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Cellulose degradation and glucose accumulation by *Clostridium thermocellum* ATCC 27405 under different cultural conditions

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Abstract - Effect of various cultural parameters on cellulose degradation, glucose accumulation and ethanol production by *Clostridium thermocellum* ATCC 27405 were investigated. Optimum pH values for glucose accumulation and ethanol production were determined as 7 and 10, respectively. Highest amount of ethanol (0.92 g/l) was obtained from the culture which contains 10 g urea/l with 34.5% decrease in glucose accumulation. Addition of 100 mM phosphate to the medium increased ethanol production while cellulose degradation and sugar accumulation decreased by 34 and 99%, respectively. Among minerals tested, Mg^{+2} was found to be the most important element which affects cellulose degradation. When the medium contained no Mg^{+2}, residual cellulose concentration was 4.3 g cellulose/l. When the cultural parameters were optimised, glucose accumulation started at early days of fermentation and glucose concentration was 60% higher than that of the control at the 10th day of fermentation.

Key words: cellulose degradation, *Clostridium thermocellum,* ethanol production, glucose accumulation.

INTRODUCTION

The abundance of plant biomass as a renewable resource has generated intense interest in studying fermentation processes in which cellulose can be converted to fuels and solvents. Cellulose and hemicellulose from the plant material can be digested by cellulolytic microorganisms or their enzymes to accomplish total liberation of simple sugars from these polysaccharides, making them available to microorganisms for fermentation to ethanol (Palmalora-Adrados *et al*., 2004).

Clostridium thermocellum is a cellulolytic, ethanologenic, thermophilic and anaerobic bacterium and produces ethanol, acetic acid, lactic acid and CO_2 as end products of fermentation (Salapack *et al*., 1985; Lynd, 1989). *Clostridium thermocellum* has a very active cellulase system (Johnson *et al*., 1982; Ng and Zeikus, 1982; Johnson, 1983) and has been extensively studied for being an important industrial organism for the conversion of cellulosic biomass to liquid fuel (Weimer and Zeikus, 1977; Lamed and Zeikus, 1980; Lynd and Grethlein, 1987; Lynd, 1989). The single step conversion of cellulosic biomass to ethanol by *C. thermocellum* has some advantages over the multi-step processes. However, the low ethanol tolerance (up to 1.5% v/v) (Herrero and Gomez, 1980; Wang *et al*., 1983; Taillez *et al*., 1989; Sai Ram and Seenayya, 1991; Sudha Rani *et al*., 1996) and low ethanol yields (0.08-0.29 g/g) (Freier *et al*., 1984; Bender *et al*., 1985; Mori, 1990; Sai Ram *et al*., 1991; Sato *et al*., 1992) of the organism are the major limiting factors for industrial use (Sudha Rani *et al*., 1996).

A reasonable quantity of glucose accumulated in the medium during fermentation with *C. thermocellum* can be furnished to noncellulolytic organisms that are capable of producing higher amount of ethanol. In this study, sugar accumulation from cellulose digestion by *C. thermocellum* 27405 was investigated. Effects of various cultural parameters on cellulose breakdown, sugar accumulation and production of fermentation end products were studied and glucose accumulation was optimised.

MATERIALS AND METHODS

Cells and media. *Clostridium thermocellum* 27405 was obtained from ATCC. All the chemicals used in the medium preparations were of Merck grade.

Russian medium (Tsoi *et al*., 1987) used for batch experiments contained (g/l): cellulose 10, urea 2, KH_2PO_4 2, K_2HPO_4 3, yeast extract 5, $MgCl_2{\cdot}6H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.05, $FeSO_4{\cdot}7H_2O$ 0.0025, and L-cysteine 1. The medium was prepared in an anaerobic cabinet (Bactron I, Shellab, Sheldon Manufacturing Inc., Oregon, USA) with an atmosphere of mixed gases (10% CO_2, 5% H_2 and 85% N_2).

The experiments were performed in 100 ml septum bottles, each filled with 55 ml culture. A three day-old culture was used for inoculation for all the experiments. Inoculum volume was 1/11 of total culture volume. Batch cultures (except for the ones used for testing temperature effect tests) were incubated at 55 °C for 2 weeks in the anaerobic cabinet.

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Cellulose degradation, sugar accumulation and concentrations of end products produced by *C. thermocellum* ATCC 27405 were analysed under different cultural conditions. The parameters tested and their ranges were as follows: inoculum amount with 1/11, 1/6, 1/4 and 2/7 of the culture volume; temperature of 50, 55, and 60 °C; atmosphere gases of argon, nitrogen and mixture; pH of 4-11; different concentrations of urea (0-20 g/l), phosphate (0-200 mM), yeast extract (1-10 g/l), $MgCl_2 \cdot 6H_2O$ (0-1 g/l), $CaCl_2 \cdot 2H_2O$ (0-0.5 g/l), $Fe_2SO_4 \cdot 7H_2O$ (0-0.25 g/l).

To test the effect of atmosphere, the media in septum bottles were flushed with appropriate gas for 5 min and the culture was incubated at 55 °C inside the cabinet. Temperature effect was tested by growing cultures in incubators at an appropriate temperature outside the cabinet.

Determination of total sugar and cellulose concentration. Total sugar content was determined by the modified phenol sulphuric acid method described by Dubois *et al*. (1956). One millilitre of a two week-old culture was centrifuged at 6000 rpm for 10 min, and pellet and supernatant were used for determination of residual cellulose and total sugar content, respectively. For determination of total sugar amount, 500 ml of supernatant was mixed with 500 ml of 5% phenol. After addition of 2.5 ml concentrated H_2SO_4 (Fluka), the mixture was incubated at 30 °C for 15 min. Colour change was determined at 490 nm by using a UV-VIS Spectrophotometer (GBC-Cintra-20, GBC Scientific Equipment Pty Ltd., Victoria, Australia).

For determination of residual cellulose concentration, 500 ml distilled water was added onto pellet and phenol sulphuric acid method was used for determination of residual cellulose as described above.

Assays were performed in duplicate. Standard deviations were calculated using the equation:

$$\sqrt{\frac{n\sum x^2-(\sum x)^2}{n(n-1)}}$$

(n: number of measurements, x: values obtained in measurements).

Determination of glucose, cellobiose and fermentation end products concentrations. Glucose, cellobiose and end product concentrations in cultures were determined by using an HPLC with a Refractive Index (RI) detector (Lab Alliance Essence System, Scientific Systems Inc., Pennsylvania, USA). One millilitre of a two week-old culture was centrifuged at 6000 rpm for 10 min and supernatant was filtered through a 0.45 mm pore sized filter. The filtrate was analysed by using a polymer IEXH form 8 mm sugar column operated at 45 °C with 9 mM sulphuric acid as running buffer and a RI detector operated at 45 °C. Measurements were performed in duplicate and standard deviations were calculated.

RESULTS

First, sugar accumulation in *C. thermocellum* culture was monitored for 30 days (data not shown). Results showed that glucose accumulation proceeded at a higher rate between 3rd and 12th days of fermentation.

It was determined that amount of inoculum was important for the rate of glucose accumulation. The highest level of glucose was obtained when inoculum amount was 1/11 of the medium volume (control). Increasing the amount of inoculum resulted in low levels of glucose accumulation in the culture (Fig. 1). Ethanol production was not affected by inoculum amount. Its concentration reached around 0.5 g/l at the third day of the fermentation and remained at around the same level during fermentation in all the cultures (data not shown).

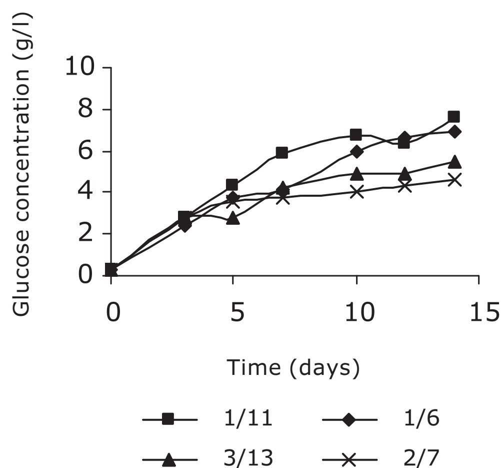

FIG. 1 - Effect of inoculum amount on glucose accumulation in *Clostridium thermocellum* 27405 culture.

Rates of cellulose utilisation, sugar accumulation and ethanol production in *C. thermocellum* 27405 culture were found to be higher at 50 °C as compared to 55 °C and 60 °C (Fig. 2A, 2B). Total sugar accumulation and ethanol production were not affected significantly by the atmosphere gases. Argon gas had an inhibitory effect on lactic acid production (Fig. 2B). Results showed that most effective cellulose degradation was observed at a pH range of 7-10. The highest amount of glucose (6.6 g/l) and ethanol (0.8 g/l) were produced at pH 7 and 10, respectively. Significant decrease in lactic acid and acetic acid productions was observed at pH 11 (Fig. 3A, 3B).

The remainder of the study included an assessment of the effect of medium ingredients on sugar accumulation and fermentation end products. Figure 4 illustrates the results obtained with different urea concentrations. Total sugar and glucose concentrations in cultures reached the highest levels when the cells were cultivated in the medium containing 2 g urea/l. Increasing urea concentration caused a decrease in cellulose utilisation and sugar accumulation. Highest level of ethanol, 0.92 g/l, was obtained from the cultures containing 10 g urea/l. Furthermore, phosphate concentrations of 32 mM (control) and 50 mM were observed to be the optimum amounts for cellulose breakdown and sugar accumulation. Ethanol production increased to 0.56 g/l in the medium containing 100 mM phosphate (2.8 fold increase compared to control) whereas cellulose degradation and glucose accumulation decreased by 34% and 99%, respectively (Fig. 5A, 5B). No fermentation end products could be detected in the 200

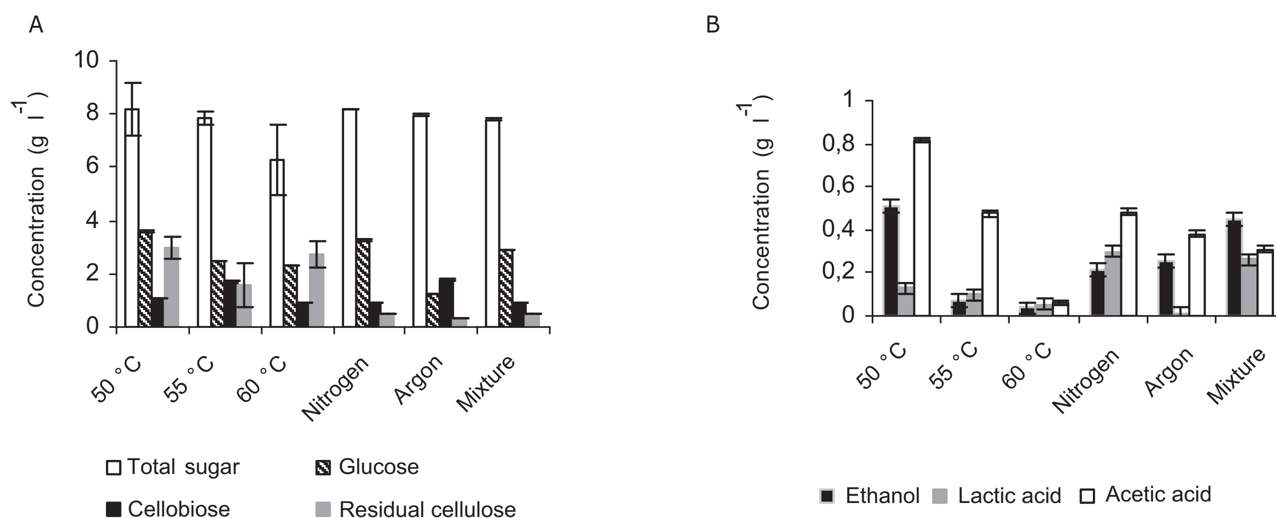

FIG. 2 - Effect of temperature and atmosphere gases on sugar accumulation, cellulose degradation (A) and concentrations of fermentation end products (B) in *Clostridium thermocellum* 27405 culture at the end of two weeks of fermentation. Vertical bars indicate standard deviations.

mM phosphate-containing culture. In most of the experiments fermentation was acetogenic (ethanol/acetate and ethanol/lactate ratios were < 1). However, increasing the urea and phosphate concentrations in medium enhanced this ratio. Ethanol/acetate and ethanol/lactate ratios increased to 2.1 and 1.5, respectively in the presence of 10 g urea /l and 1.7 and 1.3, respectively in the presence of 100 mM phosphate.

It was observed that cellulose digestion by *C. thermocellum* 27405 was affected by changes in mineral concentrations in the medium. Cellulose digestion rate and accumulated sugar amount decreased significantly (1.3 g total sugar/l) in the absence of $MgCl_2$. Highest amount of glucose, 7.6 g/l, was obtained when cells were cultivated in the presence of 0.5 g $MgCl_2 \cdot 6H_2O$/l. Cellulose utilisation and the amount of total accumulated sugar decreased when $FeSO_4 \cdot 7H_2O$ concentration was increased to 0,25 g/l. Hence, optimum concentrations of $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ for ethanol production were determined to be 0.25 and 0.5 g/l, respectively. Changing the concentration of yeast extract in the medium had no remarkable effect on the amount of metabolites (data not shown).

Optimised medium contained (g/l): cellulose 10, urea 2, KH_2PO_4 3,12, K_2HPO_4 4,68, yeast extract 5, $MgCl_2 \cdot 6H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.1, $FeSO_4 \cdot 7H_2O$ 0, L-Cysteine 1. The medium was prepared with N_2 atmosphere and pH was adjusted to 8.0. When all cultural parameters were adjusted to optimum values, glucose accumulation started at early days of fermentation and 60% more glucose was accumulated as compared to that in the control at the end

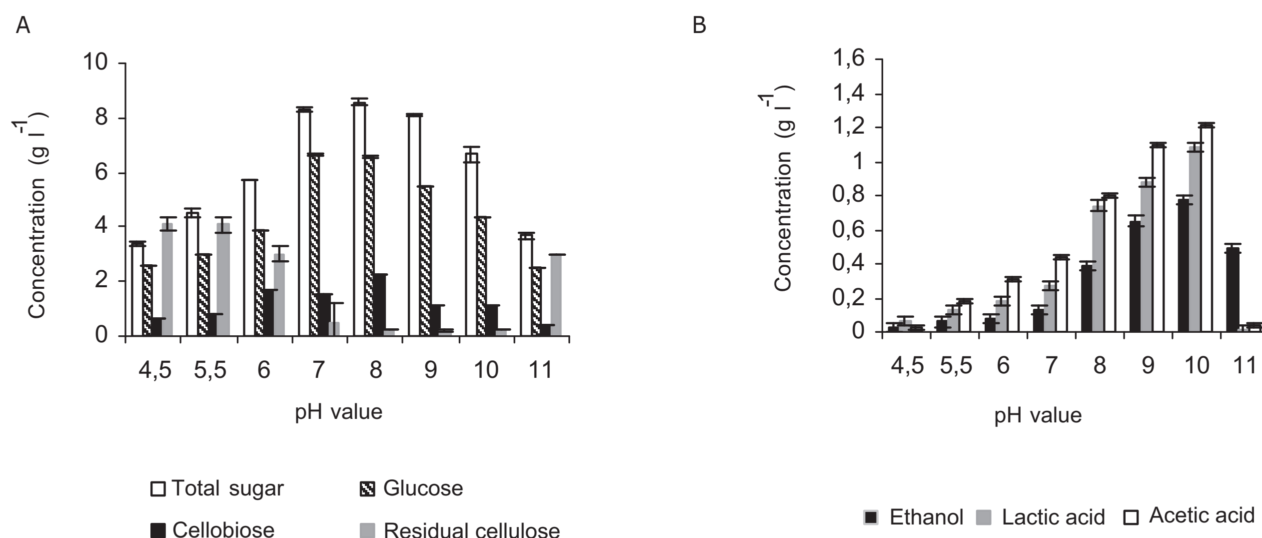

FIG. 3 - Effect of pH on sugar accumulation, cellulose degradation (A) and concentration of fermentations end products (B) in *Clostridium thermocellum* 27405 culture at the end of two weeks of fermentation. Vertical bars indicate standard deviations.

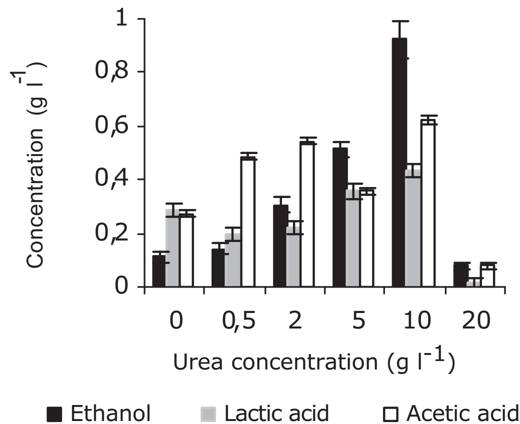

FIG. 4 - Effect of urea concentration on sugar accumulation, cellulose degradation (A) and concentrations of fermentation end products (B) in *Clostridium thermocellum* 27405 culture at the end of two weeks of fermentation. Vertical bars indicate standard deviations.

of 10th day of fermentation (Fig. 6). Ethanol concentration reached around 0.6 g/l both in control and modified medium at the third day of incubation and did not change during the remainder of the fermentation period.

DISCUSSION

Results of this study pointed that cellulose degradation and sugar accumulation by *C. thermocellum* 27405 were highly affected by the cultural conditions. Especially pH, urea, phosphate and Mg^{+2} concentrations in the medium were found to be the most important parameters.

Cellulolytic activity of *C. thermocellum* 27405 was very low below pH 6. This might be related with reduced cell growth at these pH values. The literature does not state any cellulolytic anaerobes that grow (i.e., increase in cell mass) at pH values lower than 6. However, cellulose removal by some anaerobic mixed cultures is observed at pHs as low as 4.5 (Lynd *et al*., 2002). Cellulose degradation percentages in *C. thermocellum* 27405 culture at pHs 7 and 9 (there was no pH control) were 94% and 98%, respectively. These values are quite comparable to those of mesophilic cellulolytic *Clostridium cellulolyticum* that degraded 67% of cellulose at pH 7.2 under non-pH-controlled conditions (Desvaux *et al*., 2001).

Cellulose utilisation was so sensitive to phosphate concentration that less than 10 mM and more than 100 mM phosphate concentration caused a decrease in the cellulose breakdown. This regulation might be linked to Carbon Catabolite Repression (CCR) via the inhibition of Hpr kinase and activation of Hpr phosphatase by Pi. *Clostridium thermocellum* is the only organism known to contain three different putative Hpr kinase/phosphatase genes which are regarded as key components of the CcpA-dependent CCR systems in Gram-positive bacteria (Warner and Lolkema, 2003). It was interesting that ethanol production was the highest in 100 mM phosphate-containing medium although the cells could not degrade cellulose as efficiently as they did in the control medium, and the level of accumulated glucose was very low with 100 mM phosphate. Zhang and Lynd (2004) reported that inorganic phosphate donates phosphate ions to cellobiose phosphorylase (CbP) and cellodextrin phosphorylase (CdP) for phosphorylation of glucose. Therefore, high phosphate concentration might have stimulated uptake of available cellobiose and glucose in this study.

Even though high concentrations of metal ions inhibit growth and can destroy the organisms in nutrient media, very low concentrations are known to stimulate the growth. These inorganic ions play an important role in bacterial survival (Kim *et al*., 2000). It was previously reported that Mg limitation decreases solvent production by *C. acetobutylicum* (Bahl and Gottschalk, 1984). We observed a

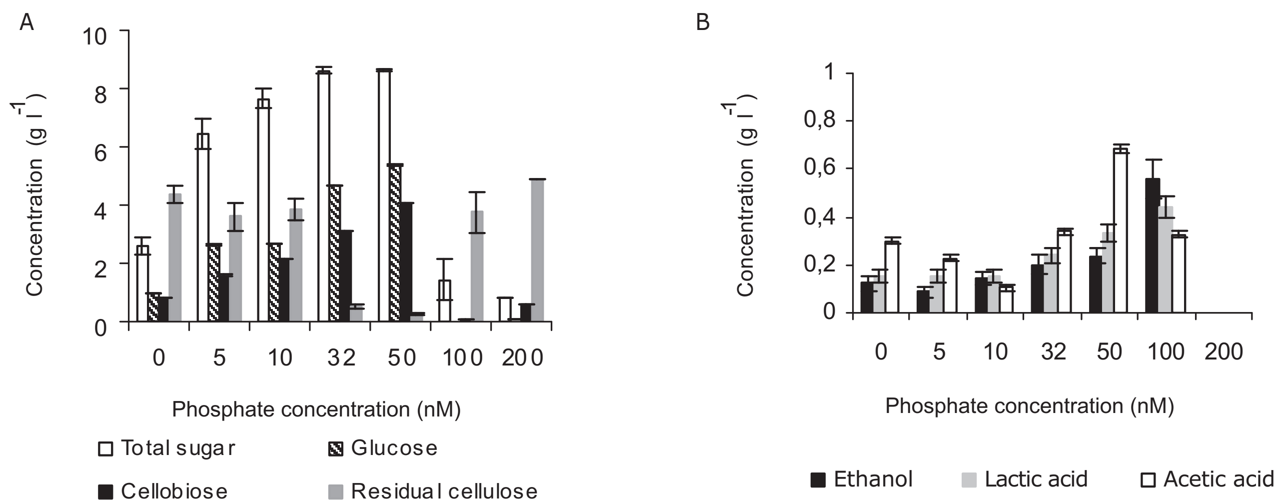

FIG. 5 - Effect of phosphate concentration on sugar accumulation, cellulose degradation (A) and concentrations of fermentation end products (B) in *Clostridium thermocellum* 27405 culture at the end of two weeks of fermentation. Vertical bars indicate standard deviations.

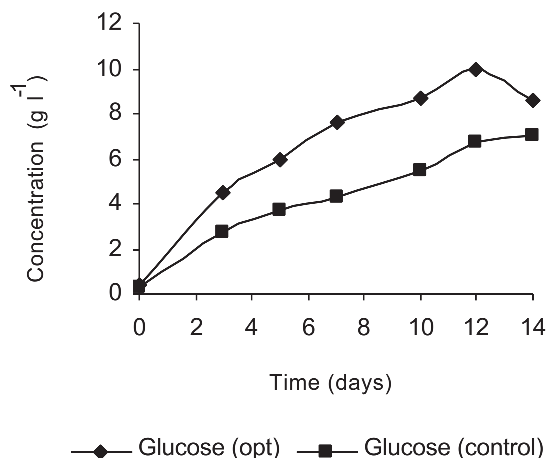

FIG. 6 - Glucose accumulation under optimum conditions in *Clostridium thermocellum* 27405 culture.

similar effect of Mg^{+2} limitation on ethanol production and cellulose degradation capacity of *C. thermocellum* 27405. Increasing $Fe_2SO_4·7H_2O$ concentration to 0.25 g/l increased ethanol production in our study. Iron might play a role as the terminal electron acceptor as suggested by Bhushan *et al*. (2006).

Increasing inoculum amount caused a decrease in glucose accumulation. Ethanol production by *C. thermocellum* 27405 was very low during this study and did not change with changing inoculum amounts (data not shown). Stevenson and Weimer (2005) reported that at low growth rates, ethanol yields were very low, and the fermentations were nearly homoacetogenic. In our study, low growth rate of *C. thermocellum* 27405 resulted in high amount of glucose production while it caused low ethanol yields.

Among three different atmosphere gases tested, mixed gas atmosphere promoted ethanol production. The previous studies reported an enhanced ethanol/acetate ratio in *C. thermocellum* culture because of the dissolved H_2 in the broth (Lamed *et al*., 1988; Bothun *et al*., 2004). Increase in ethanol production in *C. thermocellum* 27405 culture in our study might be attributed to 5% H_2 present in the mixture gas.

Glucose utilisation by *C. thermocellum* 27405 was very slow that even at 14th day of fermentation high amount of glucose was obtained in the culture. After the concentrations of medium components and culturing conditions were optimised, amount of glucose produced was 60% more than that of the control at the 10th day of fermentation

Glucose from an optimised *C. thermocellum* culture which provides continuous cellulose degradation and subseeding sugar accumulation can be fed to noncellulolytic organisms more tolerant to ethanol. Such a system can be used as a simple and cheap method of high-throughput ethanol production.

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