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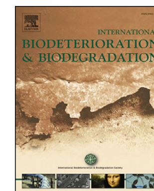

Isolation and identification of anaerobic bacteria from coconut wastewater factory for ethanol, butanol and 2, 3 butanediol production

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A B S T R A C T

Wastewater from coconut processing industry contains carbohydrates which are potential substrates for butanol and ethanol fermentation process. In this research, five isolates of anaerobic bacteria were screened from coconut wastewater. To identify the species and solvent production, the morphological characterization, 16S rRNA gene identification and biochemical test were performed. It was discovered that the isolate COCON2 belongs to the species *Pectinatus frisingensis* with 16S rRNA gene sequence identity of 98%, whereas COCON4, COCON9 and COCON11 are closely related to *Pectinatus portalensis* (with 16S rRNA sequence identity percentage of 98, 99 and 99% respectively). As for COCON6, it is similar to *Klebsiella oxytoca* with 98% 16S rRNA sequence similarity. The solvent production of *Pectinatus* spp. under anaerobic condition from 20 g/L mannose in 30 mL synthetic medium showed acetate as the main product (1.877–2.146 g/L). In conclusion, naturally selected bacteria have the ability to convert sugar into acetate and ethanol which are metabolites from cellular respiration in optimum condition.

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1. Introduction

Thailand is the sixth largest coconut producer of the world, contributing around 1.72 million tons or 2.8% of total production (Shashikumar and Chandrashekar, 2014). The food industry generates coconut waste which can be further divided into coconut solid and coconut wastewater. According to several reports, coconut wastewater is pollutant. The good news is, in biodegradation process, there are bacteria which have the ability to detoxify coconut wastewater by utilizing it as a carbon source (Khuwijitjaru et al., 2012). Yong et al. (2009) reported chemical compositions and nutrients in coconut water and compared them to those of coconut milk. As for coconut water, it contains various types of sugar such as sucrose, glucose and fructose (9.18, 7.25 and 5.25 mg/mL respectively). Previously, a coconut milk factory's in-house researcher studied environmentally-effected parameters such as chemical oxygen demanded (COD) by open reflux method and biological oxygen demanded (BOD) by Azide modification method. Earlier reports of CMS Engineering & Management Co., Ltd., 2012 indicated that the effluents from the coconut processing factory contained high levels of COD and BOD (5445 mg/L and 3100 mg/L respectively). Pollution caused by untreated wastewater release is a matter of concern as shown in the previous report of Nandana and Werellagama (2001) which stated that a factory processing 50,000 nuts per day discharges approximately 50 m^3 of wastewater per day.

Clostridium acetobutylicum, the strictly anaerobic, rod-shaped fermentative bacterium, is an efficient producer of ethanol and butanol. For this reason, many research groups have attempted to study the utilization of wastewater and ABE production from wastewater by this type of bacteria. Azimatun Nur et al. (2015) adapted coconut milk skim effluent into a growth medium for *Spirulina platensis*. However, only 20% v/v coconut milk skim

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effluent could be adapted into the synthetic medium. In 2011, Ouephanit et al. (2011) demonstrated that the solvent (ethanol and butanol) production of *C. acetobutylicum* ATCC824 and *C. butyricum* TISTR1032 could be applied with tapioca starch wastewater fermentation without substrate modification (0.144 and 1.81 g/L ethanol and butanol respectively). Ellis et al. (2012) reported the ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 using wastewater algae as the carbon source, which yielded 0.17 g/L butanol within 96 h. Nevertheless, no ethanol was gained from the process. In the more recent report of Comwien et al. (2015), ethanol and butanol (0.4 g/L and 0.6 g/L respectively) could be detected with naturally isolated *Clostridium* sp. in cellulosic ethanol wastewater fermentation. From a scientific point of view, these bacteria possess the ability to produce value-added chemicals from wastewater which is a cheap source of raw material. Despite such potential, the yields of butanol and ethanol are still limited due to low carbon concentration in wastewater substrates.

To convert this biological waste to other value-added products such as solvents by bioprocess (acetone-butanol-ethanol (ABE), 2, 3-butanediol and isopropanol), practical biocatalysts are necessary. In this research, solvent-producing *Clostridium* (*C. acetobutylicum* ATCC824 and *Klebsiella oxytoca* TISTR556) were used in wastewater application in comparison with the isolates from coconut wastewater. Appropriate microorganisms were isolated and identified for this approach, especially the consolidated bioprocess to compensate for traditional wastewater treatment. Isolates and selective media were generated for coconut waste living bacteria in anaerobic condition. All of the isolates were identified by the 16S rRNA sequence comparative analysis. Finally, the ABE production was detected by gas chromatography methods to observe the ability to ferment wastewater and produce chemical substances efficiently.

2. Material and methods

2.1. Samples analysis and bacterial strain/inoculums preparation

Sampling area is located in Nakhonpathom province in central of Thailand. Solid waste and waste samples were gifted from Thep Padungporn coconut milk factory. Physical properties of sample (pH, Biological oxygen demanded (BOD), Chemical oxygen demanded (COD), suspended solid (SS), Total dissolved solid (TDS), Total Kjeldahl nitrogen (TKN)) were analysed by environment and laboratory, CO., LTD. After screening process, culture were kept in 20% glycerol at −20 °C after incubate for 144 h. Initial cultures were transferred from frozen culture stock into serum bottle containing 30 ml of Cooked Meat medium. The cultures were incubated at 37 °C for 48 h, before three transfers to synthetic media for ABE production. *Clostridium acetobutylicum* is derived from American Culture Collection ATCC824. *Klebsiella oxytoca* is activated from lyophilized form of bacteria culture collection TISTR556.

2.2. Screening condition

Selective condition for screening was adapted by using Reinforced Clostridial Media for inoculated samples. After 3 times subculturing of pure isolate under anaerobic condition, pure isolate was collected from culture plate and activate the growth until the optical density OD600 reached to 1.0–1.2. The isolate is inoculated into synthetic media for ABE production medium containing (per L): 0.22 g $MgSO_4 \cdot 7H_2O$, 0.55 g KH_2PO_4, 0.55 g K_2HPO_4, 0.011 g $FeSO_4$, 1 mL of 1 g/L resazurin and 2.3 ml acetic acid. The mixture was adjusted to pH 6.5. Added 5 mL of 1.6 g/L para-aminobenzoic acid (PABA) and 4 mL of 0.02 g/L biotin, boiled and distributed to 100 mL bottle with butyl rubber septum. The media was degassed with N_2 gas mixture and autoclaved at 120 °C for 15 min. The medium is supplemented by 20 g/L initial different types of sugar including mannose and coconut water were used.

2.3. Biochemical test

Cell shape and spore stain of 48–72 h culture were examined by light microscope to determine cell morphology. The test for nitrate reduction, sulfite reducing, lecithinase and spore-forming were carried out by the method described in Burgey's Manual of Systematic Bacteriology (Forbes et al., 1998). Sulfite reducing ability is experimented by Differential Reinforced Clostridial Agar (Gibbs and Freame, 1965). Endospore formation was determined by Malachite-green staining under light microscope.

2.4. 16S rRNA identification and phylogenetic analysis

Genomic DNA was extracted from pure isolates with Bacterial DNA kit (OMEGA Bio-Tek, USA). The 16S rRNA fragments were amplified using universal DNA primer: 27F (5′AGAGTTTGGATCCTGGCTCAG′3) and 1492R (5′CGGTTACCTTGTTACGACTT′3) (Gillan et al., 1998). PCR amplification was performed according to the Taq DNA polymerase protocol (Biolabs, UK). The reaction condition were as follows initial denaturation at 98 °C for 30 s, 30 amplification cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 10 s, follow by a final extension at 72 °C for 10 min. The PCR products were purified and sequenced by the Pacific Science Co., Ltd. The nucleotide sequences of 16S rRNA were compared with the sequences data in the National Center of Biotechnology information (NCBI) using the basic local alignment search tool (BLAST) (Altschul et al., 1990). The evolutionary history of the isolate was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). All position containing gaps and missing data were eliminated. Evolutionary analysis were conducted in MEGA7 (Kumar et al., 2016).

2.5. Solvents production in batch culture

Inoculum preparation including three different stains, *Clostridium* sp. CNC, *C. acetobutylicum* ATCC 824 and *Klebsiella oxytoca* TISTR556, were incubated at 37 °C for 72 h, before transfers to fresh media. ABE production was performed in coconut water (CW) and wastewater from Thep Padungporn coconut milk factory including coconut wash-line wastewater (CCW) and total wastewater (TW), as substrates. The media were added to 50 mL bottles with 30 mL of media, flushed with N_2 gas mixture. The cultures were incubated with 10% inoculums under anaerobic condition without shaking at 37 °C for 168 h.

2.6. Solvents analyses methods

Liquid samples were taken from each of three replicates at 0, 24, 48, 72, 96, 120, 144 and 168 h. Growth of the isolate was measured at the optical density 600 nm (Zenyth 200rt spectrophotometer). The samples were centrifuged at 10,000 g for 10 min. Supernatant was analysed for end product by Gas chromatography (GC-2010A Shimadzu, Japan). ABE was detected by gas chromatography equipped with DB-WAX column and flame ionization detector (FID). The temperature of column, injector and detector was 45, 210 and 220 °C respectively. Helium was used as carrier gas. ABE and 2, 3-BDO concentration are calculated from the peak area after standard curve of concentration are plotted by linear regression.

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