



Universidade do Minho
Escola de Engenharia

Semana da Escola de Engenharia October 24 - 27, 2011

BUTANOL PRODUCTION BY *CLOSTRIDIUM PASTEURIANUM* USING BIODIESEL-DERIVED CRUDE GLYCEROL

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KEYWORDS

Biofuels, crude glycerol, butanol, *Clostridium pasteurianum*

INTRODUCTION

Butanol, C_4H_9OH , is an aliphatic saturated alcohol with potential as fuel/fuel additive that can also be used as intermediate in chemical synthesis and as solvent for a wide variety of industrial applications. The increasing demand for using renewable resources as feedstock for the production of chemicals, combined with advances in biotechnology, is generating a renewed interest in fermentative butanol production. In this context, glycerol, generated as a by-product during the production of biodiesel, arises as a potential substrate for butanol production. In Europe alone, the production of glycerol has tripled within the last 10 years and its price has been considerably reduced. Although fermentation of low-grade glycerol to butanol has been proven, there is still place for process optimization. The main goals of the current thesis are to improve the yield of butanol production from glycerol by *Clostridium pasteurianum* and to reduce the butanol toxicity towards this microorganism.

MATERIAL AND METHODS

Clostridium pasteurianum DSM 525 was anaerobically cultured in 500 ml serum bottles using 200 ml working volume. Ten percent volume was repeatedly transferred to increasing crude glycerol concentrations using a semi-defined medium (crude glycerol – salts – yeast extract). The initial pH was set at $7 \pm 0,2$ and cells were incubated at 37°C. Acids, glycerol and 1,3-propanediol (1,3-PDO) were measured through HPLC (Aminex cation-exchange HPX-87H column) coupled to an UV and RI detector. Butanol and ethanol were determined by GC (TR-WAX column) equipped with a flame ionization detector.

RESULTS AND DISCUSSION

Stability considerations

Besides the toxicity issues, a major problem in solvent production using *Clostridium* spp. is the so called strain degeneration, term used to describe mutant cells that produce more acids and little or no solvents (Cornillot et al., 1997).

Different strategies have been attempted to avoid strain degeneration. For instance, *C. acetobutylicum* could be subcultured in a medium containing an excess of $CaCO_3$ for more than 200 days without completely lose its ability to produce solvents; and it was possible to restore the initial solvent yield by increasing the volume of inoculum used (Hartmanis et al., 1986). However, Taconi et al. (2009) reported the loss of solvent capacity for *C. pasteurianum* ATCC 61013 using an excess of $CaCO_3$. Thus, these strategies seem to be useless for *C. pasteurianum*. The addition of acetate (Chen and Blaschek, 1999) and butyrate (Yusof et al., 2010) to the culture medium have also been suggested to prevent degeneration and to enhance solvent production.

In the current work, degeneration was observed after 4 generations when cells were transferred in a vegetative state (12-24 h after inoculation) In this case, higher production of acids, including isobutyric acid (that is not observed in normal cultures) and 1,3-PDO was observed.

However, experiences started from stock cultures, using an excess of $CaCO_3$ and transferring the cells towards the end of the fermentation, were successful and it was possible to obtain a stable strain that has been transferred for over 10 generations without degeneration. Further media optimization towards the production of butanol was conducted with this strain.

Strain adaptation to increasing crude glycerol concentrations

C. pasteurianum was serially transferred from stock cultures to media containing 5, 10, 15, 20, 25, 35 and 50

g/l crude glycerol. Besides acids (acetic, n-butyric, lactic, formic) the main products found were butanol, ethanol, and 1,3-PDO. The competitive nature of Butanol and 1,3 PDO pathways in *C. pasteurianum* is evident from Figure 1, where a shift to butanol pathway for higher glycerol concentrations was clearly observed.

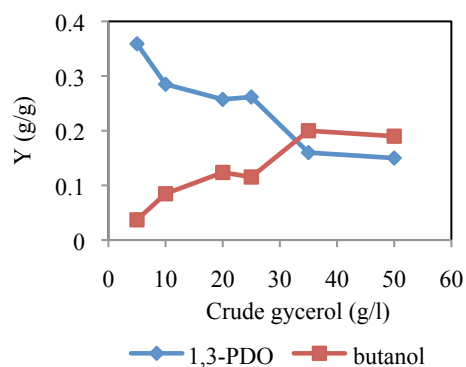


Figure 1: Butanol and 1,3-PDO yield versus crude glycerol concentration in batch fermentation.

The strain was able to consume up to 31,3 g/l of crude glycerol which is slightly higher than the maximum glycerol consumption obtained by Dabrock et al. (1992) (27,6 g/l) but lower than the values reported by Biebl (2001) (50 g/l) using pure glycerol. For 50 g/l crude glycerol a considerable amount of substrate remained in the culture medium. To overcome this situation, the concentration of different nutrients was evaluated to assess possible limitations. NH_4Cl , CaCO_3 , FeCl_2 , microelements, salts, yeast extract were increased independently and in combinations. A simultaneous increase in NH_4Cl (from 3 to 5 g/l) and FeCl_2 (from 1 to 11 mg/l) had a positive effect in the butanol yield (from 0,19 to 0,27 g/g) whereas 1,3-PDO yield decreased (from 0,15 to 0,06 g/g), which is in agreement with Dabrock et al. (1992), who found that iron limitation somehow inhibit butanol production. A 9 g/l concentration of butanol was obtained which is in accordance with the values reported for solventogenic Clostridia. Based on these results, it is likely that the glycerol consumption is not being affected by nutrient limitation but by some butanol inhibition. It is well known that butanol is very toxic to cells and maximum tolerance between 7 and 13 g/l has been reported for non-manipulated *Clostridium* strains.

Effect of butyrate

Sodium butyrate (36 mM) was added to culture media containing 5 to 50 g/l crude glycerol. In general, higher butanol titers were obtained in those cultures containing sodium butyrate, nevertheless this difference was less pronounced for increasing glycerol concentrations. A slight difference could be observed for the experiments run with 50 g/l crude glycerol (10,11 g/l of butanol).

It is important to stress that a higher butanol titer obtained as a result of butyrate addition does not necessarily imply a higher butanol on glycerol yield since butyrate can be directly converted into butanol via butyryl-CoA – butyraldehyde – butanol. The difference observed compared to the controls is close to its stoichiometric conversion.

CONCLUSION

C. pasteurianum shows a great potential for butanol production from biodiesel-derived crude glycerol.. Nevertheless, butanol toxicity seriously limits butanol titers and therefore, it is important to find ways to overcome this problem. Future work will be focused on this issue considering that the plasmatic membrane is the main target for the negative effect exerted by butanol. It would be desirable to increase the butanol yield, for example, by shutting down genes involved in lactic acid production and/or overexpressing enzymes involved in the butanol production. The complete genome of the strain has been sequenced and annotated and will be used in further work.

ACKNOWLEDGEMENT

R. Gallardo acknowledges F.C.T. for the PhD grant ref. SFRH/BD/42900/2008.

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