INTRODUCTION

Many developed countries around the world have begun to develop biomass ethanol (bioethanol) as an emerging energy source to replace depleting oil resources. However, using food crops as raw material for bioethanol leads to high production costs and also indirectly increases global food crop prices. This seriously endangers the economic survival of individuals living in developing countries [1]. The large quantity of fertilizer and the high water consumption required by food crops also results...
in negative environmental impacts. The planting of food crops in order to produce bioethanol also causes soil erosion, reduces biodiversity and produces significant amounts of pollution by highly volatile organic compounds (VOC) and nitrogen oxides (NO) [2].

Cellulose is one of the most abundant biomaterials on the planet and this biological material has a very wide range of sources. In particular, various types of forestry and agricultural residues are easily available for environmental improvement and energy application. Cellulose has a high value in terms of energy generation, which will reduce waste and greenhouse gas emissions into the environment [3]. Cellulosic ethanol is therefore an important bio-resource and it has been called the second-generation bioethanol [4]. Large amounts of cellulosic material are available in the natural environment. Nevertheless, the breakdown of this material in useful compounds requires further study in order to enhance its efficiency as a source of bioenergy.

A major unsolved problem associated with cellulosic bioethanol as bioenergy is the cost. There are two areas increase significantly the cost of cellulosic ethanol. The first factor is the materials and the transportation. The second factor that increases dramatically the cost of cellulosic bioethanol is the requirement for multiple stages for the production of ethanol. To solve the latter problem, the production processes must be simplified. A bacterial strain that can convert cellulose into sugars while surviving in an acid environment would be critical to reducing production costs. This would allow the use of bio-waste for the production of bioethanol to be increased significantly [5].

Many cellulose decomposition bacteria being isolated, including examples such as Clostridium thermocellum SS19, Thermotoga sp. FSS3-B1, Clostridium stercorarium NCIB 11754, Rhodothermus marinus ITI 378, Microbispora bispora NRRL 15568 and Thermus Z-1 [6-9], however, their efficiency in terms of biotransformation are still under investigation and their resistance to an acid environment have seldom been reported.

This study selected agricultural waste (bio-waste) as the source material of bioethanol production and used it to isolate the bacteria capable of biotransforming cellulose into fermentable sugars to allow the production of bioethanol. The sugar cane bagasse was used in this study as the raw material for isolating bacteria capable of cellulose biotransformation and the novel species isolated from the local Taiwan environment were then screened to see if they were suitable to be applied to the production of bioethanol. The first stage of the study was involved screening for bacteria capable of utilizing cellulose as a sole carbon source. Subsequently, the bacteria were acclimated to using carboxymethyl cellulose (CMC) as a sole carbon source in order to investigate the biotransformation properties of each strain. Finally, the characteristics of the strains in terms of growth kinetics, the production of reducing sugars, acid resistance were evaluated. The final aim of this study was to identify one or more bacterial strains that could both bring about efficient cellulose biotransformation and also were acid resistant. This would provide an opportunity to simplify the current procedures used for the production of cellulosic bioethanol.

MATERIALS AND METHODS

Source of the inoculums

The sources of the various inocula were as follows. (1) Volcanic soil from Yang-Ming Shan National Park in the northern part of Taiwan; the soil is soft and light with significant organic matter and mostly as small granules. (2) Hot spring drainage soil obtained from a drainage area of a hot spring in Beitou near Taipei City; the hot spring temperature is about 90 °C to 100 °C and produces hydrochloric acid containing spring water with a pH of 1.6. (3) Drainage sediment from a fuel station in Taipei City; the sediment is mainly organic material with a black outlook.

Carboxymethyl cellulose (CMC)

CMC is a cellulose derivative material in which the polymer retains the structure of cellulose material; this was purchased from Merck (Merck, USA). The medium containing CMC used in this study was minimal salt based (MSB) at different concentrations. The composition of MSB was described in a previous study [10].

Screening and isolation of the bacterial strains

The screening was conducted using sugarcane bagasse as the sole carbon source mixed with MSB medium in order to allow microbial growth and acclimation in the culture. Specifically, 3g of each soil inoculum and 5g of bagasse were added together into a 500 ml flask and add with 150ml MSB to create individual selection cultures for each soil. The flasks were then shaken in an incubator at 150 rpm and 30 °C. After 60 days of acclimatization, to further confirm that the strain present in the culture could use cellulose as a sole carbon source, inoculums were transferred to CMC liquid culture to test whether they continued to grow. After this second acclimatization, the bacteria present in the CMC liquid culture were spread on CMC plates containing the same CMC medium, but in the presence of agar to give a solid medium. Single colonies underwent ten single colony subcultures, after which they were evaluated.

The morphological evaluation of the isolates

After Gram staining [11], the morphology of each bacterial isolate was observed by electronic optical microscopy at a magnification of 1000X. The morphological information and Gram characteristic of the isolates were collected and then after sample pre-treatment, the strains were also observed by SEM (Scanning Electron Microscopy, Hitachi, S-5000, Japan)) and TEM (Transmission Electron Microscopy, Hitachi, H7500, Japan) to explore the detailed cell structure of each isolate.

Phylogenetic identification of the isolates

Phylogenetic identification of the isolates used their 16S rDNA nucleotide sequences, which were compared with the sequences of known species contained on the NCBI database and the Applied Biosystem database. Initially, chromosomal DNA was extracted from each isolate independently and the 16S rDNA amplified by PCR using the primers F8 (5’-AGAGTTTGATCCTGCTGAC-3’), and R1510 (5’-GGTTACCTCAGTTACCT-3’). The amplification products were separated by agarose gel electrophoresis, the 16S rDNA fragment purified and then it was
sent to be DNA sequenced (MingShin Biotechnology, Taiwan). The 16S sequences of the isolates were then compared to the NCBI database and the Applied Biosystem database using the BLAST program.

**Bacterial growth kinetics analysis**

Microbial growth kinetics is an important indicator of microbial activity. To further understand how the various isolates utilize cellulose, the growth rate of the various isolates were evaluated over a range of substrate concentrations. In total, the growth characteristics of five isolates were analyzed. Utilization of cellulose as sole carbon source was analyzed simultaneously use the single substrate model (Monod equation) together with the inhibition at high substrate concentrations (Haldane-Andrews model) [12]. The microbial growth was evaluated using CMC and MSB liquid culture. The strains were cultured at 150 rpm shaking and 30 °C. Every four hours the culture turbidity was measured set at OD_{600} absorbance using a spectrophotometer. Each culture was grown until stationary phase was reached.

**Reducing sugar production by the various isolates**

Reduction sugar concentration was determined by the DNS method [13] as modified by Frost [14] and because the hydrolysis of cellulose mainly produces glucose, the calibration curve prepared using glucose as the standard. Samples consisted of approximately 2ml of culture medium, which were centrifuged at 6,000 rpm for 5min; then 1ml of supernatant was added to an equal volume of DNS reagent in screw cap closed glass tube with mixing. This was placed in boiling water bath for 10 min. After rapid cooling to room temperature, a visible light spectrophotometer was used to measure absorbance at 540 nm.

**Evaluation of the acid resistance of the various isolates**

Acid resistance was measured using liquid culture and medium containing 10,000 mg/L of CMC with MSB shaken at 150 rpm and 30°C. The pH of the culture medium was then adjusted to 2, 3, 4, 5 and 6 using K_2HPO_4 and KH_2PO_4. Cell growth was assessed by measuring the OD_{600} of the culture every 2 hours.

**RESULTS AND DISCUSSION**

The isolation and the identification of the strains

As shown in (Table 1), all those five strains which can using CMC as the sole carbon source were isolated from the culture contain MSB as the basic salts nutrients. After 10^th subcultures, the strains were identified by the 16S rDNA sequences alignment. The 16S rDNA were extracted and identified the sequence. After that, this study using two databases to finish the identification of the strains. As shown in (Table 1), the strain S1 was identified as *Sphingomonas* sp. by alignment with the NCBI database with similarity of 98.56%. When using the applied biosystems database for the alignment, the strain was identified as *Pseudomonas* sp. with 96.25% similarity. The strain S1 was identified as *Pseudomonas* sp. by using both NCBI and applied biosystems databases with similarity of 99.40% and 97.52%, respectively. The strain G1 was identified as *Achromobacter* sp. using NCBI database and *Achromobacter xylosoxidans* using applied Biosystems database with similarity of 98.35% and 93.63%, respectively. The strain S2 was identified as *Pseudomonas* sp. by using both NCBI and applied biosystems databases with the similarity of 97.45% and 97.24%, respectively. The strain G2 was identified as *Stenotrophomonas* sp. by using both NCBI and applied biosystems databases with the similarity of 98.56% and 99.52%, respectively. As the identification results shown in (Table 1), this study named those five strains as *Sphingomonas* sp., *Pseudomonas* sp. M1, *Achromobacter* sp., *Pseudomonas* sp. M2, and *Stenotrophomonas* sp. according the higher similarity of the sequence alignment from those two databases. Comparing the isolation and identification results showed in (Table 1). The strain of *Pseudomonas* was the most ever appear strain in the references [16]. The other four strains in this study were not mentioned yet in the literature. So, the strains of *Sphingomonas* sp.,

**Table 1: The bacteria screening, identification and growth kinetic analysis.**

<table>
<thead>
<tr>
<th>Source of strains</th>
<th>Identification to NCBI database (Similarity)</th>
<th>Identification to Applied Biosystems database (Similarity)</th>
<th>Name in this study</th>
<th>Growth kinetic analysis using CMC as the solo carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Sphingomonas sp. (98.56%)</td>
<td>Pseudomonas sp. (96.25%)</td>
<td>Sphingomonas sp.</td>
<td>μmax(h^-1) = 0.419, K (mg/L) = 1,038, K (mg/L) = 310,000</td>
</tr>
<tr>
<td>G1</td>
<td>Pseudomonas sp. (99.40%)</td>
<td>Pseudomonas sp. (97.52%)</td>
<td>Pseudomonas sp. M1</td>
<td>μmax(h^-1) = 0.439, K (mg/L) = 776, K (mg/L) = 350,000</td>
</tr>
<tr>
<td>S2</td>
<td>Achromobacter sp. (98.35%)</td>
<td>Achromobacter xylosoxidans (93.63%)</td>
<td>Achromobacter sp.</td>
<td>μmax(h^-1) = 0.395, K (mg/L) = 2,510, K (mg/L) = 21,000</td>
</tr>
<tr>
<td>S3</td>
<td>Pseudomonas sp. (97.45%)</td>
<td>Pseudomonas sp. (97.24%)</td>
<td>Pseudomonas sp. M2</td>
<td>μmax(h^-1) = 0.436, K (mg/L) = 1,188, K (mg/L) = 368,000</td>
</tr>
<tr>
<td>G2</td>
<td>Stenotrophomonas sp. (98.56%)</td>
<td>Stenotrophomonas sp. (99.52%)</td>
<td>Stenotrophomonas sp.</td>
<td>μmax(h^-1) = 0.37, K (mg/L) = 593, K (mg/L) = 15,600</td>
</tr>
</tbody>
</table>

1. S1, S2, S3: the soil from hot spring drainage in Taipei city Baitou Dist. The hot spring belong to the carbonate acid hot water in temperature of 90°~100°.
2. G1, G2: the soil form YangMing mountain at the suburban districts of Taipei city.
3. The strains were isolated using CMC (Carboxymethyl cellulose) as the sole carbon source and the MSB as the inorganic salts.
4. The strains were all identified after 10 subcultures.
5. The appearance of the strains are all bacillus and characteristics of gram – negative.
6. μmax: the maximum specific growth rate.
7. Ks: half velocity constant or saturation constant for cell growth.
Achromobacter sp., and Stenotrophomonas sp. might be the novel discovery strains concerning the cellulose biotransformation.

The morphology of the strain for cellulose biotransformation

After gram staining, all five strains are Gram negative strains and all have the appearance of bacillus type. To further observing the morphology, the strain Pseudomonas sp. M1 was observed by SEM and TEM since it obtained higher growth rate among those five strains. The morphology of the strain Pseudomonas sp. M1 was observed by SEM and TEM using 40.0 K and 51.0 K magnification, respectively. As shown in (Figure 1), the strain of Pseudomonas sp. M1 has the length of 3μm and width of 1μm. The SEM photo (Figure 1A) indicates that the bacterium has a smooth surface and the pilus was not easy to observed. The strain was also observed without flagellum and spore (Figure 1A) by SEM and (Figure 1B) by TEM. There has one or two flange line encircle on the surface of the bacteria. The intracellular organization of the strain is clear and obviously for observed which demonstrated by the TEM photo. Accordingly, the configuration of the strains isolated from this study obtained the similar profile to the reference mentioned [17].

The evidence of the CMC biodegrading by the strain

As shown in (Figure 2), the CMC was degraded during the cell growth and the colony formation period. In that time, around the colony, there will be a white shadow around the colony. It indicated that the CMC was biodegraded by the strain of Pseudomonas sp. M1. To further approve the CMC biodegraded in the culture, the circle round of CMC was cut after the colony removed. The initial CMC concentration of the plate was 5000 mg/L. The cut gel of CMC was rediluted to the MSB medium and the concentration of CMC was measured. The result of the cut gel CMC shown the concentration of 155 mg/L. It indicates that almost 90% of CMC was use for the bacteria growth during the colony formation period. The white shadow of CMC on the gel and the concentration of CMC decline in the culture point out that the strain of Pseudomonas sp. M1 can really use CMC as the carbon source for the cell growth and the bacteria can using the cellulose as the sole carbon source.

The growth of the strains by using CMC as the sole carbon source

The growth of the strains was observed at different CMC concentrations. The strains include Sphingomonas sp., Pseudomonas sp. M1, Achromobacter sp., Pseudomonas sp. M2, and Stenotrophomonas sp. The CMC concentrations were 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, and 10000 mg/L. The bacterial growth amount was display by OD600. (Figure 3) showed the growth of the bacteria in the CMC concentration of 1000 mg/L(A) and 10000 mg/L (B). As shown in (Figure 3A), these five cellulolytic bacteria growth in the medium contain 1000 mg/L of CMC were investigated and display by OD600. The growth of the strains was not very effective in the concentration of 1000 mg/L. The amounts of OD600 for all those strains were less than 0.1. It points out that the low cellulose concentration was insufficient offer the growth requirement for the bacteria. After increase of the CMC concentration, the increase of the bacteria cell amount was observed. When increase the concentration of CMC to 2000 mg/L (data no shown), the bacteria obtained a steady growth pattern. The growth of the strain Pseudomonas sp. M1 obtained the best growth amount. The optimal growth period was about 36 hours with the OD600 value of 0.18. However, the other four strains cannot obtain good growth effect in 36 hours, the cell growth amount still less than 0.12 of OD600. When increase the CMC concentration to 10000 mg/L, the cell growth can obtain better condition for those five strains (Figure 3B). For the cellulose biotransformation, the CMC as the substrate for cell growth need achieved 10,000 mg/L then the cell of the bacteria can achieved the optimal growth level. Among those five strains, the strain of Pseudomonas sp. M1 obtain the highest growth amount with the OD600 of 0.5. In contrast, the
strain of *Stenotrophomonas* sp. got relatively low growth and the cell growth can apparent only after 44 hours for the OD_{600} of 0.13. It might characterize *Stenotrophomonas* sp. required a longer acclimation period for the cellulose biotransformation than the other four strains.

Overview, the growths of the cellulosic bacteria were actually highly depending on the concentration of the CMC. When CMC on the concentration of 1000 mg/L, most of the strains cannot growth effectively. When CMC increase to 5000 mg/L, the growth of the bacteria can obtain OD_{600} to 0.3 for the strain of *Pseudomonas* sp. M1, but the strain *Stenotrophomonas* sp. still not growth apparently. When increase CMC concentration to 9000 mg/L, the amount of the bacteria cells can be growing to the optimal results. When increase CMC to 10000 mg/L, the growth of bacteria not obviously increase simultaneously. For those five strains, the greatest growth of the bacteria was happened by the strain of *Pseudomonas* sp. M1 with the OD_{600} greater than 0.5. The smallest growth was happened by the strain of *Stenotrophomonas* sp. with the OD_{600} less than 0.3 after the growth for 44 hours. The other three strains obtain the growth effect among *Pseudomonas* sp. M1 and *Stenotrophomonas* sp. The exponential growth phase for those five strains was all finish before 48 hours culturing.

**The growth kinetic analysis**

As shown in (Table 1), the strain of *Pseudomonas* sp. M1 accessed the highest specific growth rate (μ_{max}) of 0.439 h^{-1}. It is the same level to the strain of *Pseudomonas* sp. M2 of the μ_{max} of 0.436 h^{-1}. The lowest μ_{max} was obtained by the strain of *Stenotrophomonas* sp. with the μ_{max} of 0.37 h^{-1}. The K of the strain means the affinity between the bacteria cell and the substrate (CMC). The higher of the K means the growth of the strain is higher depending on the substrate concentration. According to the results of the kinetic analysis, the strain *Achromobactor* sp. obtained the greatest K of 2510 mg/L. It indicated that the growth of *Achromobactor* sp. has higher relationship to CMC concentration than the other four strains. The lowest K obtained by the strain of *Stenotrophomonas* sp. with the K of 593 mg/L. It represent that the strain can grow when the CMC was exist, and the concentration of CMC is not too much impact to the cell growth. Concerning the substrate inhibition coefficient K_i which indicate the substrate concentration which can inhibit the growth of the cells. As shown in (Table 1), the strain *Pseudomonas* sp. M2 obtain the highest K_i indicates the strain can survive in the highest concentration of 36.8% of CMC in the culture medium. The lowest K_i was found from the strain of *Stenotrophomonas* sp. with 1.5% of CMC. All five strains obtained the K_i are over 15000 mg/L designated the strains are all feasible using CMC as the carbon source.

**The generation of the reduction sugar from the strains**

The ability for generation of the reduction sugar of a bacteria culture represented the cellulose biotransformation ability of the strains when use the cellulose as the sole carbon source. As shown in (Figure 4A), the strain increase obtain the reduction sugar accompany with the increase of the CMC concentration. The reduction sugar generation rate is positively propositional to the CMC concentration for all five strains. Among those five strains, *Pseudomonas* sp. M1 created the highest reduction sugar in most of the CMC concentration than the other four strains. In contrast, the strain *Achromobactor* sp. obtains the lowest reduction sugar than the other strains. As shown in (Figure 4B), *Pseudomonas* sp. M1 obtains 52 mg reduction sugar per 1g CMC. It was happened when the medium content 8 g/L of CMC. It is also the highest reduction sugar generation ratio in all the CMC concentration for those five strains. Among the five strains, the strain *Sphingomonas* sp. and *Pseudomonas* sp. M1 generated higher reduction sugar than the other three strains. The range of the reduction sugar generation ratio is between 16 to 52 mg/g CMC in this study. This result closely meets the consequence from the literature that the strain of *Pseudomonas* provided the ability of cellulose degradation [18].

**The acid resistant ability of *Pseudomonas* sp. M1**

The cellulose transformation can be easier happen in an acid than in a neutral environment. The acid resistant ability of the bacteria can help the strain to employ in an acid environment and promote the reaction cellulose biotransformation. According this, the strain with the ability of acid resistant can easier use for cellulose biotransformation than the others. After the acid resistant evaluation between those five strains was finished, the strain *Pseudomonas* sp. M1 had been approved the highest acid resistant ability since it can survived in low pH environment. As shown in (Figure 5), when the strain was growing in the medium with pH 6, the growth can achieve of OD_{600} to 1.0 during 20 hours. When pH value diminished to 5, the growth can achieve of OD_{600} to 1.0 during 28 hours. When pH decline to 4, the growth of the bacteria can only be achieve of OD_{600} to 0.8 during 28 hours. When the pH decline to 3, the strain need 50 hours to growing in the medium and obtain the OD_{600} to 0.8. This result showed
Mountain with the pH about 4.5. This study proposed the ability to tolerate the acid environment should be generated from the acclimation of the acid soil. The mechanism of the acid resistant ability of *Pseudomonas* sp. M1 needed further investigation.

### CONCLUSION

Five strains of cellulose biotransformation bacteria were isolated from this study. They are *Sphingomonas* sp., *Pseudomonas* sp. M1, *Achromobacter* sp., *Pseudomonas* sp. M2, and *Stenotrophomonas* sp. which isolated from the soil with abundant cellulose material and contaminated with petroleum compounds. The growth kinetic analysis of the strains shown the strain *Pseudomonas* sp. M1 obtain the greatest growth rate of 0.439 h⁻¹ with the saturation coefficient of 766 mg/L and the growth inhibition coefficient over 200,000 mg CMC/L medium. The greatest growth amount of the bacteria was *Pseudomonas* sp. M1 with OD₆₀₀ of 0.5 when the CMC concentration was 10,000 mg/L for 64 hours yield. In addition, the greatest reduction sugar generation ratio of 51 mg/g was happened when using 8 g/L CMC as the sole carbon source. The strain *Pseudomonas* sp. M1 obtain the optimal acid resistant ability in pH3 among those five strains.

### REFERENCES

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