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Glycerol fermentation to 1,3-propanediol by *Klebsiella oxytoca* NRRL B-199: study of the inhibition of the final products of both the oxydative and the reductive routes

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As an important chemical intermediate, 1,3-Propanediol (1,3-PD) is used as a monomer to produce poly(propileneterephthatlate). The microbial conversion of glycerol to 1,3-PD, has recently paid more attention because, as consequence of the growing use of biodiesel, glycerol has become a very suitable compound. In this work, the conversion of glycerol is carried out by *Klebsiella oxytoca* NRRL B-199, a non pathogenic bacteria and an excellent 1,3-PD produced. In the biological production of 1,3-PD, a number of by-products (acetic acid, ethanol, 2,3-butanediol, succinic acid and lactic acid) are simultaneously produced together with 1,3-PD. In this work, the study of product inhibition is carried out. To quantify their influences, specific growth rate, μ, yield into biomass, Y_{XG} and yield into product, Y_{PG} have been calculated. In this work, is stated that all products inhibit 1,3-PD production, being EtOH the most important inhibition agent.

1. Introduction

The recent development of a new polyester called poly(propylenterephtalate), a biodegradable polymer with unique physicochemical properties for the fibber industry has increased the attention towards the production of 1,3-propanediol (1,3-PD), a product with this and other applications in cosmetics, foods, lubricants and medicines (Deckwer, 1995).

Traditional chemical production of 1,3-PD by means of conversion of acrolein requires high temperature, high pressure and expensive catalysts. The microbial conversion of glycerol to 1,3-PD has recently received more attention because it is carried out using milder operational conditions than the chemical process and it does not generate toxic by-products (Yang et al., 2007).

Moreover, the microbial process can use glycerol as substrate; therefore, it will be a very suitable compound when the biodiesel production had increased its production as consequence of the application of the European Directive 2003/30/CE, which imposes

the growing use of biofuels. Glycerol is the main by-product obtained from biodiesel production around 10 % of biofuel

Glycerol can be naturally fermented into 1,3-PD by bacteria belonging to the genera *Klebsiella, Clostridia, Citrobacter* and *Enterobacter* under anaerobic or microaerobic conditions (Chen *et al.,* 2003). Best yields are reached with *Klebsiella genus* of *Enterobacteriaceae*, mainly with *Klebsiella pneumoniae*. *K. pneumoniae* is commonly associated with human respiratory and genitourinary infections, and also, other bacteria belonging to the genera *Klebsiella* has been implicated in urinary tract infections. The genera *Klebsiella* is more commonly regarded as an inhabitant of soil, plants, and the aquatic environment (Brown *et al*., 1973). However, *Klebsiella oxytoca*, that is closely related to *K. pneumoniae*, is a human non-pathogenic bacteria.

The fermentation of glycerol involves two parallel pathways: oxidative and reductive, as shows Figure 1. Through the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone (DHA) and then to dihydroxyacetonephosphate (DHAP). Acetic acid (HAc), succinic acid (HSucc), lactic acid (HLac), 2,3-butanediol (2,3-BD) and ethanol (EtOH) are the final products of the oxidative route. Through the reductive pathway, a glycerol dehydratase removes a water molecule from glycerol to form 3-hydroxypropionaldehyde (3-HPA) which is then reduced to 1,3-PD, that is not metabolized further, and, as a result, it is accumulated in the media (Cameron *et al*., 2002). All the final products of both routes have been observed to inhibit microbial production of 1,3-PD from glycerol in *Enterobacter agglomerans* (Barbirato et al, 1996).

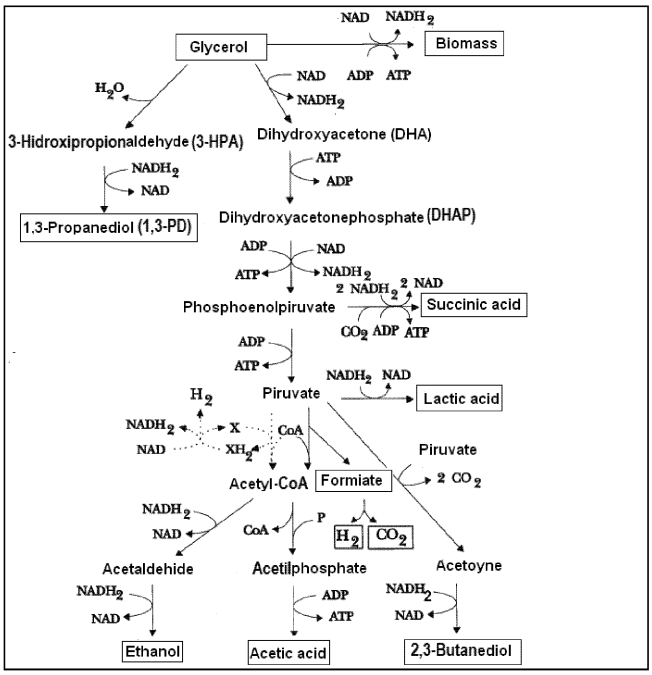

Figure 1. Routes of anaerobic glycerol metabolism in *Klebsiella sp.*

The aim of this work is to study the inhibition both in the *K oxytoca* growth and in the production of 1,3-PD by the main product of its fermentation, 1,3-PD, and also by all the by-products, using glycerol as carbon source.

2. Materials

2.1 Microorganism

The microorganism used in this work was *Klebsiella oxytoca* NRRL B-199, from the Biological Research Center (CIB, Madrid, Spain).

2.2 Medium composition

The medium composition used for the growth of *K. oxytoca* (and for the production of 1,3-PD, because it is an associated growth product) was determined and optimized by in a previous work (Galdeano *et al*, 2007). It contains (per litre): 40 g glycerol, 0.15 g yeast extract, 0.5 g NH_4NO_3, 3.5 g K_2HPO_4, 1.5 g KH_2PO_4, 0.25 g Mg SO_4 and 2 g NH_4Cl.

3. Methods

3.1 Inoculum preparation

Pre-inoculum was prepared in the culture medium in a 25-mL shake flask, containing 10 mL of culture medium which were inoculated with a loop of cells from the stock conserved in glycerol at -20ºC and incubated in orbital shaker Gallenkamp (model INR-200) at 210 rpm, 30 ºC, for 12 h, under anaerobic conditions.

3.2 Microorganism growth

Pre-inoculum was used to inoculate 10mL of culture medium in a 25mL shake flask, at an initial concentration at 0.1 g/L. Samples were withdrawn from the flasks at one hour intervals, to measure cell growth. Afterwards, cells were harvested from the sample by centrifugation at 14000×*g* for 5 min at 12ºC. The supernatant obtained after centrifugation was analyzed by HPLC.

3.3 Analytical methods

Cell growth was monitored as Optical Density at 600 nm. The concentration of glycerol, 1,3-PD and the other by-products (EtOH, HLac, HAc, HSucc, 2,3-BD) were determined by HPLC using an Aminex HPX-87H column and a refractive index detector. Operation conditions were: 0,005 M H_2SO_4 , flow rate 0,6 ml min^{-1}, column temperature 60 ºC and refractometer temperature 55ºC.

4. Results and discussion

4.1 Results

Twelve experiments have been carried out. Each experiment has been performed introducing into the growth medium one of the products of the glycerol metabolism in *Klebsiella oxytoca*, as shown in Table 1.

4.2 Discussion

To evaluate the inhibitory effect of the differents compounds, specific growth rate (μ), the yield into biomass (Y_{XG}) and into 1,3-PD (Y_{PG}) were calculated using equations (1), (2) and (3).

$$C_x = \frac{C_{Xo} \cdot \exp(\mu \cdot t)}{1 - \frac{C_{Xo}}{C_{X,m}} \cdot (1 - \exp(\mu \cdot t))} \quad (1)$$

$$Y_{XG} = \frac{C_{X,m} - C_{Xo}}{C_{Go}} \quad (2)$$

$$Y_{PG} = \frac{C_P^{max}}{C_{Go}} \quad (3)$$

Table 1. Sets of experiments, using different initial concentations of 1,3-PD, HAc, HLac, HSucc , 2,3-BD and EtOH.

Run	1,3-PD (g/L)	HAc(g/L)	HLac(g/L)	HSucc(g/L)	2,3-BD (g/L)	EtOH(g/L)
1	0	0	0	0	0	0
2	8	0	0	0	0	0
3	16	0	0	0	0	0
4	20	0	0	0	0	0
5	0	1	0	0	0	0
6	0	2	0	0	0	0
7	0	0	1	0	0	0
8	0	0	2	0	0	0
9	0	0	0	1	0	0
10	0	0	0	2	0	0
11	0	0	0	0	1	0
12	0	0	0	0	2	0

The influence of both the different products and concentrations on specific growth rate is shown in Figures 2a and 2b. It can be seen that all the studied compounds inhibit growth of *Klebsiella oxytoca*, being succinic acid the product more affecting microorganism growth. However, the different concentrations used for each product do not increase the inhibition efect in a great way.

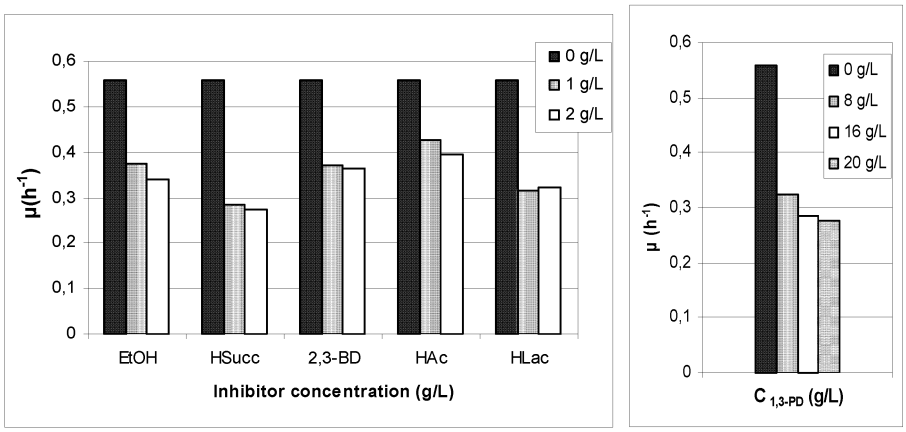

Figure 2a and 2b. Specific growth rate (μ) vs. different inhibitors.

Y_{XG} seems to decrease when the initial concentration of EtOH, HSucc, 2,3-BD, HLac and 1,3-PD is increased, as show Figures 3a and 3b. The decrease of Y_{XG} is specially significant when the EtOH is the studied inhibitor. The different concentrations of HSucc seem to affect strongly in the yield.

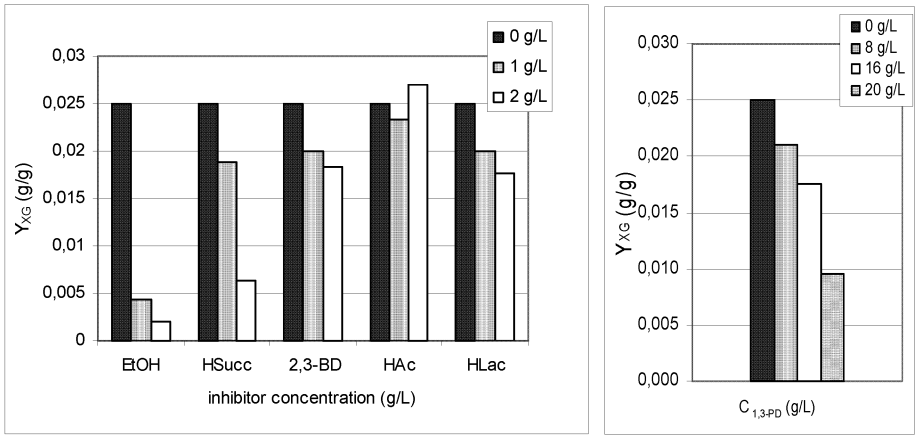

Figura 3a and 3b. Yield in biomass (Y_{XG}) vs. different inhibitors.

Figures 4a and 4b show the values of Y_{PG}. It can be seen that the higher initial concentration of the studied compound, the less Y_{PG} is obtained. Values of Y_{PG} are severely affected by the different concentrations of each product added to the medium. The highest influence in Y_{PG}, is observed when EtOH is studied as inhibition agent. Moreover, the inhibition effect is very significant when $C_{1,3\text{-}PD}$ is higher than 8g/L, reducing dramatically the value of Y_{PG}.

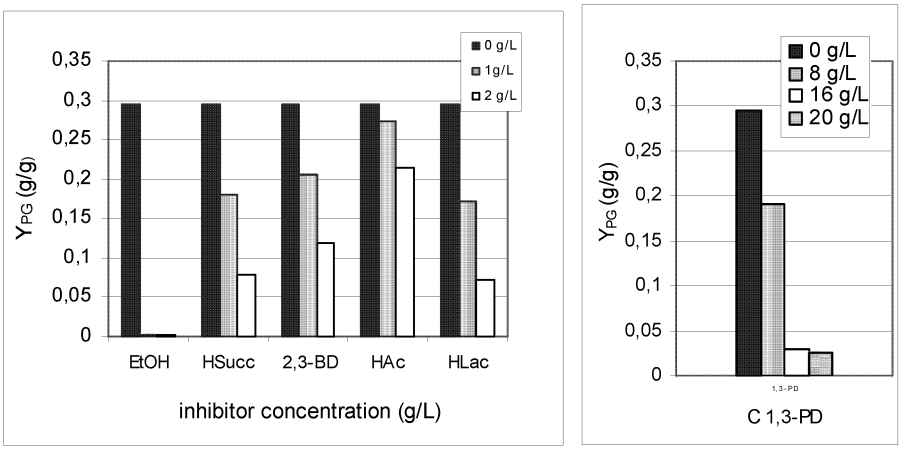

Figura 4a and 4b. Yield in 1,3-PD (Y_{PG}) vs. different inhibitors

5. Conclusion

In this work, the inhibition effect caused by all the final products of both the oxydative and the reductive routes, involve in the fermentation of glycerol, has been studied

Specific growth rate is affected by all the studied compounds, showing all of them to inhibit *K. oxytoca* growth.

Experimental results show that not only the main product, 1,3-PD, but also all the by-products, do affect to the biomass yield of *K. oxytoca.* The most important growth inhibition is observed when EtOH is studied.

Moreover, the results above showed demonstrate that exists a strong influence on the production of 1,3-PD by all the studied compounds. The different compounds and concentrations affect to values of Y_{PG}. The highest decreases of Y_{PG} have been observed when the inhibition caused by EtOH and 1,3-PD is studied.

6. Nomenclature.

1,3-PD : 1,3-Propanediol

2,3-BD: 2,3-Butanediol

Cx: Biomass concentracion (g/L)

C_{xm} : Biomass concentration reached at stacionay phase (g/L)

C_{PO}: Initial concentration of 1,3-PD (g/L)

C^{max}_{P}: maximum concentration of 1,3-PD, reached at stacionay phase (g/L)

C_{GO}: Initial concentracion of Glycerol (g/L)

C_j: Concentration of the compound j (g/L)

EtOH: Ethanol

HAc: Acetic acid

HLac: Lactic acid

HSucc: Succinic acid

Y_{PG}: Yield in 1,3-PD (g/g)

Y_{XG}: Yield in biomass (g/g)

μ: specific rate of growth (h^{-1})

7. Acknowlegments

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8. References

Barbirato F., Grivet, J.P., Soucaille, P. and Bories, A. 1996. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-Propanediol by *Enterobacter agglomerans* species. Appl. Environ. Microbiol 62, 1448-1451

Brown, C. and Seidler, R. J. 1973. Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. Appl. Microbiol. 25:900–904.

Cameron D.C., Zhu Marie M., Lawman P.D. (2002) Improving 1,3-propanediol production from glycerol in a metabolically engineered *Escherichia coli* by reducing accumulation of sn-glycerol-3-phosphate. Biotechnol Prog. Jul-Aug;1 8(4):694-699.

Chen X, Long Z., Feng J., and Zhang D. (2003). Stoichiometric analysis and experimental investigation of glycerol bioconversion to 1,3-propanediol by *Klebsiella pneumoniae* under microaerobic conditions. Enzyme and Microbial Technology, 33, 386–394

Deckwer W-D. Microbial conversion of glycerol to 1,3-Propanediol. FEMS Microbiology Reviews 16, 143-149

European Directive 2003/30/CE of the European Parliament and of the Council of 8 May 2003, relating to the promotion of the use of biofuels or fuel alternatives.

Galdeano, C.; Zazo, M.; Santos, V.E.; Garcia, J.L.; Garcia-Ochoa, F. Production of 1,3-Propanediol using *Klebsiella oxytoca* NRTL B-199 growing cells: medium composition optimization using Taguchi method. ECCE-6. Accepted communication

Yang G., Tian R. and Jilun L. (2007) Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions. Applied Microbiology and Biotechnology, 73 (5). 1017-1024.