A study of feasibility to utilize lignocellulosic biomass as materials for biodiesel production

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Abstract

Lignocellulosic biomass is a renewable, inexpensive, and abundant resource with high potential for biofuel production to implement the sustainable energy energy worldwide. The bottleneck of biofuel production is the recalcitration of ligoncellulosic biomass to fermentable sugars. Searching for a novel cellulase, a biocatalyst is one of major challenges to promote biofuel production with economic and environmental friendly. Natural microorganism is the great source of cellulase production. Therefore the objective of this research is to identify thermophilic, rapid, efficient cellulose-degrading bacteria from organic fertilizer, rice field, activated sludge, and rain forest in Thailand. More than 300 isolates were screened at 45°C on carboxy-methyl-cellulose (CMC)-containing media to observe the cellulase activity. Using standard filter paper assay, only 9 isolates (S3-10, S3-20, L2-S1, L2-S2, S3-7, L3-S1, L11-S2, L11-S3, and L11-S4) showed high total cellulase activity among other isolates. All isolates then were cultured in media containing 4 different carbon sources; CMC, filter paper, untreated rice straw, nutrient broth (NB), to see effect of substrates on cellulase production. It showed that L2-S2 in Dubois salts media with rice straw could induce high total cellulase activity to 25.57 umole/mg-protein while L11-S3 in NB could induce the highest total cellulase activity to 27.92 umole/mg-protein. Therefore untreat rice straw and NB represented as an effective inducer for cellulase production. A portion of the 16srDNA genes of cellulase positive isolates were amplified and sequenced, then BLASTed to determine species. The results showed that most of isolates are *Bacillus* sp.

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Introduction

The continuous growing of industry worldwide, including Thailand, leads to the increasing of energy demand to support their production lines and processes. Natural petroleum is the main resource to generate energy. However the price is increasing, while the accumulated crude oil supply is decreasing. To increase the national security in energy situation issue, Thai Government launched the policy to promote the research and production of biofuel by using as the alternative option of petroleum fuels. By year 2021, the production of biofuel is planned to 39.97 million liters per day, as year 2012 only 2.7 million liters per day was produced. (Source: Energy in Thailand: Facts&Figures 2012 Report, Department of Alternative Energy Development and Efficiency, Ministry of Energy).

Each year Thailand has agricultural waste more than 55 million per year. These agricultural wastes are abundant and inexpensive source of lignocellulosic biomass for conversion to biofuel. Lignocellulosic biomass is comprised of mainly cellulose (the most abundant organic polymer in the world), a homologous polymer of glucose molecules connected by β -1,4 linkages [1]. It also contains hemicellulose (a heterologous polymer of 5- and 6-carbon sugars) and even less so lignin (a complex aromatic polymer) [1]. Plant cell wall is composed of lignocellulose biomass, though with different proportion up to plant species [2]. For example, rice straw has 69.2% cellulose, 4.9% lignin, 10.2% hemicellulose [3].

Cellulase enzyme plays key importance rule in conversion of lignocellulosic biomass into fermentable sugar for biofuel production. Cellulase enzyme complex comprises of three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase, and β -glucosidase (β-D-glucoside glucohydrolase or cellobiase) [4]. Endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain [4]. Exoglucanase is necessary for cleavage of the nonreducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β -1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose [4]. To complete cellulose hydrolysis, the synergy of these three enzymes is needed. In nature, cellulose biomass is hydrolyzed and degraded by microorganisms that produce many types of cellulolytic enzymes. Synergic multienzyme systems are also expected in bacterial cellulase complexes [5]. Cellulolytic bacteria include aerobes such as *Pseudomonas* and *Actinomyces*, facultative anaerobes such as *Bacillus* and *Cellulomonas*, and strict anaerobes such as *Clostridium* [5]. A variety of *Bacillus* species secrete cellulases, including strains of *B. cereus* [6], *B. subtilis* [7]. Therefore this research was aimed to identify thermophilic, rapid, efficient cellulose-degrading bacteria from organic fertilizer, rice field, activated sludge, and rain forest in Thailand

Materials and Methods

Materials and chemicals
The organic fertilizer was obtained from rainforest park at Assumption University, Bangkok, Thailand. The activated sludge was obtained from waste water treatment system at King Mongkut's University of Technology, North Bangkok, Thailand. The soil from rice field and rain forest was obtained from Mahasarakham province, Thailand.

The growth media used in the experiments include the R2A agar (0.5g yeast extract, 0.5g protease peptone, 0.5g casamino acids, 0.5g glucose, 0.5g soluble starch, 0.3g dipotassium phosphate, 0.5g magnesium sulfate·$7H_2O$, 0.3g sodium pyruvate, 15.0g agar), the carboxymethyl cellulose (CMC) agar (0.5g CMC, 0.1g $NaNO_3$, 0.1g K_2HPO_4, 0.1g KCl, 0.05g $MgSO_4$, 0.05g yeast extract, 1.5g agar, per 100 ml ddH_2O) and the Dubois salts media (K_2PO_4 1g, KCl 0.5 g, $MgSO_4$ 0.5g, $NaNO_3$ 0.5g, $FeSO_4$ 0.01 g, pH 7.4) [1]. The other chemicals used in this study were purchased from Ajax (Bangkok, Thailand)

Isolation of microorganisms using R2A

The 1 g of each sample was suspended in 20 ml of sterile 0.1% peptone by vortexing for 2 min on maximum speed. Following, a 10X serial dilution of the suspension was made in peptone. Then, 1 ml of each dilution in the series was spread onto R2A agar using the standard spread plate technique. All plates were incubated at 45°C as the thermophilic screening. The microorganisms were isolated at 24 h. and then they were incubated for an additional 48 and 72 h to allow growth of slower growing microorganisms for further sampling [1]. From the growth observed over 24, 48 and 72 h, various colonies were selected based on their morphology, size and color [1]. The colonies selected were then streaked out on separate R2A plates to ensure purity. Colonies were further subcultured on R2A if more purification was required [1]. The plates were then photographed and described for a database (data not shown).

Screening for cellulase activity

Isolates were grown in 10 ml of NB broth at 45°C for 48 hrs. Then 5µl of each broth culture were singly dropped onto a carboxymethyl cellulose (CMC) agar and then was incubated at 45 °C for 48 h. To investigate the cellulase activity, the plates was flooded with 0.1 % Congo red solution for 15 min and washed with 0.1 M NaCl [8]. To indicate the cellulose activity of the organisms, diameter of the clear zone around colonies on CMC agar was measured. The cellulase activity screening was repeated five times to check the stability of cellulase activity.

Total cellulase activity on different carbon source

The 9 selected isolates, which shown the highest and stable cellulase activity, were choose for total cellulase activity. The total cellulase activity was performed by transferring 100 µl of an overnight culture to 20 ml of different four medias as the following; CMC media, Dubois salts media with filter paper, Dubois salts media with rice straw, and Nutrient broth (NB) to investigate the effect of carbon source on cellulase production and activity at 45 °C for 24 h. The total cellulase activity was performed by using standard filter paper assay. Protein determination was done by using Bio-Rad protein assay. The specific activity of cellulase was calculated.

16S rDNA amplification and isolates identification

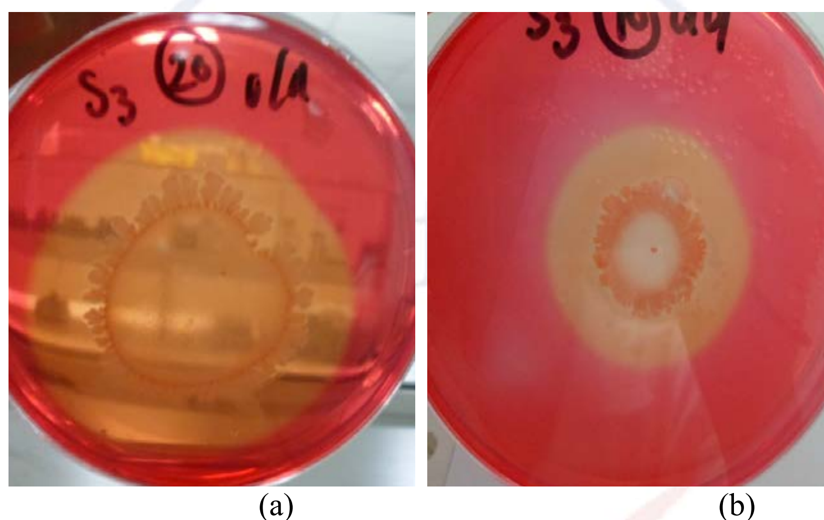
The genomic DNA of 9 selected isolates was isolated by using Genomic DNA Extraction Kit from RBCbioscience, Gibthai Co., Ltd., Thailand. The genomic DNA was used as a template in a PCR reaction to amplify a region of the 16S rDNA. The Universal primers (27F (forward) : 5'AGAGTTTGATCMTGGCTCAG 3' and 1429R (reverse) : 5' GGTTACCTTGTTACGACTT 3') were used. The PCR products were sent for sequencing.

The sequencing results were individually aligned online into the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov) to identify the possible genera of the isolates.

Results and Discussion

Isolation and Screening

Cellulolytic bacteria were screened using R2A media from various sources; organic fertilizer, activated sludge, soil from rice field and rain forest at Thailand at 45°C. A totally 330 isolates were selected and screened for cellulase activity on CMC agar by using congo red method to check clear zone as cellulase activity. After first round cellulase activity screening, only 29 isolates showed high positive cellulase activity at 45°C, 48 h. Most of isolates' cellulase activity were dramatically decrease after first round cellulase activity assay, as show in figure 1. In order to check stability of isolates' cellulase activity, it is necessary to repeat isolates' cellulase activity assay for 5 more times.



(a) (b)

Figure1: Cellulase activity assay showed the clear zone on CMC media at 45°C, 48 h. after congo red flooding indicated cellulase activity positive. The cellulase activity was decrease after first round assay. (a) showed the first round cellulase activity assay (b) showed the second round cellulase activity assay.

After 5 times cellulase activity assay, only 9 isolates showed high and stable cellulase activity as the following; S3-10 and S3-20 from organic fertilizer in rainforest park at Assumption University, Bangkok, Thailand, L2-S1 and L2-S2 from soil in rice field at Mahasarakham province, Thailand, S3-7 from soil in rain forest at Mahasarakham province, Thailand, L3-S1, L11-S2, L11-S3, and L11-S4 from activated sludge in waste water treatment system at King Mongkut's University of Technology, North Bangkok, Thailand. The results showed that clear zone and hydrolytic capacity (HC) value ranged between 45.0 to 32.0 mm and 5.3 to 8.7 for all 9 isolates as showed in table 1.

Table1: The cellulase activity as clear zone on CMC agar and hydrolytic capacity (HC) value at 45°C, 48 hrs.

Source	Isolates	Average Clear Zone (mm)	Average HC value
Organic fertilizer in rainforest park at Assumption University, Bangkok, Thailand	S3-10 S3-20	32 40	5.6 7.6
Soil in rice field at Mahasarakham province, Thailand	L2-S1 L2-S2	42 38	8.7 5.3
Soil in rain forest at Mahasarakham province, Thailand	S3-7	45	6.5
Activated sludge in waste water treatment system at King Mongkut's University of Technology	L3-S1 L11-S2 L11-S3 L11-S4	42 39 34 44	6.9 7.4 8.1 6.5

Total cellulase activity on different carbon source

The 9 selected isolates, which shown the highest and stable cellulase activity, were chosen for total cellulase activity. The total cellulase activity was performed by transferring 100 µl of an overnight culture to 20 ml of different four medias as the following; CMC media, Dubois salts media with filter paper, Dubois salts media with rice straw, and Nutrient broth (NB) to investigate the effect of carbon source inducer on cellulase production and activity at 45 °C for 24 h. The total cellulase activity was determined by standard filter paper method. The totally cellulase activity of all 9 isolates was showed in table 2. The L11-S3 in NB could induce the highest total cellulase activity, 27.92 umole/mg-protein while the L2-S2 in Dubois salts media with rice straw could induce high total cellulase activity, 25.57 umole/mg-protein. It is interesting that the L2-S2 was isolated from soil in rice field at Mahasarakham province, Thailand and showed high total cellulase activity as using rice straw as carbon source inducer. This indicated that habit of microorganism has impact on cellulase expression. The cellulase isolated from the Bacillus sp. was highly active at 50°C (24 U/mg protein) and highly stable at this temperature for at least 30 min of enzyme denaturation [10]. Glucose and milled filter paper inhibited the growth and cellulase production of *B. brevis* VS-1 [11]. Cellulose, cellobiose and lactose are effective only at high concentrations [11]. Milled filter paper inhibits cellulase due to its adsorption to the substrate [11]. Cellulase biosynthesis is regulated by a complex interaction of catabolite repression, induction and enzyme inhibition [12]. In addition, the production of extracellular endoglucanases is cAMP dependent and therefore is subject to catabolite repression by glucose accumulation [13]. Cellulose has been reported as the best inducer for the complete cellulase complex [13].

Table 2: Total cellulase activity on different carbon source as inducer

Isolates	Total cellulase Specific activity (µmole/mg protein)			
	CMC media	Dubois salts media with filter paper	Dubois salts media with rice straw	Nutrient broth
S3-10	4.90	1.16	1.92	0.82
S3-20	10.34	19.00	1.64	0.77
L2-S1	0.19	6.37	0.19	2.52
L2-S2	2.76	7.41	25.57	1.15
S3-7	2.67	1.31	1.81	1.14
L3-S1	2.93	2.06	2.80	3.03
L11-S2	2.05	1.55	1.64	2.95
L11-S3	17.99	11.14	3.10	27.92
L11-S4	2.05	3.04	1.46	5.36

16S rDNA sequencing

The sequencing results were successfully obtained for all 9 difference 16S rDNA PCR products. The sequencing results were individually aligned online into the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov) to identify the possible genera of the isolates base on homology. From BLAST search results, genera of all 9 isolates were determined based on 92-99% homology. The BLAST results are shown in table 3. The majority of sequencing belong to *Bacillus* sp.

Table 3: The BLAST results

Isolated	Strain	E Value	Present Homology
S3-10	*Bacillus subtilis* strain W48	0	99%
S3-20	*Bacillus licheniformis* strain B3-15	0	97%
L2-S1	*Bacillus methylotrophicus* strain ALK069	0	99%
L2-S2	*Bacillus* sp. JS3	0	93%
S3-7	*Bacillus safensis* strain PDRV	0	96%
L3-S1	*Bacillus pumilus* strain SEMP7	0	99%
L11-S2	*Bacillus safensis* strain PDRV	0	99%
L11-S3	*Pseudomonas brenneri* strain GGRJ16	0	92%
L11-S4	*Bacillus methylotrophicus* strain Ns2-16	0	92%

The 99% homology sequencing BLAST results shown to belong to the genus *Bacillus*; S3-10 is *B. subtilis* strain W48, L2-S1 is *B. methylotrophicus* strain ALK069, L3-S1 is *B. pumilus* strain SEMP7, and L11-S2 *B. safensis* strain PDRV. *B. subtilis* YJ1 was cultivated in the medium and only produced the Endo-β-1,4-glucanase at 37°C for 36 hrs. [9]. However, the activities of other 2 enzymes, Avicelase and β-glucosidase were very low during cultivation [9].

Over - production of CMCase from *Bacillus* sp. can be achieved by physical, chemical methods of mutagenesis and through recombinant DNA technology. This kind of study also helps to produce CMCase in commercial scale utilizing local agro-wastes in solid state culture [10].

Conclusion

From the results obtained in this study, L2-S2; *Bacillus* sp. JS3, the novel thermophile cellulase producing bacteria which rice straw is an inducer for its production was isolated. Further study is to characterize the cellulase proteins, optimize the growth condition.

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