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Original article

Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of *Clostridium acetobutylicum* YM1

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ABSTRACT

The effect of pH and butyric acid supplementation on the production of butanol by a new local isolate of *Clostridium acetobutylicum* YM1 during batch culture fermentation was investigated. The results showed that pH had a significant effect on bacterial growth and butanol yield and productivity. The optimal initial pH that maximized butanol production was pH 6.0 ± 0.2. Controlled pH was found to be unsuitable for butanol production in strain YM1, while the uncontrolled pH condition with an initial pH of 6.0 ± 0.2 was suitable for bacterial growth, butanol yield and productivity. The maximum butanol concentration of 13.5 ± 1.42 g/L was obtained from cultures grown under the uncontrolled pH condition, resulting in a butanol yield ($Y_{P/S}$) and productivity of 0.27 g/g and 0.188 g/L h, respectively. Supplementation of the pH-controlled cultures with 4.0 g/L butyric acid did not improve butanol production; however, supplementation of the uncontrolled pH cultures resulted in high butanol concentrations, yield and productivity (16.50 ± 0.8 g/L, 0.345 g/g and 0.163 g/L h, respectively). pH influenced the activity of NADH-dependent butanol dehydrogenase, with the highest activity obtained under the uncontrolled pH condition. This study revealed that pH is a very important factor in butanol fermentation by *C. acetobutylicum* YM1.

1. Introduction

Butanol is a highly efficient biofuel that has earned renewed attention recently due to its potential as a renewable and environmentally friendly biofuel substitute for gasoline. Butanol is produced by acetone-butanol-ethanol (ABE) fermentation by anaerobic solvent-producing *Clostridium* strains (Al-Shorgani et al., 2012a; Chandrasekaran and Bahkali, 2013). Butanol as a fuel offers many superior advantages to ethanol, including its high energy content, less corrosive nature, low freezing point, high octane number, high hydrophobicity and the fact that it can be blended with gasoline or used directly in current engines without any modifications (Al-Shorgani et al., 2012b,d; Dürre, 2007; Qureshi et al., 2014).

However, the production of butanol via the ABE fermentation process by *Clostridium* suffers from some set-backs, including butanol toxicity due to product accumulation, low yield and productivity, and the high cost of the substrate and product recovery. Although significant efforts have been made and newer methods have been proposed in increasing numbers to curb these limitations in the fermentation process, more research is required to address these issues (Al-Shorgani et al., 2012c).

Proposed approaches, including screening for new strains of solvent-producing microbes capable of producing higher concentrations of butanol with higher tolerance against high concentrations of solvent, using renewable and readily available wastes, and optimization of the fermentation process could contribute to solving the issues related to the low productivity of butanol production.

Typically, ABE production by *Clostridium* species is achieved during biphasic fermentation. The first phase is called the acidogenic phase. During this phase, the major products are acetate,

Abbreviations: ABE, acetone-butanol-ethanol; NADH-BDH, NADH-dependent butanol dehydrogenase.

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butyrate, hydrogen and carbon dioxide. This acidogenic phase is usually associated with the exponential growth phase of cell division. The second phase is the solventogenic phase, in which the produced acids are reconsumed by the cells and used to produce acetone, butanol and ethanol (or isopropanol instead of acetone in some strains of *Clostridium beijerinckii*) (Andersch et al., 1983; S.Y. Lee et al., 2008; Servinsky et al., 2014). However, the shift from the acidic phase to the solvent formation phase is not well understood. Undissociated butyric acid and changes in pH have been reported to be involved in triggering the shift from acid production to solvent production in solvent-producing *Clostridium* strains (Gottwald and Gottschalk, 1985; Monot et al., 1984). Although studies have investigated the mechanism behind ABE fermentation, the enzymatic regulation of the shift mechanism from the acidogenic phase to the solventogenic phase are not understood. Moreover, the role of pH-induced gene regulation on butanol fermentation needs further investigation to optimize the continuous fermentation of butanol using *Clostridium acetobutylicum* on an industrial scale (Haus et al., 2011).

In this study, *C. acetobutylicum* YM1 was used to produce butanol in a batch fermentation process under different pH strategies with and without the addition of butyric acid. The objective was to investigate the influence of pH and butyric acid on butanol production by strain YM1. This strain is one of the solvent-producing *Clostridia* strains that was recently isolated from Malaysian agricultural soil and has been used for the production of biohydrogen and butanol (Abdeshahian et al., 2014; Al-Shorgani et al., 2013, 2015).

2. Materials and methods

2.1. Microorganism

The newly isolated strain of *C. acetobutylicum* YM1 was used in this study (GenBank accession No. KC969670). This microorganism was isolated from a local agricultural soil in Malaysia. The isolate was maintained as a stock in glycerol and stored at −30 °C. Prior to the experiments, the isolate were activated in tryptone yeast-extract medium (TYA) supplemented with 20 g/L glucose.

2.2. Medium and fermentation conditions

The effect of pH on butanol fermentation in a batch culture of *C. acetobutylicum* YM1 was studied in a 5 L bioreactor (INFORS HT, Switzerland) with a working medium volume of 3 L. TYA medium supplemented with glucose as a carbon source at a concentration of 50 g/L was used in this study. The TYA consisted of tryptone (6 g/L), yeast extract (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), ammonium acetate (3 g/L), KH_2PO_4 (0.5 g/L) and $FeSO_4.7H_2O$ (0.01 g/L). Butyric acid was added in some experiments.

The medium was sterilized by autoclaving at 121 °C and 15 psi for 15 min prior to use. Nitrogen gas was used to create anaerobic conditions by flushing the medium for 4 min before inoculation. The fermentation conditions for the experiments were as follows: temperature 30 °C, inoculum size of 10% and bioreactor agitation at 200 rpm throughout the fermentation period. The pH was automatically adjusted to the desired pH value by feeding with either NaOH (5 M) or HCl (5 M). The investigation into the effect of the initial pH on butanol production was conducted in 100 mL serum bottles with a working volume of 50 mL using TYA medium supplemented with 30 g/L glucose.

2.3. Analytical methods

Samples of fermentation broth were collected at appropriate time points for the analysis of solvents, acids, cell growth and glucose. The samples were centrifuged at 7000 rpm prior to analysis for butanol, acids and glucose. Butanol and butyric acid were analyzed using a gas chromatograph (7890A GC-System, Agilent Technologies, USA) equipped with a flame ionization detector (FID). A 30 m capillary Supelco column (Equity 1^{TM} column) was used, and the detection and injection temperatures were set as 240 °C and 260 °C, respectively. Helium was used as the carrier gas with a flow rate of 1.5 mL/min.

Cell growth was measured using a UV–Vis spectrophotometer (Shimadzu, UV mini-1240, Japan) at 600 nm. Glucose was measured using a glucose kit [glucose oxidase kit; GOD, (E.C. 1.1.34), Roche Ltd., Switzerland] following the manufacturer's instructions. The intracellular protein of the cells was measured according to the protocol of Bradford (Bradford, 1976).

2.4. Butanol dehydrogenase activity

For determining the activity of NADH-dependent butanol dehydrogenase (NADH-BDH), *C. acetobutylicum* YM1 samples were collected and cell free extracts were prepared using the method described by Salleh et al. (2008). NADH-BDH was measured in the forward (butanol formation) direction at 30 °C. To measure the NADH-BDH activity, 50 mM of butyraldehyde and 0.5 mM of NADH in Tris-HCl buffer (50 mM, pH 6) was used. The volume of reaction mixture was 1 mL and it contains 6% (v/v) of cell lysate or enzyme solution except the blank control. The reaction was initiated by the addition of 60 µL enzyme solution, and the absorbance of the solution at 340 nm was recorded for 500 s using a UV–Vis spectrophotometer (Shimadzu-UV 1800, Japan). One unit (U) of activity is equivalent to amount of enzyme required for oxidization of 1 µmol of NADH/min. The specific activity of NADH-BDH was calculated as the enzyme activity (U) per mg protein.

3. Results and discussion

3.1. Effect of initial pH on butanol production

The initial pH of the fermentation medium in butanol production is an important factor that significantly affects the fermentation process. The effect of different initial pH values of 5.0, 5.5, 6.0, 6.2, 6.5 and 7.0 (adjusted by addition of either 3 M HCl or 3 M NaOH) were investigated in TYA medium supplemented with 30 g/L glucose. Fig. 1 shows the production of butanol in batch fermentation of *C. acetobutylicum* YM1. The results showed that an initial pH of 6.0 and 6.2 represented the optimal pH values for butanol production; these initial pH values resulted in the production of 6.22 and 6.28 g/L of butanol, respectively. Lower butanol

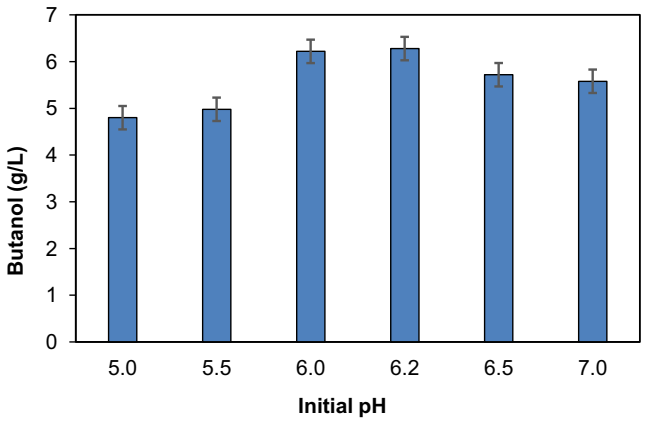

Fig. 1. Effect of initial pH on butanol production by *C. acetobutylicum* YM1.

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