Short Communication

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High-efficient production of biobutanol by a novel *Clostridium* sp. strain WST with uncontrolled pH strategy

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Abstract

A novel *Clostridium* sp. strain WST isolated from mangrove sediments demonstrated its unique characteristics of producing high titer of biobutanol from low concentration of substrates via anaerobic fermentation. The strain is able to convert glucose and galactose to high amount of biobutanol up to 16.62 and 12.11 g/L, respectively, and the yields of 0.54 and 0.55 g/g were determined to be much higher than those from the previous reports on Clostridial batch fermentation. Moreover, the inherent strong regulatory system of strain WST also prompts itself to perform the fermentation process without any requirement of pH control. In addition to tolerance of high butanol concentration and negligible production of by-products (e.g., ethanol or

acids), this strain has immense potential for the sustainable industry-scale production of biobutanol.

Keywords: *Clostridium* sp.; Butanol; High yield; pH control; Microbial fermentation

1. Introduction

In recent decades, the rapid consumption of fossil fuels (e.g., coal, petroleum and natural gas) and increasing energy demand have led the government and researchers to seek a new form of sustainable energy from renewable resources with environmentally friendly characteristics over those petroleum-based ways involved in energy production (Xin et al., 2017). Biobutanol, as one of the novel biofuel forms, possesses lower volatility, lower hygroscopicity and is less corrosive to equipment than ethanol, providing a safer and more convenient process for its storage and transportation, without any major modification to current engine systems (Lipovsky et al., 2016). In addition, the energy density and octane rating of butanol is comparable to gasoline, allowing it to be miscible with gasoline in any ratio, which make it a superior fuel extender (Luo et al., 2017).

So far, research on biobutanol production is mainly concentrated in the field of microbial fermentation, and strains from *Clostridium* genus are widely used due to their solvents-producing capability (Al-Shorgani et al., 2015). Most Clostridial strains can utilize simple sugars (e.g., glucose, galactose and xylose) to produce biobutanol via the acetone-butanol-ethanol (ABE) pathway; however, the complexity of feedstock and occurrence of by-products exponentially increase the capital cost and difficulty in separation and purification of biobutanol. Algal biomass is considered to be an untapped

renewable resource for biofuels production due to its high content of fermentable sugars (e.g., galactose and glucose) with negligible level of lignin that can further minimize the cost of feedstock pretreatment into utilizable substrates (Wu et al., 2017c). Another important issue to be addressed in biobutanol application is to enhance the production and yield. Xin et al. (2016) utilized glucose and xylose (60 g/L) to obtain butanol concentration of 5.0 g/L and 2.5 g/L, respectively, while Ou et al. (2017) employed *C. cellulovorans* to achieve butanol yield of only 0.134 g/g from glucose. Even those well-studied Clostridial strains such as *C. acetobutylicum* strain ATCC824 and *C. beijerinckii* strain NCIMB8052, has relatively lower butanol yield indicating that the industrialization of microbial fermentation process is still strongly hindered to a certain degree. Moreover, most *Clostridium* species require pH adjustment to facilitate the solventogenic process for butanol synthesis (Al-Shorgani et al., 2017). He et al. (2017) performed controlled ABE fermentation of *C. beijerinckii* strain IB4 by maintaining the pH at 6.8 to reach the optimal yield of 0.23 g/g, which further increases the cost if applied to industrial process.

In this study, a new strain WST isolated from mangrove sediments, identified to be *Clostridium* sp. via phylogenetic analysis, demonstrated exceptional butanol production with a higher yield rather than that by other reported butanol-producing strains during the glucose and galactose fermentation process. Moreover, its tolerance against the higher butanol concentration, as well as the strong pH-regulatory system of this strain without the requirement of additional pH adjustment throughout the whole fermentation demonstrate the potential of this strain for the large-scale industrial application.

2. Materials and methods

2.1. Sample collection and microbial cultivation

Soil samples collected at a depth of ~10-20 cm from the mangrove sediment located in Chenghai District (Shantou, China) were anaerobically incubated at 70 ℃ for 30 min for selection of spore-forming bacterial strains. The heat-treated samples were then transferred to the anaerobic fermentation medium containing glucose (30 g/L) as the sole carbon source for microbial enrichment (Wu et al., 2017b). After 3-4 times of transfer, the enriched bacterial consortia were serially diluted, spread onto the reinforced Clostridia medium (RCM) agar plate and incubated anaerobically for 24-48 h. The formed single colony was picked and inoculated into the same liquid culture medium for the chromatographic analysis on its fermentative product profile and strain identification.

2.2 Strain identification and phylogenetic analysis

Genomic DNA of strain WST was extracted by commercial genome extraction kit (Dongsheng, China) for the amplification of 16S rRNA gene sequence using the primer set of 27F and l492R (Wu and He, 2015). The cloned PCR fragment was sequenced by Beijing Genome Institute (Shenzhen, China), and BLAST analysis was performed to identify the obtained sequence based on comparison of the available 16S rRNA gene sequences of *Clostridium* species from GenBank. A phylogenetic tree was constructed using bootstrap analysis and neighbor-joining algorithm by MEGA 6 software (Tamura et al., 2013).

2.3 Batch fermentation for biobutanol production

The anaerobic defined fermentation medium without carbon source was sealed and flushed with N_2 before sterilization. Glucose (or galactose) was filter-sterilized and added as the sole carbon source to each bottle to achieve a final concentration of ~30 g/L. Batch fermentation of strain WST for biobutanol production was carried out in 100 mL serum bottle, containing 50 mL of the culture medium with an initial pH of 6.0, and incubated in a shaking incubator (150 rpm) at 30 °C for 120 h. For the pH-control fermentation, the culture medium was maintained at the respective pH value of 5.0-7.0 by manually adding the NaOH solution (3 M) every 12 h during the whole fermentation. At intervals of 24 h, samples were collected and treated with 2 M of HCl solution according to the method of Sun et al. (2018) before applying to the gas chromatography (GC) analysis. All of the experiments were performed in triplicate and averages of parameters were determined.

2.4 Analytical assay

Sample preparation and quantification of fermentation products, especially for hydrogen and biosolvents by gas chromatography were performed as described previously (Wu et al., 2017c; Xin et al., 2017). During fermentation, the cell density (OD_{600}) was measured with a spectrophotometer (UVmini-1240; Shimadzu, Japan), while the concentration of residual substrates (e.g., glucose and galactose) was estimated via the 3,5-dinitrosalicylic acid (DNS) method.

3. Results and discussion

Mangrove ecosystem is usually regarded as complex and dynamic with varying physiological and nutritional conditions that offers diverse and distinct microbes with

various potential biotechnological applications (Gomes et al., 2011). Through a series of enrichment and isolation process, one bacterial strain, namely WST, was obtained as an individual colony on RCM plate under anaerobic condition. According to the BLAST comparative analysis based on 16S rRNA gene sequencing, strain WST demonstrated higher similarity to *C. diolis* strain S33-KKU (KT799798), followed by *C. diolis* strain DSM 5431 (AJ458418) and *C. beijerinckii* strain DSM 791 (X68179). 16S rRNA gene sequences of this strain have been deposited into GenBank under the accession number of MG738333. The phylogenetic tree constructed via the neighbor-joining maximum likelihood and minimum-evolutionary strategy revealed that the strains from genus *Clostridium* formed a monophyletic clade (Collins et al., 1994), and strain WST and *C. diolis* strain DSM 5431 were determined to be in the same branch (Fig. 1). Previously, *C. diolis* strain DSM 5431 was reported to be able to produce butyric acid and acetic acid from glucose or generate 1,3-propanediol when utilizing glycerol as the substrate (Kaur et al., 2013); however, this is the first report to illustrate the physiological and metabolic characteristics of *C. diolis* strain WST with the capability of producing biobutanol from glucose so far.

To investigate butanol production by strain WST, batch fermentation process using glucose as carbon source was carried out at the initial pH of 6.0. As indicated in Fig. 2a, the cells of strain WST enter into exponential growth phase to attain the maximal cell density (OD_{600nm}) of 8.95 at 48 h with the consumption of ~80% of glucose. The highest production of biobutanol was observed to be up to 16.62 g/L, with 12.08 L/L of biohydrogen as byproduct detected at the cultivation time of 120 h (Fig. 2). During

varying incubation periods from 36 to 48 h, strain WST also demonstrates the typical biphasic fermentation profile of the solventogenic Clostridial strains including acid formation followed by the re-exploitation of acids for solvent generation. The concentration of acids (e.g., acetic acid and butyric acid) in the medium was finally diminished to negligible levels. The obtained yield of 0.54g/g further reveals that strain WST belongs to a highly efficient butanol-producing strain that is able to generate higher amount of butanol by utilizing lower concentration of glucose rather than other previously reported wild-type *Clostridium* strains (Wu et al., 2017a; Xin et al., 2016).

In addition, strain WST was also examined for its efficiency of butanol generation from galactose, one of the major sugars present in the marine algal biomass that can be used as another potential source of future biofuels conversion (Wu et al., 2017c). The culture medium supplemented with galactose concentration of 30 g/L resulted in the respective biobutanol and biohydrogen production up to 12.11g/L and 4.22 L/L with the butanol yield of 0.55 g/g after 120 h of fermentation (Fig. 3). In general, the butanol production by *Clostridium* sp. included the concomitant generation of $NAD(P)^+$ that is related to the biomass synthesis as well as biohydrogen production, and the release of H_2 causes the deficiency of $NAD(P)^+$ to reduce the formation of butanol (Liu et al., 2013). Thus, it explains the observation that the galactose supplemented fermentation can possess a similar butanol yield to that from glucose via a lower bacterial biomass (OD_{600nm}=5.20) and H_2 production. With regard to the butanol yield, Xin et al. (2014) reported the yield of 0.25 g/g was obtained from 60 g/L of xylose by *Clostridium* sp. strain BOH3 when maintaining the culture pH at 5.0; while Wu et al. (2017a) employed

C. beijerinckii strain IB4 to generate the yield of 0.23 g/g from 60 g/L of glucose. In addition, Sun et al. (2018) recently utilized two newly isolated Clostridial strains to achieve a butanol yield up to 0.28 g/g from 60 g/L of galactose. *C. pasteurianum* was observed to produce butanol using glycerol as the substrate (Lin et al., 2015; Malaviya et al., 2012), and the most comparable study achieved a butanol yield of 0.41 g/g of butanol when applying *C. pasteurianum* strain GL11 to utilize 60 g/L of glycerol (Xin et al., 2016). To the best knowledge, the butanol yield by strain WST using glucose or galactose in present study represents the highest production in available reported batch fermentation with comparatively lower concentration of substrates strongly demonstrating the potential of applying this strain to the large-scale industrial applications.

On the other hand, most Clostridial strains need additional pH control to maintain the external pH to facilitate solventogenesis (Lin et al., 2015; Wu et al., 2017a; Xin et al., 2016), as the pH of ABE fermentation medium has a major impact on the intracellular biosynthetic pathways and biophysical characteristics. Thus, the effect of controlled pH over the butanol production by strain WST was investigated in batch fermentation by maintaining pH at the range from 5.0 to 7.0. It was indicated in Fig. 4 that the controlled pH strategy did not have a significant effect on butanol production, whether glucose or galactose was used as the substrate, and the fermentation profile under the controlled pH of 5.0 represented the similar production levels of (16.40 and 11.86 g/L) to that without pH adjustment (16.62 and 12.11 g/L) from glucose and galactose, respectively.

However, further increase in pH up to 7.0 inhibited the cell growth, lowering the butanol production accompanied by a higher generation of acetic and/or butyric acid, which was similarly observed by Al-Shorgani et al. (2017). In most cases, maintaining the Clostridial fermentation at low pH or uncontrolled is easy to generate the "acid-crush" phenomenon that will affect the bacterial growth and product formation, and the regulation of external pH is highly ascribed to the balance between butanol and butyrate converted from butyryl-CoA (Maddox et al., 2000). Owing to the presence of the strong self-regulatory system, strain WST favors its bacterial growth and butanol production without any procedure of pH adjustment, demonstrating a more natural and low-cost approach than other butanol producers.

The commercial application of biobutanol production through the ABE fermentation is mainly hindered by the low yield of Clostridial strains, the cost of fermentation pH maintenance as well as the generation of competitive by-products (Ndaba et al., 2015). *Clostridium* sp. strain WST was able to address the above problems by utilizing glucose and galactose to obtain the highest yield in reported batch fermentation studies. With the advantages of higher butanol tolerance and less by-product generation, strain WST can further accelerate its potential for cost-effective industrial production of butanol from galactose-rich algal biomass, with an uncontrolled pH strategy.

4. Conclusion

A newly isolated *Clostridium* sp. WST was identified to be able to produce highest titer of biobutanol from lower concentration of substrates, and the respective butanol yields of 0.54 and 0.55 g/g from glucose and galactose were determined to be highest

within the reported batch fermentation by Clostridial strains. The inherent strong regulatory system of strain WST also facilitates its fermentation process without the requirement of pH control, with the negligible level of generated ethanol and acids, further verifying the great potential of this strain to offer an economically feasible option for the large-scale sustainable biobutanol production.

Acknowledgements

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Figure legends:

Fig. 1 A neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of *Clostridium* sp. WST using MEGA 6. The tree was constructed based on the sequences from various *Clostridium* species with *Rhodobacter veldkampii* strain DSM 11550 as the outgroup. Bootstrap values greater than 50% are shown at nodes, and the scale bar indicates evolutionary distance.

Fig. 2 The batch fermentation of Clostridium sp. WST using glucose (30 g/L) as substrate with an initial pH of 6.0. (a) Biosolvents fermentation process; (b) Profile of bacterial cell growth, substrate utilization and biohydrogen production.

Fig. 3 Batch fermentation of *Clostridium* sp. WST using galactose (30 g/L) as substrate with an initial pH of 6.0. (a) Biosolvents fermentation process; (b) Profile of bacterial cell growth, substrate utilization and biohydrogen production.

Fig. 4 Effect of pH control on butanol production by *Clostridium* sp. WST using 30 g/L of glucose or galactose. UC: the uncontrolled pH of culture medium.

Figures:

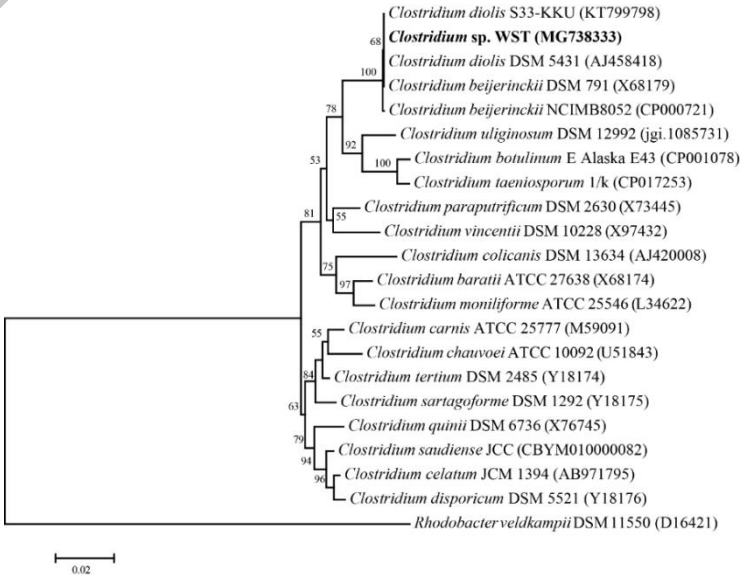
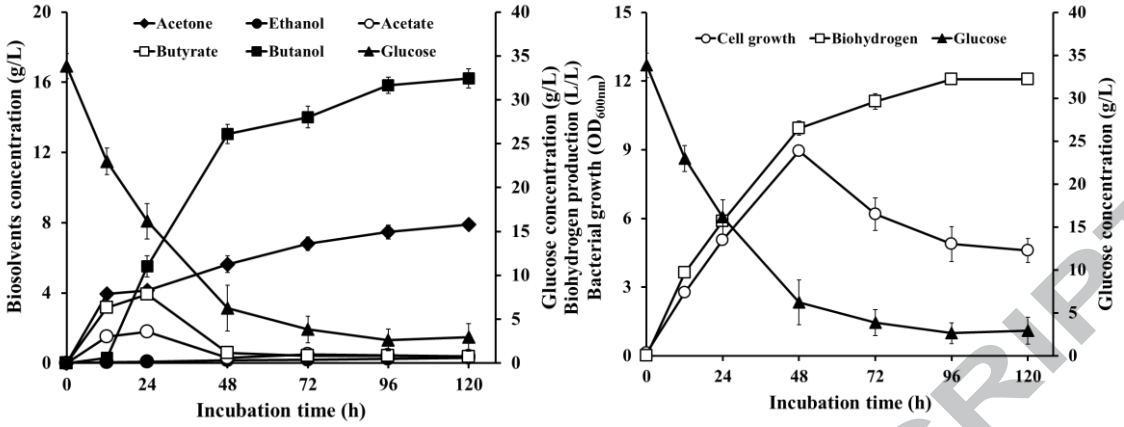
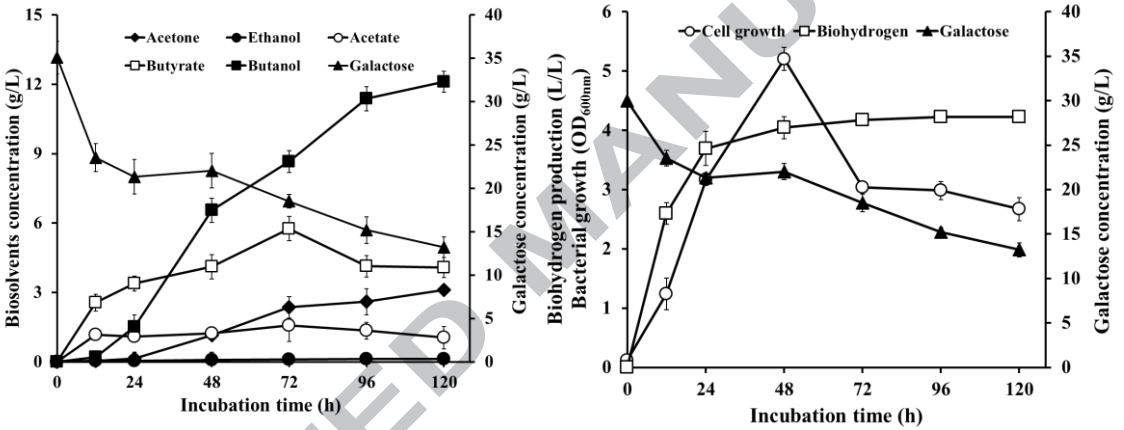


Fig. 1



(a) **(b)**

Fig. 2



(a) **(b)**

Fig. 3

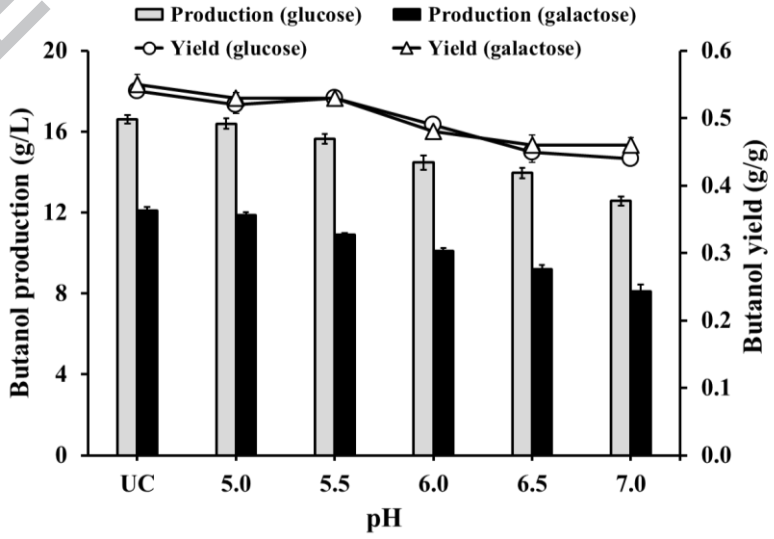


Fig. 4

Highlights

- A newly isolated *Clostridium* sp. WST with efficient butanol-producing capability was identified.
- The biobutanol production of 16.62 g/L can be archived by using 30 g/L of glucose as the substrate.
- The biobutanol yield of 0.54 g/g was much higher than those from the reported Clostridial strains.
- Neglectable amount of ethanol was detected during the fermentation by strain WST.
- The self-regulatory system enables this strain to be without the requirement of pH control.