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Isolation of a *Clostridium acetobutylicum* strain and characterization of its fermentation performance on agricultural wastes

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ABSTRACT

A new solvent-producing *Clostridium* has been isolated from soil used in intensive rice cultivation. The 16S rRNA analysis of the isolate indicates that it is closely related to *Clostridium acetobutylicum*, with a sequence identity of 96%. The new isolate, named *C. acetobutylicum* YM1, produces biobutanol from multiple carbon sources, including glucose, fructose, xylose, arabinose, glycerol, lactose, cellobiose, mannitol, maltose, galactose, sucrose and mannose. This isolate can also utilize polysaccharides such as starch and carboxylmethyl cellulose (CMC) for the production of biobutanol. The ability of isolate YM1 to produce biobutanol from agro-industrial wastes was also evaluated for rice bran, de-oiled rice bran, palm oil mill effluent and palm kernel cake. The highest concentration of biobutanol (7.27 g/L) was obtained from the fermentation medium containing 2% (w/v) fructose, with a total acetone–butanol–ethanol (ABE) concentration of 10.23 g/L. The ability of isolate YM1 to produce biobutanol from various carbon sources and agro-wastes indicates the promise of the use of this isolate for the production of biobutanol, a renewable energy resource, from readily available renewable feedstocks.

1. Introduction

Increasing global demand for alternative liquid fuels has drawn the attention of the scientific community to the production of biofuels from renewable resources via biological processes [1,2]. The production of butanol as a liquid fuel has also prompted several companies, such as DuPont, BP, Gevo Inc. and Green Biologics, to launch research and industrial scale production programs [3,4]. Butanol is the most suitable liquid biofuel to replace gasoline. Compared to ethanol, butanol has advantageous fuel properties, including a higher energy content and reduced sensitivity to temperature. Butanol is also less corrosive and does not require any modification for use in combustion engines [4–6]. Butanol can be produced by an anaerobic fermentation process called acetone–butanol–ethanol (ABE) fermentation using solvent-producing *Clostridium* [7,8]. One of the most significant problems in ABE fermentation by *Clostridium* strains is the low yield, attributed to the toxicity of butanol, which results in low final concentrations of fermentation products produced by batch culturing [9,10].

Although there have been numerous efforts to improve biobutanol production by genetically manipulating solvent-producing *Clostridium* species [9], genetically manipulated clostridia can be unstable [10]. Thus, screening for novel solvent-producing *Clostridium* strains that have the ability to produce large quantities of biobutanol and are able to ferment a wide range of sugars and agriculture wastes is of great importance to the field of biofuels.

The substrate cost in butanol fermentation contributes to 63% of the total production cost [7]. The use of low cost and renewable feedstock can reduce butanol production costs. Utilization of agricultural biomass in butanol production have already been achieved including palm oil mill effluent, rice bran, deoiled rice bran, corn stover, corn fiber, wheat barley, wheat bran, palm kernel cake, sago starch waste, rice straw, and wood chips [11–14]. Glycerol is a byproduct of the process of the transesterification of oils to biodiesel, and glycerol has been found to be a promising substrate for butanol fermentation because of its low-cost [15]. Approximately 10% of byproducts of biodiesel production is crude glycerol, which can be used for butanol production by Clostridia [16]. In this study,

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available agro-wastes and glycerol were fermented to biobutanol by the newly isolated strain of YM1 as inexpensive and renewable substrates.

Moreover, a number of studies have provided new methods for reducing the difficulties encountered in the biobutanol production process, which include the procurement of strains with a higher tolerance to butanol and the enhancement of yields through mutagenesis or the periodic collection of solvent, as well as extractive fermentation, to alleviate butanol toxicity [9,17,18].

For this study, we isolated 28 isolates of locally sourced biobutanol-producing *Clostridia*. Among the 28 solvent-producing cultures isolated, only one culture was selected for its ability to produce the highest concentrations of biobutanol as indicated by gas chromatography analysis. This isolate was identified as *Clostridium acetobutylicum* YM1 from the soil of a paddy field managed under a system of rice intensification (SRI). Moreover, the strain was demonstrated to utilize various carbon sources (mono, di, and polysaccharides) and agro-industrial wastes, suggesting a potential value for this isolate to produce biobutanol as an alternative, renewable and environmentally friendly fuel.

2. Materials and methods

2.1. Isolation of solvent-producing Clostridium

A solvent-producing *Clostridium* was isolated from the soils of the SRI paddy fields located in Ban 9, Parit 3, Sekinchan, Selangor, Malaysia. The submerged soil samples were inoculated after collection in sterilized Reinforced Clostridia Medium (RCM) in 100 mL serum bottles with a working volume of 50 mL, which were flushed with nitrogen to impart anaerobic conditions.

The RCM medium was thereafter incubated at 30 °C, and the gas production was observed for 5 days. The gas-producing cultures were then selected to inoculate RCM agar plates at 30 °C under anaerobic conditions using an anaerobic generation kit for 2 days. Single colonies were transferred to new plates with RCM agar and incubated at 30 °C under anaerobic conditions. The pure cultures were then Gram stained for microscopic characterization. Only Gram positive, rod shaped cells and gas-producing cultures were selected for further study.

To investigate their solvent production capabilities, an acetone test was administered to each pure culture. Positive acetone production was indicated by a change in the color of a colony or culture suspension from yellow to purple after the addition of 1 mL of a 5% sodium nitroperosside solution and drops of a 40% ammonium solution.

Twenty-eight isolates were showed similar Gram stain reaction (positive), rod-shaped, spore-forming ability and were able to produce acetone. Furthermore, all the 28 isolates were tested to produce biobutanol as indicated by gas chromatograph analysis. Among the 28 solvent-producing cultures isolated, only one culture was selected for its ability to produce the highest concentrations of biobutanol which named YM1.

2.2. Characterization

The genomic DNA of *C. acetobutylicum* strain YM1 was extracted and purified using a commercial DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer's protocol.

The 16S rRNA genes were amplified by PCR (Eppendorf, Germany) using a pair of universal primers; forward primer 8F (5′-AGA GTT TGATCC TGG-3′) and reverse primer 1392R (5′-CTC AGACGG GCG GTG TGT-3′) [19].

The PCR amplified DNA fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and then sequenced using a Biosystems Genetic Analyzer (BigDye® Terminator v3.1 cycle sequencing kit chemistry) by 1st Base, Malaysia.

A Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis was performed on the 16S rRNA gene sequence to identify the bacterial isolate. The sequence of the *C. acetobutylicum* YM1 isolate was deposited in the GenBank database with the accession number KC969670.

Using the MEGA 5 software [20], a phylogenetic tree of *C. acetobutylicum* strain YM1 was created based on the 16S rRNA sequence, which provides a suitable method for classification [21]. A bootstrap consensus tree was constructed using the neighbor-joining algorithm provided in the MEGA 5 program.

2.3. Media preparation and biobutanol fermentation

Gram-positive, rod-shaped, spore-forming and acetone-producing isolates were cultured in RCM medium (at 30 °C, 10% inoculum size and under anaerobic conditions) to evaluate their capabilities to produce ABE.

A high ABE-producer strain was considered and chosen for ABE production using multiple media, such as reinforced clostridial medium (RCM), P2 medium, tryptone yeast-extract acetate medium (TYA) and anaerobic sugar medium (AnS).

The RCM medium contains 30 g/L glucose, 10 g/L peptone, 10 g/L beef extract, 3 g/L yeast extract, 5 g/L sodium chloride, 0.5 g/L cysteine HCl, 3 g/L sodium acetate and 0.5 g/L agar.

TYA medium, which was also used as a fermentation medium to culture the inoculum, consisted of the following components: 30 g/L glucose, 6 g/L tryptone, 2 g/L yeast extract, 3 g/L ammonium acetate, 0.5 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$ and 0.01 g/L $FeSO_4{\cdot}7H_2O$. The AnS medium consisted of the following components: 30 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 3 g/L K_2HPO_4, 1 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgCl_2{\cdot}6H_2O$, 0.2 g/L $CaCl{\cdot}2H_2O$, and 1 g/L Na_2CO_3. The P2 medium was used as a fermentation medium and consisted of the following components: 30 g/L glucose, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}4H_2O$, 0.01 g/L $FeSO_4{\cdot}5H_2O$, 1.0 g/L yeast extract and 0.5 g/L cysteine. A final concentration of 80 μg/L biotin and 1 mL of a solution containing 1 mg/L 4-aminobenzoic acid were added to 1 L of P2 medium.

2.4. Analysis

Samples were collected and centrifuged at 7000 rpm for 10 min and then the supernatants were collected for analysis. The ABE, acetic acid, and butyric acid concentrations were measured using a gas chromatograph (7890A GC-System, Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a 30 m capillary column (Equity1; 30 m × 0.32 mm × 1.0 μm film thickness; Supelco Co, Bellefonate, PA, USA). The oven temperature was programmed to increase from 40 to 130 °C at a rate of 8 °C/min. The injector and detector temperatures were set at 250 and 280 °C, respectively. The carrier gas was helium (99.99%; NIG Gases Sdn. Bhd., Malaysia) at a flow rate of 1.5 mL/min [22].

The residual glucose concentration was determined using a GOD test kit according to the manufacturer's instructions.

3. Results and discussion

3.1. Isolation and characterization of C. acetobutylicum strain YM1

All of the 28 isolates were tested for ABE and biobutanol production in batch culture using RCM medium (glucose 20 g/L) under

anaerobic conditions, initial pH 6.2 and mesophilic temperature (30 °C). All isolates exhibited the capability to produce ABE with the range between 0.12 and 5.78 g/L while the biobutanol production was ranged between 0.01 and 3.98 g/L.

The highest production of ABE and biobutanol (5.78 g/L and 3.98 g/L, respectively) was obtained from culture YM1 which was selected for its ability to produce the highest concentrations of biobutanol as indicated by GC-FID analysis. Microscopic examination revealed that this isolate is a rod-shaped, Gram-positive, spore-forming bacteria. The genomic DNA from a culture of *C. acetobutylicum* YM1 was isolated and the 16S rRNA genes were amplified for characterization. Matching The BLAST analysis of the obtained sequence of culture *C. acetobutylicum* YM1 against the bacterial sequences in the GenBank database demonstrated that the sequence of this isolate is identical to those of *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* EA 2018, with 96% sequence identity. Subsequently, this isolate was identified as *C. acetobutylicum* strain YM1.

The 16S rRNA gene sequence analysis of the *C. acetobutylicum* YM1 isolate revealed that the isolate belongs to the genus *Clostridium*. The phylogenetic analysis further indicated that this strain clusters with the *Clostridium* group I because of its strong similarity to the amylolytic type strain *C. acetobutylicum* ATCC 824, as revealed by the analysis of the 16S rRNA [21]. The phylogenetic tree is presented in Fig. 1, including the relationships among strain YM1 and related *Clostridium* species.

3.2. Biobutanol production from multiple carbon sources

In this study, the *C. acetobutylicum* YM1 isolate produced biobutanol when cultivated in media containing various carbon sources (pentoses, hexoses, disaccharides and polysaccharides). The sugars evaluated (2% w/v) included xylose, arabinose, glucose, galactose, fructose, mannose, sucrose, starch and carboxymethyl cellulose (CMC). The isolate was cultured in TYA media (at 30 °C and an initial pH of 6.5, with a 10% inoculum size) under anaerobic conditions by sparging nitrogen gas through the media before inoculation. Fermentation was assessed over 72 h using 100-mL serum bottles with a working volume of 50 mL.

The newly isolated *C. acetobutylicum* YM1 strain can produce solvents (acetone, butanol and ethanol) and acids (acetic and butyric acids) from all of the above-mentioned sugars (Table 1). The *C. acetobutylicum* YM1 strain demonstrated the ability to grow and produce solvents over a range of incubation temperatures, but 30 °C was the optimum incubation temperature for solvent production (data not shown).

The maximum biobutanol production (7.27 g/L) was observed when the culture was grown on 2% fructose.

C. acetobutylicum YM1 exhibited the ability to consume various sugars. Notably, the highest amounts of biobutanol and ABE production, 7.27 and 10.22 g/L, respectively, were achieved when fructose (2% w/v) was used as the sole carbon source. These values were 2-fold the levels of biobutanol and ABE produced in cultures relying on glucose (2% w/v). In contrast, Isar and Rangaswamy (2012) reported that only negligible amounts of biobutanol were produced from 20 g/L of fructose by a batch culture of *Clostridium beijerinckii* ATCC 10132 [23].

Fructose is more preferable for biobutanol production by *C. acetobutylicum*. In biobutanol fermentation using mixed sugars of glucose and fructose by *C. acetobutylicum*, the fructose utilization was significantly improved comparing to glucose under same conditions of oxidation–reduction potential [24].

It was found that when *Clostridium thermoaceticum* was grown in a mixture of glucose and fructose, the utilization of glucose does not start until the fructose is almost completely depleted [25]. The study concluded that fructose inhibited the fermentation of glucose due to the competition between glucose and fructose as substrates for the enzyme involved in active transport of the sugars into the cell, or a repression by fructose of the synthesis of an enzyme needed for glucose utilization [25].

The first reaction in fructose catabolism is the formation of fructose 1-phosphate by phosphoenolpyruvate (PEP):fructose phosphotransferase while the first catabolism of glucose is the formation of glucose 6-phosphate via phosphoenolpyruvate (PEP):glucose phosphotransferase then to fructose 6-phosphate by glucose-6-phosphate isomerase [26].

When xylose and arabinose were assessed as carbon sources, greater amounts of biobutanol and ABE were produced by *C. acetobutylicum* YM1 compared to glucose, 4.57 and 6.24 g/L, respectively. The ability of this strain to utilize pentose sugars such as xylose and arabinose as carbon sources is an important characteristic because this indicates the potential of this strain to use inexpensive lingocellulosic materials as substrates. This potential is based on the production of pentoses via the degradation or hydrolysis of lingocellulosic materials results.

Strain YM1 grown, in a medium containing 20 g/L of glycerol, is

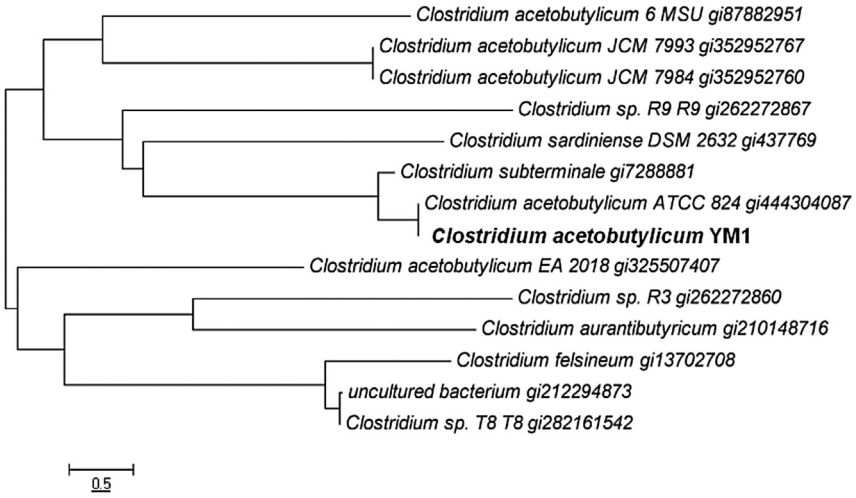

Fig. 1. Phylogenetic tree of *C. acetobutylicum* strain YM1 based on 16S rRNA gene sequences using the Neighbour-joining algorithm provided in MEGA 5 program. The scale bar indicates evolutionary distance.

Table 1
Biobutanol production from different carbon sources (2%) by *C. acetobutylicum* YM1 isolate.

Sugar type (2% w/v)	Solvent production (g/L)				Acid production (g/L)			Butanol productivity (g/L.h)
	Acetone	Butanol	Ethanol	ABE	Acetic	Butyric	Total acids	
Glucose	1.35 ± 0.11	3.71 ± 0.25	0.17 ± 0.04	5.23 ± 0.33	2.01 ± 0.19	1.16 ± 0.32	3.17 ± 0.14	0.05
Glycerol	1.64 ± 0.10	3.13 ± 0.07	0.33 ± 0.06	5.10 ± 0.08	0.09 ± 0.01	0.10 ± 0.06	0.19 ± 0.07	0.04
Maltose	1.78 ± 0.11	4.32 ± 0.13	0.35 ± 0.07	6.45 ± 0.06	0.10 ± 0.01	1.05 ± 0.14	1.15 ± 0.08	0.06
Cellobiose	1.81 ± 0.02	4.22 ± 0.17	0.31 ± 0.11	6.34 ± 0.04	0.16 ± 0.03	2.23 ± 0.12	2.39 ± 0.10	0.06
Lactose	1.65 ± 0.07	1.44 ± 0.06	0.24 ± 0.06	3.33 ± 0.08	0.11 ± 0.01	3.84 ± 0.08	3.95 ± 0.08	0.02
Mannitol	1.59 ± 0.06	3.66 ± 0.06	0.30 ± 0.04	5.55 ± 0.08	0.10 ± 0.01	1.99 ± 0.06	2.09 ± 0.08	0.05
Galactose	0.11 ± 0.01	0.36 ± 0.01	0.10 ± 0.04	0.57 ± 0.03	3.45 ± 0.23	4.22 ± 0.40	7.67 ± 0.64	0.01
Fructose	2.31 ± 0.32	7.27 ± 0.65	0.64 ± 0.02	10.22 ± 0.93	0.90 ± 0.06	0.55 ± 0.17	1.45 ± 0.23	0.10
Mannose	0.88 ± 0.01	2.60 ± 0.01	0.13 ± 0.05	3.61 ± 0.08	2.21 ± 0.09	2.49 ± 0.48	4.70 ± 0.58	0.04
Xylose	1.55 ± 0.004	4.57 ± 0.09	0.29 ± 0.19	6.41 ± 0.28	1.33 ± 0.27	0.73 ± 0.12	2.06 ± 0.38	0.06
Arabinose	2.28 ± 0.06	6.24 ± 0.08	0.44 ± 0.05	8.96 ± 0.19	1.64 ± 0.40	0.68 ± 0.11	2.32 ± 0.51	0.09
Sucrose	0.50 ± 0.02	0.98 ± 0.08	0.09 ± 0.02	1.57 ± 0.09	3.12 ± 0.05	3.72 ± 0.94	6.84 ± 1.0	0.01
CMC	1.12 ± 0.02	3.67 ± 0.03	0.23 ± 0.06	5.02 ± 0.01	0.72 ± 0.04	0.79 ± 0.30	1.51 ± 0.34	0.05
Starch	1.38 ± 0.19	3.14 ± 0.08	0.15 ± 0.10	4.67 ± 0.17	1.43 ± 0.25	1.30 ± 0.27	2.73 ± 0.52	0.04

capable of producing 3.13 g/L of biobutanol, and this capability of this strain represents a potential mechanism for biobutanol production from the waste of the biodiesel industry.

Strain YM1 can utilize cellobiose (2% w/v) efficiently and produces 4.22 g/L of biobutanol with 6.34 g/L of total ABE. *C. acetobutylicum* ATCC 824 has been reported to produce 2.4 g/L of biobutanol from 2% (w/v) cellobiose [27]. Isolate YM1 demonstrated a higher level of biobutanol production from cellobiose compared to *C. acetobutylicum* ATCC 824. Servinskt et al. (2010) reported that *C. acetobutylicum* ATCC 824 is capable of utilizing cellobiose directly via phosphotransferase systems (PTSs) and that cellobiose is hydrolyzed to glucose by intracellular glucosidases for further metabolism. The use of cellobiose and glucose by phosphotransferase systems in *C. acetobutylicum* ATCC 824 have also been reported [28]. The direct uptake of cellobiose by strain YM1 is very important because cellobiose can be generated from cellulose by endo-glucanase and cellobiohydrolase without the activity of β-glucosidase. The direct utilization of cellobiose and xylose in the biobutanol fermentation process leads to the simplification of the saccharification process and decreases the cost of hydrolysis by enzymes, in addition to achieving efficient production of biobutanol [29]. Recently, *Clostridium saccharoperbutylacetonicum* N1-4 was reported to utilize cellobiose and xylose to produce biobutanol efficiently without carbon catabolite repression [29].

The use of CMC as a polysaccharide resulted in the production of a similar concentration of biobutanol as from glucose (3.67 g/L *vs.* 3.71 g/L). This similarity indicates that isolate YM1 may possess cellulosomes, which are extracellular multienzyme complexes that aid in the hydrolysis of cellulose to simple sugars. The ability to hydrolyze cellulose and produce quantities of biobutanol comparable to those produced from glucose is of unique potential value.

Recently, Bramono et al. [19], reported that strain *Clostridium* sp. BOH3 produced hydrogen and negligible amounts of biobutanol (0.02 g/L biobutanol) from cellulose and xylan, whereas our strain can produce biobutanol (3.67 g/L) from 2% (w/v) CMC under similar conditions. It has been reported that solvent-producing *Clostridia* are characterized by a weak ability to utilize cellulosic substrates because the enzymes produced do not adhere to cellulose [30,31].

The use of disaccharides, such as sucrose, resulted in low concentrations of biobutanol but the highest amounts of biogas (data not shown). Disaccharides such as sucrose need to be degraded first to produce glucose and fructose and this activity required energy. As a result, the biobutanol yield and growth of isolate YM1 were lower than monosaccharides.

Wang and Blaschek (2011) reported that glucose and fructose were consumed earlier than sucrose when *C. beijerinckii* was grown in a mixture of glucose, fructose and sucrose and in the end of the fermentation; only 50% of the sucrose was utilized while 70% of the glucose and fructose were consumed. They also found that *C. beijerinckii* capable to degrade sucrose but the monosaccharides glucose and fructose were more preferable for biobutanol production [32].

A small amount of biobutanol was produced from galactose compared to other hexoses. Isolate YM1 was also capable of utilizing mannose to produce a moderate concentration of biobutanol (2.6 g/L).

The concentrations of biobutanol produced by strain YM1 from 2% (w/v) of maltose, mannitol or lactose were 4.32, 3.66 and 1.44 g/L, respectively. The fermentation of maltose resulted in higher amounts of biobutanol compared to that produced from glucose, while mannitol fermentation generated a similar amount of biobutanol to those produced from CMC and glucose.

The low biobutanol concentrations produced from galactose, sucrose and lactose can be attributed to the limitation of reutilization of butyric acid and acetic acid as indicated by the residual amounts of these acids (Table 1). This may be ascribed to the acid crash phenomenon. Acid crash is a phenomenon rarely occurring if the pH is not controlled in the non-dissociated acid concentration in batch fermentation. When non-dissociated acid concentration in the broth reaches more than 50–60 mmol/L, ABE fermentation completely stopped. A decrease in pH due to the accumulation of acids causes a decrease in the unsaturated to the saturated ratio of membrane lipids (U/S ratio) and in an increase in cyclopropane fatty acids [33]. The accumulations of organic acids in acidogenic phase decreased the culture pH, while a decrease in the level of residual acids related to the reutilization of acids can result in a rise in culture pH [34]. In solvent-producing Clostridia, galactose, inulin, and mannitol are partially utilized [7].

The utilization of starch by isolate *C. acetobutylicum* YM1 indicates that this strain exhibits amylolytic activity, with 3.14 g/L biobutanol produced from 20 g/L starch. Secretions of amylolytic enzymes during the growth of *Clostridium* species grown on starch have been reported [35,36]. Starch can represent an alternative and inexpensive carbon source for ABE fermentation because of its high abundance and low cost [37–39].

3.3. Biobutanol production from agro-wastes

The use of agricultural residues for the production of biofuels such as biobutanol is one potential alternative to reduce the cost of biobutanol production because agrowastes are characterized by a high availability and very low cost. The cost of the substrate has

been reported to contribute up to 63% of the total production cost of ABE fermentation [7].

In this study, agroindustrial wastes, including rice bran (RB), de-oiled rice bran (DRB), palm oil mill effluent (POME) and palm kernel cake (PKC), that are readily available in Malaysia were investigated for their utilization by the *C. acetobutylicum* YM1 isolate to produce biobutanol without any pre-treatment or enzymatic hydrolysis.

The results demonstrated that isolate *C. acetobutylicum* YM1 can utilize these substrates, but the amounts of biobutanol production varied from 0.2 to 3.49 g/L depending on the nature of the sugar and its concentration in the substrate (Table 2). The highest concentrations of ABE and biobutanol were obtained from DRB (5.31 and 3.49 g/L, respectively). *C. saccharoperbutylacetonicum* N1-4 has also been reported to produce 5.16 and 3.31 g/L of ABE and biobutanol, respectively, when DRB was supplemented with P2 medium [12].

The low production levels of biobutanol from POME and PKC can be ascribed to their low sugar contents. The initial reducing sugar contents in POME and PKC were 5.05 and 10 g/L, respectively, and POME has been reported to inhibit the growth of *C. saccharoperbutylacetonicum* N1-4 [40]. The growth of *C. saccharoperbutylacetonicum* N1-4 and the production of ABE were significantly improved only when the POME hydrolysate was treated with a XAD4 resin to remove growth inhibitors.

A comparable content of reducing sugar was produced from DRB and PKC, but the amount of biobutanol production was greater in the presence of DRB. These findings can be attributed to the nature of the substrate. DRB contains additional carbon sources such as starch that are absent from PKC. Moreover, limitation by the carbon source in the fermentation medium of solvent-producing *Clostridium* limits the stimulation of solvent production and only acids are produced [41]. A concentration of greater than 10 g/L of glucose is required to shift from acid to solvent production in batch cultures of *C. acetobutylicum* [42]. These results demonstrate that the YM1 isolate can utilize agrowastes as carbon sources for the efficient production of biobutanol.

3.4. Effects of medium composition on biobutanol production by isolate YM1

To investigate the effect of the choice of nutrient media on biobutanol production by the newly isolated *C. acetobutylicum* YM1, four media were evaluated. These media included RCM, AnS, P2 and TYA, and all media contained 30 g/L glucose. Among these four media, growth was most stable and rapid in RCM, while TYA was the best medium for biobutanol production, followed by P2 medium (6.2 and 5.69 g/L, respectively) (Fig. 2). In the TYA medium, the production of biobutanol was 6.2 g/L and the total solvent production (ABE) was 8.93 g/L (Fig. 2). Compared to the RCM and AnS media, the concentrations of biobutanol and ABE produced in the TYA medium were 2-fold and 3-fold those generated from RCM and AnS, respectively (Fig. 2).

Table 3 compares the results obtained in this study to those from previous reports. It is clear that *C. acetobutylicum* strain YM1 can produce a greater amount of biobutanol from 20 g/L glucose (3.71 g/L) compared to the corresponding levels of production by either *C. beijerinckii* 10132 (3.2 g/L) [23] or *C. saccharoperbutylacetonicum* N1-4 (2.11 g/L) [43]. Moreover, Bramono et al. (2011) reported that *Clostridium butyricum* strain BOH3 produced only 4.67 g/L biobutanol from 30 g/L glucose, whereas our isolate can produce a higher concentration of biobutanol (6.2 g/L) from 30 g/L glucose within 96 h (Fig. 3).

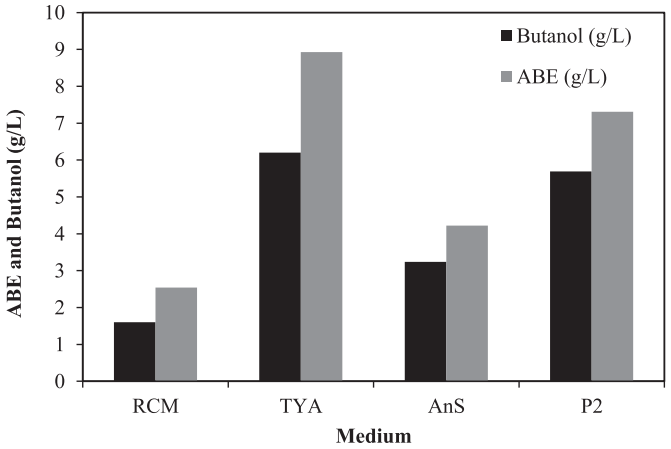

Fig. 2. Biobutanol production using different media containing 30 g/L glucose by isolate YM1.

Our isolate *C. acetobutylicum* YM1 can also utilize xylose successfully to produce biobutanol. The amount of biobutanol produced by the *C. acetobutylicum* YM1 isolate from 20 g/L xylose was similar to that produced by *C. butyricum* strain BOH3 from 30 g/L xylose as reported by Bramono et al. (2011). These findings indicate the ability of *C. acetobutylicum* YM1 to produce relatively larger amounts of biobutanol from smaller amounts of substrates compared to other previously characterized strains.

In addition, fermentation of 50 g/L glucose by *C. acetobutylicum* YM1 produced higher biobutanol concentration (17.01 g/L) compared with biobutanol produced by *C. beijerinckii* BA101 [44], *C. acetobutylicum* ABE 1201 [45] and *C. beijerinckii* P260 [46] from 60 g/L glucose (13.7, 11.92 and 13.89 g/L, respectively). *C. acetobutylicum* YM1 showed the highest productions and yields of biobutanol among other solventogenic strains (Table 3). Such result indicates its superior potential for application in production of biobutanol.

4. Conclusions

A new mesophilic and biobutanol-producing bacterial strain, *C. acetobutylicum* YM1, was isolated from agriculture soil. This strain can consume a broad range of sugars and agro-wastes and efficiently produce biobutanol. The strain also has the ability to produce biobutanol from xylose, arabinose and cellulose, indicating its potential capability to utilize agricultural wastes. Its ability to

Table 2
Biobutanol production from available agroindustrial wastes in Malaysia by *C. acetobutylicum* YM1.

Medium	Reducing sugars (g/L)	Solvent (g/L)				Acids (g/L)			ABE productivity (g/L.h)
		Acetone	Butanol	Ethanol	ABE	Butyric	Acetic	Total acids	
RB 10%	6.70	1.00 ± 0.07	1.42 ± 0.017	0.04 ± 0.01	2.46 ± 0.11	2.50 ± 0.07	0.22 ± 0.04	2.72 ± 0.03	0.03
DRB 10%	10.5	1.77 ± 0.03	3.49 ± 0.10	0.05 ± 0.08	5.31 ± 0.21	2.05 ± 0.66	0.17 ± 1.55	2.22 ± 2.21	0.07
POME	5.05	0.05 ± 0.0	0.22 ± 0.01	0.02 ± 0.0	0.29 ± 0.02	0.32 ± 0.0	0.06 ± 0.03	0.38 ± 0.03	0.003
PKC 10%	10.0	0.07 ± 0.0	0.30 ± 0.01	0.03 ± 0.0	0.40 ± 0.01	0.27 ± 0.01	0.02 ± 0.0	0.29 ± 0.01	0.01

Table 3
Comparison of biobutanol production from isolate YM1 and others *Clostridium* strains.

Culture	Medium	Substrate	Fermentation time (h)	Fermentation conditions			Solvent production (g/L)			Reference
				Temperature	Inoculum	pH	Acetone	Butanol	Ethanol	
C. beijerinckii 10132	AnS	Glucose 20 g/L	84	37 °C	2%	6.5	–	3.20	–	[23]
C. saccharoperbutylacetonicum N1-4	TYA	Glucose 20 g/L	72	30 °C	10%	6.5	0.9	2.11	0.02	[43]
Strain YM1	TYA	Glucose 20 g/L	72	30 °C	10%	6.5	1.35	3.71	0.17	This study
C. butyricum strain BOH3	MSM	Glucose 30 g/L	120	35 °C	10%	5 −5.5	2.65	4.67	0.36	[19]
Strain YM1	TYA	Glucose 30 g/L	96	30 °C	10%	6.5	2.66	6.20	0.07	This study
C. saccharoperbutylacetonicum N1-4	TYA	Xylose 30 g/L	96	30 °C	10%	6.5	0.84	1.23	0.15	[43]
C. butyricum strain BOH3	MSM	Xylose 30 g/L	144	35 °C	10%	5 −5.5	2.88	4.63	0.61	[19]
Strain YM1	TYA	Xylose 20 g/L	96	30 °C	10%	6.5	1.55	4.57	0.29	This study
C. beijerinckii BA101	P2	Glucose 60 g/L	68	35 °C	5%	6.5	4.4	13.70	0.5	[44]
C. acetobutylicum ABE 1201	P2	Glucose 60 g/L	88	37 °C	10%	6.5	5.21	11.92	2.08	[45]
C. beijerinckii P260	P2	Glucose 62.5 g/L	70	35 °C	6%	6.5	7.70	13.89	1.66	[46]
Strain YM1	OM	Glucose 50 g/L	96	30 °C	10%	6.2	3.88	17.01	0.83	This study

AnS: Anaerobic Sugar Medium.
MSM: Mineral Salts Medium.
OM: Optimized Medium.
TYA: Tryptone Yeast-Extract Acetate Medium.

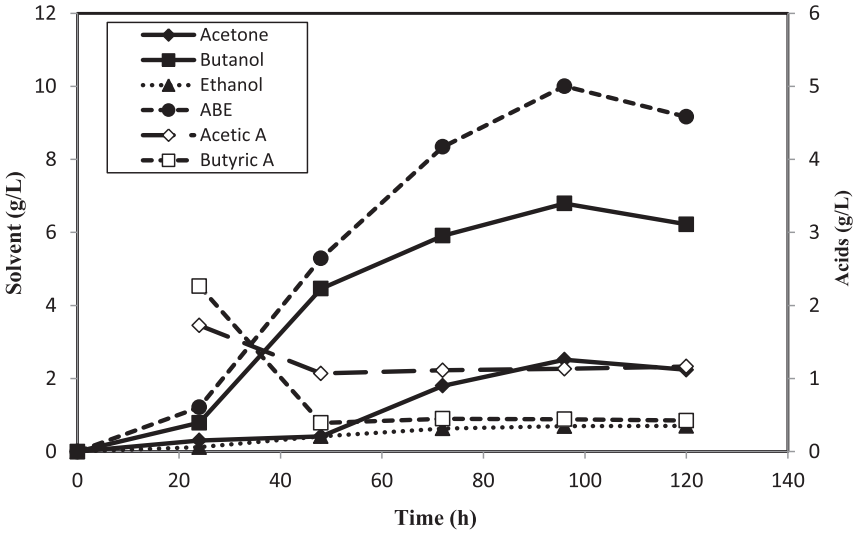

Fig. 3. Biobutanol fermentation profile from 30 g/L glucose in batch culture of *C. acetobutylicum* YM1.

utilize mannose suggests that this isolate also has potential value for the production of biobutanol from Palm Kernel Cake (PKC), which is inexpensive and abundant. Starch was also consumed efficiently by this strain, indicating its ability to use starch as an inexpensive substrate for biobutanol production.

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List of abbreviations:

ABE:: Acetone–Butanol–Ethanol
SRI:: System of Rice Intensification
POME:: Palm Oil Mill Effluent
PKC:: Palm Kernel Cake
RB:: Rice Bran
DRB:: De-oiled Rice Bran
GC-FID:: Gas Chromatograph equipped with a Flame Ionization Detector
TYA:: Tryptone Yeast-Extract Acetate Medium
RCM:: Reinforced Clostridial Media
AnS:: Anaerobic Sugar Medium
OM:: Optimized Medium
MSM:: Mineral Salts Medium