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Fermentative production of butyric acid from paper mill sludge hydrolysates using *Clostridium tyrobutyricum* NRRL B-67062/RPT 4213☆

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ABSTRACT

The pulp and paper industry produces about 300–350 million tons of paper mill sludge (PMS) annually and majority is disposed of by landfill. PMS contains up to 75% carbohydrates. In this study, PMS was treated by de-ashing, fiber regeneration and enzymatic hydrolysis. The PMS hydrolysate was used for butyric acid production by *Clostridium tyrobutyricum* B-67062/RPT 4213. We reported that 8.52 and 8.35 gL^{-1} butyric acid was produced from 4 L batch fermentation in MRS and RCM4 medium respectively. Moreover, nearly 7 gL^{-1} butyric acid was produced by using PMS hydrolysates from two different mills when combined with a fraction of MRS, and over 6 gL^{-1} butyric acid was produced from hydrolysate combined with a fraction of RCM4 medium. This study suggested that beside agricultural lignocellulosic biomass feedstocks, low valued waste materials from the pulp and paper industries could also be used for the sustainable production of butyric acid.

1. Introduction

Butyric acid (C_3H_7COOH) is a short-chain fatty acid. The concentrated butyric acid has it's distinguished unpleasant foul-smelling characteristics, and esters of butyric acid can be found naturally in animals and some plants (Fayolle et al., 1990). Esters and salts of butyric acid have been used in animal feeds and as flavoring agents in various food products (Zhang et al., 2009). Other applications of butyric acid include varnishes, perfumes, pharmaceuticals and disinfectants, as well as a feedstock for plastics, plasticizers, surfactants and textile auxiliaries (Zhang et al., 2009).

Butyric acid is being produced on a commercial industrial scale through chemical synthesis using feedstock derived from crude oil. Due to limited petroleum resources, plus some specific medical and food related applications, the production of butyric acid from microbial fermentation has attracted a lot of attention in recent decades (Fayolle et al., 1990; Huang et al., 2002; Zhu et al., 2002; Liu et al., 2006; Jiang et al., 2011; Sjoblom et al., 2015). Most of the butyric acid fermentation studies have been carried out by using *Clostridium* species under strict anaerobic fermentation conditions (Zhang et al., 2009).

Previously, we have reported the isolation and identification of a highly efficient *Clostridium tyrobutyricum* strain RPT-4213 that produces 9.47 $g\,L^{-1}$ butyric acid using nutrient media in batch fermentation (0.48 g/g glucose) under strict anaerobic conditions (Liu et al., 2013). Aimed at converting various lignocellulosic biomass feedstocks to butyric acid, this strain was used to ferment dilute acid pretreated hydrolysates including wheat straw (WSH), corn fiber (CFH), corn stover (CSH), rice hull (RHH) and switchgrass (SGH). Among the hydrolysates tested, this strain utilized WSH and SGH better than other strains and produced 9.87 and 7.05 $g\,L^{-1}$ butyric acid respectively (Liu et al., 2013). This earlier study has been the subject of a US patent application (application serial number 15/173,602).

Beside agricultural biomass, woody biomass and waste materials from the pulp and paper industries could also be used as feedstocks for renewable bioproducts (Jeffries, 1983; Deeba et al., 2016). The pulp and paper making process produces about 300–350 million tons of paper mill sludge (PMS) every year which is a huge volume of industrial waste. This waste stream is an economic burden and also an environmental concern since the majority of the PMS is disposed of by landfill (Negi and Suthar, 2013; Gurram et al., 2015). PMS contains up to 75% carbohydrates, moreover, most of the lignins are removed during the pulping step of the paper making process. Therefore, PMS could be used as a low valued substrate for fermentative production of high valued bioproducts (Bengtsson et al., 2008; Gurram et al., 2015; Deeba et al., 2016).

In this current study, we report the utilization of PMS hydrolysates

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Table 1.1
Culture media compositions.

Composition	Media name			
	RCM1 gL^{-1}	RCM2 gL^{-1}	RCM3 gL^{-1}	RCM4 gL^{-1}
Glucose	20	20	20	20
Beef extract	10	10	10	10
Peptone	5	5	5	5
Tryptone	5	5	5	5
Starch	1	1	1	1
Sodium Acetate	3		3	
Sodium Lactate		3		3
Sodium Chloride	5	5	1	1
Cysteine HCl	3	3	3	3

to produce butyric acid through anaerobic fermentation using the proprietary *C. tyrobutyricum* RPT-4213 strain.

2. Results and discussion

Side by side fermentation analyses were carried out in flasks to compare the *C. tyrobutyricum* ATCC 25755 strain with the proprietary strain *C. tyrobutyricum* NRRL B-67062 for butyric acid production. Fermentation results showed that under the conditions used in this study, NRRL B-67062 produced 9.32 g L^{-1} butyric acid while ATCC 25755 strain produced 6.28 g L^{-1} butyric acid from glucose in batch fermentation under anaerobic conditions. Although the ATCC 25755 strain has been used by several research groups (Zhu et al., 2002; Sjoblom et al., 2015; Huang et al., 2016), based on the result obtained, the NRRL B-67062 strain was used for additional butyric acid fermentations as described below.

Studies of data published by other laboratories reported the use of various media and as it is expected that culture media plays a critical role in the outcome of specific fermentations. We made and tested 9 different media. The compositions of these culture media are listed in Table 1.1 and Table 1.2. For the RCM media series (Table 1.1), the basic nutrient components remain unchanged, and only salt composition and concentrations were modified. For the other five media (Table 1.2), more nutrient rich components with complex nitrogen/peptides/ amino acids/organic acids/minerals & vitamins were added.

The effects of different media on anaerobic fermentation by *C. tyrobutyricum* NRRL B-67062 were tested, and Table 2 summarized the butyric acid production under different media conditions. For these studies, the culture was grown and maintained under anaerobic condition in MRS at 37 °C. Approximately 50 µl of an overnight grown culture was inoculated into 5 ml of each specific medium and strict anaerobic fermentations were carried out for 48 h. HPLC analyses (Table 2) suggested that the best medium for butyric acid fermentation is the nutrient rich MRS + 30 G (9.32 g L^{-1} butyric acid) and the standard MRS that contains 20 g L^{-1} glucose (8.12 g L^{-1} butyric acid). Although the Ct medium was frequently used by other researchers for butyric acid fermentations (Michel-Savin et al., 1990; Huang et al., 2002; Jiang et al., 2009), it used only half of the glucose and produced 4.5 g L^{-1} butyric acid. Under the current conditions used, Ct medium did not result in the anticipated high butyric acid production. Somewhat unexpected was that the second best medium RCM4 produced 8.05 g L^{-1} butyric acid in batch fermentation, and the remaining RCM1, RCM2 and RCM3 produced low levels of butyric acid (Table 2). The only differences among the RCM series were the variations of the amount of sodium acetate, sodium lactate and sodium chloride (Table 2). Considering RCM4 has only half the peptone and no yeast extract, this medium appeared to be the most efficient medium for butyric acid fermentation. Therefore RCM4 was used for the further studies described below.

Table 2
The effects of media on butyric acid fermentation using *C. tyrobutyricum* NRRL B-67062.

Media name	Glucose used (gL^{-1})	Butryic acid produced (gL^{-1})
MRS + 30 G	25.90	9.32
MRS	19.15	8.12
MRS w/o Tween 80	15.31	7.09
RCM 1	3.49	0.69
RCM 2	2.57	0.43
RCM 3	7.27	2.39
RCM 4	17.16	8.05
CT	10.12	4.50
GM	1.86	0.48

The cultures were fermented under anaerobic condition for 48 h.

Laboratory scale butyric acid fermentations (up to 4 l) were carried out in an anaerobic chamber using *C. tyrobutyricum* NRRL B-67062. The fermentation scale was increased gradually from 5 ml to 4 L. Results of fermentations in 4 L indicated that RCM4 medium produced a total of 34.08 g butyric acid (8.52 g L^{-1}), only slightly better than MRS medium that produced 33.4 g butyric acid (8.35 g L^{-1}) in the same scale. It was noted that there was still residual glucose (2.34 g L^{-1}) in the RCM4 fermentation broth when compared to the situation when MRS medium was used. It is reasonable that the nutrient rich MRS medium is better suited for bacterial growth and multiplication; some glucose is utilized for cell mass accumulation instead of fermentative conversion to butyric acid.

To increase efficiency and decrease cost of fermentative production of butyric acid, the low valued PMS samples were obtained and pretreated. The physical and enzymatic pretreatment process used here resulted in effective release of glucose from the PMS fiber. HPLC analysis results showed that PMS Mill A hydrolysate contained about 34 g L^{-1} glucose and Mill C hydrolysate contained about 29 g L^{-1} glucose. These hydrolysates were tested for butyric acid fermentation analyses by using *C. tyrobutyricum* NRRL B-67062 under anaerobic conditions at 37 °C. It was noted that when the hydrolysates were used alone, poor growth led to failed fermentations (data not shown). Additional media was prepared to contain half of MRS (or RCM4) and half of Mill A (or Mill C) hydrolysate. The final glucose concentration for MRS + Mill A and RCM4 + Mill A media was about 37 (g L^{-1}) and the final glucose for the MRS + Mill C and RCM4 + Mill C media was about 34.5 (g L^{-1}).

The 5 ml cultures were grown in 50 ml flasks in an anaerobic chamber and fermentation was initiated by addition of a 10% inoculum. It was noted that the fermentation took about 120 h, that is 72 h longer than using nutrient media, and still most of the fermentations

Table 1.2
Culture media compositions continued.

Composition	Media name				
	GM gL^{-1}	CT gL^{-1} (Michel-Savin et al., 1990; Jiang et al., 2009)	MRS	MRS + 30 G	MRS w/o Tween 80
Glucose	20	30	20	30	20
Beef Extract			10	10	10
Protease Peptone	10	5	10	10	10
Yeast Extract	5	5	5	5	5
Polysorbate 80			1	1	
Ammonium Citrate	2		2	2	2
NH_4SO_4		3			
Sodium Acetate	5		5	5	5
$MgSO_4$ $7H_2O$	0.1	0.6	0.1	0.1	0.1
$MgSO_4$ $7H_2O$	0.05	0.03	0.05	0.05	0.05
Disodium Phosphate	2				
Dipotassium Phosphate		1.5	2	2	2