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),

Nomenclature

$C_{1,3\text{-PD}}$	1,3-PD concentration (g/L)
C_{Gly}	Glycerol concentration (g/L)
C_{Gly}^{0}	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)
$\overline{\overline{SN}}_{j}$	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$_2$SO$_4$ 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

3. Calculation

The Taguchi method was employed for experimental design [34]. Traditional full or fractional factorial design and response surface methods involve a higher amount of experiments than the Taguchi method, especially when the number of factors or the levels at which they are to be applied are greater than two (that is the case in this work). The Taguchi method, on the other hand allows for the detection of the relationship between factors and responses with less experimental work. In this work, this method has been implemented to optimize the culture medium composition. First, it is necessary to establish the nutrients requirements, taking into account both responses, biomass growth and glycerol utilization for 1,3-PD production. All of the variables and parameters presented in this section are appropriately described in the nomenclature section of this work.

The steps for the implementation of the experimental design in this work are as follows: (1) selection of the output variables to be optimized, (2) identification of the factors and choice of the levels to be tested, (3) selection of the adequate orthogonal design, (4) performance of experiments, (5) completion of a statistical analysis of the data and the signal-to-noise ratio and determination of the optimum factor levels and (6) conduction of confirmatory experiments.

To establish which of the selected factors have an effect on the chosen variables or responses, the signal-to-noise ratio (SN) has to be determined. In its simplest form, SN is the ratio of the mean (signal) to the standard deviation (noise), calculated in this work according to:

$$SN_i = -10 \times \frac{\log 1}{y_i^2} \tag{1}$$
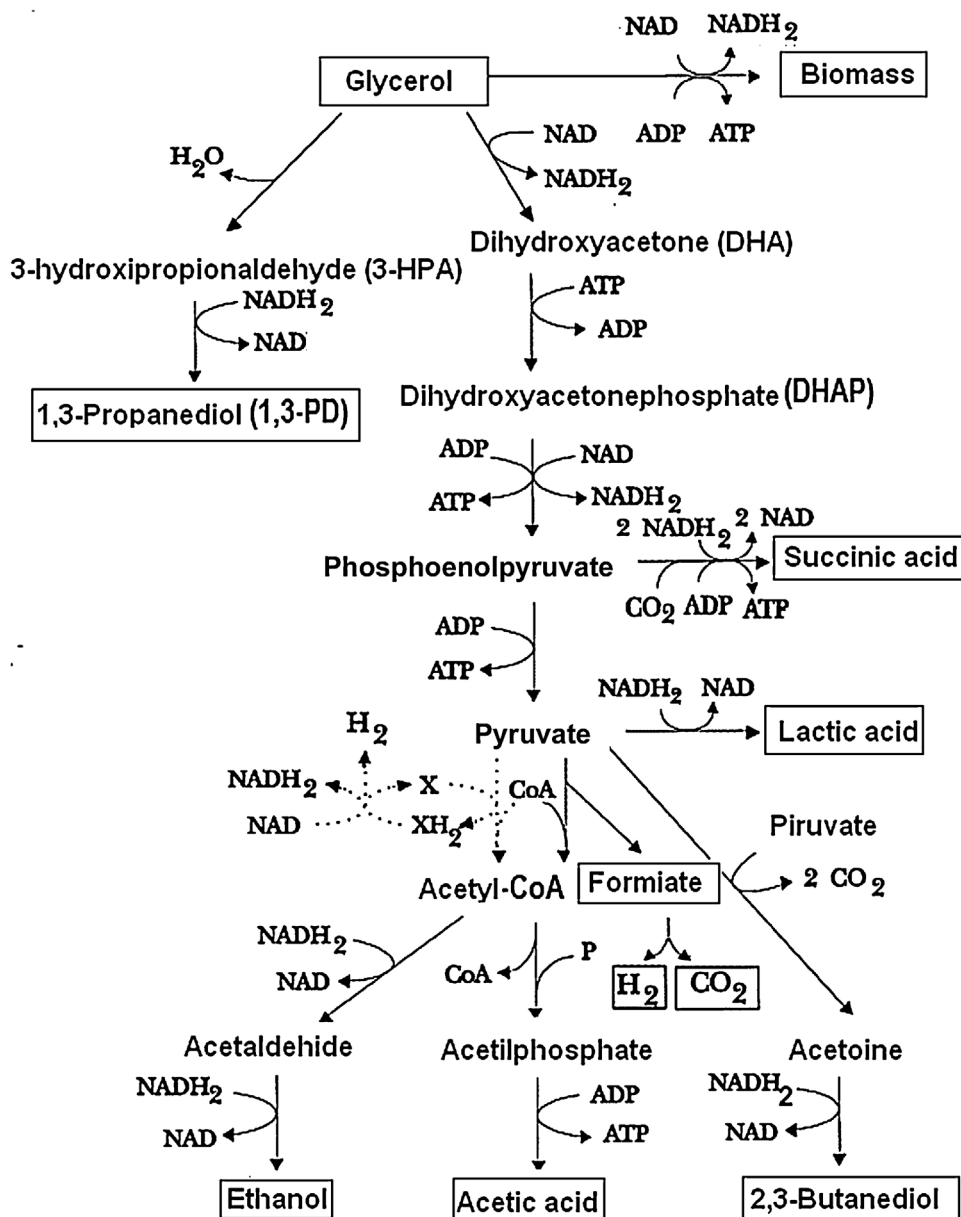

Fig. 1. Pathways for the glycerol metabolism in *Klebsiella* genus.

Once SN_i values have been calculated for each experimental data, the average value for each level ($j = 1, N_L$) is calculated by:

$$\overline{SN}_j = \frac{1}{N_L} \sum_{j=1}^{N_L} SN_j \tag{2}$$

Finally, the average value, $\overline{SN}$, is calculated for all the values following the next equation:
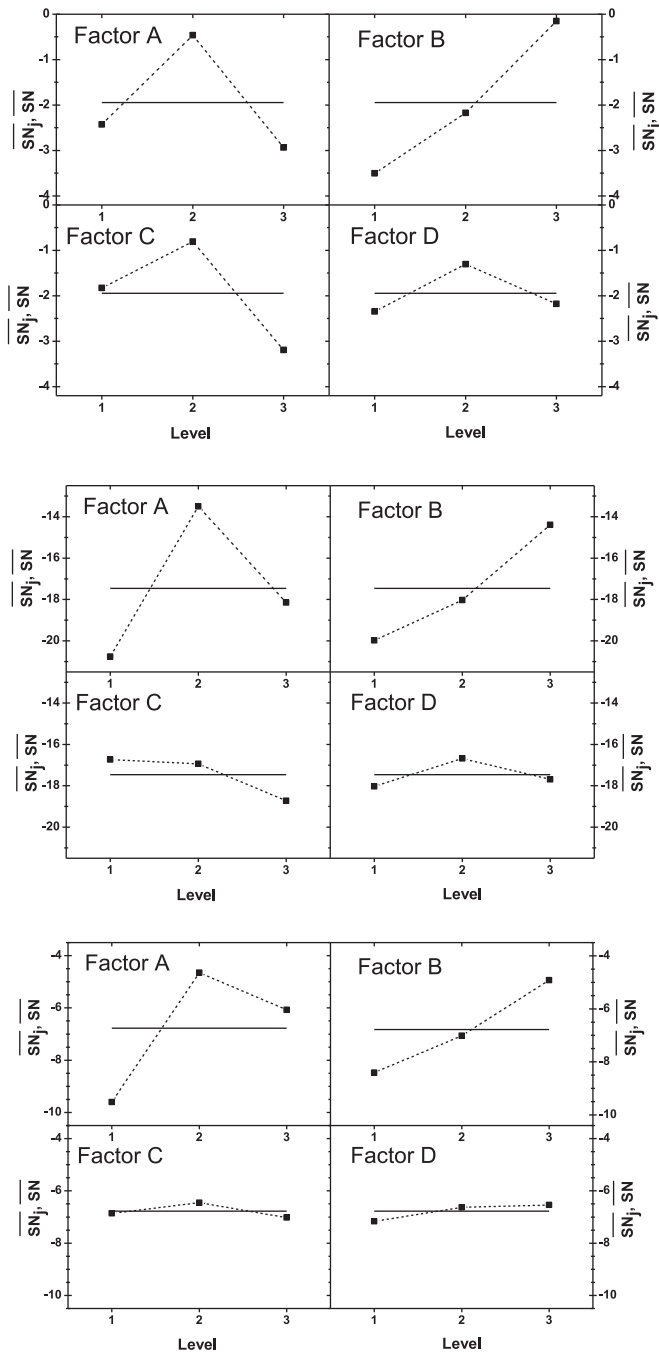
$$\overline{SN} = \frac{1}{N} \sum_{j=1}^{N} SN_j \tag{3}$$

Fig. 2. Application of Taguchi method. Values of $\overline{SN}_j$ and $\overline{SN}$ (A) for C_X^{max} values, (B) for Y_{PG} values, (C) for S_{PG} values.

Table 3
Factors and levels taken into account for the optimization of the medium composition.

Factor		Level	Value	Units
A	Gly	1	20	g/L
		2	40	
		3	80	
B	PO_4^{3-}	1	1.6	g/L
		2	3.1	
		3	6.2	
C	Mg^{2+}	1	0.5	mM
		2	1	
		3	2	
D	K:Na	1	0.33	g/g
		2	1	
		3	3	

As indicated above, three variables or responses have been considered, which are defined as follows:

C_X^{max}: maximum concentration of biomass reached at the stationary phase of growth. Y_{PG}: product yield referred to the initial glycerol concentration, given by:

$$Y_{PG} = \frac{C_{1,3-PD}}{C_{Gly_0}} \tag{4}$$

S_{PG}: product selectivity, taking into the account the amount of glycerol converted, defined by:

$$S_{PG} = \frac{C_{1,3-PD}}{C_{Gly_0} - C_{Gly}} \tag{5}$$

These calculations have been repeated for all variables (C_X^{max}, Y_{PG} and S_{PG}). Fig. 2A–C shows the results obtained in theses analyses, where the solid line depicts the value of $\overline{SN}$, Eq. (3), and the dotted line represents the values of $\overline{SN}_j$, as defined by Eq. (2).

4. Results and discussion

4.1. Study of the medium composition

To favor the optimal growth of *K. oxytoca* NRRL-B199 and the highest production of 1,3-PD, three variables or responses (C_X^{max}, Y_{PG} and S_{PG}) have been considered. The experimental conditions (factors) considered in this study are the concentrations of glycerol, phosphate and magnesium, as well as the K:Na ratio. Glycerol is expected to strongly affect both the growth and the production of 1,3-PD because it is the carbon source for bacterial growth and the raw material to produce 1,3-PD. Phosphate concentration is usually

Table 4
Experimental design and results according to the Taguchi method.

	Factor/level				Experimental results		
Run	A	B	C	D	C_X^{max} (g/L)	Y_{PG} (g/g)	S_{PG} (g/g)
1	1	1	1	1	0.61	0.07	0.26
2	1	2	2	2	0.90	0.10	0.34
3	1	3	3	3	0.78	0.11	0.41
4	2	1	3	2	0.74	0.15	0.48
5	2	2	1	3	0.91	0.21	0.58
6	2	3	2	1	1.27	0.29	0.72
7	3	1	2	3	0.66	0.10	0.44
8	3	2	3	1	0.57	0.09	0.45
9	3	3	1	2	0.96	0.21	0.62

Table 5
Experimental values of the three objective functions (C_X^{max}, Y_{PG} and S_{PG}) in runs carried out to check the wellness of the optimized medium.

Run	Factor/level				C_X^{max} (g/L)	Y_{PG}(g/g)	S_{PG} (g/g)
10	2	3	2	2	1.29	0.31	0.72
6	2	3	2	1	1.27	0.29	0.72
11	3	3	2	2	1.15	0.24	0.69
9	3	3	1	2	0.96	0.21	0.62

a significant factor, being an essential nutrient for all the bio-synthetic and bio-energetic requirements of the cells; additionally, phosphate is also known to be a frequent limiting nutrient. Magnesium concentration was chosen because of its great influence in cell metabolism. The study of the influence of K:Na ratio was considered due to its possible influence on the transport of 1,3-PD through the cell membrane.

Three levels for each control factor were chosen to detect any quadratic or non-linear relation between the factors and the output variables, all of which are shown in Table 3. The selection of these levels was based on previous results [20,12,27,35,29]. Thus, an experimental design consisting of four control factors at three levels containing eight degrees of freedom has been employed; therefore, it can be fitted to the L9 orthogonal array, which provides the greatest amount of information from the minimum number of experiments (Table 4). The nine runs resulting from this design were conducted in triplicate; the factor/level employed and the experimental results of each one of these runs are compiled in Table 4.

The choice of the optimal set of variable levels on the object of the optimization. In order to maximize the values of the variables (C_X^{max}, Y_{PG} and S_{PG}), the optimal level of each factor is that which yields the highest values of the response variables. Fig. 2A shows that the four control factors (i.e., glycerol, phosphate, magnesium concentrations and K:Na ratio) affect the output variable C_X^{max}. The optimal levels of the different factors resulted from the combination 2-3-2-2 for factors A, B, C and D, respectively.

Fig. 2B and C shows that, for the two latter responses (Y_{PG} and S_{PG}), the influence of the four different factors is very similar. Glycerol and phosphate initial concentrations strongly affect the values of both variables, while the other two factors, magnesium initial concentration and the K:Na ratio, hardly affect the product yield and selectivity obtained. With respect to the optimal values of the different factors, the combination 2-3-2-2 can be selected (see Fig. 2B and C).
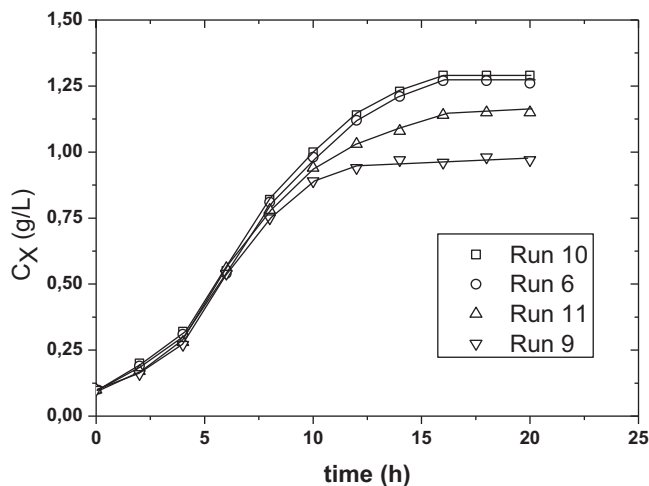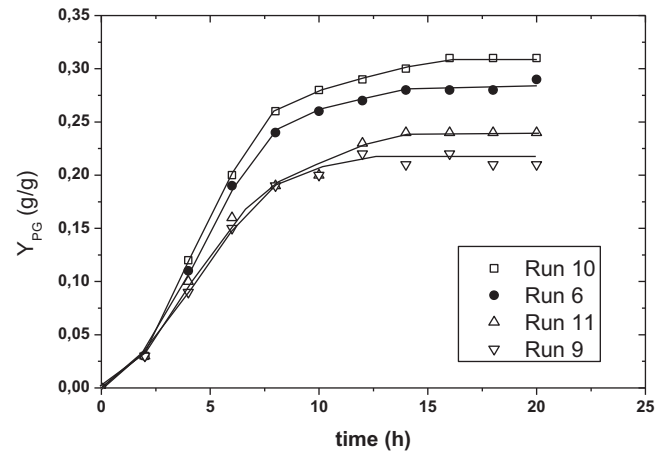
Fig. 4. Y_{PG} values versus time course for runs of Table 5.
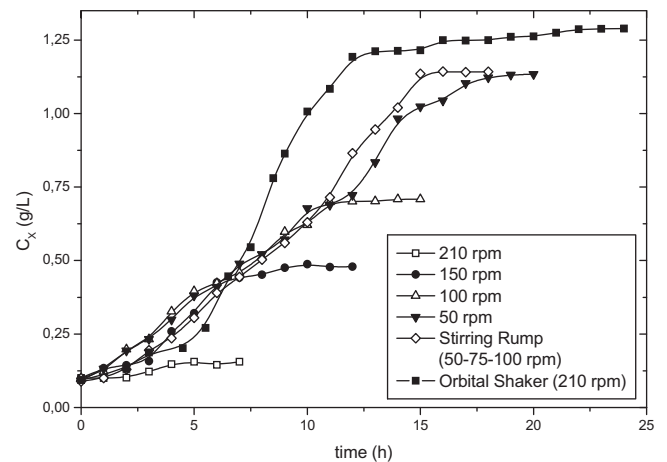
Fig. 5. Variation of biomass concentration with time course in the runs of the stirring study (Table 6).
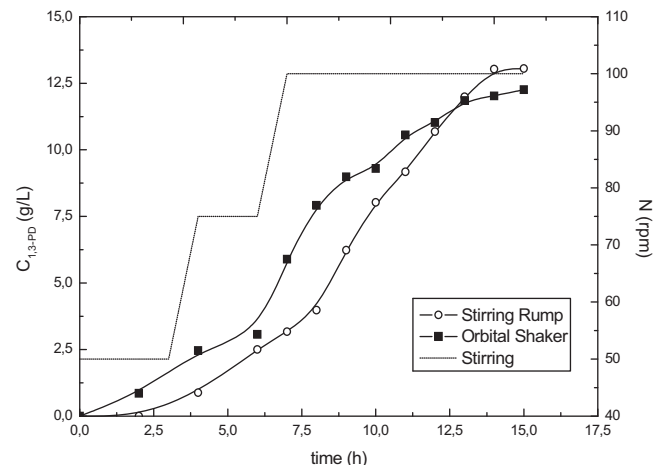
Fig. 3. Variation of biomass concentration throughout the time course in the runs described in Table 5.

Fig. 6. Variation of 1,3-PD concentration with time course in the runs of the stirring study (Table 6).

Table 6
Experimental results obtained in the stirring study.

Run	Bioreactor: stirrer speed (rpm)	C_X^{max} (g/L)	$C_{1,3-PD\ max}$ (g/L)	Y_{PG} (g/g)	S_{PG} (g/g)
12	STBR: 50	1.12	10.12	0.25	0.77
13	STBR: 100	0.71	6.24	0.16	1.06
14	STBR: 150	0.48	5.11	0.13	1.22
15	STBR: 210	0.16	4.58	0.11	1.17
16	Orbital shaker: 210	1.26	12.26	0.31	0.88
17	STBR: stirring ramp	1.14	13.06	0.33	0.86

Table 7
Experimental results obtained in the temperature study.

Run	Temperature (°C)	C_X^{max} (g/L)	$C_{1,3-PDmax}$ (g/L)	Y_{PG} (g/g)	S_{PG} (g/g)
18	30	1.14	13.06	0.33	0.86
19	32	1.12	11.24	0.28	0.89
20	34	1.18	12.23	0.31	0.81
21	37	1.26	13.51	0.34	0.86
22	38	1.07	12.9	0.32	0.94
23	39	0.51	3.09	0.08	0.59

According to these results, the optimal combination of the four levels in order to obtain the highest values of C_X^{max}, Y_{PG} and S_{PG} is that corresponding to the factor levels: 2-3-2-2. To validate this optimization process, two new runs were carried out, which were not conducted initially following the experimental design given in Table 4. Thus, run 10 corresponds to the best conditions deduced from the optimization method, run 11 changes the initial glycerol concentration, and runs 6 and 9 (included in the previously performed statistical design) have been selected due to their good results. As can be seen in Table 5, the results provided by the optimized medium are better than those reached with other media, although the results of runs 6 and 10 are very similar. Figs. 3 and 4 show the evolution of both responses C_X (Fig. 3) and Y_{PG} (Fig. 4) throughout the performance of the runs featured in Table 5. With the optimized medium (run 10), a concentration of 1,3-PD of 12.4 g/L is reached at 16–20 h, showing a yield (Y_{PG}) of 0.31 and a selectivity value (S_{PG}) of 0.72.

4.2. Study of the operating conditions in STBR

Once the culture medium composition was determined by means of the Taguchi method, the operational conditions of the process were studied in a 2 L SBTR under anaerobic conditions (1 L/min nitrogen flow rate), taking advantage of the experimental conditions determined for the operation in shaken bottles. Initially, four experiments employing different stirring speeds were carried out., all of which were performed with an initial glycerol concentration of 40 g/L, with 0.1 g/L of initial biomass concentration and a temperature of 30 °C (see Figs. 5 and 6). Comparing the biomass concentration time course obtained both in shaken bottles and in SBTR experiments, it was surprising that the best results were obtained in the bottles agitated in an orbital shaker. The results of the runs carried out in SBTR show that low stirring improves the production of biomass, yet a lower production of 1,3-PD is achieved, as can be seen in Table 6.

Based on these results, a different experimental approach was performed by changing the stirring speed throughout the time course (run identified as *stirring ramp*). The stirring speed of this run was changed as follows: for the first 5 h, stirring was fixed at 50 rpm, from 5 to 8 h the stirrer speed was increased to 75 rpm, and from that time throughout the remaining of the run, it was escalated to a value of 100 rpm. Employing this program of the stirrer speed, the maximum biomass concentration reached was similar to that obtained in orbital shaker; while the maximum 1,3-PD concentration was increased by 6% (see Table 6). Therefore, the 1,3-PD yield (Y_{PG}) was 0.33 and the selectivity value was 0.86. Figs. 5 and 6 show the results in all the runs carried out in the stirring study. As demonstrated by these figures, the strategy employed in this run named as *stirring ramp* improves the orbital shaker results, hence, this strategy was chosen for further studies.

The next variable analysed was temperature. To determine the optimal value of temperature for this process, six experiments using different values (from 30 to 39 °C) of this variable were performed, as shown in Table 7. These runs were carried out employing the best conditions previously determined: stirring strategy (*stirring ramp*) as described above, using 40 g/L of initial glycerol concentration and 0.1 g/L of initial biomass concentration. The experimental results are given in Figs. 7 and 8. As can be seen, the maximum biomass (Fig. 7) and 1,3-PD concentrations (Fig. 8)
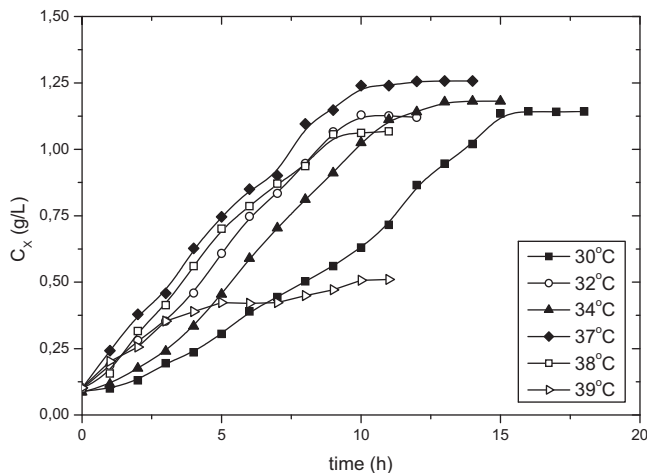

Fig. 7. Variation of biomass concentration with time course in the runs corresponding to the temperature study (Table 7).
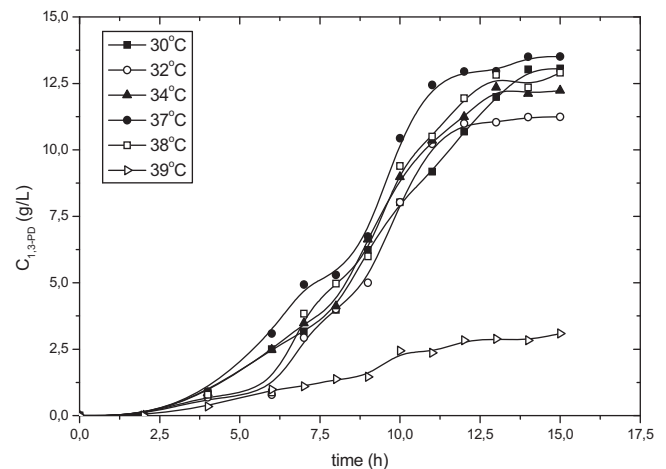

Fig. 8. Variation of 1,3-PD concentration with time course in the runs corresponding to the temperature study (Table 7).

are reached when a temperature of 37 °C is employed. Moreover, the growth and production rates are also maximal for this temperature.

Under these conditions, the highest biomass and 1,3-PD concentrations were 1.26 g/L and 13.5 g/L, respectively; the 1,3-PD yield (Y_{PG}) was 0.34, and the substrate uptake was 39.1%, with a selectivity value of 0.86. All of these values are included in Table 7. Remarkably, it was surprising that the transition from 38 °C to 39 °C caused a dramatic change in the growth and 1,3-PD production rates.

5. Conclusions

A new experimental procedure has been developed to optimize the production of 1,3-PD in *K. oxytoca*. The Taguchi method was applied to optimize medium composition, taking into account the biomass growth and glycerol utilization to yield 1,3-PD as response variables. The results obtained using this method showed a clear influence of two of the selected factors (glycerol and phosphate concentrations) on both responses.

The optimal composition of the culture medium was fixed at the following amounts of components on a per liter basis: 40 g of glycerol, 15.2 g of $Na_2H(PO_4) \cdot 12H_2O$, 3.1 g of KH_2PO_4, 0.5 g of KCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CaCl_2$, 2 g of NH_4Cl and 1.5 g of yeast extract.

Employing this culture medium, the highest biomass (1.29 g/L) and 1,3-PD (12.4 g/L) concentrations were reached after 16 h of culture with an additional Also, a high value of 1,3-PD selectivity is obtained (S_{PG} of 0.72). These S_{PG} and 1,3-PD concentration values using the optimized culture medium were higher than those previously reported for other bacteria with this fermentative capability, like *K. pneumoniae* or *C. butyricum* (see Table 2).

When the operational conditions were studied in a SBTR, the results showed that the best operational conditions were obtained using a varying stirring rate (from 50 to 100 rpm) and a temperature of 37 °C. Under these conditions, a concentration of 13.5 g/L of 1,3-PD was obtained, and the values of Y_{PG} and S_{PG} were 0.31 and 0.81, respectively. Although the yield (Y_{PG}) obtained in this work is within the same range as the values obtained in literature, the selectivity (S_{PG}) value is higher than that in other previous works reported in literature, operating in similar STBR batch conditions, where this variable reaches only 0.55–0.57 g/g [11,37,1] (see Table 2).

The results presented above represent a proof of concept to demonstrate that the rational experimental approach developed in this work can contribute with minimum time consumption and cost to further improve the production of important industrial metabolites like 1,3-PD in other still under explored bacterial producers.

Acknowledgements

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