corresponding with *T. reesei* growth from day 2.

The incremental ethanol concentrations at day 8 compared to those of the initial day for the Keller and Cowley cultivars were 20.46 g/L (i.e., 41.82–21.36 g/L) and 35.30 g/L (i.e., 56.82–21.52 g/L), respectively due to enzyme hydrolysis by cellulase of *T. reesei* RT-P1 during its exponential growth and to reducing sugar converted to ethanol (Lever et al., 2010; Mamma et al., 1996).

Ethanol, reducing sugar and cell concentration profiles of SSF from the Keller and Cowley cultivars with 10% v/v *S. cerevisiae* yeast starter mixed with *T. reesei* crude enzyme powder are depicted in Figs. 3 and 4, respectively. The results were similar to those of batch fermentation. The reducing sugar and cell mass profiles of SSF for one day fermentation were different from those of one-day batch fermentation using only *T. reesei* crude enzyme powder because the SSF fermentation contained *S. cerevisiae* and *T. reesei*, both of which rapidly consumed sugar by approximately 63% w/w for cell growth. However, at day 8 fermentation the maximum ethanol concentration and cell mass in SSF were insignificantly different relative to those of batch fermentation. This implied that the addition of *S. cerevisiae* promoted merely a meager increase in the ethanol yield while unnecessarily adding to the production cost of ethanol.

T. reesei RT-P1 growth profiles of the Keller and Cowley cultivars had two exponential phases as shown in Figs. 1–4. The first and second exponential phases were from 0 to 3 days and 5 to 7 days fermentation. Double time (t_d) of each batch was calculated based on the first and second exponential phases. The reducing sugar concentrations of both cultivars for batch fermentation and SSF, i.e., 71.87 g/L and 82.56 g/L for batch fermentation and SSF of the Keller cultivar; and 93.62 g/L and 95.62 g/L for batch fermentation and SSF of the Cowley cultivar, in Table 5 were computed by extrapolating from the weight of fresh stalks and volume of liquid media based on the data that whole sweet sorghums contained approximately 58.2% w/w dry basis of combined sucrose (55 g) and glucose (3.2 g) (Billa et al., 1997) and fresh stalks 80% w/w initial moisture. The results were used in conjunction with the data in

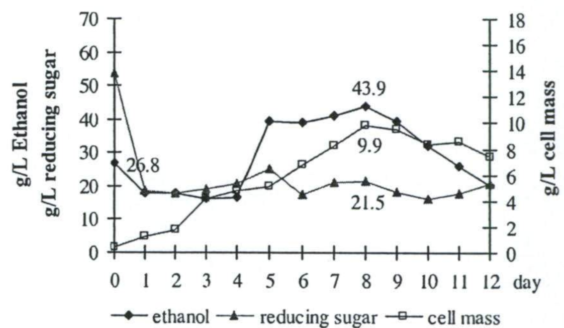

Fig. 3. Ethanol, reducing sugar and cell concentration profiles of Keller cultivar in simultaneous saccharification and fermentation (SD between duplicates = 3%).

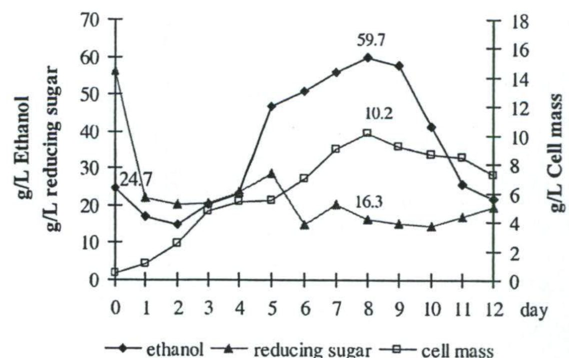

Fig. 4. Ethanol, reducing sugar and cell concentration profiles of Cowley cultivar simultaneous saccharification and fermentation (SD between duplicates = 3%).

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Table 6
The comparison between previous study and this study.

Items	Previous study (Yu et al., 2008)	This study
Fermentation	Solid-state	Submerge state
Strain	Mutant strain of baker yeast AF37X	*T. reesei* RT-P1 crude enzyme powder
Sorghum cultivar	Rio	Keller & Cowley
g ethanol/100 g fresh stalks	9.7	45.5
g ethanol/g total sugar	0.46	0.50
Theoretic ethanol yield %	91	97
Fermentation time, h	50	192

Figs. 1–4 to calculate kinetic parameters of ethanol fermentation of various batches of the Keller and Cowley cultivars at the optimal conditions as shown in Table 5.

Although ethanol yield efficiency of both Keller and Cowley cultivars for batch fermentation was less than that of SSF, ethanol productivity of both cultivars in batch fermentation was the same as that of SSF. This implied that utilization of mere *T. reesei* crude enzyme powder yielded a better result than using 10% v/v yeast starter mixed with this enzyme for ethanol fermentation, which was due to the fact that yeast starter increased consumption of sugar for cell growth, particularly in this experiment which was conducted under aerobic fermentation (Liu and Shen, 2008). Besides, utilization of *T. reesei* crude enzyme powder in batch fermentation was more convenient while reducing the experiment period by one day since yeast starter preparation was no longer required. The comparison of a previous study of ethanol production from sweet sorghum fresh stalks (Yu et al., 2008) with this study is presented in Table 6.

4. Conclusions

The optimal conditions for ethanol fermentation were 25 g Keller cultivar with 4% w/w crude enzyme powder loading and 30 g Cowley cultivar with 5% w/w crude enzyme loading. Fermentation time of both cultivars was 8 days. Ethanol obtained was 20.46 g/L and 35.3 g/L for the Keller and Cowley cultivars, respectively.

T. reesei crude enzyme powder produced under solid state fermentation can be an attractive alternative enzyme for ethanol fermentation of un-pretreated lignocellulosic materials due to its being bred from native culture with low cost substrates, e.g. cassava waste or pineapple peel waste; and simple and effective media composition.

Acknowledgements

This research was financially supported by Rajamangala University of Technology Thanyaburi, Phathumtani, Thailand. Sincere appreciation is also extended to Ms. Umaporn Kitsanit and Ms. Banjamas Sooksai for their technical assistance.

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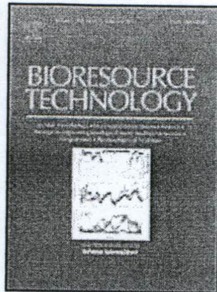

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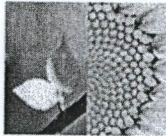

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Article revised:	4 Nov 2011
Article accepted for publication:	4 Dec 2011
Received at Elsevier:	5 Dec 2011
Journal publishing agreement sent to author:	8 Dec 2011
Offprint order form sent to author:	8 Dec 2011
PDF offprint:	Yes
Proofs available:	26 Dec 2011
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