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DOI: 10.2225/vol16-issue2-fulltext-4 *RESEARCH ARTICLE*

Evaluating Colombian *Clostridium* spp. strains' hydrogen production using glycerol as substrate

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Abstract

Background: The ability for hydrogen production of 13 native strains of *Clostridium* spp. strains isolated from Colombian soil was evaluated using glycerol substrate. Glycerol to hydrogen conversion was investigated using a batch fermentation reactor and industrial glycerol source (50 $g.l^{-1}$, pH 7.00).
Results: The results were quantified regarding acids, hydrogen, biomass and solvent production. The selected strain gave good hydrogen over production output at 14.4 mmol $H_2.l^{-1}$, productivity 0.3167 mg $H_2.h^{-1}$ $l^{-}1$ culture mediumand yield 0.1962 mol $H_2.mol^{-1}$ glycerol. A further fermentation assay a 4.0 liter batch reactor let to being 0.26 $mg.l^{-1}.h^{-1}$ after 18 hrs of fermentation. Logistic model, Luedeking-Piret model and Luedeking-Piret modified models were used for modeling changes in cell growth, hydrogen production and substrate consumption (Correlation coefficients R^2 = 0.95 for biomass substrate, R^2 = 0.77 hydrogen production).
Conclusions: Our results indicate that hydrogen production through glycerol bioconversion by native strains is possible.

Keywords: anaerobic fermentation, biofuel, *Clostridium* spp., glycerol, hydrogen, mathematical model.

INTRODUCTION

The international community has considered hydrogen to be the fuel of the future, receiving attention due energy content per unit weight (141.86 KJ.g-1) being 2.75 times greater than hydrocarbon fuel (Pattra et al. 2008). Hydrogen can be easily converted into electricity by fuel cells, and only produces water during combustion; it contributes towards reducing greenhouse gases (Martínez, 2009).

Around 95% of hydrogen is produced from fossil raw materials, such as reforming natural gas and gasifying of coal; only 4% is produced by electrolysis of water from renewable-energy sources and the remaining 1% is generated by biological processes (Das et al. 2008). Biological technologies include direct biophotolysis (green algae), indirect bio-photolysis (cyanobacteria), photo fermentation (photosynthetic bacteria) and dark fermentation (fermentative bacteria) (Sen and Das, 2005). Some advantages over other processes include low-cost and using renewable raw materials and simplicity, leading to higher hydrogen production rates (Levin et al. 2006).

Glycerol is the main by-product from the biodiesel industry. Biodiesel production is increasing exponentially; transesterification of vegetable oils has let to raw glycerol being produced in a large quantities (100 kg glycerol per ton biodiesel). Despite the wide application of pure glycerol in food, pharmaceutical and cosmetic industries, glycerol by-products should be processed to high purity

glycerol (Pachauri and He, 2006). However, organisms from the *Clostridium* genera can use crude glycerol as a carbon source which is then converted into organic acids (acetic acid and butyric acid) intermediate products prior to solvent formation (acetone, butanol, ethanol and 1,3-propanediol) (Jones and Woods, 1986) and volatile products such as carbon dioxide and hydrogen (Figure 1), (Biebl et al. 1999).

Recent experiments have studied hydrogen production using the *Clostridium* genera, such as *C. acetobutylicum* (Chin et al. 2003; Zhang et al. 2006), C. *butyricum* (Pattra et al. 2008; Chong et al. 2009), *C*. *tyrobutyricum* (Jo et al. 2008; Mitchell et. al. 2009) and *C*. *thermocellum* (Levin et al. 2006). Hydrogen release by *Clostridium* results from pyruvate molecules division to acetyl-CoA; this reaction is catalyzed by pyruvate-ferredoxin oxydoreductase. Ferredoxin plays a key role as an electron carrier in electron distribution within a cell the reduced ferredoxin is able to transfer electrons to an iron-containing hydrogenase allowing protons to be used a final electron acceptor, thereby leading to molecular hydrogen production, as can be seen Figure 2 (Bedoya et al. 2008).

Mathematical models are valuable tools for providing an insight into processes to enable optimizing them; modeling has thus brought about an improvement in hydrogen production (Chong et al. 2009). The Gompertz, Luedeking-Piret and modified Monod models have been used to describe hydrogen production by *Clostridium* (Chong et al. 2009; Nath and Das, 2011).

This research thus studied 13 native *Clostridium* strains to obtain a hydrogen overproducing strain using glycerol as an alternative substrate, to take advantage of biodiesel by-products. This study has developed a competitive low cost fermentation.

MATERIALS AND METHODS

Microorganisms

13 native strains and 2 control strains from the Insituto de Biología, Universidad Nacional de Colombia (IBUN) were used (Table 1). These isolated from Colombian soils and biochemically and molecularly characterized in previous studies (Montoya et al. 2000). The strains were classified as *Clostridium* genus, Gram-positive, obligatory anaerobic, non-sulphate-reducing, spore-forming, rod-shaped bacteria (Cato et al. 1986).

Table 1. Strains available from the IBUN.

Designation	Classification
IBUN 13A	*Clostridium* sp -native
IBUN 137K	*Clostridium* sp - native
IBUN 140B	*Clostridium* sp - native
IBUN 158B	*Clostridium* sp - native
IBUN 64A	*Clostridium* sp - native
IBUN 95 B	*Clostridium* sp - native
IBUN 18S	*Clostridium* sp - native
IBUN 62B	*Clostridium* sp - native
IBUN 125C	*Clostridium* sp - native
IBUN 18A	*Clostridium* sp - native
IBUN 22A	*Clostridium* sp - native
IBUN 62F	*Clostridium* sp - native
IBUN 18Q	*Clostridium* sp - native
ATCC 824	*C. acetobutylicum*- control
DSM 2478	*C. butyricum*-control

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Medium and culture conditions

The strains were activated by transferring 10% (v/v) of stock culture to 25 ml of RCM (reinforced clostridial medium, Oxoid) to obtain fresh culture before an experiment. The *Clostridium* strains were incubated at 37ºC for 24 hrs in anaerobic conditions to keep microorganism spores germinating and promote pre-inoculum growth. The inoculum was obtained by extracting 10% (v/v) from a pre-inoculum to culture it in industrial medium (IM), 5 g yeast extract, 1.8 gK_2HPO_4, 0.5 g L-cysteine, 50 g glycerol, 4 mL mineral solution and 0.1 µL resazurine. The mineral solution consisted of the following per litre of ionized water: 5 g $MgCl_2$, 3 g $CaCl_2$, 3.7 g $FeSO_4.7H_2O$, 3.3 g $MnSO_4.H_2O$, 3.8 g $CoCl_2.6H_2O$, 1.3 g Na_2MoO_4, 2.8 g $ZnSO_4$, 0.12 H_3BO_3, 0.18 g $CuSO_4.5H_2O$, 0.13 g $NiCl_2.6H_2O$. Medium pH was initially adjusted to 7.00. The strains were inoculated with 7% v/v inoculum into 25 ml industrial medium in vials and cultured at 37ºC using an orbital shaker at 200 rpm.

Batch reactor conditions

The selected strain was fermented in a R'ALF Bioengineering AG Plus reactor (4 liter working volume); IM was used for culturing the organisms. The reactor was gassed overnight with nitrogen (99.999% purity). The fermentation conditions used were standardized on previous work by the research group (Unpublished data) (37ºC, 200 rpm agitation, initial pH 7.00). The strain culture used 10% (v/v) from the inoculum. Fermentation was carried out in duplicate for 72 hrs taking samples every 2 hrs. The amount of displaced gas was quantified by using a system volume measuring by water displacement.

Analytical methods

Cell growth. Cell concentration was quantified by the dry weight method. 1 ml of culture broth was centrifuged at 14.000 rpm, for 10 min, 15ºC. Cell pellets were dried to reach steady state (weight) at 50ºC. Batch reactor, biomass was estimated by filtering through 0.22 µm cellulose acetate membrane, using the procedure described above, until weight became steady.

Acids solvents and substrate consumption. Organic acids concentration, including acetic (HAc), butyric (HBu), lactic (HLa) acids, solvents such as butanol (BuOH), 1,3-propanediol (1,3-PD) and glycerol substrate was analyzed by high-performance liquid chromatography (HPLC). HPLC analysis was carried out using a Varian Star Chromatography with an AMINEX HPX - 87H (Biorad) column (50ºC oven temperature), 5 mM H_2SO_4 as mobile phase, 0.6 $mL.min^{-1}$ flow rate and a refraction index detector (Waters).

Gas measurements. Hydrogen (H_2) was measured by gas chromatograph (GC). The devices were equipped with a thermal conductivity detector (TCD) and the column was packed with Packed Porapak Q (80/100 mesh) using nitrogen as the carrier gas at 20 $mL.min^{-1}$ flow rate. Stainless column temperature was 90ºC and injection/detector points were 100ºC and 150ºC respectively. Standard hydrogen curves were plotted using normal hydrogen gas (Sen and Das, 2005; Chong et al. 2009).

Model parameters. An algorithm was generated using MATLAB software to obtain model parameters and establish an objective function based on the solution of differential equations from the mathematical model describing cell growth, formed product and substrate consumption. These parameters were then optimized using MATLAB statistical toolbox *fmincon* function based on minimization with residual sum of squares (RSS) Equation 1 (Martínez, 2005):

$$RSS = \sum_i (Y_i - \hat{Y}_i)^2 = \sum e_i^2$$

[Equation 1]

where Y_i was observed biomass, substrate and products value, $\hat{Y}_i$ the estimated value by model and e_i the error.

RESULTS AND DISCUSSION

Vial fermentation results (Table 2) led to the IBUN 18S strain being selected as the best hydrogen producer owing to its 0.1962 mol.mol^{-1} yield and 0.31669 mg.L^{-1}.h^{-1} molar productive. Figure 3 shows the hydrogen production of all native strains evaluated, highlighting the IBUN 18S strain which had maximum production at 14.4 mmolH_2.L^{-1}.

Hydrogen is a primary metabolite which is correlated with cell growth (Pattra et al. 2008). Gas production began within the first 24 hrs fermentation during the exponential phase (Figure 4a); pH became lowered to 4.5 during this stage by the formation of organic acids affecting microorganism membrane load and hydrogenase activity. This inhibited hydrogen production through stationary phase (Figure 4b).

Table 2. Native strains' vial fermentation (72 hrs, industrial medium, pH 7.00, 200 rpm and 37ºC).

Strain	Glycerol consumption (g.L^{-1})	Yp/s $_{H2}$ (mol.mol^{-1})	Q_{H2} (mg.l^{-1}.h^{-1})
95B	5.1317	0.04601	0.07122
125C	6.1591	0.04028	0.07482
18Q	5.3161	0.06393	0.10250
18S	**5.3513**	**0.19621**	**0.31669**
18A	6.5946	0.04573	0.09096
137K	12.6153	0.02435	0.09265
140B	6.8270	0.04509	0.09284
158B	11.1806	0.02646	0.08924
13A	11.7644	0.02844	0.10091
22A	4.7193	0.07839	0.11158
62F	12.8869	0.07387	0.28714
62B	6.7563	0.04924	0.10034
64A	8.2273	0.04178	0.10369
ATCC824	8.9555	0.03765	0.10169
DSM2478	6.9340	0.04017	0.08402

Batch reactor fermentation

Figure 5 illustrates cell growth during exponential phase (0-18 hrs) and pH decreasing to 3.95 units due to lactic, acetic and butyric acid formation. The lag phase did not occur and stationary phase started at 18-72 hrs. This fermentation also produced 1,3-propanediol as solvent. Products were generated a parallel to cell growth, thus being considered primary metabolites (Chong et al. 2009).

Figure 6 shows the hydrogen production profile during *Clostridium* sp IBUN 18S batch fermentation on industrial medium. Gas production began at the same time with 11.58 mmol.L^{-1} maximum biomass growth and 10 g.L^{-1} glycerol consumption. Bio-hydrogen generation was explained as excess proton being reduced to molecular hydrogen to dispose of the reducing equivalent. Medium pH affects yield, biogas composition, the type of organic acid formed and the volumetric rate of hydrogen production (Sen et al. 2008).

The highest yield was 0.1095 mmol.L^{-1} and 0.2595 mg.L^{-1}.h^{-1} for molar production maximum 11.56 mmol.l^{-1} gas release being obtained during exponential phase (18 hrs). Production was slightly less than the 14.2 mmol.l^{-1} reported for the *Clostridium* sp. URNW strain (Ramachandran et al. 2011). Gas production rates were affected by lactate synthesis producing 1.027 g.L^{-1}, causing a drop in pH which had a significant influence on hydrogen production rate. The lactate metabolic pathway only appeared to operate as a less efficiency alternative to allow energy generation regarding NADH+H oxidation, which can be used to produce hydrogen. The drop in hydrogen generation has also been reported to occur when hydrogenase activity has been inhibited when ferredoxin levels become reduced (Jones and Woods, 1986). Lactate presence considerably affected ferredoxin levels as well as hydrogen released.

Different end-product production by *Clostridium* sp. IBUN 18S was significantly affeted by corresponding changes in pH in the medium after 0-18 hrs growth. A significant drop in pH was observed from 7.00 to 3.95 (Figure 5), correponding to higher butyrate and acetate levels (Figure 7). pH dropped below 4.00-4.80 which is the optimum reported for biogas production (Jones and Woods, 1986; Sen et al. 2008); lower pH affected vital activities such as changing cell membrane charge causing low hydrogen yield. During the stationary phase, hydrogen production plummet graduallly until reaching a minimum 5.13 mmol.L^{-1} (Figure 6) and pH remained steady because acids so generated were not assimilate, total solvents (acetone-butanol-ethanol) were not detected. pH acts as inductive factor for solventogenic enzimes synthesis and expression catalyzing reduction reactions (Rajchenberg et al. 2009).

Modelling hydrogen production in batch fermentation

An unstructured model provide a good approach fementation profile, even though the complete cell mechanism is not considered in the models (Rajendran et al. 2007). Table 3 shows the stimated parameters for the unstructered models that were fitted for experimental cell growth values (logistic model), sustrate comsumption (modified Luedeking-Piret

Cell growth may be characterized by a logistic equation Equation 2, which can be estated as follows:

$$\frac{dX}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right) X$$

[Equation 2]

Logistic equation, describing cell growth, where dX/dt was growth rate ($g.L^{-1}h^{-1}$), X biomass concentration ($g.L^{-1}$), μ specific cell growth rate (h^{-1}), μ_m initial specific growth rate and X_m maximum cell mass concentration ($g.L^{-1}$). Equation 2 for integration using $X_O = X\ (t) = 0$, gave sigmoidal variation X (t) empirically representing both exponential and stationary phase (Wang et al. 2006).

Table 3. Kinetic parameters of unstructure model fitting MATLAB software, using batch fermentation experimental values.

Parameters	Value	R^2
Biomass: logistic model		
$\mu_m(h^{-1})$	0.2959	0.97
$X_m(g.l^{-1})$	1.0153	
Hidrogen: Luedeking-Piret model		
$\alpha(gH_2.gx^{-1})$	12.4842	0.77
$\beta(gH_2.gx^{-1}.h^{-1})$	-0.0666	
Sustrate: modified Luedeking-Piret model		
$\gamma(gS.gx^{-1})$	13.5839	0.96
$\delta(gS.gx^{-1}.h^{-1})$	0.0497	

The model's cell growth prediction (Figure 8) was consistent with experimental results from exponential phase to stationary phase. The model's goodness of fit was checked by the determination coefficient (R^2) a measuring the strength of linear relationship between empirical data and predicted values. Table 3 shows that cell growth R^2 was 0.97 having good correlation, demonstrating that this model was applicable for predicting experimental results. The values for parameters provided by the model μ_m and X_m were 0.296 $h^{-1}$and 1.053 $g.l^{-1}$ respectively and experimental data were μ_m = 0.226 $h^{-1}$and X_m = 1.005 $g.l^{-1}$. Model prediction was compared to the experimental parameters to verify the model, showing that this model was very suitable for describing cell growth (Figure 9).

Luedeking-Piret (Equation 3), describing the hydrogen production, stated that product formation rate depends upon instantaneous biomass concentration (X) and growth rate (dx/dt) in a linear fashion (Rajendran et al. 2007).

$$\frac{dp_i}{dt} = \alpha_i \frac{dx}{dt} + \beta_i x$$

[Equation 3]

Where α($gH_2.gx^{-1}$) and $\hat{a}$($gH_2.gx^{-1}.h^{-1}$) were empirical constants which might have varied with fermentation conditions *á* was a growth associated parameter and *â* a non-growth associated parameter.

R^2 value in the present study was 0.77, indicating that 23.3% of total variation was not explained by the model. Figure 10 shows that exponential phase experimental result did not agree with model prediction. Non-growth associated constant β was much more associated than growth magnitude associated parameter α, this have been attributed to the comparison between experimental (α = 17.04, β = -0.075) and model data. Comparing growth-associated constant α = 12.42 with non-growth-associated constant β = -0.0666. α showed that hydrogen production rate was high throughout exponential growth phase, by contrast constant β indicated that hydrogen production decreased during the stationary growth phase.

Glycerol is used to form cell components and metabolic products as well as cells maintenance (Wang et al. 2006).

Substrate material balance equation,

$$-\frac{dS}{dt} = \left(\frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}}\right)\frac{dX}{dt} + \left(\frac{\beta}{Y_{P/S}} + m_s\right)X$$

[Equation 4]

Where $Y_{x/s}$ and $Y_{p/s}$ were cell mass and product yields regarding substrate and m_s cells maintenances coefficient. Rearranging substrate material balance Equation 4 gave:

$$-\frac{ds}{dt} = \gamma\frac{dx}{dt} + \delta x$$

[Equation 5]

The glycerol consumption equation (Equation 4) was a modified Luedeking-Piret equation. Model prediction data and experimental results are shown in Figure 10. This model was clearly very suitable for describing glycerol consumption rate (R^2 = 0.96).

CONCLUDING REMARKS

Hydrogen production through glycerol bioconversion by native strains is possible; the IBUN 18S native strain was identified here as being suitable overproducing potentially reaching a maximum 11.59 $mmol.L^{-1}$ after 18 hrs fermentation in batch reactor. Hydrogen may be generated in a parallel way cell growth (biomass production) which was appropriately associated with the unstructured cell kinetic models and modified Luedeking-Piret model. These models effectively predicted the fermentation profile with higher accuracy. However, prediction using the Luedeking-Piret model for hydrogen production did not totally agree with the experimental results; this model thus needs to be enhanced adding further parameters to achieve a better fit.

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Figures

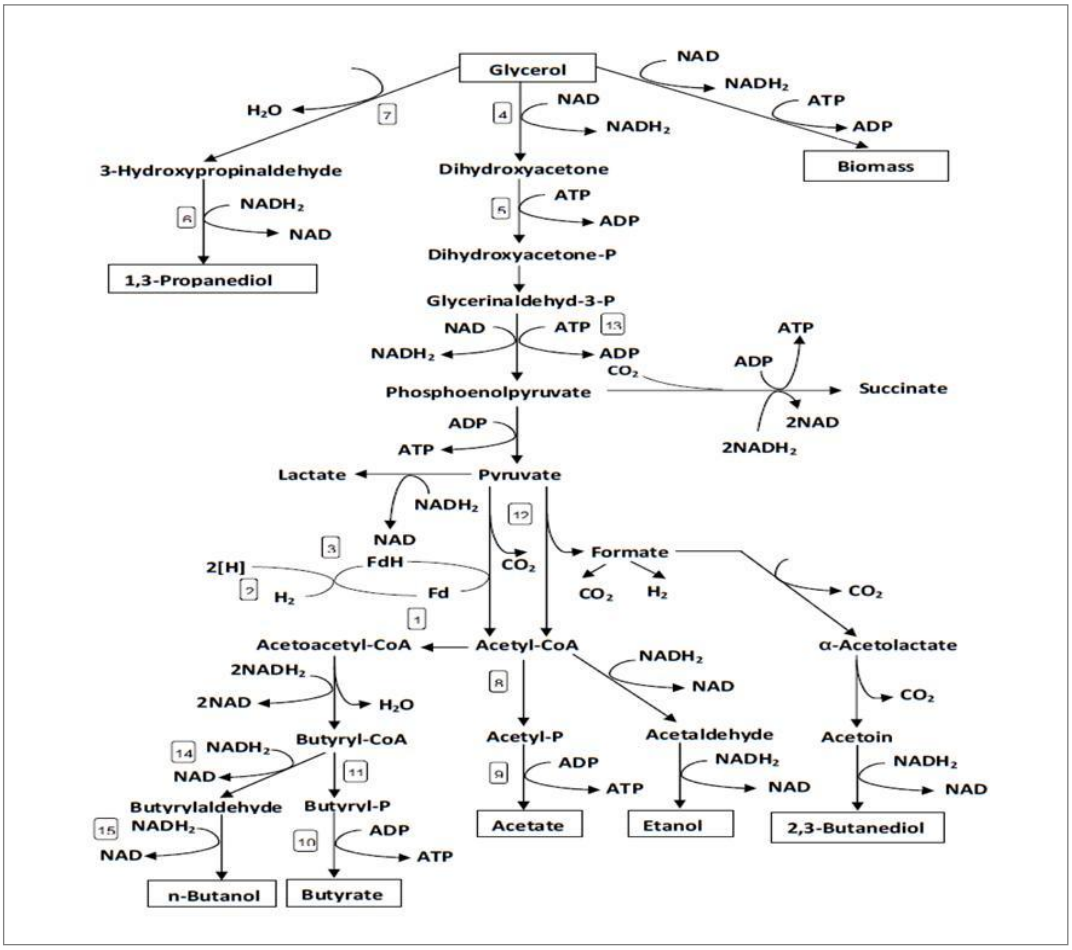

Fig. 1 Clostridium spp metabolic pathway using glycerol as carbon source. (1) Hydrogenase; (2) ferredoxin-NADP + reductase; (3) NADPH-ferredoxin reductase; (4) glycerol dehydrogenase; (5) DHA-kinase; (6) 1,3-PD dehydrogenase; (7) glycerol dehydratase; (8) phosphotransacetylase; (9) acetate kinase; (10) phosphotransbutyrylase; (11) butyrate kinase; (12) pyruvate-ferredoxin oxidoreductase; (13) glyceraldehyde-3P-dehydrogenase; (14) butyraldehyde dehydrogenase and (15) butanol dehydrogenase.

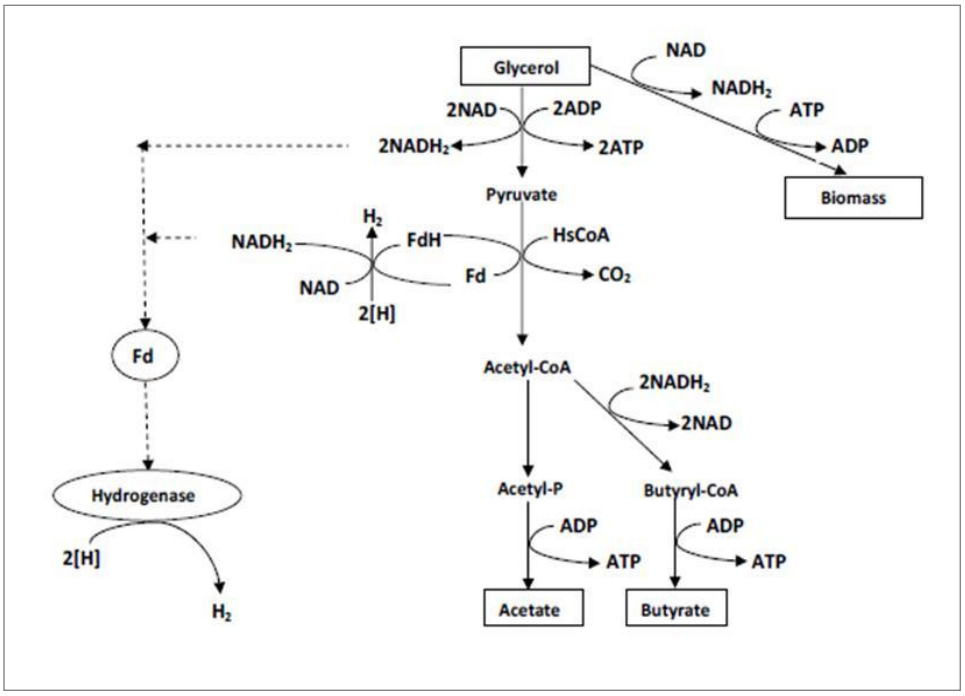

Fig. 2 Clostridium spp metabolic pathway for hydrogen production using glycerol as substrate.

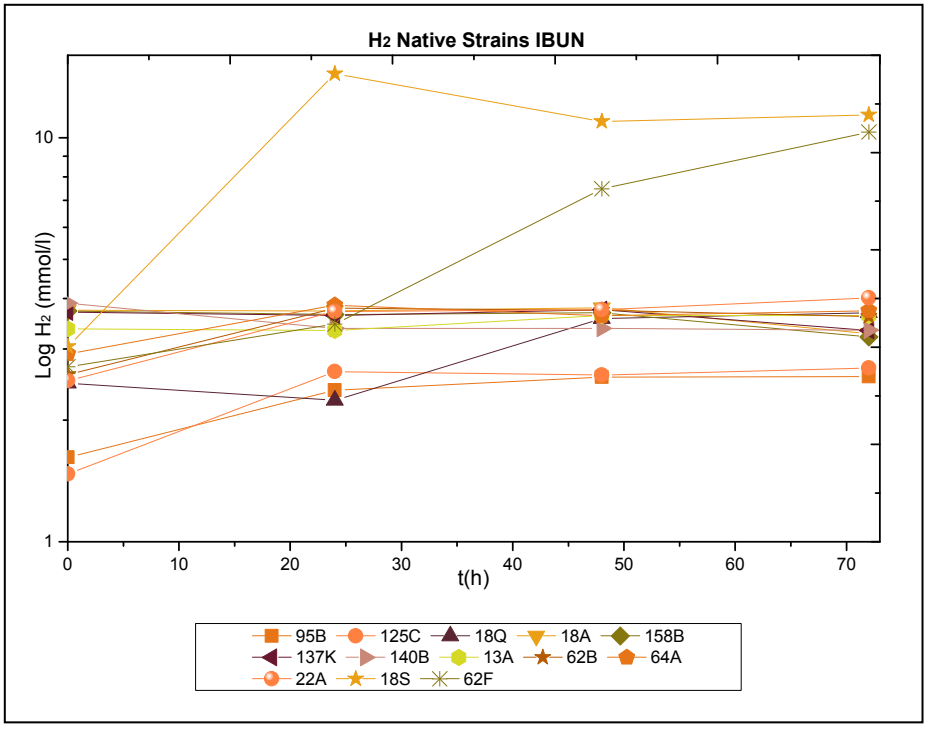

Fig. 3 Hydrogen production by 13 native strains, vial fermentation. (25 mL, 200 rpm, initial pH 7.00, 37ºC and industrial medium).

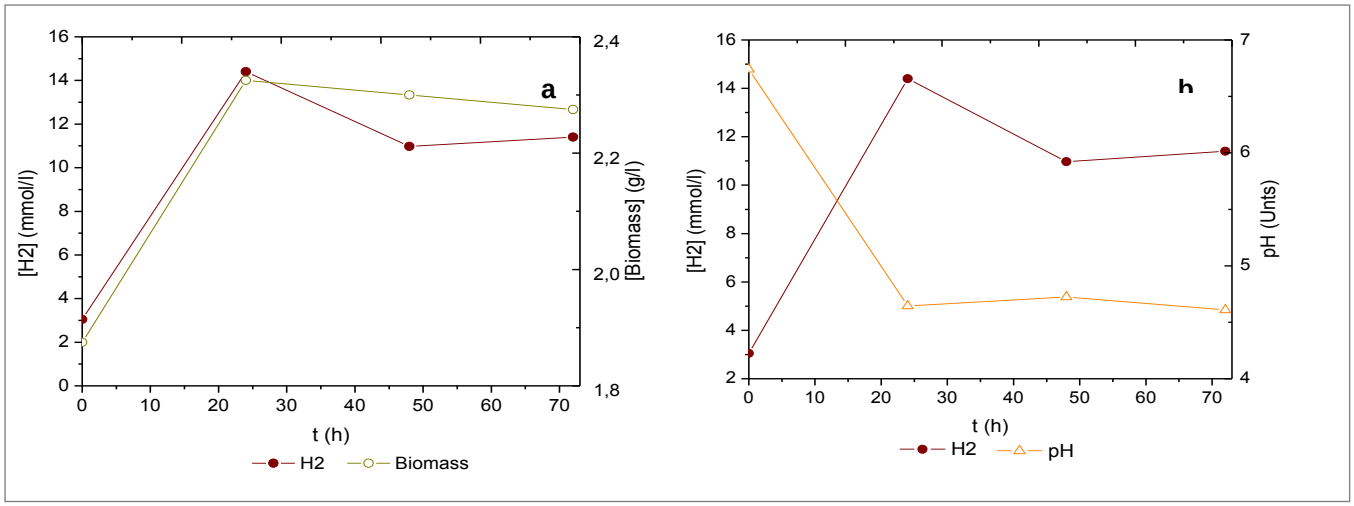

Fig. 4 Hydrogen production by IBUN 18S strain (a) H_2 *cf* Biomass (b) H_2 *cf* pH.

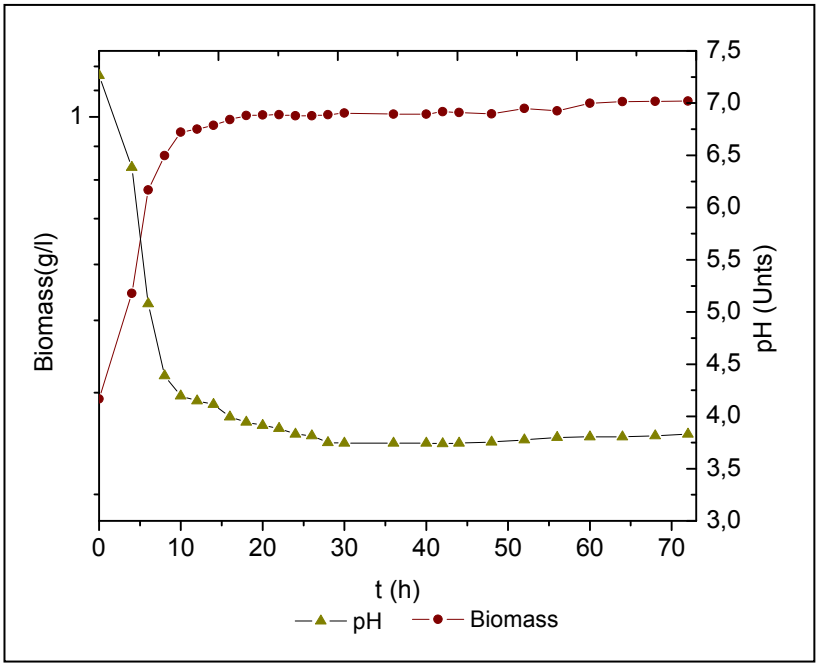

Fig. 5 *Clostridium* sp. IBUN 18S cell growth profile and pH during 72 hrs fermentation with industrial medium.

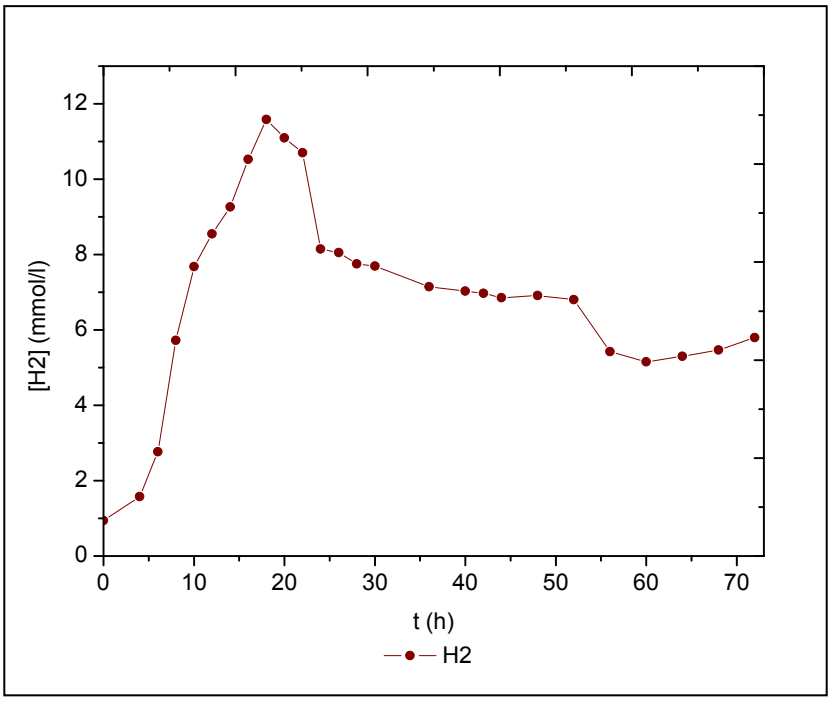

Fig. 6 *Clostridium* sp. IBUN 18S profiles for solvent and acid production during 72 hrs fermentation using industrial medium.

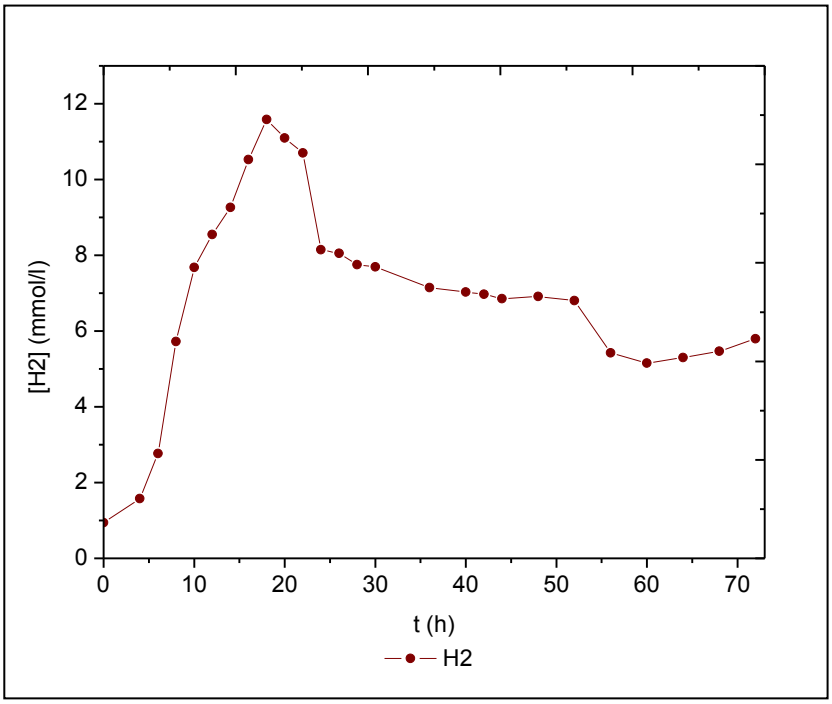

Fig. 7 *Clostridium* sp IBUN 18S hydrogen production during 72 hrs fermentation with industrial medium, at initial pH 7.00.

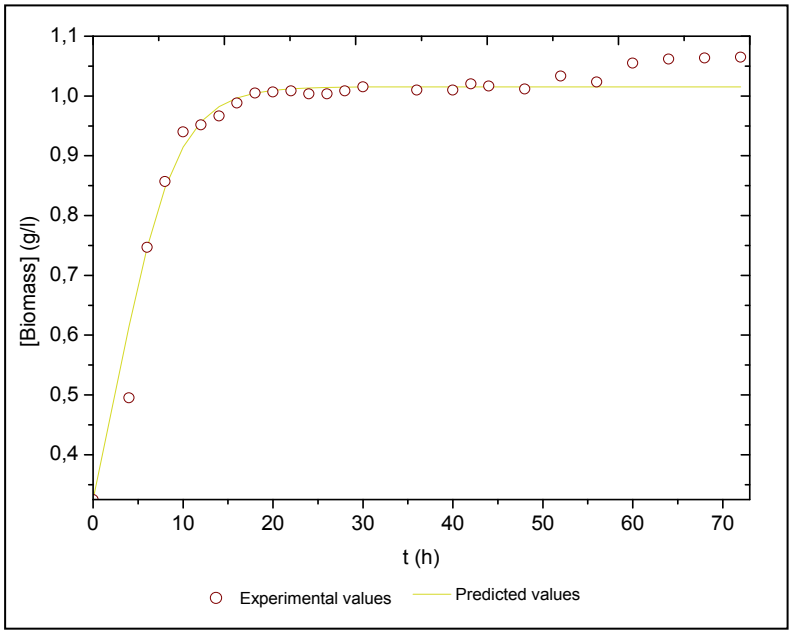

Fig. 8 Clostridium sp IBUN 18S cell growth simulated using logistic equation.