

Full Length Research Paper

Potential of *Klebsiella oxytoca* for 1,3-propanediol production from glycerol under excess substrate conditions

Miguel Angel Aparicio Rodriguez^{1*}, Aline Gozzi², Benício Alves de Abreu Filho³,
Gisella Maria Zanin⁴ and Flavio Faria de Moraes⁴

¹Department of Food Engineering and Technology, Federal University of Technology of Paraná, CEP 87301-006, Campo Mourão - PR, Brazil.

²Medical School, University of São Paulo, CEP 14049-900, Riberão Preto - SP, Brazil.

³Department of Clinical Analysis, State University of Maringá, CEP 87020-900, Maringá-PR, Brazil.

⁴Department of Chemical Engineering, State University of Maringá, CEP 87020-900, Maringá - PR, Brazil.

Accepted 29 June, 2012

Glycerol, a by-product of biodiesel, is a source of carbon for the bioproduction of chemical intermediates with high commercial value, such as 1,3-propanediol. One strain of *Klebsiella oxytoca*, isolated from soil samples, was tested to determine its potential to produce 1,3-propanediol through fermentation of a glycerol solution, under excess substrate conditions. The microbial growth curve was evaluated by optical density reading using a spectrophotometer (600 nm), while glycerol consumption and concentration of the products were determined by high performance liquid chromatography (HPLC) with HPX 87H column (BioRad). Formate, 3-hydroxypropionaldehyde and ethanol were produced in the early hours of fermentation. The increased rate of glycerol consumption and the formation of 1,3-propanediol coincides with formate degradation. This indicates that formate degradation likely works as an alternative means to generate part of the nicotinic adenine dinucleotide (NADH) used by the 1,3-propanediol-dehydrogenase enzyme. Yield in mole of product per 100 mol of substrate reached 48.5, which is higher than that of previously investigated *K. oxytoca* strains.

Key words: *Klebsiella oxytoca*, glycerol, fermentation, 1,3-propanediol, biodiesel.

INTRODUCTION

Glycerol degradation pathways have been widely studied in recent decades, under microaerobic and anaerobic conditions. The main reason behind that interest is centered on the need to identify new possibilities of use for that substrate, especially as a source of by-products with high commercial value. Glycerol is the main by-product of the biodiesel production process, in which every 1,000 kg of processed oil yields approximately 100 kg of glycerol. Therefore, as worldwide biodiesel production grows due to global biofuels policies, the

amount of glycerol produced increases yearly. The most evident effects of that phenomenon are: increased devaluation of the market price of glycerin and the potentially adverse environmental impact linked to that overproduction (Chen et al., 2003; Liu et al., 2007; Johnson and Taconi, 2007; Behr et al., 2008; Willke and Vorlop, 2008).

One of the by-products of greatest interest is 1,3-propanediol, a monomer used to produce polymers, polyester resins, paints, lubricants and antifreeze (Paster et al., 2003; Saxena et al., 2009; Maervoet et al., 2011; Leja et al., 2011). Under anaerobic conditions, glycerol is used as substrate by species *Klebsiella* through two linked metabolic pathways: one oxidative and another reductive. In the oxidative pathway, the glycerol-

*Corresponding author. E-mail: migrod@utfpr.edu.br. Tel: +55 44 35181482. Fax: +55 44 35181405.

dehydrogenase enzyme (EC 1.1.1.6) converts glycerol into dihydroxyacetone (DHA) with a concomitant reduction of nicotine adenine dinucleotide (NAD⁺) to NADH. Dihydroxyacetone is then phosphorylated by the action of the dihydroxyacetone-kinase enzyme (EC 2.7.1.29), and the obtained product, dihydroxyacetone phosphate, enters the glycolytic pathway.

The parallel reductive pathway consists of the glycerol-dehydratase enzyme (EC 4.2.1.30), which converts glycerol into 3-hydroxypropionaldehyde (3-HPA), and by the NADH-dependent 1,3 propanediol-dehydrogenase enzyme (EC 1.1.1.202), which converts 3-HPA into 1,3-propanediol (Lin, 1976; Johnson et al., 1985; Zeng et al., 1994; Bouvet et al., 1995; Ahrens et al., 1998; Yazdani and Gonzalez, 2007). The metabolic pathway under microaerobic conditions has not been fully explained (Huang et al., 2002; Chen et al., 2003; Wang et al., 2003; Hongwen et al., 2005).

MATERIALS AND METHODS

The composition of the growth medium, in g/L, was: 75.60 glycerol, 0.650 KH₂PO₄, 0.200 MgCl₂·6H₂O, 4.300 KCl, 0.200 K₂SO₄, 4.000 NaCl, 0.600 sodium thioglycolate, 4.9 (NH₂)₂CO. In addition, 4 ml of mineral solution and 4 ml of vitamin solution were added per liter of growth medium. The composition of the mineral solution in g/L was: 2.085 FeSO₄·7H₂O, 10.000 citric acid monohydrate, 0.735 CaCl₂·2H₂O, 0.422 MnSO₄·H₂O, 1.363 ZnCl₂, 0.242 Na₂MoO₄·2H₂O, 0.044 Na₂SeO₃ and 1.190 CoCl₂·6H₂O, while the composition of the vitamin solution in mg/L was: 100 pyridoxal-HCl, 50 riboflavin, 100 thiamine-HCl, and 20 biotin.

Medium pH was buffered at 7.4 using 3-(N-morpholino) propanesulfonic acid – MOPS (45 mM final concentration) and NaOH. The medium was sterilized by autoclaving at 121°C for 15 min. Sterilization time was set at 15 min to avoid glycerol caramelization. Thermolabile components were prepared as stock solutions, sterilized by filtration in cellulose acetate membranes (0.22 µm) and added later under aseptic conditions to the previously sterilized medium in order to reach the desired concentration. Fermentation tubes were prepared in triplicate by adding 30 ml of sterilized medium and 3 ml of mineral oil in flasks of penicillin (50 ml capacity) under aseptic conditions. The tubes were then sealed with rubber stopper and aluminum ring.

A strain of *Klebsiella oxytoca*, given the nomenclature MIG 01, was isolated and identified from soil samples collected at the campus of Maringá State University, showing great glycerol degradation potential under microaerobic conditions. The inoculum used in the assays was prepared from pure colonies of this bacterium. The volume of inoculum applied to each flask was 1.5 ml with approximate bacterial concentration of 1.5 × 10⁸ CFU/ml, prior to inoculation; all flasks were tested to verify the sterility of the medium. To that end, the flasks were incubated under the same conditions as the assays: 37°C for 48 h; after that period, the medium was checked for clouding.

During the fermentation assays, the flasks were coupled to a type of breather, using a needle with sterile syringe and cotton buffer, to lessen the pressure created by the gases produced during the fermentation process (CO₂ and H₂). Samplings during the fermentation process were carried out using sterile syringes and needles. 1.5 ml aliquots were taken from the fermentation tubes at 0, 3, 12, 24, 36, 48 and 72 h. Immediately following the optical density (OD) reading, samples were centrifuged for 15 min at 12000 rpm and 4°C (Jouan - Model GR 2022). They were then filtered (0.22 µm) and stocked in a freezer (-20°C) for later

determination of formed metabolites.

Bacterial growth was accompanied by a change in optical density (OD) of the medium, determined by absorbance (λ of 600 nm) in a UV/VIS spectrophotometer (Shimadzu - Model UVMMini 1240). The concentrations of the fermentation products were determined by high performance liquid chromatography (HPLC) analysis, using a Varian device model 920-LC with HPX 87H column (BioRad) measuring 300 × 7.8 mm. Two methodologies were used for HPLC analysis: (1st) ACID (adapted from the methodologies of Galdeano-Villegas et al. (2007) and Du et al., 2006), and (2nd) Chen (TALARICO et al., 1988; Chen et al., 2007). In the former, the mobile phase was H₂SO₄ 5 mM with column temperature set at 65°C, while in the latter methodology, the mobile phase consisted of an acidified mixture (0.5 mM of H₂SO₄) of acetonitrile and water at a ratio of 35:65 (v/v) with column temperature set at 25°C.

In both cases, the flow rate was 0.5 ml/min. The detector used to determine HPLC was an RI type, but a photodiode array (PDA) detector was also used in order to verify the possible overlap in the peaks of lactate, glycerol and formate, especially when the ACID technique was used. The great advantage of the second methodology proposed by Talarico et al. (1988) and perfected by Chen et al. (2007) consists in the fact that the peak signals for organic acids are quite small and are located within a short time interval at the start of elution (11-13 min); thus, the peaks of glycerol, 1,3-propanediol, 2,3-butanediol, ethanol and 3-HPA were defined quite well in the chromatograms.

RESULTS AND DISCUSSION

Figure 1 shows that the lag phase lasted approximately 12 h, and the culture reached the stationary phase after 48 h. There was no significant increase in bacterial population during the lag phase (Figure 1). According to Madigan et al. (2003) and Tortora et al. (2003), the substrate consumed in the lag phase only contributes to support the adaptation process of the inoculum by synthesizing proteins and other metabolites or components necessary for the satisfactory use of nutrients present in the medium. Based on this assumption and in the principle of conservation of mass, the concentration of 3-HPA was calculated for 3 h.

Thus, the calculation factor for 3-HPA can be determined by taking in consideration the area observed in the chromatogram for that time, as in this case HPLC analysis identified only three peaks (Chen and Acid methods): one glycerol peak was overlapped with the formate peak, one of ethanol, and another of a metabolic intermediate identified in previous works as 3-HPA (Barbirato et al., 1996, Chen et al., 2007). Data on the evolution in the concentration of products and substrate throughout the degradation process, under microaerobic conditions, are represented in Table 1. The residual concentration of glycerol was determined by the Chen method, as there is no peak overlap. Meanwhile, the amount of formate produced was calculated from the observation that the peak determined as being glycerol by the acid method also included formate. This was confirmed by the formation of an asymmetric tail and by the signal from the PDA detector. Therefore, the difference between the concentrations calculated by the acid and Chen methods represents the amount of formate

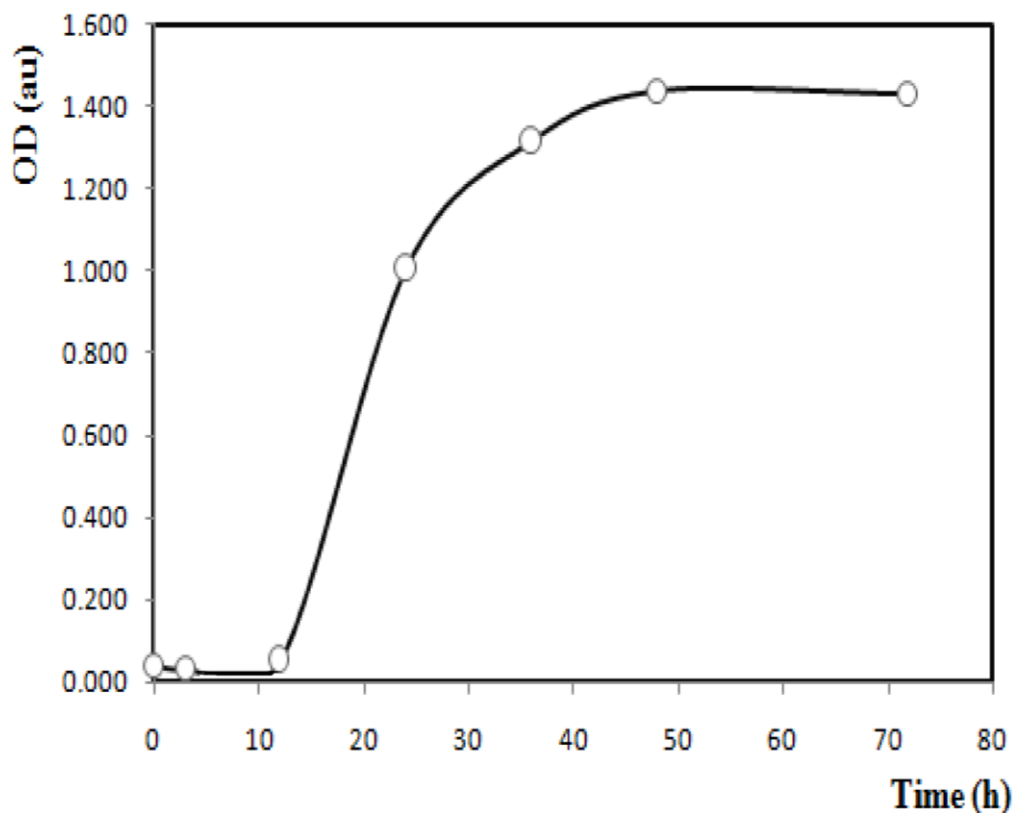


Figure 1. Growth curve obtained for bacterium *Klebsiella oxytoca*. Optical density was measured in terms of absorbance (dimensionless quantity) at a wavelength of 600 nm.

Table 1. Evolution of the concentrations of products and substrate expressed as g/L. HPLC analysis used the following methodologies: Acid (adapted from the methodologies by Galdeano-Villegas et al., 2008 and DU et al., 2006) and Chen (Talarico et al., 1988; Chen et al., 2007).

Product	Method	Concentration (g/L)						
		0	3 h	12 h	24 h	36 h	48 h	72 h
SUCC	Acid	0.00	0.00	0.00	0.23	0.47	0.70	0,93
LACT	Acid	0.00	0.00	0.00	0.33	1.30	2.55	3,90
GLYresd	Chen	75.60	70.65	66.16	62.44	46.36	39.88	36,02
%GLYcons	Chen	0.00	6.55	12.49	17.41	38.68	47.25	52,35
GLYresd	Acid	75.60	74.85	73.41	68.82	51.69	44.76	40,66
ACET	Acid	0.00	0.00	0.00	1.23	1.95	1.84	1,84
FORM	Dif ^a	0.00	4.21	7.25	6.38	5.33	4.88	4,64
1,3-PROP	Chen	0.00	0.00	0.00	3.08	10.36	13.47	15,85
2,3-BUT	Acid	0.00	0.00	0.00	0.00	2.55	3.88	4,18
ETH	Chen	0.00	0.24	0.71	0.75	0.74	0.72	0,73
3-HPA	Chen	0.00	0.51	1.24	1.14	1.12	1.09	1,06
Bacterial mass (g)		0.00	0.00 ^b	0.47	0.66	1.46	1.79	1.98
TOTAL MASS (g)		75.60	75.60	78.66	78.89	73.86	74.51	74.34
Carbon recovery (%)		100.00	100.00	100.44	102.12	97.42	96.91	97.67

^a the amount of formate is calculated as the difference between the values given by the acid and Chen methods. ^b up to time=3 h, it is assumed that the bacteria are in the lag phase and that glycerol consumption only supports the adaptation of the inoculum. SUCC=succinate; LACT=lactate; GLYresd=residual glycerol; %GLYcons=percentage of consumed glycerol; ACET=acetate; FORM=formate; 1,3-PROP=1,3-propanediol, 2,3; BUT=2,3-butanediol; 3-HPA=3-hydroxypropionaldehyde; ETH=ethanol.

produced.

The other organic acids and 2,3-butanediol were determined by the acid methodology. Meanwhile, compounds 1,3-propanediol, 3-HPA and ethanol were determined by the Chen method. Additionally, according to Homman et al. (1990), bacterial mass can be calculated as 5% of consumed glycerol. This hypothesis was applied for $t \geq 12$ h. The disappearance of formate over time was also considered in obtaining the total mass of each sampling. For example, for the time of 48 h, total mass was calculated as the sum of residual glycerol, the products identified in the chromatograms and the difference of the amount of formate in relation to the value recorded at 12 h.

Formate degradation produces CO_2 and H_2 . Moreover, the production of 2,3-butanediol and acetyl-CoA – the latter through pyruvate dehydrogenase complex (micro-aerobic system) are also linked to the production of CO_2 , whereas succinate production leads to CO_2 consumption (Zhang et al., 2008; Zhang et al., 2009). Therefore, only a quantitative and quantitative analysis of the gaseous phase contained in the fermentation flasks could allow a precise assessment of these components. In the present case, the experiments were performed so that the formed CO_2 and H_2 were expelled from the fermentation recipient through a “breathing hole”. Thus, the mass balance as proposed in this work is not exact. Nevertheless, the mass balance was used as a valuable direct determination tool, as demonstrated by the carbon recovery values presented in Table 1.

Figure 2 describes the residual values of glycerol determined by the acid and Chen methods, and describes the percentage of glycerol consumption. The data in this figure makes it possible to affirm that the residual glycerol value calculated using the acid methodology was always higher than the value determined by the Chen methodology, at any sampling time. This confirms the overlapping of formate and glycerol peaks, for all sampling times different from zero, whenever the acid methodology was used.

The glycerol consumption curve, described in Figure 2, can be divided into three phases: between 0 and 24 h, a period that includes the lag phase and practically the entire logarithmic growth phase see (Figure 1); glycerol consumption equals 17.41 % (Table 1). This represents a consumption rate of approximately $6.0 \text{ mmol L}^{-1} \text{ h}^{-1}$. Also, between 24 and 36 h, the rate of glycerol consumption increases significantly despite being at the end of the logarithmic growth phase (Figure 1). In this case, the consumption rate increased to $14.6 \text{ mmol L}^{-1} \text{ h}^{-1}$, more than doubling in value. Between 36 h and 72 h, when the stationary growth phase was established (Figure 1), the rate of glycerol consumption fell to $3.1 \text{ mmol L}^{-1} \text{ h}^{-1}$. After 72 h of fermentation, 52.35% of the substrate was consumed.

The evolution of the fermentation products is represented in Figures 3 and 4. A few important observations

about these graphs were: 1,3-propanediol production showed a faster phase during the period between 24 and 36 h (Figure 3); formate accumulated until 12 h, and was consumed continuously from then on (Figure 3); the amount of ethanol in the medium remained stable, around 0.73 g/L , after 12 h; the intermediate 3-HPA accumulated until 12 h; after that period, the amount of that product decreased very slowly (Figure 4); measurable amounts of succinate (Figure 4), lactate and 2,3-butanediol (Figure 3) were detected only after 24 h; acetate production stagnated after 36 h of inoculum incubation (Figure 4).

The data indicated the existence of a relationship between the phases of faster glycerol consumption, 1,3-propanediol production, equilibrium between the rates of 3-HPA formation and consumption, and the conversion of formate into CO_2 and H_2 . Formate degradation in enterobacteria happens through the formate-hydrogen-lyase (FHL) complex. This complex is composed by selenopolypeptides known as formate-dehydrogenase-H (FDH-H), hydrogenases (Hyd) and certain proteins of membranes that serve to anchor or carry electrons (Leonhartsberger et al., 2002). Several studies indicate that activation of the FHL complex, with action of the FDH-H enzyme, happens under anaerobic or low oxygenation conditions and in the absence of other electron acceptors (Leonhartsberger et al., 2002; Bagramyan et al., 2002; Hakobyan et al., 2005).

According to information available in the UniProtKB database (2011), the presence of an FDH-H- NAD^+ -dependent enzyme (EC 1.2.1.2) was identified in *K. pneumoniae* bacteria, strain 342, and in *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884. It should be mentioned that these species are quite close in genetic terms to *K. oxytoca*. The yield of 1,3-propanediol from the strain used herein (MIG 01) was 0.485 mol/mol of substrate; that is, 48.5% for 72 h of fermentation. Two strains of *K. oxytoca* (NRCC 3006 and Lin) and one strain of *K. pneumoniae* (DSM 2026) tested by Homman et al. (1990), who also used glycerol PA as substrate, had yields of 41, 44 and 54.1%, respectively. Furthermore, the obtained data indicate that the *Klebsiella oxytoca* strain used in this work behaved similarly to strains of *Klebsiella pneumoniae* under excess substrate conditions (Zeng et al., 1994; Solomon et al., 1994; Menzel et al., 1997; Biebl et al., 1998).

Conclusion

Evaluating the data represented in Figures 2, 3 and 4, it was possible to observe that the increase rate of glycerol consumption and higher 1,3-propanediol production that took place between 24 and 36 h coincided with the phase of formate consumption and stability in 3-HPA concentration. This allows the assumption that a relationship exists between these phenomena; that is, formate

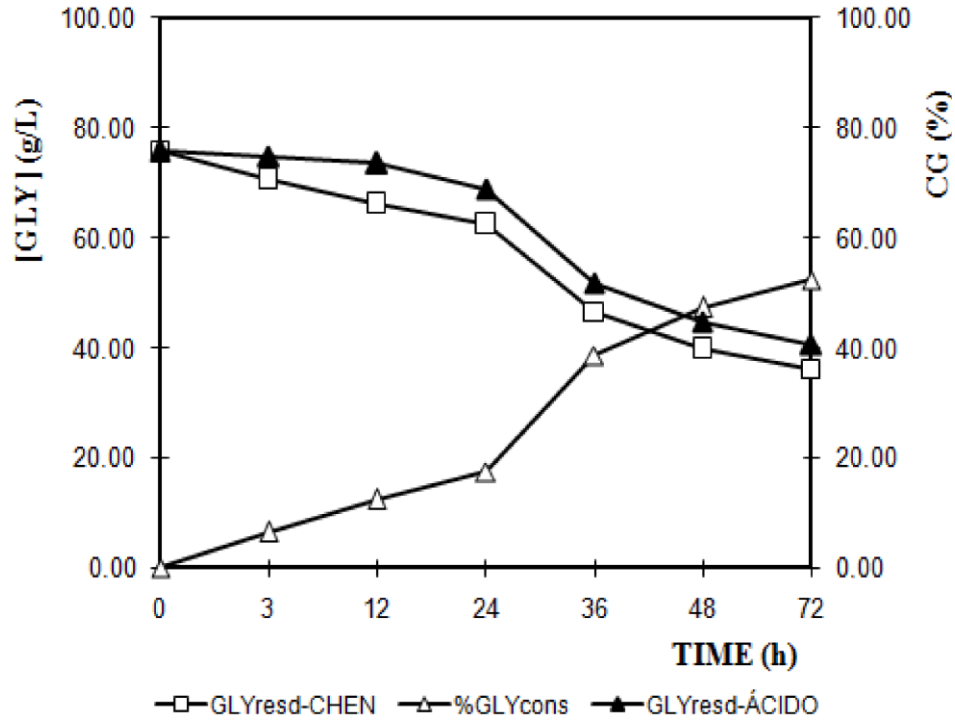


Figure 2. Evolution of the residual glycerol concentration determined by the methods: Acid (adapted from the methodologies by Galdeano-Villegas et al., 2008 and Du et al., 2006) and Chen (Talarico et al., 1988; Chen et al., 2007), and glycerol, consumption (CG) by the Chen technique for the assays throughout the 72 h fermentation time.

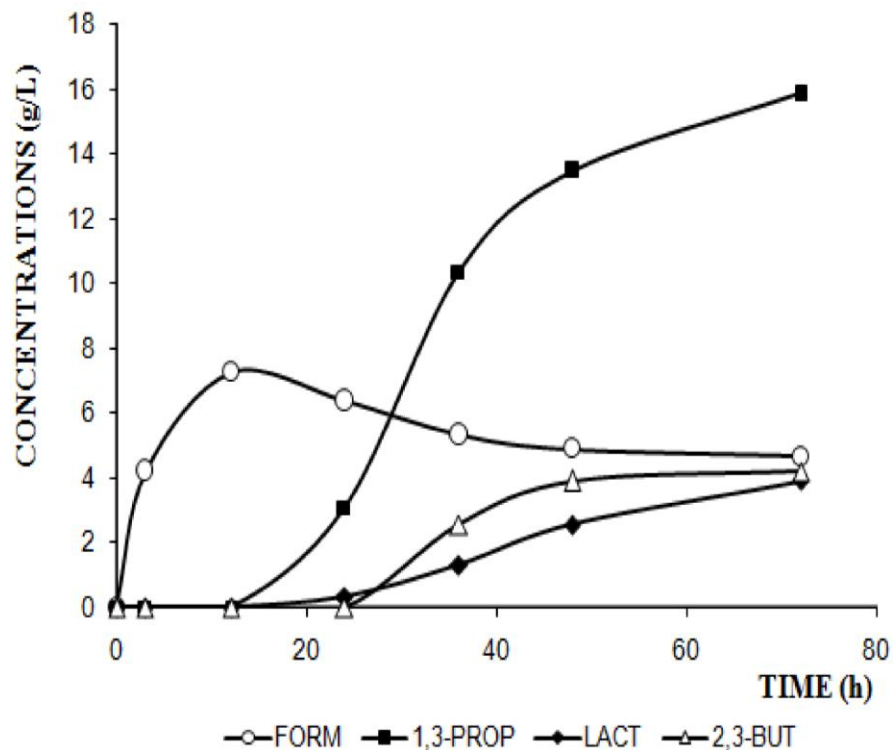


Figure 3. Production of formate (FORM), 1,3-propanediol (1,3-PROP), lactate (LACT) and 2,3-butanediol (2,3-BUT) for the assays throughout the 72 h fermentation time.

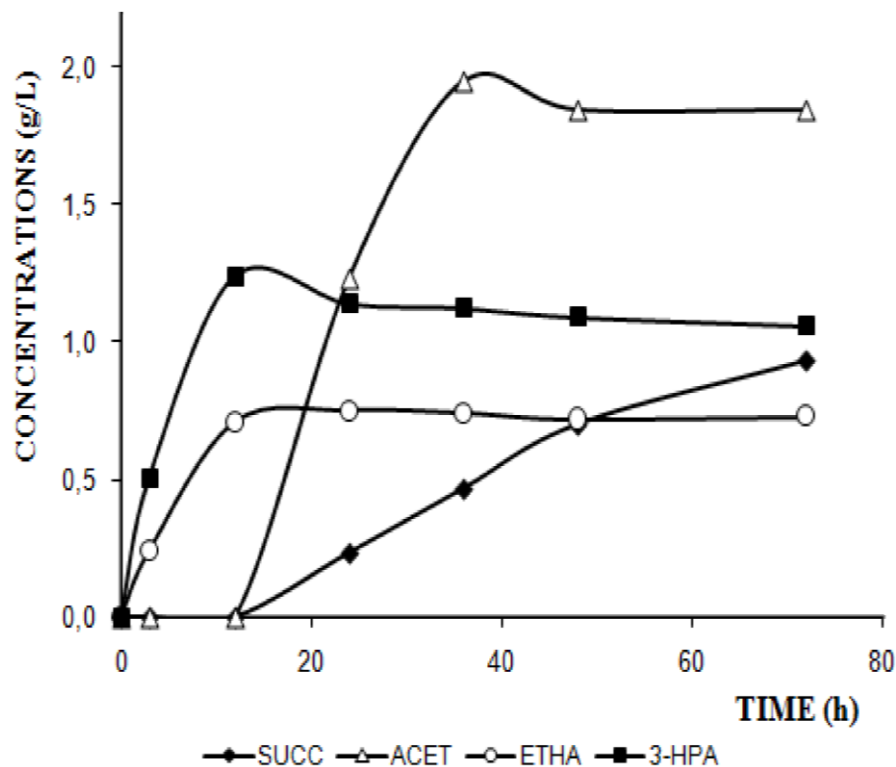


Figure 4. Production of succinate (SUCC), acetate (ACET), 3-HPA and ethanol (ETHA) for the assays throughout the 72 h fermentation time

degradation may be linked to the NADH regeneration process. This mechanism would function as an alternative to maintain the system's redox equilibrium, and was most active during the final stage of the logarithmic growth phase and in the early stationary phase.

However, a more precise evaluation would require monitoring of the evolution in the concentration of NAD⁺, NADH, of the composition of the gases produced and the enzymes involved in the degradation process. The main products of glycerol fermentation by the strain of *K. oxytoca* isolated in this work were 1,3-propanediol, formate, 2,3-butanediol, lactate and acetate, with very small produced amounts of succinate and ethanol. The yield in terms of glycerol conversion into 1,3-propanediol stayed above values observed in the literature (48.5 versus 41 and 44%). The reported yield of 1,3-propanediol was higher (48.5 versus 54.1%) for only one strain of *K. pneumoniae*. Thus, it can be affirmed that the tested strain has similar performance to that observed for other strains of *K. oxytoca* and even of *K. pneumoniae*, but with quite favorable yields.

ACKNOWLEDGEMENT

This study was supported by the National Council for Scientific and Technological Development (CNPq-Brazil).

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