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Production and productivity of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* GLC29

Gervásio Paulo da Silva [a,*], Cristian J. Bolner de Lima [b], Jonas Contiero [b]

[a] *Microorganism Biotechnology Laboratory, Education Department, Bahia State University (UNEB), BR 407, Km 127, CEP 48970-000, Senhor do Bonfim, BA, Brazil*

[b] *Industrial Microbiology Laboratory, Biochemistry and Microbiology Department, Biological Sciences Institute, São Paulo State University (UNESP), Av 24A, 1515, Bela Vista, CEP 13506-900, Rio Claro, SP, Brazil*

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ABSTRACT

Interest in the development of the bioproduction of 1,3-propanediol, an important chemical intermediate with various industrial applications, has increased in recent years. *Klebsiella pneumoniae* is one of the most studied and efficient bacteria for 1,3-propanediol production from glycerol. A new isolate of *K. pneumoniae* was investigated using response surface methodology by central composite design for the production of 1,3-propanediol using glycerol. The effects of pH, temperature, stirrer speed, and glycerol concentration on the production and productivity of 1,3-propanediol were examined. Considering both production and productivity, the best conditions for glycerol conversion in 1,3-propanediol are: a pH range of 6.9–7.1, a temperature between 33 and 38.5 °C, a stirrer speed of 110–180 rpm, and a glycerol concentration of 39–49 g l^{-1}. Batch fermentation carried out at a pH of 7.0, a temperature of 35 °C, a stirrer speed of 150 rpm, and a glycerol concentration of 40 g l^{-1} produced 20.4 g 1,3-propanediol l^{-1}, with a maximum volumetric productivity of 2.92 g l^{-1} h^{-1} and a yield of 0.51 g g^{-1}. The main byproducts were acetic acid (approximately 7.0 g l^{-1}) and formate (approximately 3.7 g l^{-1}). The newly isolated *K. pneumoniae* GLC29 showed potential for the conversion of glycerol into 1,3-propanediol, with high production and productivity.

1. Introduction

In recent years, many countries have adopted biofuels, such as biodiesel, an alternative diesel fuel produced from renewable sources. Their increased use is related to environmental pollution caused by the emission of greenhouse gases into the atmosphere mainly through the burning of fossil fuels. Fossil oil is a non-renewable resource, thus leading to price instability in the international market. Biodiesel is a mono-alkyl ester of long chain fatty acids produced from vegetable oils and animal fats. A transesterification reaction between the oil and an alcohol in the presence of a catalyst produces esters (biodiesel) and about 10% glycerol (w/w) as a byproduct [1]. Glycerol, a substance recognized as safe for human and animal health, is a chemical with widespread uses in several industrial sectors, like food, cosmetics and pharmaceutical industries, but the increasing biodiesel production worldwide is responsible for the generation of a glycerol surplus in the market. Crude glycerol from biodiesel refineries consists of glycerol, water, salts such as potassium, and residual alcohol [2,3]. Glycerol must be highly purified to be used in products such as drugs and food, and the high cost of purification makes this process impractical for the glycerol derived from biodiesel industries [4].

This glycerol surplus is an environmental problem for biorefineries since its disposal and/or storage generates economic costs, raising the final biodiesel price. Therefore, the search for new glycerol applications is one objective of researchers and industries. Possibilities for new glycerol utilization by chemicals [5] or industrial microbiology [1] have been reviewed. Among possible new applications in the chemical industry is the use of glycerol as a raw material for the production of various chemicals, including mesoxalic acid, 1,3-dicloropropanol, glyceryl ethers, glycerol carbonate, glyceryl esters, hydroxypyruvic acid, and polyglycerol [5]. Biotechnologically, glycerol can be converted by microorganisms in a number of valuable chemicals such as 1,3-propanediol, dihydroxyacetone, succinic, propionic, and citric acids, ethanol, pigments, polyhydroxyalkanoates, biosurfactants, 2,3-butanediol, amino acids, glyceric acids, D-xylulose, hydrogen, 3-hydroxypropionaldehyde, and fatty acids [1,6–16]. Microbial bioconversion of glycerol has a number of advantages over chemical synthesis, including a greater selectivity on the final products,

* Corresponding author. Tel.: +55 74 3541 8929.
E-mail addresses: gpsilva@uneb.br, gpaulosilva@yahoo.com.br (G.P. da Silva).

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efficiency, and a shorter reaction time. In general, chemical synthesis requires several intermediate steps before reaching the final product, specific catalysts, and extreme and well controlled conditions such as high temperatures and high pressures [5,9], which favor the biotechnological route of glycerol bioconversion [17].

One of the main products obtained through microbial bioconversion of glycerol is 1,3-propanediol (1,3-PDO), a polyol with applications in the cosmetics, food, lubricant, and pharmaceutical industries [18–21]. The development of polypropylene terephthalate (PPT), a thermoplastic with superior physicochemical properties to those of polyethylene terephthalate (PET), used in the production of fabrics, carpets, and engineered plastics has created a new demand for 1,3-propanediol. Interest in the commercial production of 1,3-PDO through either chemical synthesis or microbial conversion of glycerol has increased in recent years. Chemically, 1,3-PDO is produced through two different routes. One of them uses acrolein (2-propenal) as a raw material, which is hydrated to 3-hydroxypropionic acid, which is then hydrogenated in the presence of a catalyst to 1,3-PDO. Another route involves the hydroformylation of ethylene oxide with CO and H under high pressure in the presence of a catalyst and a solvent [18]. This reaction produces a dioxane that is hydrogenated to 1,3-PDO. These methods are expensive, create pollutants, [22] and are dependent on crude oil.

Bioconversion of glycerol into 1,3-propanediol has been demonstrated only in bacteria, such as *Citrobacter freundii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, *Clostridium butyricum*, and *Enterobacter agglomerans* [1,18,23,24]. One of the most studied and efficient bacteria for 1,3-propanediol production is *K. pneumoniae* [18,24]. A number works have focused on improving the production and productivity of 1,3-propanediol using glycerol. Yang et al. [25], in fed-batch fermentation at a pH of 7.0, a temperature of 37 °C, and microaerobiosis, using mutants of *K. oxytoca* deficient in lactate formation and sucrose as a co-substrate, obtained 83.56 g 1,3-PDO l^{-1}, with a yield of 0.62 mol mol^{-1}, and a productivity of 1.61 g l^{-1} h^{-1}, with 60.11 g l^{-1} of 2,3-butanediol (2,3-BDO) produced in parallel with 1,3-PDO. Seo et al. [26] obtained mutants deficient in the oxidative pathway, however, the production of 1,3-PDO was not improved, probably due to a redox imbalance. Zhu et al. [27] cloned the *yqh*D gene encoding 1,3-propanediol oxidoreductase isoenzyme (PDORI) from *Escherichia coli* in *K. pneumoniae.* The overexpression of PDORI led to a higher 1,3-PDO production, reaching 67.6 g l^{-1}. In addition, the concentration of the toxic intermediate 3-hydroxypropionaldehyde was reduced by 22.4% when compared to the original strain. Huang et al. [15] examined the effects of an over-expressed aldehyde dehydrogenase gene on the simultaneous production of 3-hydroxypropionic acid and 1,3-PDO by *K. pneumoniae*, obtaining 24.4 g l^{-1} and 49.3 g l^{-1}, respectively. A high production of 1,3-PDO using co-substrates is recorded by Oh et al. [28] using *K. pneumoniae* mutant deficient in carbon catabolite repression. Under optimized conditions, the concentration of 1,3-PDO from glycerol was 81.2 g l^{-1} using molasses as a co-substrate. Rossi et al. [20] reported concentrations up to 23.80 g 1,3-PDO l^{-1} in batch fermentations under controlled pH, while in fed-batch cultivations the 1,3-PDO production was 36.86 g l^{-1} using a new strain of *K. pneumoniae*. Sattayasamitsathit et al. [29] applied a statistical optimization for the simultaneous production of 1,3-PDO and 2,3-BDO using crude glycerol by a new *K. pneumoniae* isolate. They reported a yield of 24.98 g 1,3-PDO l^{-1} and 9.54 g 2,3-BDO l^{-1}. A high production of 1,3-PDO by a D-lactate deficient mutant of *K. pneumoniae* was reported by Xu et al. [30], obtaining 102.06 g 1,3-PDO l^{-1} from aerobic fed-batch fermentation. The same approach was tested by Durgapal et al. [31], which was used to construct a *K. pneumoniae* mutant for lactate formation. In glycerol fed-batch fermentation, the mutant strain produced 58.0 g 1,3-PDO l^{-1} with a yield of 0.35 g g^{-1} and 2,3-BDO as the main byproduct (26.6 g l^{-1}).

In this work, response surface methodology was used to determine the interaction effect of four independent variables (pH, temperature, stirrer speed, and glycerol concentration) on 1,3-propanediol production and productivity by a new *K. pneumoniae* isolate.

2. Materials and methods

2.1. Isolation and identification of microorganisms

For the isolation of microorganisms that use glycerol as their only carbon and energy source, Erlenmeyer flasks containing an enrichment minimal medium (g l^{-1} of deionized water: $NH_4H_2PO_4$ 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.2, NaCl 5.0, glycerol 20) were directly inoculated with different natural samples (soil, decaying plant, leaves, mosses, etc.) and incubated at 30 °C and 100 rpm on a rotatory shaker. Pure cultures were obtained by inoculating Petri dishes containing the same enrichment medium. The potential of different isolates were determined by evaluating glycerol bioconversion in high value-added products (data not shown). The isolate GLC29 showed high potential for glycerol fermentation and was identified as a *K. pneumoniae* strain using the Enterobacteriaceae identification kit API 20E (Biomerieux, France). The new isolate GLC29 was characterized for ethanol tolerance, growth pH, osmotolerance, and carbon sources used. Cultures were maintained refrigerated in agar slants containing minimal medium, with periodical transference to new media; long time maintenance was performed in cryotubes containing glycerol 40% (v/v) at −20 °C.

2.2. Glycerol fermentation

For the preparation of the inoculum, the bacterium *K. pneumoniae* GLC29 from agar slants was grown in 500 ml Erlenmeyer flasks containing 200 ml of autoclaved (121 °C/15 min) minimal medium (g l^{-1} of deionized water: $NH_4H_2PO_4$ 5.0, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.2; NaCl 1.0, yeast extract 1.0, glycerol 20) and 200 μl of trace element solution (g l^{-1} of deionized water: EDTA 0.5, $CaCl_2{\cdot}2H_2O$ 0.5, $CoCl_2{\cdot}6H_2O$ 0.16, $MoNH_4{\cdot}4H_2O$ 0.1, $CuSO_4{\cdot}5H_2O$ 0.16, $FeSO_4{\cdot}7H_2O$ 0.5, $MnSO_4{\cdot}H_2O$ 0.5, $ZnSO_4{\cdot}7H_2O$ 0.22, $NiCl_2{\cdot}6H_2O$ 0.03, H_3BO_3 0.12). Seed culture flasks were incubated overnight on a rotatory shaker at 30 °C and 100 rpm.

The fermentation medium was the same as for seed inocula. Glycerol was added in different concentrations, and the pH was adjusted with NaOH, in agreement with each experiment (Table 1). Batch fermentations were carried out on a 2 l reactor containing 500 ml final working volume, with a 5% (v/v) inoculum. The pH was maintained through the automatic addition of 5 M NaOH, and the temperature was controlled by a water bath with microprocessor-based temperature control. The fermentation medium was stirred magnetically with cylindrical stir bars (12 mm × 55 mm). Each fermentation experiment was run in duplicate or triplicate.

2.3. Analytical methods

Fermentation samples were withdrawn periodically for growth monitoring and to determine residual substrates and metabolites. Samples for HPLC were centrifuged (approximately 12,000 × *g*/8 min), and the supernatant was frozen for posterior analysis. Cell growth was monitored at 600 nm (OD_{600}) on a Bel Photonics SP-220 UV/vis spectrophotometer. A standard curve was constructed relating OD_{600} to cell dry weight (CDW). The pH was recorded for fermentation monitoring (pH rises after carbon source depletion), and 5 M NaOH consumption was recorded for the correction of dilution due to base addition in the reactors. Residual substrate and metabolites (succinate, lactate, formate,

acetate, ethanol, 1,3-propanediol, and 2,3-butanediol) were quantified through high-performance liquid chromatography (HPLC Thermo Separation Products) equipped with an in-line UV/vis and RI detectors and an ion-exchange column (300 mm × 7.8 mm Rezex Roa organic acid – Phenomenex, Torrance, CA, USA). The samples were run at 60 °C and the mobile phase was 0.005 M H_2SO_4 at 0.4 ml min^{-1}.

2.4. Experimental design

The central composite design (CCD) was performed to test the effect of the independent variables pH, temperature, stirrer speed, and glycerol concentration on the production and productivity of 1,3-PDO by *K. pneumoniae* GLC29 using glycerol as the only carbon and energy source. Each independent variable was arranged at five levels with eight star points and three replicates at the center point. A second order polynomial model was used to determine the optimal interval of each variable for 1,3-PDO production and productivity. The variables of the experiments were coded according to the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \quad \text{where} \quad i = 1, 2, \ldots, K \tag{1}$$

where x_i is the coded value of an independent variable; X_i is the real value of an independent variable; X_{cp} is the real value of an independent variable at the center point; and ΔX_i is the step change value.

The behavior of the system was explained by the following quadratic equation:

$$Y = B_0 + \sum B_i X_i + \sum B_{ii} X_{i^2} + \sum B_{ij} X_i X_j \tag{2}$$

where Y is the predicted response, i.e., 1,3-PDO concentration and/or productivity; B_0 is the offset term; B_i is the linear effect; B_{ii} is the squared effect; and B_{ij} is the interaction effect. X_i is ith independent variable. A total of 27 experiments were carried out using the CCD method, with five levels each, namely pH (X_1), temperature (X_2), stirrer speed (X_3), and glycerol concentration (X_4) (Tables 1 and 2).

Table 1
Experimental range and levels of the independent variables used in central composite design.

Independent variables		Range and levels				
		$-\alpha$	−1	0	+1	$+\alpha$
pH	X_1	5.5	6.0	6.5	7.0	7.5
Temperature (°C)	X_2	26.5	30	33.5	37	40.5
Agitation (rpm)	X_3	70	120	170	220	270
Glycerol (g l^{-1})	X_4	15	25	35	45	55

2.4.1. Statistical analysis

The Statistica 7.0 software package (StatSoft, Tulsa, USA) was used for the experimental design and regression analysis of the experimental data. Response surface and contour plots were generated to understand the interaction between different variables. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was statistically tested by the coefficient of determination R^2 and the statistical significance determined by an F-test. The significance of the regression coefficients was tested by a t-test.

3. Results

3.1. K. pneumoniae *GLC29*

The isolated GLC29 showed a high potential for glycerol fermentation. GLC29 was isolated from bryophytes collected near the soil, growing at the base of *Terminalia catappa* and was identified as a newly isolated *K. pneumoniae* strain. This bacterium grows quickly in minimal medium containing only glycerol as its

Table 2
CCD values for the optimization of four mathematically predicted and experimentally observed variables for production and productivity of 1,3-propanediol by *K. pneumoniae* GLC29 using glycerol.

Essay	pH	Temperature (°C)	Stirrer speed (rpm)	Glycerol (g l^{-1})	1,3-Propanediol			
	X_1	X_2	X_3	X_4	Production (g l^{-1})		Productivity (g l^{-1} h^{-1})	
					Observed	Expected	Observed	Expected
1	6.0	30.0	120	25	13.22	12.33	1.47	1.66
2	6.0	30.0	120	45	21.88	20.92	1.62	1.29
3	6.0	30.0	220	25	14.30	13.5	1.94	2.17
4	6.0	30.0	220	45	22.91	22	1.62	1.67
5	6.0	37.0	120	25	14.19	12.17	1.95	1.89
6	6.0	37.0	120	45	20.98	20.14	2.22	2.25
7	6.0	37.0	220	25	12.53	11.54	2.04	1.76
8	6.0	37.0	220	45	20.50	19.43	1.84	1.99
9	7.0	30.0	120	25	13.10	12.99	2.46	2.17
10	7.0	30.0	120	45	23.55	23.91	2.82	3.13
11	7.0	30.0	220	25	11.98	12.19	1.93	1.92
12	7.0	30.0	220	45	22.19	23.02	2.82	2.74
13	7.0	37.0	120	25	14.09	14.37	3	2.97
14	7.0	37.0	120	45	25.05	24.67	5.03	4.66
15	7.0	37.0	220	25	11.99	11.77	1.89	2.08
16	7.0	37.0	220	45	21.73	21.99	3.8	3.63
17	5.5	33.5	170	35	10.29	13.62	1.33	1.28
18	7.5	33.5	170	35	18.36	16.84	3.27	3.43
19	6.5	26.5	170	35	17.01	17.24	1.86	1.76
20	6.5	40.5	170	35	14.45	16.04	2.66	2.87
21	6.5	33.5	70	35	18.25	19.62	2.98	3.19
22	6.5	33.5	270	35	17.65	18.1	2.78	2.68
23	6.5	33.5	170	15	7.73	9.1	1.34	1.31
24	6.5	33.5	170	55	27.46	27.91	2.35	2.49
25C	6.5	33.5	170	35	18.96	19.21	3.36	3.53
26C	6.5	33.5	170	35	19.89	19.21	3.96	3.53
27C	6.5	33.5	170	35	18.78	19.21	3.28	3.53

Table 3
Model coefficient estimated by multiple linear regression for 1,3-propanediol production by *Klebsiella pneumoniae* GLC29.

Factor	Regression coefficient	Standard error	*t* value	*P* value
Intercept	19.2100	0.9452	20.3237	0.0000
X_1	0.8046	0.3342	2.4076	*0.0330*
X_1^2	−0.9943	0.3544	−2.8051	*0.0158*
X_2	−0.2996	0.3342	−0.8964	0.3876
X_2^2	−0.6430	0.3544	−1.8141	0.0947
X_3	−0.3804	0.3342	−1.1383	0.2772
X_3^2	−0.0880	0.3544	−0.2483	0.8080
X_4	4.7020	0.3342	14.0705	*0.0000*
X_4^2	−0.1767	0.3544	−0.4987	0.6269
X_1X_2	0.3844	0.4093	0.9391	0.3661
X_1X_3	−0.4918	0.4093	−1.2018	0.2526
X_1X_4	0.5831	0.4093	1.4247	0.1797
X_2X_3	−0.4494	0.4093	−1.0979	0.2937
X_2X_4	−0.1543	0.4093	−0.3772	0.7126
X_3X_4	−0.0206	0.4093	−0.0504	0.9606

X_1 = pH; X_2 = temperature; X_3 = agitation; X_4 = glycerol concentration. Meaningful values at 95% confidence are in italics.

carbon and energy source. GLC29 grows between pH 4 and 10, with optimal growth between pH 5 and 8. NaCl osmotolerance varies between 0 and 4.0% (p/v) after 24 h of incubation, but still grows in higher NaCl concentrations when incubated for a longer time, that is, 5.5% in 44 h, up to a maximum of 6.5% in 72 h, and not growing in higher NaCl concentrations. *K. pneumoniae* GLC29 grows in up to 8% ethanol in mineral medium containing a glycerol concentration of 20 g l^{-1}. In a phenol red carbohydrate broth containing Durham tubes, this bacterium produces acid and gas from the following tested carbon sources: glucose, xylose, sucrose, maltose, lactose, mannitol, mannose, sorbitol, and glycerol. The products from glycerol fermentation are the 1,3-PDO, 2,3-BDO, ethanol, and the organic acids acetate, formate, lactate, and succinate.

3.2. *Production of 1,3-propanediol by* K. pneumoniae *GLC29*

Preliminary fermentations in a rotatory shaker showed that GLC29 consumed glycerol quickly, converting it to 1,3-propanediol as the main fermentation product (data not shown). The central composite design was used to determine values of pH, temperature, agitation, and glycerol concentration for optimal bioconversion of this substrate to 1,3-PDO. Linear and quadratic effects of each variable and the effect of interaction between them on 1,3-PDO production and productivity were analyzed. Table 2 presents the matrix of experiments, observed and expected production (g l^{-1}), and productivity (g l^{-1} h^{-1}) of 1,3-PDO. Results of the dependent variable were used to determine regression coefficients for 1,3-PDO production (Table 3), to calculate ANOVA (Table 4), and to construct response surface and contour plots (Fig. 1) for significant results. Eq. (3) is presented the complete equation as a function ofregression coefficients for 1,3-PDO production.

$$Y_{(\text{Production})} = 19.210 + 0.804X_1 - 0.299X_2 - 0.380X_3 + 4.702X_4 + 0.384X_1X_2 - 0.491X_1X_3 + 0.583X_1X_4 - 0.449X_2X_3 - 0.154X_2X_4 - 0.020X_3X_4 - 0.994X_1X_1 - 0.643X_2X_2 - 0.088X_3X_3 - 0.176X_4X_4 \quad (3)$$

The quadratic model in Eq. (3) with fourteen terms contains four linear terms, six factorial interactions and four quadratic terms, where $Y_{(\text{Production})}$ is the predicted response, i.e., the 1,3-PDO concentration in the fermentation medium, and X_1, X_2, X_3, and X_4 are respectively the coded values of the tested variables pH, temperature, stirrer speed, and glycerol concentration.

Table 3 shows the Student's *t* distribution and the corresponding values, along with the estimated parameters. The probability (*P*) values were used as a tool to check the significance of each coefficient. A larger magnitude of the *t*-test and a smaller *P*-value denote greater significance of the corresponding coefficient. As seen in Table 3, temperature of fermentation (X_2) and stirrer speed (X_3) had no effect on the final 1,3-PDO concentration, while the independent variables pH (X_1) and glycerol concentration (X_4) had a strong positive linear effect on the response ($P < 0.05$). The same is observed with the squared variable (X_1^2), where the negative sign revealed a reduction in 1,3-propanediol production when pH was increased in the system. The non-significant terms (on the basis of *P*-values above 0.05) were neglected, and the model Eq. (3) was modified to the reduced fitted model ($Y_{\text{Adjusted production}}$) of Eq. (4).

$$Y_{\text{Adjusted production}} = 18.241 + 0.804X_1 + 4.702X_4 - 0.812X_1X_1 \quad (4)$$

The statistical significance of Eq. (4) was checked using an *F*-test and the analysis of variance (ANOVA) for the response surface quadratic model, which is summarized in Table 4. Fisher's *F*-test ($F_{(14,12)} = S_m^2/S_S^2 = 15.828 > \text{Ft}_{(14,12)} = 2.62$ with a very low probability value [$(P_{\text{model}} > F) < 0.0000$] indicated that the model was significant. The goodness-of-fit of the model was checked by the determination coefficient (R^2) and the multiple correlation coefficient (R). In this case, the R^2 value (0.943) indicates that the sample variation of 94.3% for 1,3-propanediol was attributed to the independent variables, and only 5.7% of the total variation cannot be explained by the model. The adjusted determination coefficient (Adj. $R^2 = 0.888$) was also satisfactory for confirming the significance of the model.

The surface and contour plots were constructed from the ANOVA results (Table 4), showing the best pH interval for 1,3-PDO production and the interaction effect between pH and glycerol concentration (Fig. 1). Analysis of Fig. 1 shows that the best pH interval for glycerol conversion in 1,3-PDO was 6.4–7.1, and a higher production of 1,3-PDO occurred in higher concentrations of glycerol, in spite of the fact that productivity is negatively affected by high glycerol concentrations, as can be seen in the following section.

Table 4
Analysis of variance of the quadratic model for 1,3-propanediol yield by *Klebsiella pneumoniae* GLC29 using glycerol.

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* > *F*
Model	593.9063	14	42.4218	15.8278	0.0000
Residual	32.1624	12	2.6802		
Lack of Fit	21.5609	9	2.3957	6.750	0.1356
Pure error	0.7098	2	0.3549		
Total	626.0688	26			

$R^2 = 0.948$; Adj. $R^2 = 0.888$; $R = 0.973$.

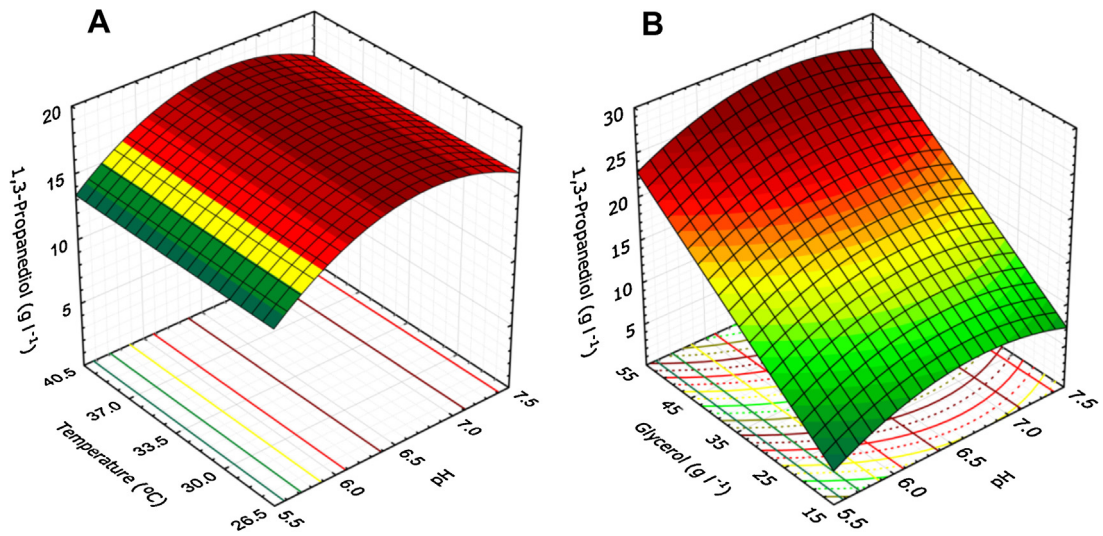

Fig. 1. Contour and surface plots of glycerol fermentation by *K. pneumoniae* GLC29, showing the best pH intervals (A) and the effect of glycerol concentration on 1,3-propanediol production (B). Temperature and stirrer speed were not significant for fermentation.

3.3. *Productivity of 1,3-propanediol by* K. pneumoniae *GLC29*

Glycerol bioconversion in 1,3-PDO was also analyzed with respect to maximum productivity, determined in the interval of time where the largest slope of the straight line plotting 1,3-PDO productivity versus fermentation time was observed. The results for productivity, observed and predicted by the statistical model, are shown in Table 2. At 95% probability, there were statistically significant effects on 1,3-PDO productivity from pH, temperature, glycerol concentration, and the quadratic coefficients of all independent variables, and interactions between pH and stirrer speed, pH and glycerol concentration, and temperature and glycerol concentration (Table 5). Table 6 presents regression coefficients for the productivity of 1,3-PDO by *K. pneumoniae* GLC29 using glycerol.

In order to determine the maximum 1,3-propanediol productivity corresponding to the optimum interval of pH, temperature, stirrer speed, and glycerol concentration, a second order polynomial model was proposed to calculate the optimum levels of these variables. By applying a multiple regression analysis on experimental data, a second order polynomial model explains the role of each variable and their second order interactions in producing 1,3-PDO. The quadratic model in Eq. (5) with 14 terms contains four linear terms, six factorial interactions, and four quadratic terms.

$$\begin{aligned} Y_{(\text{Productivity})} = {} & 3.533 + 0.539X_1 + 0.279X_2 - 0.129X_3 + 0.296X_4 \\ & + 0.143X_1X_2 - 0.191X_1X_3 + 0.331X_1X_4 \\ & - 0.161X_2X_3 + 0.183X_2X_4 - 0.033X_3X_4 \\ & - 0.295X_1X_1 - 0.305X_2X_2 - 0.150X_3X_3 \\ & - 0.408X_4X_4 \end{aligned} \quad (5)$$

Non-significant terms (on the basis of *P*-values above 0.05 as shown in Table 5) were neglected and the model Eq. (5) was modified to the reduced fitted model Eq. (6):

$$\begin{aligned} Y_{(\text{Adjusted productivity})} = {} & 3.533 + 0.539X_1 + 0.279X_2 + 0.296X_4 \\ & - 0.191X_1X_3 + 0.331X_1X_4 - 0.161X_2X_3 \\ & + 0.183X_2X_4 - 0.295X_1X_1 - 0.305X_2X_2 \\ & - 0.150X_3X_3 - 0.408X_4X_4 \end{aligned} \quad (6)$$

Surface and contour plots for interaction effects between independent variables were constructed from ANOVA results (Table 6). The model can be considered suitable because the percentage of variation explained is 94.5% for the complete equation and 88.2% for the adjusted model. The Fisher's *F*-test ($F_{(14,12)} = S_m^2/S_S^2 = 14.892 > \text{Ft}_{(14,12)} = 2.62$ with a very low probability value [$(P_{\text{model}} > F) < 0.0000$] indicated that the model was significant. These results allowed defining the best intervals for independent variables to obtain the highest 1,3-PDO productivity

Table 5
Model coefficient estimated by multiple linear regression for 1,3-propanediol productivity by *Klebsiella pneumoniae* GLC29.

Factor	Regression coefficient	Standard error	Computed *t* value	*P* value
Intercept	3.5333	0.1771	19.9496	0.0000
X_1	0.5387	0.0626	8.6036	*0.0000*
X_1^2	−0.2946	0.0664	−4.4369	*0.0008*
X_2	0.2787	0.0626	4.4515	*0.0007*
X_2^2	−0.3046	0.06647	−4.5875	*0.0006*
X_3	−0.1287	0.0626	−2.0561	0.0621
X_3^2	−0.1496	0.0664	−2.2537	*0.0437*
X_4	0.2962	0.0626	4.7310	*0.0004*
X_4^2	−0.4084	0.0664	−6.1495	*0.0000*
X_1X_2	0.1431	0.0767	1.8662	0.0866
X_1X_3	−0.1906	0.0767	−2.4856	*0.0286*
X_1X_4	0.3306	0.0767	4.3111	*0.0010*
X_2X_3	−0.1606	0.0767	−2.0944	0.0581
X_2X_4	0.1831	0.0767	2.3878	*0.0342*
X_3X_4	−0.0331	0.0767	−0.4319	0.6734

X_1 = pH, X_2 = temperature, X_3 = stirrer speed, X_4 = glycerol concentration. Meaningful values at 95% confidence are in italic.

Table 6
Analysis of variance for 1,3-propanediol productivity by *Klebsiella pneumoniae* GLC29 from glycerol.

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* > *F*
Model	19.6194	14	1.4013	14.8915	0.0000
Residual	1.1293	12	0.0941		
Lack of fit	15.7774	19	0.8304	6.0115	0.1521
Pure error	0.2762	2	0.1381		
Total	20.7486	26			

R^2 = 0.945, Adj. R^2 = 0.882.

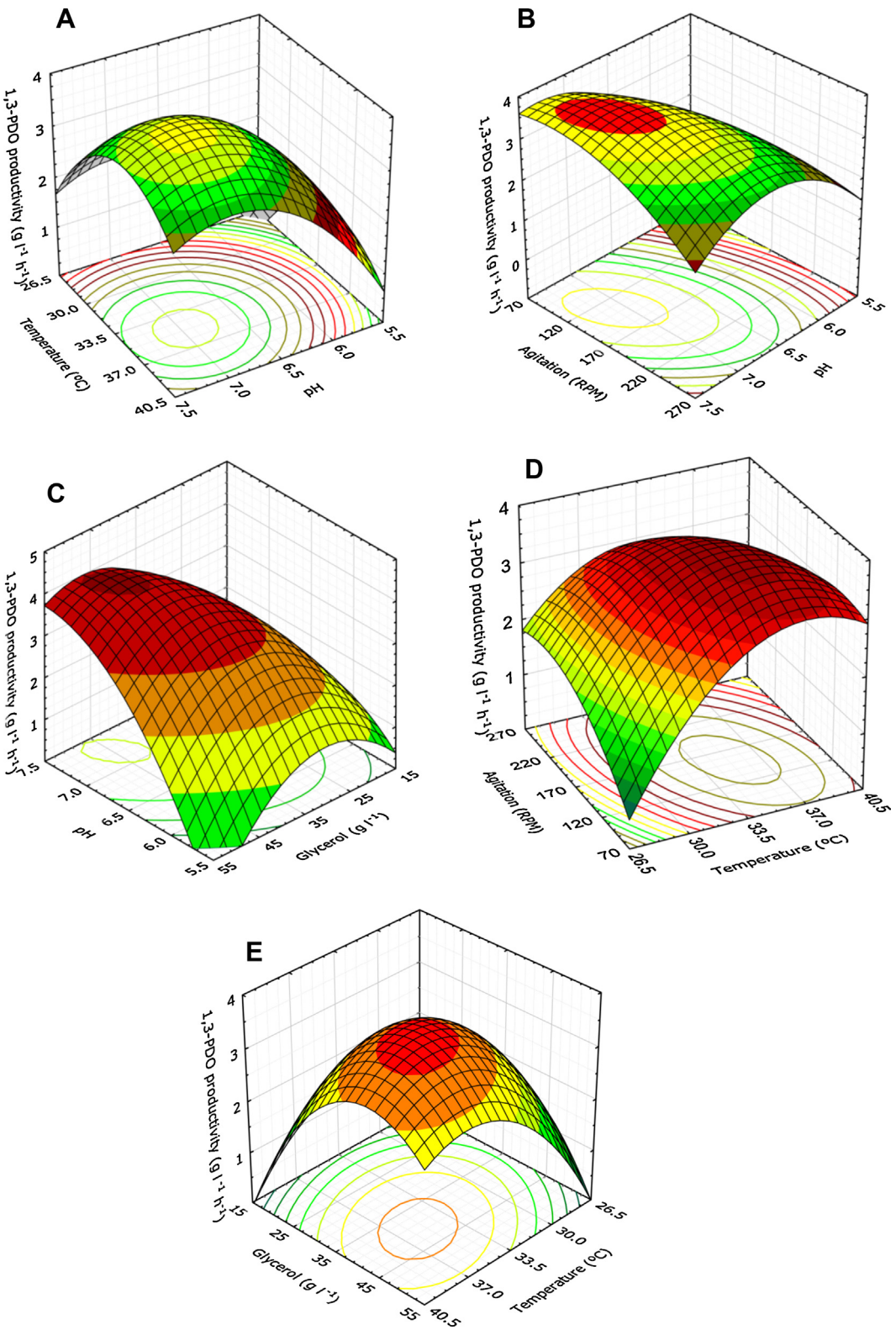

Fig. 2. Contour and surface plots of 1,3-propanediol productivity by *K. pneumoniae* GLC29 using glycerol. Effects of the interaction between pH and temperature (A), pH and agitation (B), glycerol and pH (C), temperature and agitation (D) and temperature and glycerol (E).

(Fig. 2). Analyzing contour and surface plots, the best intervals for higher 1,3-PDO productivity were as follows: a pH between 6.7 and 7.5 (Fig. 2a–c), a temperature ranging from 33 to 38.5 °C (Fig. 2a, d and e), a stirrer speed ranging from 110 to 180 rpm (Fig. 2b and d), and a glycerol concentration between 39 and 49 g l^{-1} (Fig. 2c and e). Considering these results, batch fermentation was carried out using the best intervals for 1,3-PDO production and productivity.

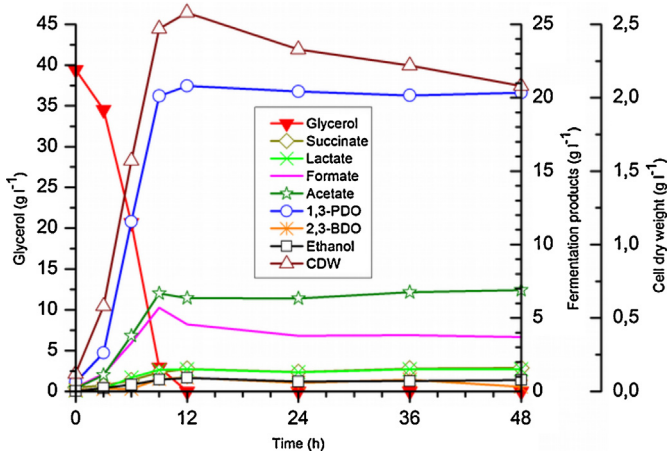

Fig. 3. Batch glycerol fermentation by *K. pneumoniae* GLC29 at pH 7.0, 35 °C, 150 rpm and 40 g of glycerol l^{-1}.

3.4. Batch fermentation

Considering the results obtained for production ($g\,l^{-1}$) and productivity ($g\,l^{-1}\,h^{-1}$) of 1,3-PDO by *K. pneumoniae* GLC29 using glycerol, batch fermentation was performed at a pH 7.0, a temperature of 35 °C, a stirrer speed of 150 rpm and a glycerol concentration of 40 $g\,l^{-1}$, which are within the optimal intervals for both parameters as seen in Figs. 1 and 2. In these conditions, GLC29 grew well producing about 2.5 g CDW l^{-1}. Glycerol was quickly consumed, taking just over 9 h of fermentation (Fig. 3). The final concentration of 1,3-PDO was about 20.4 $g\,l^{-1}$, with a yield of 0.51 g 1,3-PDO/g glycerol. Maximal productivity was 2.92 $g\,l^{-1}\,h^{-1}$, and the maximum rate of consumption of glycerol was 5.26 $g\,l^{-1}\,h^{-1}$. The main byproducts obtained were acetic acid (approximately 7 $g\,l^{-1}$) and formate (approximately 3.7 $g\,l^{-1}$). Small amounts of succinate (approximately 1.59 $g\,l^{-1}$) and lactate (1.51 $g\,l^{-1}$) were produced. The production of 2,3-BDO and ethanol was less than 1.0 $g\,l^{-1}$.

4. Discussion

The bacteria *K. pneumoniae* and *C. butyricum* are the most efficient microbial producers of 1,3-PDO using glycerol. Recently, these bacteria have been extensively studied for industrial applications in glycerol fermentation for their high production and productivity. In this work, preliminary batch fermentations in Erlenmeyer flasks and a rotatory shaker showed a new strain to be efficient in fermenting glycerol. This microorganism was identified as *K. pneumoniae* strain GLC29. The fermentation products were 1,3-PDO, 2,3-BDO, ethanol, acetate, lactate, succinate, and formate. In order to optimize the conditions for 1,3-PDO production, response surface methodology was performed to determine the effect of the independent variables pH, temperature, stirrer speed, and glycerol concentration on 1,3-PDO production and productivity.

The fermentation temperature and stirrer speed had no significant effect on 1,3-PDO production from glycerol, while the best pH was between 6.4 and 7.1. A higher production of 1,3-PDO occurred in higher concentrations of glycerol (Fig. 1a and b and Table 3). The fact that temperature did not interfere significantly with the 1,3-PDO concentration is interesting for industrial applications, because it allows working within a wide range of temperatures without losing yield in the process, reducing the cost of fermentation with respect to processes for which strict control of this parameter is required. However, productivity is negatively affected by both high and low temperatures. These results are in accordance with Rossi et al. [20], who observed that the variation of temperature had shown no effect on 1,3-PDO production by *K. pneumoniae* Blh-1. The results for temperature and pH for 1,3-PDO production found in this work is different from that of Zhang et al. [32], who observed that optimal cultivation parameters for temperature and pH using *K. pneumoniae* XJ-Li were 40 °C and pH 8.0, respectively. Zhang et al. [32] obtained 12.2 g of 1,3-PDO l^{-1} from 20 g glycerol l^{-1} consumed in about 8 h, with a productivity of 1.53 $g\,l^{-1}\,h^{-1}$ and a yield of 0.75 mol mol^{-1} (0.61 $g\,g^{-1}$). In batch fermentation at pH 7.0 and 35 °C, *K. pneumoniae* GLC29 produced 20.4 g of 1,3-PDO l^{-1} from 40 g glycerol l^{-1} consumed in about 9 h, with 2.92 $g\,l^{-1}\,h^{-1}$ of maximal productivity and a yield of 0.51 g 1,3-PDO g^{-1} glycerol (0.61 mol mol^{-1}). The difference in pH found between the XJ-Li and GLC29 strains could be explained by the origin of the isolates, since XJ-Li was isolated from micro-alkaline soil. A negative effect on 1,3-PDO production when pH was increased was also observed by Rossi et al. [20].

The pH is one of the parameters that determines the profile products in fermentation. For example, 2,3-BDO formation starts at pH 6.6 and reaches a maximum yield at pH 5.5, thereby producing fewer toxic products such as alcohols or glycols as a strategy to escape progressive acidity of the medium [33]. However, when acetate (and formate) is the main byproduct of fermentation, the 1,3-PDO yield is higher, because conversion of pyruvate to acetate produces ATP but does not consume the reducing equivalents (NADH) required for 1,3-PDO production [24,34]. In order to minimize the formation of 2,3-BDO (produced between pH 5.0 and 6.5) and lactic acid (produced between pH 7.1 and 8.0), Ji et al. [35] evaluated the production of 1,3-PDO, periodically alternating the pH of the medium to between 6.3 and 7.3 during fermentation. The best results obtained were 70 g of 1,3-PDO l^{-1}, a yield of 0.70 mol mol^{-1}, and a productivity of 0.97 $g\,l^{-1}\,h^{-1}$, with low concentrations of 2,3-BDO and lactate. Another parameter that increases lactate as well as 2,3-BDO production is an excess of glycerol in the medium [24,33].

The best intervals for a higher 1,3-PDO productivity were between pH 6.7 and 7.5, a temperature range of 33 and 38.5 °C, a stirrer speed of 110–180 rpm, and a glycerol concentration of between 39 and 49 $g\,l^{-1}$. When considering medium agitation, a higher stirrer speed is harmful to productivity, probably due to a higher aeration of the medium, which impairs fermentation. According to Biebl et al. [33], at a neutral pH the 1,3-PDO formation is reduced in the presence of oxygen, which is probably caused by the oxygen sensitivity of the two enzymes of the 1,3-PDO pathway. The authors speculate that under microaerobiosis and low pH, glycerol is converted to 2,3-BDO only, i.e., the NADH is recycled by oxygen and no longer by glycerol. According to Seifert et al. [36], inactivation of diol dehydratase by O_2 in the absence of a substrate occurs by a reaction of the activated Co-C bond of the enzyme-bound coenzyme B_{12} with O_2. Hongwen et al. [37] recorded maximal activity of key enzymes for 1,3-PDO production at pH 7.0 and that adequate oxygen favors cell growth, whereas excess oxygen is not suitable for accumulation of 1,3-PDO. Activity of the three key enzymes and biomass increased with shaker speed, with maximum biomass and activities of GDH and PDOR attained with a shaker speed of 200 rpm. Maximal activities of key enzymes were obtained at 37 °C; in temperatures above 37 °C, biomass and activities greatly decreased [37].

Productivity decreases with lower and higher glycerol concentrations. At high concentrations, glycerol could pass the cell membrane by passive diffusion, and at low concentrations, by glycerol facilitators [38]. Low substrate concentration has an obvious effect on transport across the cell membrane and, consequently, there is little substrate remaining for enzymatic reactions. At high concentrations, the enzyme glycerol dehydratase (GDHt, E.C. 4.2.1.30, convert glycerol to 3-hydroxypropionaldehyde) is the limiting step for the consumption of glycerol and consequently to produce 1,3-PDO in *K. pneumoniae* [39]. However, GDHt is subject to suicide

inactivation by glycerol during catalysis, which involves irreversible cleavage of the Co-C—C bond of the coenzyme B_{12}, forming 5′-deoxyadenosine and an alkylcobalamin-like species [36].

The newly isolated *K. pneumoniae* GLC29 strain contains several characteristics of a good industrial strain. For example, glycerol was fermented without strict anaerobic conditions, and no rich nutrient was required for fermentation. These characteristics make the fermentation process less expensive, which is pursued in industrial strains. Nevertheless, there are possibilities for better fermentation results, evaluating new medium compositions, supplementations, use of co-substrates, inocula, and by genetic engineering. Currently, the excess of glycerol produced as a byproduct of the biodiesel industry is of global concern, and several alternatives are being researched. One of the potential applications is to use glycerol as a carbon source in industrial microbiology to obtain products of high added value, and 1,3-propanediol appears to be one of the most promising alternatives. In this study, *K. pneumoniae* GLC29 presented a high potential for industrial application.

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