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ISOLATION OF NOVEL CELLULASE FROM AGRICULTURAL SOIL AND APPLICATION FOR ETHANOL PRODUCTION

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ABSTRACT:

The objective of this study is to improve the production of cellulase by screening novel cellulose-hydrolytic bacteria and optimizing the cultivation conditions for cellulase production from cellulosic feedstock. Finally, sugar hydrolysate from cellulosic material was utilized as substrate for ethanol production by SHF process using *Saccharomyces cerevisiae*. Five novel cellulase-producing bacteria were isolated and identified through 16S rRNA sequence as *Cellulomonas* sp. The activity of enzymes (mainly xylanase and endoglucanase) produced from isolated strains was almost present extracellularly and the production of enzyme was dependent on cellulosic substrate (xylan, rice straw and wastepaper) used for growth. The optimal condition for cellulase production consisted of 4% of wastepaper with controlled pH at 6 and cultivation temperature at 35^{o}C. *Cellulomonas* sp. strain TSU-03 produced the highest activity of xylanase and endoglucanse at 1860.1 and 388.5 U mg^{-1} protein, respectively. At 50^{o}C, cellulase was highly stable and losing less than 20% of initial activity after 24 h of incubation. Cellulase production from strain TSU-03 can be an advantage as the activity of enzymes is the highest value ever reported from *Cellulomonas* sp. Therefore, wastepaper hydrolysate was utilized as substrate for ethanol production using *S. cerevisiae*, the highest ethanol production was 12.5 g L^{-1} after 48 h of cultivation under separate hydrolysis and fermentation (SHF) process.

Keywords: *Bacterial hydrolysis, Cellulomonas sp., Ethanol, Sugar production, Wastepaper*

[I] INTRODUCTION

Currently, production of bioethanol from agricultural residues is a suitable alternative, in view of fast depletion of fossil fuels [1]. However, bioethanol is not extensively utilized with the common argument that the cost of fermentation of ethanol could not be reduced to a level competitive with that of gasoline. Industrial bioconversion of lignocelluloses to ethanol occurs in multiple steps, where hydrolyzing enzymes are added after pre-treatment of the lignocelluloses (saccharification) and then in an additional step, microorganisms capable of fermentation are added to produce bioethanol from sugar hydrolysate [2]. Cost of cellulase in enzymatic hydrolysis is regarded as a major factor [3]. Research and development to reduce the cost of bioethanol has been carried out in various aspects. Recently, the cost of ethanol production from cellulosic material is US$1.8 per gallon. However, development of enzymatic processing can decrease the ethanol cost as low as US$0.2 per gallon [4]. Therefore, the chance to obtain cheap ethanol will depend on the successful screening of novel cellulase-producing strain. Since industrial bioconversions of lignocelluloses requires multifunctional cellulase with broader substrate utilization as well as the application of enzymes that can work efficiently in a wide range of temperatures and pH conditions used in the bioconversion of cellulosic material to bioethanol [5]. Numerous investigations have reported the degradation of cellulolytic materials, but few studies have examined which microorganisms had met the industrial requirement. So there, new industrial relevant cellulolytic and hemicellulolytic enzymes are being considered [2, 6].

Therefore, the objective of this study was to investigate novel cellulase-producing bacteria

which answer industrial requirement (high cellulase activity, broader substrate utilization and work in wide range of temperature and pH) as well as to provide useful information for assessing the feasibility of SHF process involving bacterial hydrolysis and ethanol fermentation from cellulosic substrate, which are all vital to reduce the processing cost of bioethanol.

[II] MATERIALS AND METHODS

2.1. Bacterial isolation

Soil samples were collected from different locations of Phatthalung, Thailand for the isolation of cellulase-producing bacteria. Samples were transported to laboratory and stored at 4^{o}C until used. Ten-fold serial dilutions of each soil sample were prepared in sterilized distilled water and 0.1 mL diluted sample was spread on the surface of Bushnell Haas medium (BHM) supplemented with carboxymethyl cellulose (CMC) as the sole carbon source. The CMC-containing medium contained (g L^{-1}): CMC 10, $MgSO_4.7H_2O$ 0.2, K_2HPO_4 1, NH_4NO_3 1, $FeCl_3.6H_2O$ 0.05, $CaCl_2$ 0.02. The plates were strained by Congo red to see the cellulolytic activity of isolated strain after incubated at 35^{o}C for 48 h [1, 7]. The cellulase-producing microorganism showed the zone of clearance on this agar. The single colony having significant clear zone was sub-cultured and re-streaked on CMC-containing medium again to ensure purity. The purified microorganism was maintained at -80^{o}C in glycerol solution and kept on CMC-containing agar slant at 4^{o}C for further study.

Similar criteria were followed for the identification of xylan-hydrolytic activity of the isolates obtained. Bacterial strains were grown on BHM medium amended with xylan as sole carbon source and also strained by Congo red [1].

2.2. Identification of cellulolytic-producing bacteria

The selected isolates were identified to the genus level using morphological and biochemical methods. Morphological examination was observed by a light microscope and Gram strain. The Rapid ANA II microtests (Remel) for the anaerobic isolates and API 20E microtests (bioMérieux) for the facultative isolate were utilized for biochemical identifications [1].

2.3. 16S rRNA gene analysis and phylogenetic analysis

Pure bacterial cultures were obtained and the nucleotide sequence of the 16S rRNA gene of isolated bacteria was amplified by PCR employing DNA polymerase. The universal primers 27F (5'–GAGTTTGATCCTGGCTCA-3') and 1525R (5'-AGAAAGGAGGTGATCCAG-3') numbered according to the *E. coli* 16S rRNA gene sequence were used. Amplification and sequence analysis of the 16s rRNA was performed as previous described [8].

Nucleotide and deduced amino acid sequences were analyzed with Heidelberg UNIX Sequence Analysis Resources (HUSAR), release 4.0, and the Wisconsin program package (Unix-8.1). BLAST search of the National Centre for Biotechnology Information database was used for homology search. For alignment of nucleotide sequences, as well as alignment of deduced amino acid sequences and construction of phylogenetic trees, the PHYLIP program package with global rearrangement was used [9].

2.4. Nucleotide sequence accession numbers

The DNA sequences of isolated bacteria were deposited in the EMBL database under accession numbers HQ670717–HQ670721.

2.5. The production of cellulase enzyme on different cellulosic materials

Lignocellulosic materials obtained from Southern of Thailand (Phatthalung, Thailand) such as rice straw and wastepaper were selected as the carbon source. Rice straw and wastepaper was pretreated before used following of the method described by Sangkharak [10]. Isolated bacterial strains were grown in modified BHM medium supplemented with different carbon

sources (1% of xylan, rice straw and wastepaper) at 35°C 48 h. Samples were periodically withdrawn from the cultures to examine the production of cellulolytic enzymes.

Three sources of cellulose-degradation enzyme including extracellular, intracellular and cell-bound enzyme were prepared according to the method described by Lo and his co-worker [1]. The culture broth was centrifuged at 5000 *x g* for 20 min and the supernatant was used as extracellular source of enzyme. The cells were re-suspended in McIlvaine's buffer (containing 0.1 M citric acid and 0.2 M phosphate buffer) at pH 5 for sonication (Sonics-Vibra cell, USA) at 4°C. Supernatant from this disruption mixture was used as the intracellular source of enzyme. The particulate or cell associated fraction obtained as a pellet after centrifugation was suspended in buffer and used for cell bound enzyme source.

2.6. Enzyme assay

Endoglucanase and xylanase activity was determined following to the method described by Nitisinprasert and Temmes [11] using a reaction mixture containing 1 mL of enzyme solution with 1 mL of 1% CMC (incubated at 40°C for 30 min) and xylan (incubated at 50°C for 10 min) , respectively in McIlvaine's buffer (pH 5). The termination of xylanase reaction was done by adding of 2 mL of dinitrosalicylic acid reagent and heating in boiling water for 5 min. The amount of reducing sugars releases was determined using D-xylose as standard. One unit of xyalanase activity was defined as 1 µmol of xylose equivalent release per minute.

Exoglucanase (avicelase) activity was determined according to the method described by Lo et al. [1] and Miller [12]. The reaction mixture containing 2 mL of enzyme solution with 1 mL of 1% avicel cellulose in McIlvaine's buffer (pH 5) was incubated at 40°C for 2 h. The reaction was terminated by filtration through a 0.45 µm membrane filter (Millipore) and adding of 2 mL of dinitrosalicyclic acid reagent. In these test, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard

Cellobiase activity was determined colorimetrically by the glucostat enzyme assay method [13]. In general, 1 mL of enzyme solution was incubated with 0.2% cellobiose in McIlvaine's buffer (pH 5.0) at 40°C for 1 h. The glucose released was measured by using HPLC. One unit of enzyme activity in each case was defined as 1 µmol of glucose release per minute.

2.7. Optimization on cellulase production by isolated bacteria

Office paper obtained from Thaksin University (Phatthalung) was selected as carbon source. Wastepaper pretreated with steam explosion, selected previously because it exhibits high cellulose content (>88%) in low amount of hemicelluloses (10%) and lignin (1%) [10].

Starter culture of isolated bacteria was prepared by cultivating in BHM medium at 35°C, 100 rpm for 24 h. Periodically, aliquots were removed to determine cell growth by measurement of optical density at 660 nm. The starter culture (5%) was added into the BHM medium supplemented with office paper and cultivated at 35°C, 100 rpm for 96 h. Samples were taken to measure for the enzyme activity. Moreover, wastepaper hydrolysate was analyzed by HPLC for the presence of sugars. The effect of different amounts of office paper (1-10%), temperatures (25-100°C), pH (2-10) on enzymatic hydrolysis from isolated bacteria was also investigated.

The stabilities of the cellulase complex were examined by incubating the crude enzyme at three different temperatures (37, 50 and 60°C) [6]. Enzymatic quantifications were done under standard condition.

2.8. Analytical method

The bacterial culture broth (5-10 mL) was centrifuged at 12846 *x g* for 10 min at 4°C. The pellet was washed twice with distilled water and

then suspended in 5-10 mL distilled water. After mixing, growth will be monitored by measuring absorbance at 660 nm [14].

Reducing sugar content in the hydrolysate was determined qualitative by reverse phase-HPLC (Agilent 1110 Series HPLC, USA). The column used in HPLC analysis was Zorbax NH_2 (4.6x250 mm, 5 µm) with acetonitrile:water (75:25) as mobile phase with flow rate 0.5 ml/min keeping column oven temperature 25^oC with RI detectors [1]. For the quantitatively, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard [15].

The hydrolysis yield is defined as the amount of sugar produced per the amount of cellulosic material added.

2.9. Ethanol producer, fermentation medium and condition for ethanol production

Flocculation yeast strain *Saccharomyces cerevisiae* TISTR 5048 (Culture collection, Thailand Institute of Scientific and Technological Research, Thailand) was used. The strain was maintained on yeast malt peptone (YMP) agar plates made of yeast extract 10 g L^{-1}, soy peptone 20 g L^{-1} and agar 20 g L^{-1} with D-glucose 20 g L^{-1} as an additional carbon source [16].

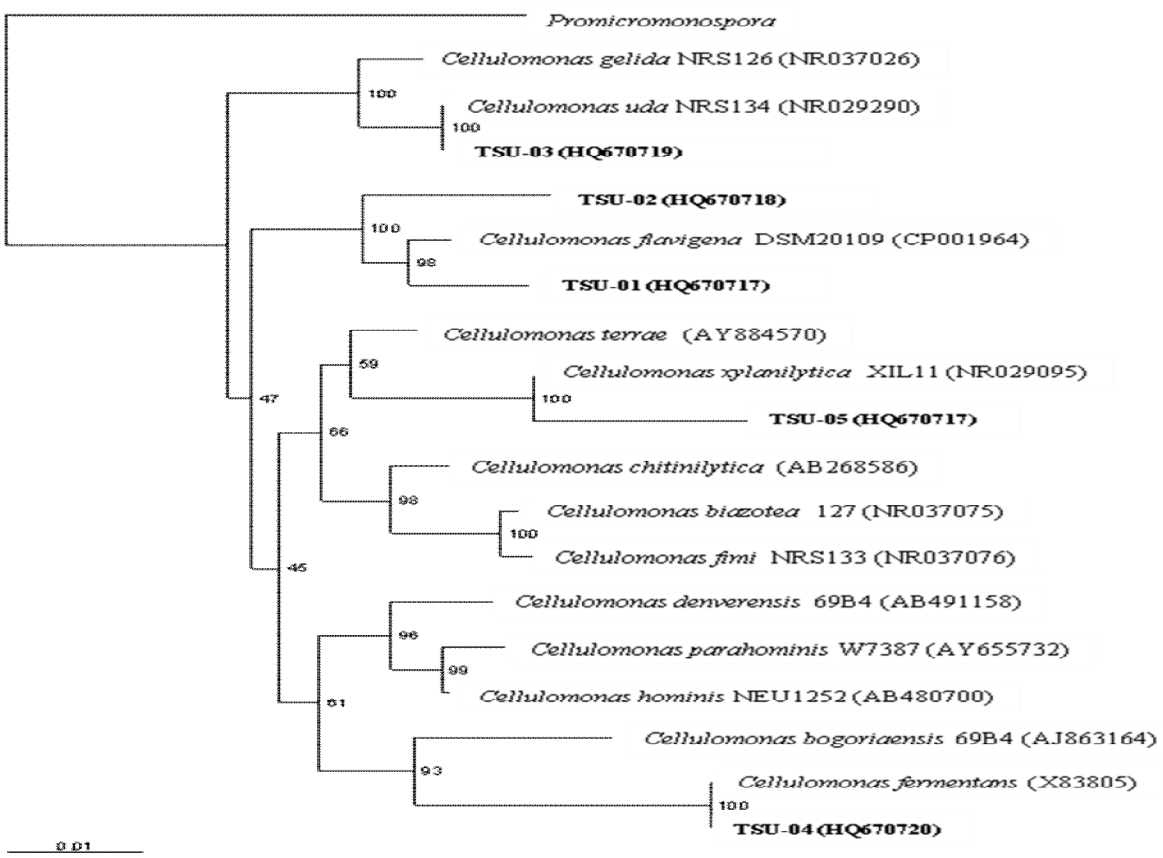
The starter (5%) of isolated bacteria added into a 5-L fermentor containing wastepaper hydrolysate (from above section) for 96 h. A 5-L fermentor (MD300, Eyela) containing 3-L of culture medium with three six bladed Rushton turbine impellers (40 mm dia.) and equipped with pH, DO, antifoam and temperature probes which were connected to the controller (B.E. Marubishi). Controlling parameters such as agitation speed, aeration rate, pH set point were selected to give desired culture conditions.

[III] RESULTS

3.1. Isolation and identification of the isolated bacterial strains

Five strains of cellulose-hydrolytic bacteria were isolated from agricultural soil and designated as strain TSU 01-05. All strains grew well at 35^oC on CMC-containing medium under aerobic condition. All strains were a gram positive and rod-shaped bacterium. The 1.5-kb fragments comprising of 16S rRNA gene were also determined for novel isolates. The nearly full length sequences were obtained by PCR, cloned and sequenced. All sequences showed >95% identity with 16S rRNA gene of a previously analyzed *Cellulomonas* sp. as well as a high identities of amino acid sequence of 16S rRNA gene from *Cellulomonas* sp. was also detected. In **[Figure-1]** Interestingly, strains of *Cellulomonas* genus found to be effective secretors of cellulolytic enzymes and render a promising, industrially relevant alternative to fungal systems because of its high productivity and stability [1, 17-18].

Fig: 1. Phylogenetic analysis of isolated bacteria TSU 01-05 and selected bacteria in alpha subdivision of the division *Cellulomonadaceae* generated from an alignment of 1.5 kb of 16S rRNA obtained from GenBank database (accession number in parentheses).



3.2. Production of cellulase from isolated strains with cellulosic materials

In this study, cellulosic materials were selected and utilized as carbon sources for cellulase production. Therefore, five isolated strains belong under *Cellulomonas* sp. were cultivated on BHM medium containing various cellulosic wastes including rice straw and wastepaper as well as purified xylan to determine their production of cellulolytic enzyme. Rice straw and wastepaper were pretreated before utilize following of the method described by Sangkharak [10].

The effect of different carbon sources (rice straw, wastepaper and xylan) on the production of cellulolytic enzyme by *Cellulomonas* sp. TSU 01-05 was summarized in **Table-1**. Xylanase was significant extracellular by strain TSU-03, -04 and -05 with all substrates tested. However, the highest activity of xylanase was observed by *Cellulomonas* sp. strain TSU-03 (885.5 U mg^{-1} protein) when rice straw were utilized as carbon source followed by wastepaper (856.2 U mg^{-1} protein) and xylan (820.0 U mg^{-1} protein), respectively. Interestingly, bacterial strains TSU-01 and TSU-02 gave significant induction in xylanase activity (200.6 and 202.4 U mg^{-1} protein, respectively) at intracellular position. The highest extracellular endoglucanase was observed by strain TSU-03 while utilized xylan (620.0 U mg^{-1} protein) as sole substrate. However, a desire level of endoglucanase (280.9-258.5 U mg^{-1} protein) was also observed by strain TSU-03 cultivation under rice straw and wastepaper. It was interesting to note that *Cellulomonas* sp TSU 01-05, isolated from rice

and pineapple soil, produced relatively high level of xylanase (111.6-885.5U mg^{-1} protein), endoglucanase (125.4-620.0 U mg^{-1} protein), exoglucanase (10.0-25.8 U mg^{-1} protein) as well as cellobiase (8.8-19.6 U mg^{-1} protein) when cellulosic waste was utilized as sole carbon source.

Cellulomonas sp. strain TSU-03 showed the ability to produce high level of enzyme activity compared to other *Cellulomonas* sp. as previous reported [1, 19-24]. This indicated that this strain offer a good prospect for cellulolytic enzyme production and can be applied this process as a part of industrial application using lignocellulosic materials (rice straw, wastepaper, xylan). It can provide double benefits because environmentally polluting waste is converted into environmentally friendly bioenergy. In an economical point of view, the cost of substrate (mainly carbon source), can decrease if waste product is used as a substrate [1, 25-26]. From above results, prominent strain *Cellulomonas* sp TSU-03 was used throughout in this study.

Cellulomonas sp.	Enzyme activity (U mg^{-1} protein)											
	Xylanase			Endoglucanase			Exoglucanase			Cellobiase		
	Extra-cellular	Intra-cellular	Cell bound	Extra-cellular	Intra-cellular	Cell bound	Extra-cellular	Intra-cellular	Cell bound	Extra-cellular	Intra-cellular	Cell bound
(a) Rice straw												
TSU 01	12.4	20.7	25.9	32.5	16.6	10.0	12.0	25.8	10.5	10.0	10.2	10.2
TSU 02	10.1	15.4	32.2	33.3	14.5	10.6	14.7	20.3	10.0	11.1	10.0	10.0
TSU 03	885.5	11.2	132.2	280.9	12.2	12.4	17.6	19.6	14.3	10.1	11.3	10.0
TSU 04	198.3	12.8	10.0	125.4	11.0	14.0	12.2	22.3	10.5	16.4	10.5	11.5
TSU 05	550.2	18.5	111.6	131.6	11.5	12.2	10.2	20.6	11.2	14.0	10.0	10.4
(b) Wastepaper												
TSU 01	22.1	10.4	12.5	50.1	10.2	11.1	20.1	12.3	11.5	14.2	10.5	15.6
TSU 02	19.1	12.1	10.3	45.2	10.2	10.3	21.2	11.4	10.6	12.6	19.6	11.5
TSU 03	856.2	11.5	12.0	258.5	12.1	10.2	10.5	14.5	12.1	11.4	11.5	10.6
TSU 04	214.7	11.6	10.2	139.2	10.5	12.1	15.4	19.6	13.5	13.8	12.4	11.5
TSU 05	375.4	10.2	10.4	140.5	10.3	12.4	10.2	10.8	10.5	10.2	13.0	10.5
(c) Xylan												
TSU 01	45.1	200.6	10.2	74.4	33.5	22.5	10.9	12.2	10.0	10.0	10.5	8.8
TSU 02	32.9	202.4	10.0	42.2	29.9	39.3	11.0	10.3	10.2	10.5	11.4	9.1
TSU 03	820.0	26.3	19.2	620.0	10.4	18.7	10.5	15.1	10.2	8.8	10.5	10.2
TSU 04	40.2	30.0	20.5	55.4	33.4	19.6	12.1	10.0	11.1	8.8	8.9	10.0
TSU 05	250.0	21.1	12.4	155.1	22.6	21.0	22.4	12.1	10.2	11.2	12.2	9.5

Table: 1. Production of cellulolytic enzyme including xylanase, endoglucanase, exoglucanase and cellobias by *Cellulomonas* sp. TSU 01 – 05 with rice straw (a), wastepaper (b) and xylan (c) as sole carbon source at different location (extracellular, intracellular and cell bound).

3.3. Optimization on cellulase production by *Cellulomonas* sp. strain TSU-03

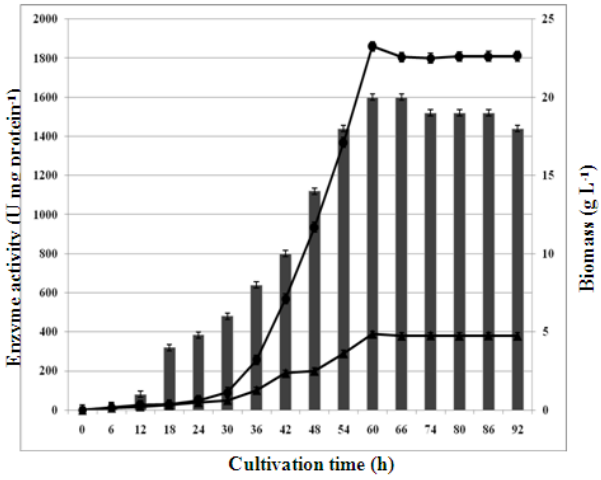
Due to the account of large amount of wastepaper (55%) in municipal waste in combination with our previous study [10] have been demonstrated treated wastepaper contained high cellulose content (>88%) in low amount of hemicelluloses (10%) and lignin (1%). After hydrolysis with commercial cellulase treated wastepaper yield high percentage conversion of glucose (43.68%) [10]. Therefore, wastepaper was selected as sole carbon source for optimization study. Effect of

wastepaper amounts on enzyme activity was studies from *Cellulomonas* sp TSU-03 in BHM medium where CMC was supplemented by wastepaper at 1-10%. The biomass and enzyme activity increased with the increase of wastepaper concentration up to a value of 4% substrate which the resulted was followed the law of the mass action. The highest activity of xylanase and endoglucanase was 1860.1 and 388.5 U mg^{-1} protein, respectively from the concentration of wastepaper at 4%. However, a slight decrease in enzyme activity was observed when concentration of substrate more than 4%. The result indicated that increase of wastepaper may produce high amount of reducing sugar which significant inhibit the enzyme activity during cellulose hydrolysis.

The effect of temperatures on biomass and enzyme activity at various temperatures ranging from 20-100^{o}C was also investigated under optimal concentration of wastepaper. The strain TSU-03 could grow and produce high activity of xylanase and endoglucanase in the temperature range of 30-70^{o}C. Effect of initial pH on biomass and enzyme activity was investigated by adjusting the pH of optimal medium at 2-10. Under acidic condition (pH 2-5) neither cell growth nor enzyme activity was significantly affected. The highest productivity of biomass and enzyme activity was achieved at pH 6 for biomass and enzyme activity. However, enzyme activity decreased under alkali condition (pH 8-10). Interestingly, cellulolytic enzyme from strain *Cellulomonas* sp. TSU-03 showed an attractive property which the enzyme was maintained at broad range of temperature (30-70^{o}C) and pH (2-7).

Cellulomonas sp TSU-03 was cultivated in optimal medium (containing 4% wastepaper) with controlled pH and cultivation temperature at 6 and 35^{o}C, respectively. The cultivation was performed in a 5-L fermentor with aeration rate of 1.0 vvm and agitation speed of 150 rpm. The results were given in **Figure-2**. The results suggested that *Cellulomonas* sp TSU-03 had a good potential for production of cellulolytic enzyme using wastepaper as the main substrate. The xylananse activity and endoglucanase, achieved from this strain, is the highest value ever reported from *Cellulomonas* sp.

Fig: 2. Biomass and enzyme production of *Cellulomonas* sp. strain TSU-03 culture under optimal medium (containing 4% wastepaper) at 35^{o}C, pH 6. The cultivation was performed in a 5-L fermentor with aeration rate of 1.0 vvm and agitation speed of 150 rpm. ■ biomass, ● xylanase activity, ▲ endoglucanase activity.



Thermal stable of cellulase produced from *Cellulomonas* sp. strain TSU-03 was also investigated. Crude enzyme was incubated at different temperatures. Cellulase was incubated under 37, 50 and 60^{o}C in order to predict the behavior of the enzymes under SSF condition, SHF process and to determine the applicability of the enzymes to processes where high temperatures are required, respectively [6].

Cellulase from *Cellulomonas* sp. TSU-03 was highly stable at 50^{o}C throughout the incubation time (24 h). However, 50% of initial enzyme activity reduced when incubated at 37^{o}C and 60^{o}C after 24 h of incubation period. These results suggest that the cellulase produced shows the great potential for the SHF process (due to its high stability at 50^{o}C) as well as for applications that require enzyme activity maintained at high

temperature such as paper industry, biofuel and bioethanol process.

3.4. Identification of wastepaper hydrolysate composition

Sugar content obtained from wastepaper hydrolysate (40 g L^{-1} wastepaper after 96 h of incubation) was identified by HPLC analysis. Glucose was found to be a major end product (30.31 g L^{-1}) with the trace of cellobiose (1.07 g L^{-1}) and xylose (2.55 g L^{-1}) in enzymatic hydrolysate. In the corresponded with hydrolysis rate and hydrolysis yield at 0.32 g L^{-1} h^{-1} and 0.76 g g^{-1} wastepaper, respectively. The high present of glucose and cellobiose suggests the sequential action of endoglucanase, exoglucanse and cellobiase as well as the formation of xylose involved with the action of xylanase during lignocellulose hydrolysis by *Cellulomonas* sp. TSU-03.

3.5. The production of ethanol by SHF using wastepaper hydrolysate as a sole carbon source

The hydrolysate obtained from wastepaper under optimal condition using *Cellulomonas* sp. TSU-03 was utilized as substrate, with no nutritional supplementation, for ethanol production using *Saccharomyces cerevisiae* TISTR 5048. As aforementioned, wastepaper hydrolysate contained high content of glucose (30.31 g L^{-1}). Therefore, the suspension offers a great opportunity as feedstock for bioethanol.

Currently, the production of ethanol can efficiently be used either by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). However, two bacterial strains (*Cellulomonas* sp. and *S. cerevisiae*) used in this study preferred different optimal condition. Therefore, SSF may not offer the suitable condition for both which might result in lower efficiency and productivity. Hence, the better efficiency of ethanol production, the approach of SHF was selected for the present study [27-28].

The highest ethanol production obtained from fermentation process was 12.5 g L^{-1} after 48 h of cultivation, corresponding to an ethanol volumetric production rate of 0.26 g ethanol L^{-1} h^{-1}. The ethanol production, substrate and fermentation process performance using wastepaper or cellulosic substrate was compared to the previous reports **(Table 2)**. The ethanol yield and production rate obtained in this study is higher than the previous results of 9.5 g L^{-1} from 40.8 g of paper sludge using SSF process [28]. However, the highest production of ethanol at 21.02 g L^{-1} was obtained from SSF process utilized office paper as substrate [10].

Table: 2. Comparison of fermentation process, microorganism, substrate and ethanol production performance using wastepaper or cellulosic materials as the substrate.

Ethanol producer	Fermentation process	Cellulase source	Ethanol (g kg^{-1} substrate)	Reference
Saccharomyces cerevisiae	SSF[a]	Cellulase (28.14 FPU) (Sigma, E.C.3.2.1.4)	525 g kg^{-1} wastepaper	[22]
S. cerevisiae	SHF[b]	Cellulase (18.1 FPU) (Novozyme 342)	190 g kg^{-1} paper sludge	[24]
S. cerevisiae	SHF	*Gloeophyllum trabeum*	40 g kg^{-1} corn fiber	[3]
Kluveromyces marxianus	SSF	Cellulase (8 FPU) (Iogen)	177-184 g kg^{-1} recycled paper	[10]
Pichia stipitis	SHF	Celluclast® 1.5L (243.4 U mL^{-1})	109.7 g kg^{-1} recycled paper	[14]
	SSF	Novozym® 188 (855.5 U mL^{-1})	104.1 g kg^{-1} recycled paper	
S. cerevisiae	SHF	*Cellulomonas* sp. TSU-03	312.5 g kg^{-1} wastepaper	This study

[V] CONCLUSION

Cellulomonas sp. isolated in his study offer a good prospect for cellulolytic enzyme production and this method can be applied as a part of bioethanol process using lignocellulosic materials (rice straw, wastepaper, xylan). Five cellulolytic bacterial strains were isolated from soil samples and identified as *Cellulomonas* sp. by 16S rRNA method. All isolated strains showed the great ability to hydrolyzed cellulosic compound and produced extracellular xylanase and endoglucanase. Under the optimal condition containing 4% of wastepaper with controlled pH at 6 and cultivation temperature at 35^{o}C, the strain TSU-03 yielded highest xylanase and endoglucanse at 1860.1 and 388.5 U mg^{-1} protein, respectively. Cellulase production from isolated bacteria giving the highest enzyme activity which ever reported from *Cellulomonas* sp. Glucose (30.31 g L^{-1}) was a major sugar component present in wastepaper hydrolysate. Therefore, wastepaper hydrolysate was utilized as carbon source for ethanol production by *Saccharomyces cerevisiae*, the highest ethanol production was 12.5 g L^{-1} after 48 h of cultivation under SHF process.

Interestingly, cellulolytic enzyme from strain *Cellulomonas* sp. TSU-03 maintained at broad range of temperature (30-70^{o}C) and pH (6-8) which all properties are required in various industrial applications. As well as cellulase was highly stable at high temperature (50^{o}C).This study is the first report indicated the feasibility of the ethanol production from wastepaper using bacterial hydrolysis and yeast fermentation instead of fungi hydrolysis. As aforementioned, cellulase production level is the major factors involved ethanol prices. Therefore, using wastepaper as substrate for cellulase can significantly decrease of both cellulase and ethanol cost. Even so this method appears to be a realistic goal for the bioethanol process in the future.

Enzyme characterization of novel cellulase from isolated strain such as their molecular weight, the optimal temperature and pH for hydrolysis will be presented in further study.

FINANCIAL DISCLOSURE

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