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1,3-Propanediol production by *Klebsiella oxytoca* NRRL-B199 from glycerol. Medium composition and operational conditions☆

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ABSTRACT

Production of 1,3-propanediol from glycerol using *Klebsiella oxytoca* NRRL-B199 has been studied. Medium composition has been optimized by means of a statistical design based on the Taguchi method. Strong influences of glycerol and phosphate concentrations have been detected on biomass and product yields. Other factors, such as magnesium concentration and K:Na ratio, have shown a small influence on both responses, biomass and product concentrations. An optimized medium composition has been proposed, leading to a final 1,3-propanediol concentration of 12.4 g/L with a selectivity of 72% with respect to glycerol consumed at shaken bottle-scale. Once the medium composition had been optimized, the scale-up from shaken bottles to STBR was conducted. Several experiments in a 2 L STBR have been conducted in order to determine the best operating conditions concerning temperature and agitation.

Under the best operating conditions, i.e., a programmed variable stirring rate ranging from 50 to 100 rpm and a temperature of 37 °C, a final concentration of 13.5 g/L of 1,3-propanediol with a selectivity of 86% with respect to the glycerol consumed was obtained.

1. Introduction

1,3-Propanediol (1,3-PD) is one of those compounds nominated as a platform chemical, since it can be used in numerous synthesis reactions (e.g., as a monomer for poly-condensation reactions, as well as to produce polyesters, polyethers and polyurethanes). Furthermore, the development of polypropylene-terephthalate, with unique properties for the fiber industry, demands a drastic increase in the production of 1,3-PD [32,23].

The traditional chemical production of 1,3-PD can be carried out from the conversion of acrolein; this process requires high temperature, high pressure and expensive catalysts. Moreover, toxic by-products are produced in this process, which requires an additional chemical reduction step also under high pressures and temperatures [20,37,23].

The microbial conversion of glycerol to 1,3-PD has recently received increasing attention because it can be carried out at low temperatures and pressures, thus avoiding the generation of toxic by-products [38]. In addition, the microbial process can use glycerol as substrate [28,32], which is a very suitable compound considering the fact that its production has remarkably increased in recent years due to the escalation of biodiesel production, of which glycerol is the main by-product, representing around 10% (w/w) of biodiesel produced [23].

In the biological production of 1,3-PD from glycerol, several by-products are formed (e.g., acetic acid, ethanol, 2,3-butanediol and succinic acid), which contribute to the reduction of the final yield of the process. Taking into account that microbial growth, production rates and product distribution are affected by operational conditions and media composition, these variables must be optimized in order to develop a cost-efficient bioprocess at industrial scale.

Glycerol can be naturally fermented into 1,3-PD under anaerobic or micro-aerobic conditions by different bacteria belonging to several genera, e.g., *Klebsiella, Clostridia, Citrobacter* and *Enterobacter* [12,13,32,23]. The metabolic pathways and the fermentative capabilities of these bacteria have mainly been analysed for the *Klebsiella* and *Clostridium* genera (see Table 1),

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Nomenclature

$C_{1,3\text{-PD}}$	1,3-PD concentration (g/L)
C_{Gly}	Glycerol concentration (g/L)
C^0_{Gly}	Initial glycerol concentration (g/L)
$C_X{}^{max}$	Maximum biomass concentration (g/L)
N	Number of experiments
N_L	Number of levels
$\overline{SN}$	Average of all the SN values, given by Eq. (5)
SN_i	Signal-to-noise ratio for each experimental data i, Eq. (3)
$\overline{SN}_j$	Average of the SN values for each level of the factors, Eq. (4)
S_{PG}	Product selectivity with respect to consumed glycerol, Eq. (2) ($g_{1,3\text{-PD}}/g_{Gly}$)
t	Time (h)
y_i	Experimental data (different units)
Y_{PG}	Product yield referred to initial glycerol concentration, Eq. (1) ($g_{1,3\text{-PD}}/g_{Gly}$)

showing that their selectivity values are very similar to each other (see Table 2).

Bacteria of the genus *Klebsiella* are widely distributed in nature, both in soil and water, though they are also part of the regular flora of the intestinal tract. Although *Klebsiella pneumoniae* is commonly associated with human infections [7], *Klebsiella oxytoca* NRRL-B199, which is the strain used in this work, is a non-pathogenic strain that can been used safely due to its lacks of the polysaccharide capsule [38].

The fermentation of glycerol by *K. oxytoca* involves two parallel and coupled pathways (i.e., oxidative and reductive) as shown in Fig. 1. Through the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone (DHA) and then to dihydroxy-acetone-phosphate (DHAP), being acetic, succinic and lactic acids, as well as 2,3-butanediol, ethanol, carbon dioxide and hydrogen the final products. Through the reductive pathway, a glycerol-dehydratase enzyme removes a water molecule from glycerol to obtain 3-hydroxy-propionaldehyde (3-HPA). This product is further reduced to 1,3-PD, which cannot metabolized and is subsequently released to the medium [40]. All the mentioned by-products pertaining to both pathways are potential inhibitors of 1,3-PD production [4].

Although *K. oxytoca* is an excellent 1,3-PD producer, only a few studies have been published on its utilization for such purpose [38]. For this reason, the optimization of the medium composition maximizing both the growth of the microorganism and the production of 1,3-PD is yet to be studied. Moreover, only studies at shaken bottle-scale have been reported [38].

Therefore, the aim of this work is the optimization of the medium composition to obtain the maximum biomass concentration of *K. oxytoca* and the highest 1,3-PD yield and selectivity using glycerol as substrate. Three variables or responses have been considered for process optimization: maximal concentration of biomass in the stationary phase of growth ($C_X{}^{max}$), product yield (Y_{PG}) and product selectivity (S_{PG}). Once the medium composition had been optimized, the scale-up from shaken bottle-scale to stirred tank bioreactor (STBR) was studied. Operating conditions, stirring speed and temperature, have been studied in a 2 L STBR.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The microorganism used in this work, *K. oxytoca* NRRL-B199, was initially grown in minimal M9 × 2 medium [31]. Pre-inocula were prepared in 25 mL anaerobic bottles containing 10 mL of culture medium. which was inoculated with cells from the bacterial stock conserved in 20% glycerol at −20 °C. To ensure anaerobic conditions, commercial nitrogen was bubbled into the culture medium-filled bottles before sterilization. The inoculated anaerobic bottle was incubated in a Gallenkamp (model INR-200) orbital shaker under anaerobic conditions at 210 rpm and 30 °C for 12 h. Pre-inocula were employed to inoculate 10 mL of culture medium in 25 mL anaerobic bottles at an initial concentration of 0.1 g/L. Samples were withdrawn every hour to measure biomass growth, for which they were subjected to centrifugation at 14,000 × *g* for 5 min at 12 °C. The supernatant obtained after centrifugation was analysed by HPLC to quantify the concentration of different products. In addition, pre-inocula were utilized to inoculate a 2 L stirred tank BIOSTAT B. The initial biomass concentration was fixed at 0.1 g/L. Cell cultivation was carried out under anaerobic conditions, by introducing a 1 L/min nitrogen flow rate through the broth. Again, samples were taken at 1 h intervals and analysed by HPLC.

2.2. Analytical methods

A Shimadzu UV–visible spectrophotometer (model UV-1603) was used to measure biomass concentration by measuring the optical density at 600 nm. Glycerol and the main reaction products, such as 1,3-PD, 2,3-butanediol, lactic acid, acetic acid, succinic acid and ethanol were determined by HPLC (Agilent Technologies, series 1100) using an Aminex HPX-87H Organic Acid Analysis Column (Phenomenex), employing a Waters 2414 Refractive Index Detector. The column temperature was maintained at 65 °C and that of the detector was 45 °C. A solution of 5 mmol L^{-1} H_2SO_4 was used as mobile phase at a flow rate of 0.8 mL/min.

Table 1
Bacteria with fermentative capacity to transform glycerol into 1,3-PD.

Genus	Species	Reference nos.
Klebsiella	*K. pneumoniae*	[27,15,22,25,33,41,2,17,19,35,16,21,29,18,30]
	K. oxytoca	[38,39]
Clostridium	*C. butyricum*	[5,11,14,24,36]
	C. diolis	[23]
Enterobacter	*E. agglomerans*	[4,3,26]
Citrobacter	*C. freundii*	[6,1,8]
Lactobacillus	*L. brevis*	[9]

Table 2
Selectivity and yield values obtained with *K. pneumoniae*, *C. butyricum* and *C. diolis* using an experimental set up like the one employed in this work.

Microorganism	S_{PG} (g/g)	Y_{PG} (g/g)	Reference
K. pneumoniae	0.41	0.33	[29]
	0.46	0.34	[30]
C. butyricum	0.57	0.34	[10]
C. diolis	0.47	0.32	[23]
K. oxytoca	0.72	0.31	This work