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Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production

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ABSTRACT

Nine cellulolytic bacterial strains were isolated from soil sample taken in southern Taiwan. Through 16S rRNA sequence matching; eight of those isolates belong to *Cellulomonas* sp., while the other one belongs to *Cellulosimicrobium cellulans*. The activity of cellulolytic enzymes (cellulases and xylanase) produced from those strains was mainly present extracellularly and the enzyme production was dependent on cellulosic substrates (xylan, rice husk and rice straw) used for growth. HPLC analysis confirmed the bacterial hydrolysis of these cellulosic substrates for soluble sugars production. The efficiency of fermentative H_2 production from the enzymatically hydrolyzed rice husk was examined with seven H_2-producing pure bacterial isolates. With an initial reducing sugar concentration of 0.36 g l^{-1}, only *Clostridium butyricum* CGS5 exhibited efficient H_2 production from the rice husk hydrolysates with a cumulative H_2 production and H_2 yield of 88.1 ml l^{-1} and 19.15 mmol H_2 (g reducing sugar)$^{-1}$ (or 17.24 mmol H_2 (g cellulose)$^{-1}$), respectively.

1. Introduction

Lignocellulosic biomass is being considered as the largest renewable biological resources continually replenished by photosynthetic reduction of carbon dioxide using sunlight energy [1]. On this planet its annual biosynthesis of cellulose by both land plants and marine algae occurs at a rate of 0.85×10^{11} tonnes per annum equivalent to more than four times the world's yearly total energy consumption [2]. Nowadays, there is rekindled enormous worldwide interest in the development of new and cost-efficient processes for converting plant-derived biomass to bioenergy in view of fast depletion of our oil reserves and food shortages [3]. As cellulosic materials are the most promising feedstock for the production of energy and some value-added chemicals, their utilization could solve modern waste disposal problems and also diminish the dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose [4].

The factors affecting the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, crystallinity of cellulose fiber and lignin, and hemicellulose content [5]. Formation of soluble sugars from cellulose in agricultural residues relies on the sequential/coordinated action of individual components of cellulase complex (i.e., exoglucanase; EC 3.2.1.91, endoglucanase; EC 3.2.1.4 and β-D-glucosidase; EC 3.2.1.21) derived from cellulolytic microorganisms [3]. The cellulases-catalyzed hydrolysis has been suggested as a feasible process to produce reducing sugar (or glucose) from cellulose for the production of biofuels or other value-added products via microbial fermentation [6]. On the other hand, xylan acts as a major ingredient of hemicellulose in nature, so there have been a surge of interest in microbial xylanase (EC 3.2.1.8), having significance in a variety of well documented industrial applications (especially in paper and food industries) [7]. Cellulolytic bacteria belonging to the genus of *Cellulomonas*, *Clostridium*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases effectively [8].

Recently, intensive research and industrial interest goes to the generation of clean, sustainable energy sources from renewable carbon sources (such as lignocellulosic biomass) for the substitution of fossil fuels [1]. Among these energy alternatives, hydrogen is considered as a clean and renewable energy carrier, not contributing to the greenhouse effect with high energy yield (up to 122 MJ kg^{-1}) [4]. Fermentative H_2 production from cellulosic materials could be achieved via direct processes, in which cellulose hydrolysis and sequential hydrogen production was carried out by the same or co-existing microorganisms [9]. However, the direct processes suffer a major limitation of lower H_2 yield arising from the consumption of reducing sugar by non-cellulolytic microorganisms.

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The generation of H_2 from lignocellulosic material by using dark fermentation usually requires substrate pretreatment procedures, which contribute significantly to the production cost [4]. For that purpose some investigators demonstrated two-stage process (i.e., hydrolysis-H_2 production process) where cellulose hydrolysis can be done by using mixed or pure microbial culture under mild conditions relative to most chemical hydrolysis and enzymatic hydrolysis that require high temperatures [10,11]. After certain period the hydrolysates (rich in reducing sugars) are used for sequential hydrogen production by using efficient hydrogen producer. In this way, the hydrogen yield could be increased thus the process becoming more advantageous in practical applications due to being more economically feasible and less energy intensive [10–12].

In this study, we isolated nine microbial strains having good efficiency in cellulose hydrolysis and determined the effects of different substrates (xylan, rice husk and rice straw) on the production of cellulase enzymes (endoglucanase, exoglucanase, cellobiase and xylanase) at different locations (extracellular, intracellular and cell bound) of the cellulolytic bacterial isolates. The composition of hydrolysate resulting from enzymatic hydrolysis of cellulosic feedstock was determined with HPLC. Finally, the rice husk hydrolysate was used as the carbon substrate for dark fermentative H_2 production using various isolated *Clostridium* strains known to produce H_2 efficiently from sugar substrates. This work is expected to provide useful information for assessing the feasibility of using two-stage process involving sequential enzymatic cellulose hydrolysis and hydrogen fermentation for cellulosic biohydrogen production.

2. Materials and methods

2.1. Lignocellulosic substrates

Lignocellulosic materials such as rice husk, rice straw, and xylan were chosen as the carbon substrate in this study because of their abundance in the local area. Coverage of the huge extensive area of cultivable land with rice crop in Taiwan generates high volumes of rice vegetative biomass. Rice husk and rice straw were collected from local farmers; whereas xylan was obtained from Sigma Chemical Co. (USA). The raw substrates were individually sieved through a 2 mm screen for uniform particle size.

2.2. Bacterial isolation and morphological test

To isolate microorganisms producing cellulases, soil sample of rice field taken in southern Taiwan was used as a screening source. The cellulose-hydrolytic bacteria were isolated by using Bushnell Haas medium (BHM) amended with carboxymethyl cellulose (CMC) as the sole carbon source. The CMC-amended BHM medium consisted of (g l^{-1}): CMC, 10; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1; KH_2PO_4, 1; NH_4NO_3, 1.0; $FeCl_3 \cdot 6H_2O$, 0.05; $CaCl_2$, 0.02. For isolation, 2 g of different soil samples were transferred to the fresh 100 ml BHM medium containing CMC as the sole carbon source in 120 ml sealed bottles for incubation. After enrichment in CMC-amended medium for more than five times, the inoculum (0.1 ml; successively diluted to 10^{-5} times) was repeatedly streaking on BHM agar plates containing CMC as a sole carbon source. After certain incubation the plates were stained by Congo red to see the cellulolytic activity of isolated strains. The cellulase activity of each culture was determined by measuring the zone of clearance on agar plate. After that the individual colony having significant clear zone was selected and transferred to a fresh CMC-amended BHM medium and again the inoculum was serially diluted 10^{-5} times and streaked over BHM agar plates repeatedly and the bacteria were re-isolated. Through several such processes, nine pure bacterial cultures were obtained. Similar procedures were followed for the identification of xylan-hydrolytic activity of the isolates obtained; the bacterial strains were grown on BHM medium supplemented with xylan (sole carbon source) and also stained by Congo red.

Morphological examination was observed by a light microscope (Zeiss Axioskop). Gram Stain Set S (BD, USA) and the Ryu non-staining KOH method [13] were used to ascertain the Gram reaction. Biochemical identifications presented in the Rapid ANA II microtests (Remel) for the anaerobic isolates and API 20E microtests (bioMérieux) for the facultative isolate were determined according to the recommendation of the manufacturers.

2.3. 16S rRNA gene sequencing and phylogenetic analysis

Amplification and sequence analysis of the 16S rRNA gene was performed as described elsewhere [14]. The sequence was compared with others available in GenBank. The multiple-sequence alignment including nine cellulose degrading strains and their closest relatives were performed using the BioEdit program [15]. The phylogenetic reconstruction was inferred by using the neighbor-joining method UPGMA, maximum-likelihood and Fitch–Margoliash methods in the BioEdit software in the BioEdit program [15]. A bootstrap analysis (confidence values estimated from 1000 replications of each sequence) was performed for the neighbor-joining analysis using the CLUSTAL w 1.7 program [16]. A phylogenetic tree was drawn using the TREEVIEW program [17]. Sequence identities were calculated using the BioEdit program.

2.4. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the bacterial isolates used in this study have been deposited in the NCBI nucleotide sequence databases under the following accession number: ANA-NS1 (EU-303276), ANA-NS2 (EU-303277), ANA-NS6 (EU-303278), I-1 (EU-420064), RP-3 (EU-303279), ANA-WS2 (EU-303275), L-133 (EU-420065), ANA-FP2 (EU-303273) and ANA-FP4 (EU-303274). Those pure strains were pre-cultured under anaerobic conditions on a preculture medium consisting of (g l^{-1}): CMC, 10; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1; KH_2PO_4, 1; NH_4NO_3, 1.0; $FeCl_3 \cdot 6H_2O$, 0.05; $CaCl_2$, 0.02.

2.5. Preparation of enzyme source

Isolated microbial strains were grown in BHM medium supplemented with different carbon sources (1% of xylan, rice husk, or rice straw) at 35 °C. After cell growth reached early stationary phase, the culture broth was centrifuged at 5000 × *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 mol l^{-1} citric acid and 0.2 mol l^{-1} phosphate buffer) at pH 5 for sonication (Sonics-Vibracell ultrasonic processor, USA), keeping sonifier output at 70 amp and giving 20 stroke of 30 s with 10 s interval each 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 study.

2.6. Enzyme assay

Endoglucanase activity was determined according to the method described by Nitisinprasert and Temmes [18] using a reaction mixture containing 1 ml of enzyme solution with 1 ml of 1% carboxymethyl cellulose (CMC) in McIlvaine's buffer (pH 5) and incubated at 40 °C for 30 min. Exoglucanase (avicelase) activity was assayed also followed the method developed from Nitisinprasert and Temmes [18]. In this assay method, the reaction mixture containing 2 ml 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 dinitrosalicylic acid reagent. In these tests, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard [19]. Cellobiase activity was determined colorimetrically by the glucostat enzyme assay method [20]. In general, 1 ml of the enzyme solution was incubated with 0.2% cellobiose in McIlvaine's buffer (pH 5) 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 released per minute. Xylanase activity was determined according to the method [18] using a reaction mixture containing 1 ml of enzyme solution appropriately diluted in McIlvaine's buffer (pH 5) with 1 ml of aqueous suspension of 1% xylan at 50 °C for 10 min. The reaction was terminated by adding 2 ml of dinitrosalicylic acid reagent and heating in boiling water for 5 min. The amount of reducing sugars released was determined using D-xylose as standard. One unit of xylanase activity was defined as 1 μmol of xylose equivalent released per minute.

2.7. Protein concentration measurement

The protein concentration was measured by the dye binding method of Bradford using the Bio-Rad dye reagent concentrate (500-0006, Bio-Rad) in microtiter plates. A standard curve was generated using solutions containing 0.1 g l^{-1} bovine serum albumin (BSA). Absorbances were measured in triplicate at 595 nm after 20 min of incubation at room temperature.

2.8. H_2-producing bacterial strains and culture medium

Hydrogen-producing bacterial strains were isolated from effluent sludge of a continuous dark fermentation bioreactor capable of producing H_2 from synthetic wastewater containing sucrose (20 g COD l^{-1}) or xylose (20–40 g COD l^{-1}) as the sole carbon source [21,22]. The pure H_2-producing strains were identified as *Clostridium butyricum* CGS2, *Cl. butyricum* CGS5, *Cl. pasteurianum* CH1, *Cl. pasteurianum* CH4, *Cl. pasteurianum* CH5, *Cl. pasteurianum* CH7 and *Klebsiella* sp. HE1. The detailed procedures for strain isolation and identification were described in our recent work [23,24]. The 16S rRNA gene sequences of the bacterial isolates used in this study have been deposited in the NCBI nucleotide sequence databases under the following accession number: AY540106 (strain CGS2), AY540109 (strain CGS5), EF140980 (strain CH1), EF140981 (strain CH4), EF140982 (strain CH5), EF140983 (strain CH7), and AY540111 (strain HE1). Those pure strains were pre-cultured on the preculture medium (g l^{-1}): sucrose, 17.8; NH_4HCO_3, 6.72; $NaHCO_3$, 5.24; K_2HPO_4, 0.125;

$MgCl_2{\cdot}6H_2O$, 0.1; $MnSO_4{\cdot}6H_2O$, 0.015; $FeSO_4{\cdot}7H_2O$, 0.025; $CuSO_4{\cdot}5H_2O$, 0.005; $CoCl_2{\cdot}5H_2O$, 1.25×10^{-4}, under anaerobic conditions [24].

2.9. Fermentation medium and condition for bioH_2 production

The medium for dark H_2 fermentation with the pure cultures was ($g\,l^{-1}$): initial reducing sugar of hydrolyzed rice husk hydrolysate, 0.36; NH_4HCO_3, 6.72; $NaHCO_3$, 5.24; K_2HPO_4, 0.125; $MgCl_2{\cdot}6H_2O$, 0.1; $MnSO_4{\cdot}6H_2O$, 0.015; $FeSO_4{\cdot}7H_2O$, 0.025; $CuSO_4{\cdot}5H_2O$, 0.005; $CoCl_2{\cdot}5H_2O$, 1.25×10^{-4}. The culture temperature and pH was 37 °C and 7.5, respectively. Batch fermentation was carried out by static incubation to investigate the effectiveness of using cellulolytic hydrolysate for H_2 production with the seven pure strains. During the course of fermentation, cell concentration, pH, residual reducing sugar, production of biogas and soluble metabolites were monitored with respect to culture time.

2.10. Analytical methods

The cellulose hydrolysates and soluble metabolites in fermented broth were analyzed using HPLC LC-10AT (RID-10A, Shimadzu, Japan) for the presence of sugars, acid content and ethanol. The column used in HPLC analysis was Transgenomic, USA (ICSep ICE-COREGEL 87H3) column with 0.008 N_2SO_4 as eluent with flow rate of 0.4 ml min^{-1} keeping column oven temperature at 55 °C with RI detectors [24]. The gas products (mainly H_2 and CO_2) was analyzed by gas chromatography (GC) using a thermal conductivity detector. The detailed procedures for GC analyses were described in our recent work [25]. Standard Methods [26] were used to determine biomass concentration.

3. Results and discussion

3.1. Isolation of bacterial strains utilizing cellulose and xylan as the sole carbon source

Nine strains (ANA-NS1, ANA-NS2, ANA-NS6, I-1, RP-3, ANA-FP2, FP-4, ANA-WS2 and L-133) capable of producing cellulase and xylan hydrolytic activities were obtained from soil of rice field. All strains grew well at 35 °C on CMC-amended BHM medium under aerobic or facultatively anaerobic conditions. Colonies on CMC agar plates are circular, smooth, creamy yellow circles within 3 days at 35 °C. Microscopic examination showed that the seven strains were in straight rods with Gram positive reaction. The nearly full-length sequences of 16S rRNA gene were also determined for the nine isolates. Based on the sequence identity of 16S rRNA gene, strains ANA-NS1, ANA-NS2, ANA-FP2, FP4 and ANA-NS6 resemble to each other with a 99.9% identity and have the highest similarity towards the type strain of *Cellulomonas uda* DSM 20107^T (99.7% identity). Moreover, stains I-1, RP-3 and ANA-WS2 were also found to belong to genus *Cellulomonas*, but represent different sublines within the genus *Cellulomonas* (Fig. 1). The highest similarity values of strains I-1, RP-3 and ANA-WS2 were obtained towards type strains of *Cellulomonas gelida* DSM 20111^T (99.5% identity), *Cellulomonas flavigena* DSM 20109^T (98.5% identity) and *Cellulomonas fermentans* DSM 3133^T (97.8% identity), respectively. A comparison of the 16S rRNA gene sequence of strain L-133 with those of members of genera listed under class *Actinobacteria* shows that strain L-133 formed a distinct lineage and fell within the evolutionary radiation of the genus *Cellulosimicrobium* (Fig. 1) with the highest similarity towards type strain of *Cellulosimicrobium cellulans* DSM 43879^T (99.8% identity). The 16S rRNA gene phylogenic tree was constructed for the nine strains as indicated in Fig. 1. According to the results of physiological and 16S rRNA gene sequence examinations, the eight strains ANA-NS1, ANA-NS2, ANA-NS6, I-1, RP-3, ANA-FP2, FP-4 and ANA-WS2 belong to *Cellulomonas* species, while strain L-133 belongs to *Cellulosimicrobium* species.

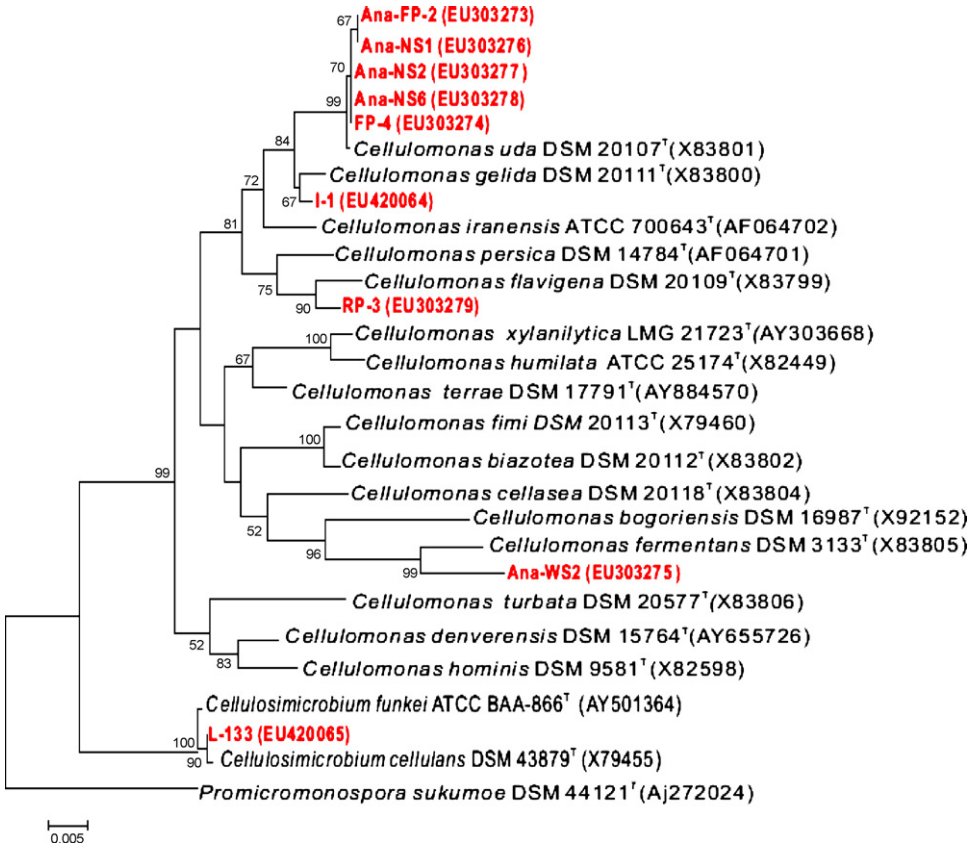

Fig. 1. Neighbor-joining showing phylogenetic positions of seven strains and *Cellulomonas* and *Cellulosimicrobium* species based on 16S rRNA gene sequence comparisons. *Promicromonospora sukumoe* DSM 44121 was used as an out group. Bootstrap values are indicated at nodes. Only bootstrap values >50% are shown. Scale bar, 1% sequence dissimilarity (one substitution per 100 nt). Representative sequences in the dendrogram were obtained from GenBank (accession number in parentheses).

3.2. Cellulolytic enzymes production from isolated strains using different cellulosic substrates

Enzymatic hydrolysis of cellulosic feedstock has several advantages over chemical processes because of its potentially high saccharification efficiency, less energy consumption and avoidance of pollution [8]. Microbial cellulase production has been influenced by a number of factors including the type of strain used, reaction conditions (temperature, pH, etc.) and inducer/substrate types. The relationship between these variables has a marked effect on the production of the cellulase enzymes [27]. 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 [28]. The regulation and mechanism of cellulolytic enzymes from bacteria (e.g., *Myrothecium verrucaria*, *Clostridium thermocellum* and *Cellulomonas* sp.) have been well studied [29].

Moreover, cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in the preparation of cellulosic feedstock. For economic point of view, there is a need to increase cellulase enzyme volumetric productivity by using cheaper substrates, with higher stability and specificity (substrates) for specific processes [2]. Therefore, in this study natural agricultural residues were used as a carbon sources for cellulase production from the isolated bacterial strains. The bacterial isolates were grown on BHM medium containing two agricultural residues (rice husk and rice straw) and purified xylan as substrates. As shown in Figs. 2–4, these isolated strains have ability to metabolize the cellulosic substrates for growth by expressing cellulolytic enzyme activities (e.g., endoglucanase, exoglucanase, cellobiase and xylanase) at different locations (e.g., extracellular, intracellular and cell bound).

3.2.1. Endoglucanase production

Endoglucanase production was significant extracellularly in the presence of xylan and rice straw by all isolated strains (Figs. 2a, 3a, and 4a). In the presence of xylan, significant induction of endoglucanase activity (534.8 and 551.7 U mg protein^{-1}) by strains L-133 and ANA-NS2 was observed, whereas the other strains gave moderate endoglucanase production extracellularly (Fig. 2a). For *Cellulomonas* species, xylan is known as a good inducer for endoglucanase activity, as similar performance was observed in L-133 and ANA-NS2 strains [30]. In contrast, limited endoglucanase activity was observed at the intracellular and cell bound locations while xylan was used as sole carbon source (Fig. 2b and c). When rice straw was used the endoglucanase activity was significant extracellularly by all strains, while the maximum endoglucanase activity (135.7 and 221 U mg protein^{-1}) was observed by strain ANA-NS2 and I-1, respectively (Fig. 3a). Like using xylan as carbon source, negligible intracellular and cell bound endoglucanase activity was found for most of the strains (Fig. 3b and c) while using rice straw as the substrate. Interestingly, in the presence of rice husk, some strains (I-1 and ANA-WS2) gave significantly higher endoglucanase activity (1194.7 and 751.2 U mg protein^{-1}) at extracellular compartment (Fig. 4a) when compared to using xylan and rice straw as substrate. This might be during hydrolysis of rice husk some disaccharides and oligosaccharides liberated and induce the endoglucanase activity. Endoglucanase production by *Cellulomonas biazotea* in the presence of different cellulosic substrates including pure and agricultural waste was reported [2]. Some strains in the presence of rice straw (RP3, I-1, L-133), xylan (ANA-NS-1, FP-2, ANA-WS2) and rice husk (L-133) can produce endoglucanase both at extracellular and cell bound locations. Similarly, endoglucanase production at extracellular and cell bound location was reported in *C. uda* and *Cellulomonas* sp. grown on CMC, avicel and boiled newspaper and wheat straw, respectively [31,32].

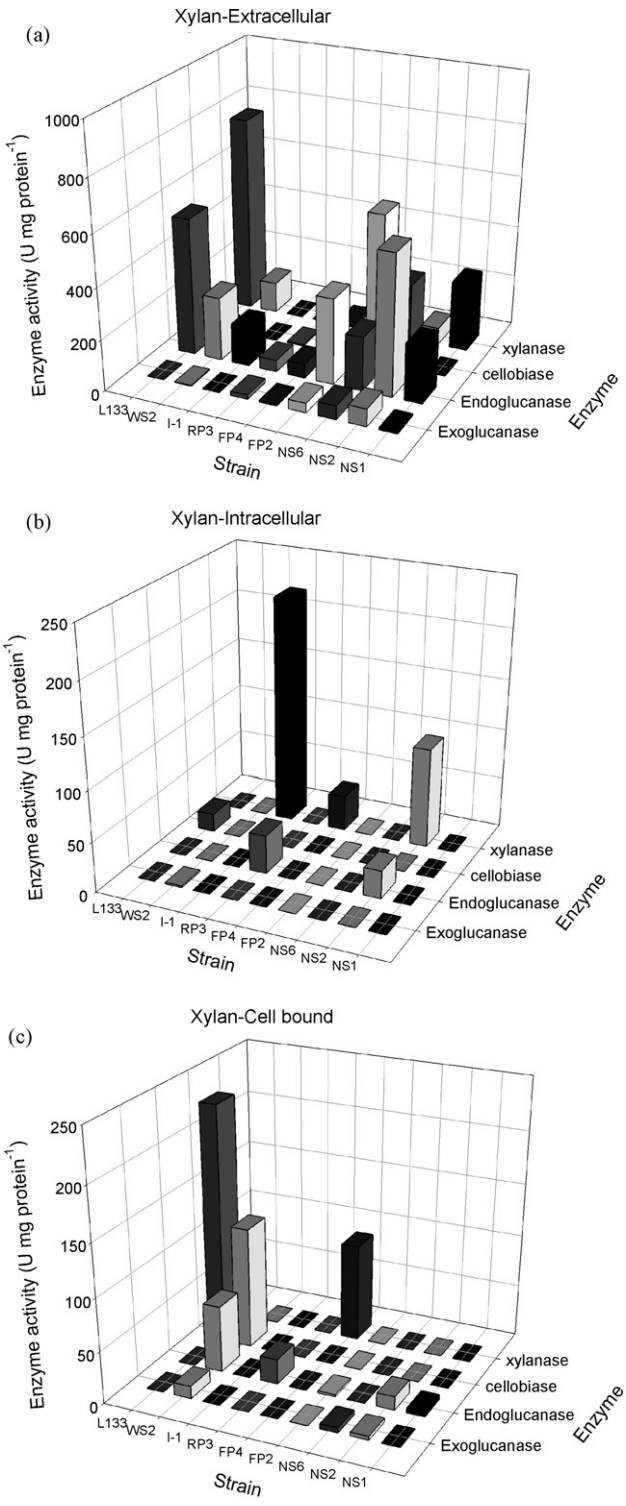

Fig. 2. Effect of xylan on the production of cellulase (endoglucanase, exoglucanase, cellobiase and xylanase) by isolated nine cellulolytic efficient strains at different locations. (a) Extracellular, (b) intracellular, and (c) cell bound.

In addition, there are also some strains (ANA-NS-1 on rice straw, ANA-NS-2 and RP3 on xylan, ANA-WS2 on rice husk) exhibited endoglucanase activity at all three locations regardless of the substrate used (Figs. 2–4). The highest endoglucanase activity by strain I-1 (1194.7 U mg protein^{-1}) in the presence of rice husk as well as the production of endoglucanase by other strains are also several folds higher than that reported with *C. uda* JC3 (142 U mg protein^{-1}) [33], mutant of *C. flavigena* (1.63 U mg protein^{-1}) [29], *Cellulomonas*

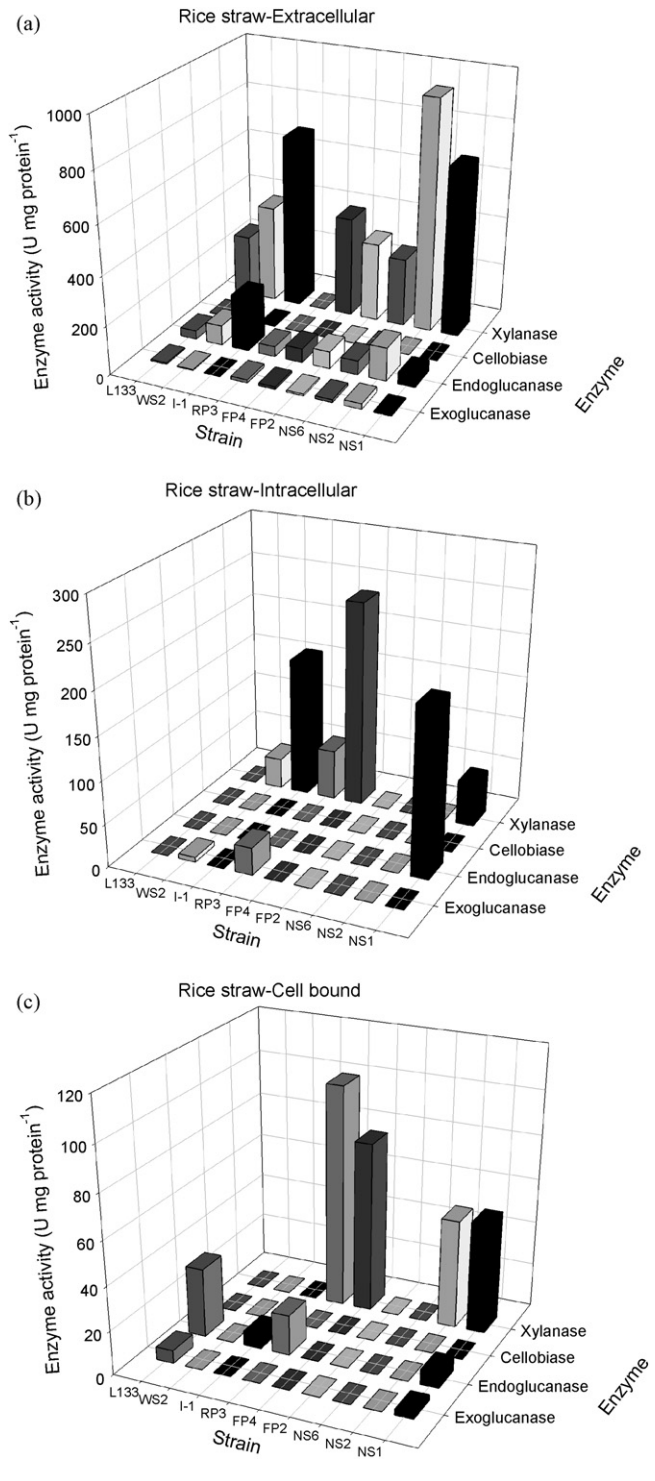

Fig. 3. Effect of rice straw on the production of cellulase (endoglucanase, exoglucanase, cellobiase and xylanase) by isolated nine cellulolytic efficient strains at different locations. (a) Extracellular, (b) intracellular, and (c) cell bound.

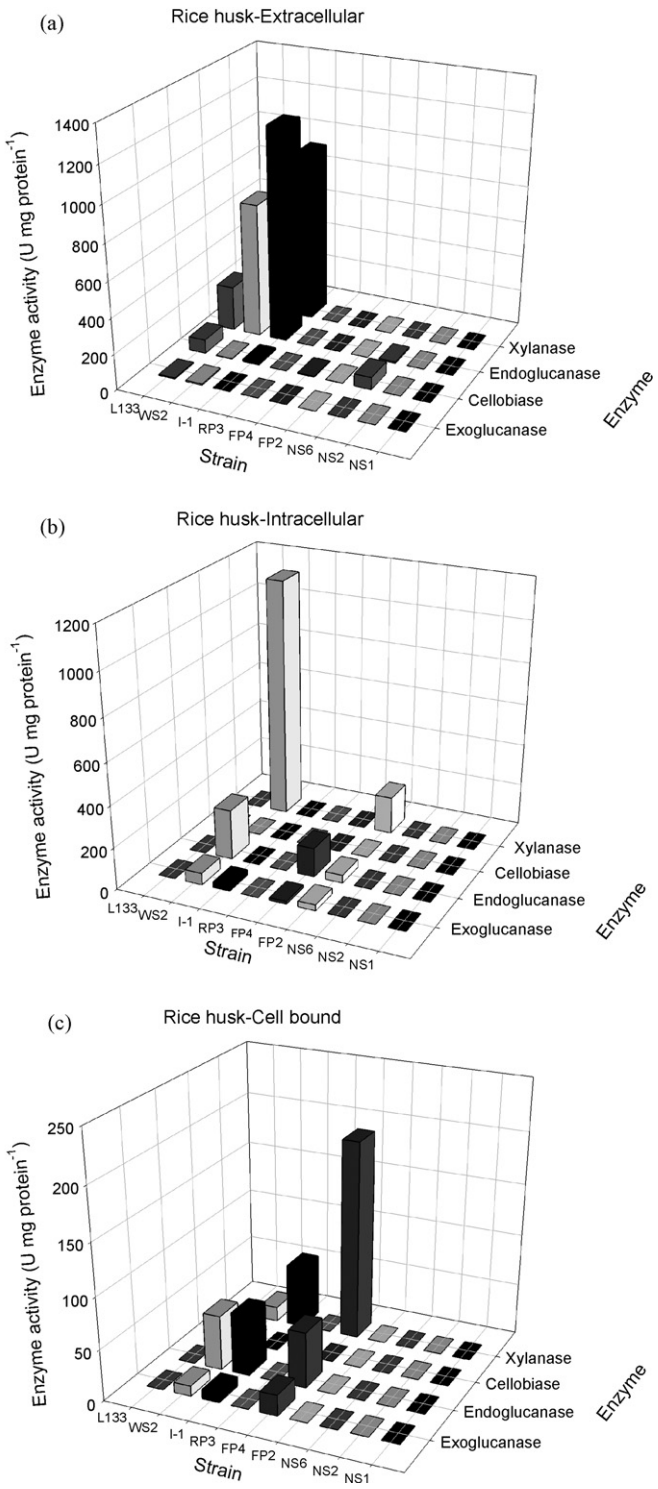

Fig. 4. Effect of rice husk on the production of cellulase (endoglucanase, exoglucanase, cellobiase and xylanase) by isolated nine cellulolytic efficient strains at different locations. (a) Extracellular, (b) intracellular, and (c) cell bound.

sp. (9.0 U mg protein^{-1}) [32], *C. uda* (2.6 U mg protein^{-1}) [34], *C. flavigena* (10.70 U mg protein^{-1}) [35], *Bacillus pumilus* EB3 (5.9 U mg protein^{-1}) [36] and also comparable with mutant fungus *Penicillium janthenellum* NCIM 1171 (97.0 U ml^{-1}) [37].

3.2.2. Exoglucanase production

The effect of different carbon sources on the production of exoglucanase by *C. flavigena* was reported earlier [28]. In this study, all isolated strains (except I-1 and L-133 on xylan and I-1 on rice straw) produced exoglucanase mainly extracellularly in the presence of xylan and rice straw. When xylan was used as the substrate significant induction of exoglucanase activity (68.95 and 53.69 U mg protein^{-1}) was observed by strain ANA-NS2 and ANA-NS6, whereas the other strains give moderate exoglucanase induction at extracellular location (Fig. 2a). It was noticed that strain ANA-WS-2 can produce exoglucanase at all extracellular, intracellular and cell bound locations. However, negligible intra-

cellular and cell bound exoglucanase activity was observed for some strains (Fig. 2b and c). It was reported that *Cellulomonas* sp. produced exoglucanase at extracellular and cell bound location in the presence of wheat straw [32]. When rice straw was used the extracellular exoglucanase activity was significant for all strains (except strain I-1) (Fig. 3a). Amazingly, RP-3 strain exhibited a maximum exoglucanase activity of 31.94 U mg protein^{-1} at intracellular location and also shows better performance at extracellular as well as cell bound locations (Fig. 3a–c); in contrast negligible performance was observed by some strains at intracellular and cell bound locations (Fig. 3b and c). It was observed that during the hydrolysis of lignocellulosic biomass disaccharides and oligosaccharides liberated and found to be strong inducers of cellulases [37]. Similarly, in the presence of rice husk a maximum exoglucanase activity of 56.15 U mg protein^{-1} was observed intracellularly for strain ANA-WS-2, which also showed exoglucanase activity at extracellular and cell bound locations (Fig. 4a). Some strains (I-1 and FP-4) produced exoglucanase significantly at intracellular and cell bound locations, whereas strain FP-2 can produce exoglucanase only intracellularly (Fig. 4b and c). In some cellulolytic bacteria the cellulolytic activity was found to be less extracellularly compared to whole cultures. This may be because of one or more important hydrolytic enzymes are membrane bound in such kind of bacteria [28,38]. The exoglucanase production of our isolated strains shows significant performance compared to the exoglucanase activity of other *Cellulomonas* species; for example, *C. flavigena* PN-120 (28.72 U mg protein^{-1}) [29], *C. uda* JC3 (17.33 U mg protein^{-1}) [33], *Cellulomonas* sp. ATCC-21399 (31.29 U mg protein^{-1}) [39], and *C. flavigena* (1.33 and 1.12 U mg protein^{-1} in the presence of α-cellulose and Kallar grass, respectively) [28].

3.2.3. Cellobiase production

Very poor cellobiase activity was observed in the presence of xylan and rice straw as the growth substrate. In the presence of all three cellulosic substrates there is no obvious production of cellobiase at intracellular as well as cell bound locations, except for strains ANA-WS-2 and L-133 in the presence of xylan as the substrate (Fig. 2). Significant production of cellobiase extracellularly observed in the presence of rice husk by all strains (Fig. 4a) (except ANA-NS1, RP3) and in the presence of xylan and rice straw some strains can produce cellobiase extracellularly (Figs. 2a and 3a). In the presence of rice husk, the maximum activity (69.3 and 77.8 U mg protein^{-1}) was observed extracellularly by strain ANA-NS-6 and L-133, respectively. Surprisingly, in the presence of xylan fantabulous cellobiase production performance (115.9 and 228.2 U mg protein^{-1}) was observed by ANA-WS-2 (cell bound) and L-133 (intracellular) (Fig. 2b and c). Therefore, L-133 seemed to be a good cellobiase producing strain. Likewise, Stoppok et al. [31] determined the presence of cellobiase activity at extracellular, intracellular and cell bound location by *C. uda*. The cellobiase activity recorded in isolated strains was higher than that of *Cellulomonas* sp. (7.5 U mg protein^{-1}) [32], *C. biazotea*; (6.1 U ml^{-1}) [2] and also higher than that of fungus and *Trichoderma reesei* (106 U mg protein^{-1}) [40].

3.2.4. Xylanase production

In the presence of xylan and rice straw, xylanase activity was significant extracellularly. When xylan was used most strains gave significant induction in xylanase activity (except for RP-3 and I-1). The maximum activity of 764 and 483.94 U mg protein^{-1} was observed for strain L-133 and ANA-FP2, respectively, whereas other strains gave moderate xylanase activity at extracellular location (Fig. 2a) and poor performance observed by some strains at intracellular and cell bound locations (Fig. 2b and c). In the presence of rice straw the xylanase activity was most significant extracellularly by all strains (except RP-3), as a maximum xylanase activity of 941, 702 and 687 U mg protein^{-1} was observed for strain ANA-NS2, I-1, and ANA-NS1, respectively. However, other strains show moderate xylanase production while using rice straw as the substrate (Fig. 3a). Some strains can produce xylanase intracellularly in the presence of rice straw, as a maximum activity of 572 U mg protein^{-1} was observed by strain FP-4, whereas strain ANA-NS1 and FP-4 have the ability to produce xylanase at all extracellular, intracellular and cell bound locations (Fig. 3a–c). Similar results were observed by *C. uda* which can produce xylanase at all cellular location in which maximum activity was observed at intracellular location [41]. In the presence of rice husk, strain I-1, ANA-WS2, and L-133 gave magnificent xylanase production extracellularly, as a maximum activity of 948.2 and 851.8 U mg protein^{-1} was obtained from strain I-1 and ANA-WS2, respectively (Fig. 4a). Surprisingly, the highest xylanase activity was observed at intracellular location by strain ANA-WS-2 which can secretes xylanase at extracellular as well as cell bound locations. Moreover, some strains showed better intracellular and cell bound xylanase activity when compared to extracellular location (Fig. 4b and c). The activity recorded in isolated strains found to be higher compared to *Cellulomonas* sp. (375 U mg protein^{-1}) [32], *C. flavigena* (12.8 U mg protein^{-1}) [35], *Cellulomonas* sp. ATCC-21399 (35.41 U mg protein^{-1}) [39], *C. flavigena* (64 U mg protein^{-1}) [42], *C. flavigena* (13.6 U mg protein^{-1}) [43], *C. flavigena* mutant P-120 and M9-82 (95 and 223 U mg protein^{-1}, respectively) [44].

From this study we found that significant cellulase and xylanase production by isolated *Cellulomonas* sp. at different cellular location, among which maximum activity was observed extracellularly even though some strains can produce enzyme effectively at intracellular and cell bound compartments. The enzyme activity was found to be higher compared to activities of other *Cellulomonas* sp., indicating the advantage of our isolates. These strains could be useful to produce efficient cellulose/xylanase by using agricultural residue (rice husk, rice straw), thereby being useful for the preparation of cellulosic feedstock to obtain a maximum amount of reducing sugars for biohydrogen production.

3.3. Identification of cellulosic hydrolysates composition via HPLC analysis

The composition of cellulosic hydrolysates has close connection with the utilizability of the hydrolyzed substrate in energy-producing fermentation. Hence, HPLC analysis was conducted to determine the composition of reducing sugars (e.g., glucose, cellobiose, cellotriose, xylose, and L-arabinose, etc.) obtained after bacterial hydrolysis and saccharification of cellulosic substrates (rice husk, rice straw, and purified xylan), as well as other fermentation products such as lactic acid, acetic acid, formic acid and ethanol. After cultivation on BHM medium amended with rice husk and rice straw, the isolated *C. uda* NS1 produced saccharides and acidogenic

Table 1
Composition of hydrolysates resulting from bacterial hydrolysis of xylan, rice husk, and rice straw by *Cellulomonas uda* NS1.

Cellulosic substrate	Hydrolysate composition (mg l^{-1})							
	Glucose	Cellobiose	Xylose	Lactate	Formate	Acetate	Propionate	Ethanol
Xylan	0	0	262	220	30	200	20	3120
Rice husk	140	143	0	0	380	100	30	0
Rice straw	152	22	32	0	90	30	20	0

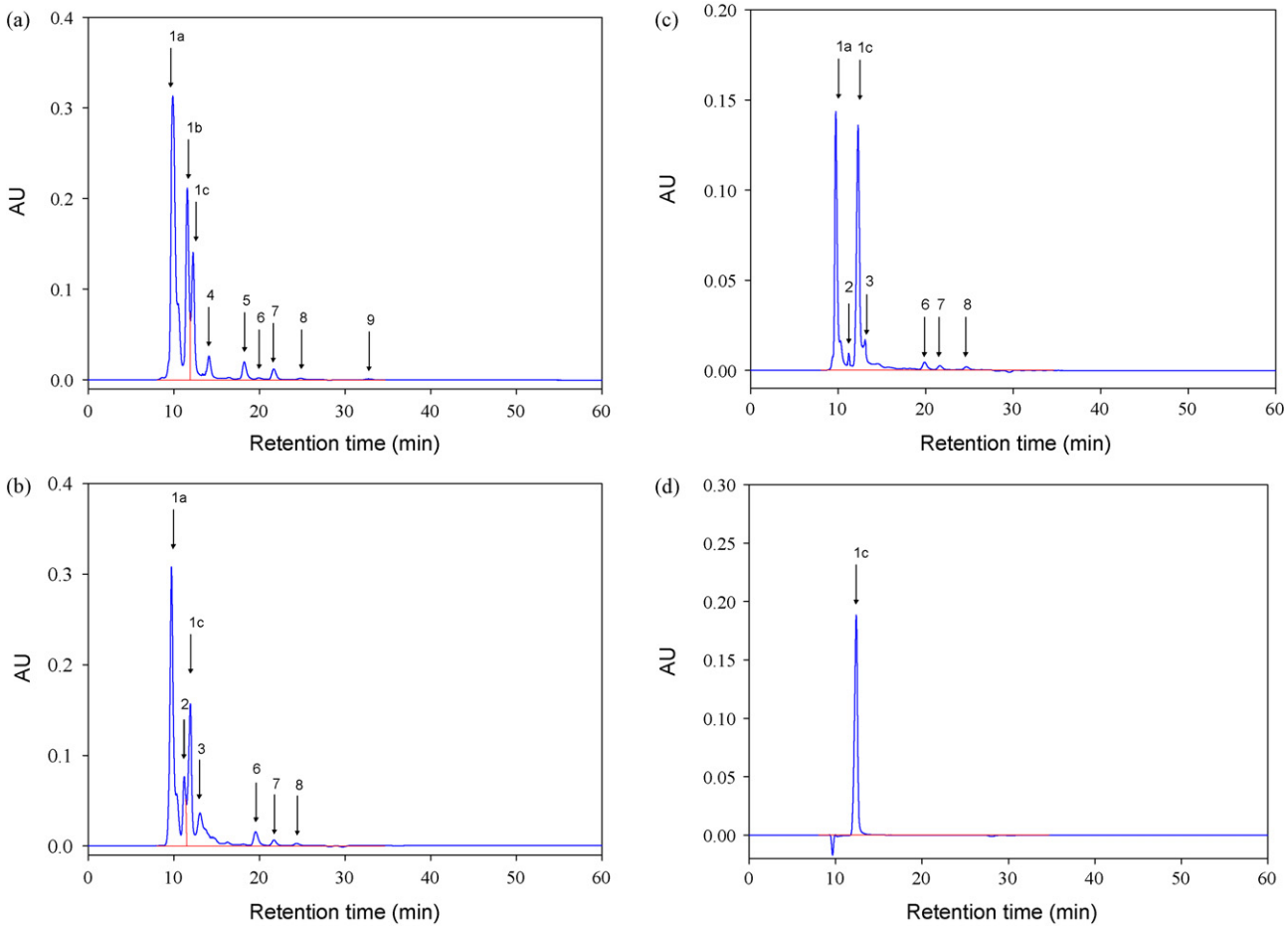

Fig. 5. HPLC analysis of hydrolysates obtained from bacterial hydrolysis (using *Cellulomonas uda* NS1) of (a) xylan, (b) rice husk, (c) rice straw, and (d) BHM media (peaks 1a and 1b: unidentified; 1c: BHM medium (phosphate ion) (12.30 min); 2: cellobiose (11.04 min); 3: glucose (13.71 min); 4: xylose (14.72 min); 5: lactate (19.20 min); 6: formate (20.87 min); 7: acetate (22.77 min); 8: propionate (26.65 min); and 9: ethanol (33.04 min)).

secondary metabolites. As indicated in Table 1, HPLC analysis identified the formation of glucose, cellobiose, and xylose as saccharide products as well as formation of lactate, acetate, formate and ethanol, indicating cellular metabolism of rice husk and rice straw by isolated strains (Fig. 5). Still, some complex components such as larger oligosaccharides seemed to be present in the hydrolysates as indicated in HPLC results as unidentified peaks (Fig. 5).

The formation of glucose and cellobiose suggests the sequential action of endoglucanase, exoglucanase and cellobiase, whereas formation of xylose suggests the involvement of xylanase in the degradation of these cellulosic raw materials. In the presence of purified xylan all the bacterial isolates produced xylose as saccharide product, while other fermentation products such as lactate, acetate, formate and ethanol were also formed. These results suggest that different carbon sources give different enzymatic performance as well as different saccharide products.

3.4. Biohydrogen production from cellulosic feedstock using two-stage processes

Due to containing higher glucose and cellobiose content, rice husk hydrolysate instead of rice straw hydrolysate was selected for fermentative hydrogen production test. The efficiency of fermentative conversion of hydrolysates from rice husk into H_2 was examined with seven H_2-producing pure strains (*Clostridium butyricum* CGS2, *Cl. butyricum* CGS5, *Cl. pasteurianum* CH1, *Cl. pasteurianum* CH4, *Cl. pasteurianum* CH5, *Cl. pasteurianum* CH7 and *Klebsiella* sp. HE1.) isolated from effluent sludge of high-rate H_2-producing bioreactors developed in our recent work [21,22]. Only *Cl. butyricum* CGS5 could use the rice husk hydrolysate to produce hydrogen, while the other species failed to produce hydrogen from rice husk hydrolysate. With an initial reducing sugar concentration of 0.36 g l^{-1}, the best H_2 production and yield by using *Clostridium butyricum* CGS5 was approximately 88.1 ml l^{-1} and 19.2 mmol H_2 g reducing sugar^{-1} (or 17.24 mmol H_2 g cellulose^{-1}), respectively (Fig. 6a). The results demonstrate the feasibility of using the two-stage system converting natural cellulosic feedstock to hydrogen under mild operating condition. The soluble metabolites formed along with cellulosic H_2 production consisted mainly of ethanol, followed by formate, acetate, and lactate (Fig. 6b). This soluble metabolite composition produced by *Cl. butyricum* CGS5 is quite different from that obtained from using sucrose or xylose for H_2 production, in which the soluble metabolites contained mainly butyrate and acetate [24]. In addition, this metabolite composition also seemed to be unfavorable for dark H_2 fermentation since alcohol production was predominant [45], probably arising from the complex composition of rice husk hydrolysates. Nevertheless, Table 2 shows that the efficiency of our cellulosic H_2 production system is still comparable to that reported in the relevant studies, once again indicating the feasibility of the proposed two-stage bioH_2 production process. However, it would require further optimization of bioreactor design and operation conditions to favor the H_2 production metabolism and also to reduce the cost of bioH_2 production via a more efficient sequencing hydrolysis-fermentation approach.

Table 2
Comparison of bioH_2 production performance using cellulose or hydrolyzed cellulose as the substrate.

H_2 producer	Culture type	Cellulosic substrate	Temperature (°C)	Initial pH	H_2 yield	References
Thermotoga maritime (DSM 3109)	Batch	Cellulose (5 g l^{-1}) CMC (5 g l^{-1})	80	6.5	0.96 mmol H_2 g cellulose^{-1} [b] 3.29 mmol H_2 g cellulose^{-1}	[46]
Thermotoga neapolitana (DSM 4359)	Batch	Cellulose (5 g l^{-1}) CMC (5 g l^{-1})	75	7.0	1.07 mmol H_2 g cellulose^{-1} [b] 3.37 mmol H_2 g cellulose^{-1}	[46]
Clostridium sp. strain No. 2	Batch	Glucose (3 g l^{-1}) Xylose (3 g l^{-1}) Avicel hydrolysate[a] (3 g l^{-1}) Xylan hydrolysate (3 g l^{-1})	36	6.8	14.6 mmol H_2 g substrate^{-1} 16.1 mmol H_2 g substrate^{-1} 19.6 mmol H_2 g substrate^{-1} 18.6 mmol H_2 g substrate^{-1}	[12]
Anaerobic digested sludge	Batch	Microcrystalline cellulose (12.5 g l^{-1}) Microcrystalline cellulose (25 g l^{-1})	37	7.0	2.18 mmol H_2 g cellulose^{-1} 1.60 mmol H_2 g cellulose^{-1}	[47]
Clostridium acetobutylicum X_9 and *Ethanoigenens harbinense* B_{49}	Batch	Microcrystalline cellulose (10 g l^{-1})	37	5.0	3.66 mmol H_2 g cellulose^{-1}	[9]
Clostridium thermocellum JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17	Batch	Microcrystalline cellulose (10 g l^{-1}) Corn stalk powder (0.5%) Corn cob powder (0.5%)	60	7.0	10 mmol H_2 g glucose^{-1} 16.1 mmol l corn stalk powder^{-1} 20.4 mmol l corn cob powder^{-1}	[48]
Clostridium thermocellum JN4	Batch	Microcrystalline cellulose	60	7.0	0.8 mol H_2 mol glucose^{-1}	[48]
Clostridium acetobutylicum X9	Batch	Microcrystalline cellulose (10 g l^{-1})	37	7.0	0.17 mmol H_2 g cellulose^{-1}	[9]
Clostridium pasteurianum	Batch	Hydrolyzed carboxymethyl cellulose (10 g l^{-1})	35	7.0	1.09 mmol H_2 g cellulose^{-1}	[10]
Clostridium butyricum CGS5	Batch	Rice husk hydrolysate	35	7.0	17.24 mmol H_2 g rice husk hydrolysate^{-1}	This study

[a] Obtained from enzymatic hydrolysis.
[b] Converted from original data.

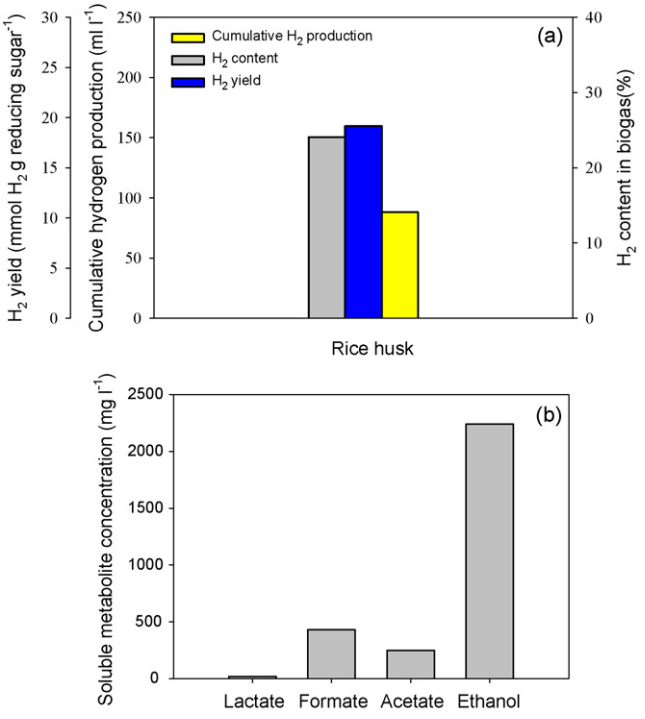

Fig. 6. Performance of dark H_2 fermentation from rice husk hydrolysate (initial reducing sugar concentration = 0.36 g l^{-1}) by using *Clostridium butyricum* CGS5. (a) Cumulative H_2 production, H_2 yield, and H_2 content, (b) metabolite formation.

4. Conclusions

The isolated and identified nine efficient cellulolytic microorganisms have the ability to hydrolyze different cellulosic substrates (i.e., rice husk, rice straw, xylan) and produce cellulase enzymes mainly extracellularly. HPLC analysis confirmed the degradation of these cellulosic substrates into soluble sugars. Using natural cellulosic feedstock (i.e., rice husk), biohydrogen production was successfully achieved via a two-stage approach, in which bacterial hydrolysis and dark fermentation were conducted sequentially. From rice husk hydrolysate containing 0.36 g l^{-1} reducing sugar, *Clostridium butyricum* CGS5 attained the best hydrogen production performance with a cumulative H_2 production of 88.1 ml l^{-1} and a yield of 19.15 mmol H_2 g^{-1} reducing sugar (or 17.24 mmol H_2 g^{-1} rice husk hydrolysate).

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