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# Optimization of culture media for ethanol production from glycerol by *Escherichia coli*

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## ABSTRACT

The culture media for glycerol to ethanol biotransformation by *Escherichia coli* under anaerobic conditions was optimized. A Plackett-Burman screening design was used to determine which factors were significant with a 95% confidence interval. A full factorial 2<sup>2</sup> and response surface model were employed to determine the optimum conditions for the selected response variable. The response variable considered in this work was biomass productivity ( $Q_x$ ). Profiles for biomass growth, glycerol consumption and ethanol production were obtained at optimum conditions and fermentation parameters were calculated. Glycerol to ethanol yield and ethanol specific productivity were determined to be 845 g kg<sup>-1</sup> of glycerol and 212 g kg<sup>-1</sup> h<sup>-1</sup> of cell mass respectively. Optimized culture media is presented along with the main results and experimental profiles obtained from this work.

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## 1. Introduction

Bioenergy is recognized as the most important renewable energy source by the OECD (Organization for Economic Co-operation and Development). It is considered GHG (greenhouse gas) neutral because the CO<sub>2</sub> produced from the energy generation process was previously fixed from the atmosphere by plants. Inside this group of energy sources, biofuels (bioethanol and biodiesel) have been the most developed [1]. Biodiesel is made from renewable sources such as vegetable oils and animal fats via a transesterification reaction that generates glycerol as a co-product at a rate of 100 g kg<sup>-1</sup> of

biodiesel. The biodiesel industry created a glycerol surplus and a dramatic 10-fold decrease in crude glycerol prices [2]. Glycerol is now an attractive carbon source due to its low price and greater degree of reduction relative to sugars. Reduced chemicals, such as succinate, ethanol, and hydrogen, can be produced in higher yields from glycerol than sugars [3,4].

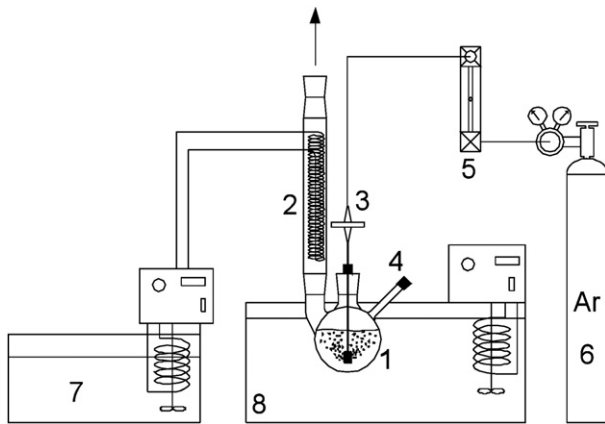
The biotransformation process facilitates the conversion of one compound (raw material) into another of technical or commercial interest (product) by means of enzymes or microorganisms that catalyze the reactions. This process presents advantages over traditional chemical synthesis in the areas of selectivity, specificity and the moderate

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**Fig. 1** – Six reactor system. 1) reactor 500 mL working volume, 2) condenser, 3) inlet gas filter, 4) sample pipe, 5) rotameter, 6) argon bottle, 7) cooling system (ethylene glycol/water at 4 °C), 8) heating immersion bath system (water at 37 °C).

**Table 1** – Culture media component studied PB design.

Component	Level (-) (kg m <sup>-3</sup> )	Level (0) (kg m <sup>-3</sup> )	Level (+) (kg m <sup>-3</sup> )
Glycerol	10	20	30
NH <sub>4</sub> Cl	0.325	0.650	0.976
KH <sub>2</sub> PO <sub>4</sub>	0.102	0.204	0.306
Na <sub>2</sub> SO <sub>4</sub>	0.0268	0.0538	0.0806
NaCl	0.0152	0.0270	0.0458
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.0310	0.0621	0.0931
Peptone	2	4	6
Salts	A	B	C
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	0	0.00225	0.0045
MnSO <sub>4</sub> ·H <sub>2</sub> O	0	0.00185	0.0037
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0	0.00235	0.0047
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0	0.00265	0.0053
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0	0.000275	0.00055
CoCl <sub>3</sub> ·6H <sub>2</sub> O	0	0.0005	0.001

conditions of the process (pressure, temperature and pH). The benefits include avoiding the production of undesirable isomers and a lower energy cost, which is translated into a more efficient and feasible process from a technical and

**Table 3** – Full factorial design for two factors, (X<sub>1</sub>: Peptone, X<sub>2</sub>: NH<sub>4</sub>Cl).

Std. order	Run order	X <sub>1</sub>	X <sub>2</sub>	Peptone, kg m <sup>-3</sup>	NH <sub>4</sub> Cl, kg m <sup>-3</sup>
1	4	-	-	0.50	0.081
2	1	+	-	2.00	0.081
3	6	-	+	0.50	0.325
4	5	+	+	2.00	0.325
5	8	0	0	1.25	0.203
6	7	0	0	1.25	0.203
7	2	0	0	1.25	0.203
8	3	0	0	1.25	0.203

economic point of view. Diverse microorganisms are able to metabolize glycerin as a carbon source. Examples of these microorganisms include *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium* and *Anaerobiospirillum*. However, the commercial use of these organisms is limited by their degree of pathogenicity, nutritional requirements and susceptibility to genetic manipulation [5]. *Escherichia coli* has many important advantages relative to the organisms previously mentioned. Under anaerobic conditions, it is able to metabolize glycerin as a carbon source [2] and can produce both ethanol and hydrogen from formate.

The K-12 MG1655 strain of *E. coli* has been sequenced [6,7]. As a consequence, this microorganism has become an attractive tool for the biotransformation processes. Utilizing genetic information, new possibilities have been opened in the fields of metabolic engineering and fermentation. The blocking or over-expression of genes in metabolic routes of interest [8–11] could potentially increase yield and/or productivity for biotransformation processes.

Culture media optimization is an important step in the design of a fermentation process. A desirable outcome is the reduction in the cost of materials and/or energy. Classical planning (one variable at time while the others remain constant) requires much effort and time, and when factor interactions take place, it can sometimes be inapplicable [12–15].

In this work, the optimization of culture media for this microorganism under anaerobic conditions was studied. A Plackett-Burman design was utilized to identify significant components in the culture media. Three significant positive factors (of eight factors studied) for biomass productivity were

**Table 2** – PB screening design.

Experiment	Glycerol	NH <sub>4</sub> Cl	KH <sub>2</sub> PO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	NaCl	MgSO <sub>4</sub> ·7H <sub>2</sub> O	Peptone	Salts
1	-	-	-	-	-	-	-	-
2	-	+	+	+	-	+	+	-
3	+	+	-	+	-	-	-	+
4	-	+	+	-	+	-	-	-
5	-	-	+	+	+	-	+	+
6	+	+	-	+	+	-	+	-
7	+	-	+	+	-	+	-	-
8	+	-	+	-	-	-	+	+
9	+	-	-	-	+	+	+	-
10	+	+	+	-	+	+	-	+
11	-	+	-	-	-	+	+	+
12	-	-	-	+	+	+	-	+

observed. A full factorial design,  $2^2$ , and a response surface model were used to determine the optimal level for these factors.

## 2. Materials and methods

### 2.1. Microorganism

*E. coli* wild-type MG1655, CGSC#6300, was obtained from *E. coli* Genetic Resource Center, Coli Genetic Store Center (CGSC), Dept. of Molecular, Cellular & Developmental Biology (Yale University). The strain was kept in 15% glycerol stocks at  $-80^\circ\text{C}$ . Plates were prepared using LB medium (peptone  $10\text{ kg m}^{-3}$ , yeast extract  $5\text{ kg m}^{-3}$ , NaCl  $10\text{ kg m}^{-3}$ ) containing 1.5% agar.

### 2.2. Inoculum preparation and reactors

Plates were prepared and incubated at  $37^\circ\text{C}$  overnight. A single colony was placed in a 10 mL tube filled with LB medium and incubated at  $37^\circ\text{C}$  and 1.7 Hz for 2 h. Afterward, the content of the tube was mixed with 326 mL LB medium and 24 mL of phosphate buffer (pH 6.3) containing glycerol  $150\text{ kg m}^{-3}$ ,  $\text{NH}_4\text{Cl}$   $4.88\text{ kg m}^{-3}$ ,  $\text{KH}_2\text{PO}_4$   $1.53\text{ kg m}^{-3}$ ,  $\text{Na}_2\text{SO}_4$   $0.40\text{ kg m}^{-3}$ , NaCl  $0.23\text{ kg m}^{-3}$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   $0.46\text{ kg m}^{-3}$ . Chemicals were obtained from Panreac (PANREAC QUIMICA S.A., Spain) and Scharlau (Scharlab S.L., Spain). The glycerol quality was food grade, 99% extra pure. This medium was proposed in this work to aid in the adaptation of *E. coli* to glycerol as a main carbon source. The medium was called

LB + G and was formulated according to the chemical elementary composition of the bacteria and previously reported requirements [16,17]. The concentration of each component was calculated from theoretical element yields. Bottles with a butyl septum aluminum cap seal were filled with 50 mL of inoculated LB + G medium and incubated anaerobically at  $37^\circ\text{C}$  and 2.5 Hz for 14 h until an optical density of 0.6 (at 600 nm) was reached. Each of these bottles was used to inoculate a reactor of 500 mL of working volume. A six reactor system, shown in Fig. 1, was utilized for the media culture optimization experiments. Each reactor was equipped with a condenser that circulated ethylene glycol/water at  $4^\circ\text{C}$  to prevent  $\text{H}_2\text{O}$  losses by evaporation and stripping phenomena. The pH was adjusted to 6.3 with phosphate buffer. Temperature was fixed at  $37^\circ\text{C}$  using an immersion thermal bath. An inert gas (Ar quality 5.0, Abelló Linde S.A, Spain) was bubbled continuously at  $0.04\text{ min}^{-1}$  through a tube sparging system equipped with a  $0.2\text{ }\mu\text{m}$  filter (MILLIPORE Filter SLG05010) to produce  $\text{H}_2$  displacement and stir the media. The effluent gas was passed through a sterile cotton filter. In the set of six reactors, the concentration of each of the components of the media culture was modified to complete the experiment design.

### 2.3. Analytical methods

Biomass was determined by optical density at 600 nm. Glycerol and ethanol were determined by HPLC using a LaChrom Elite® VWR-Hitachi equipped with an HPX-87H organic acid

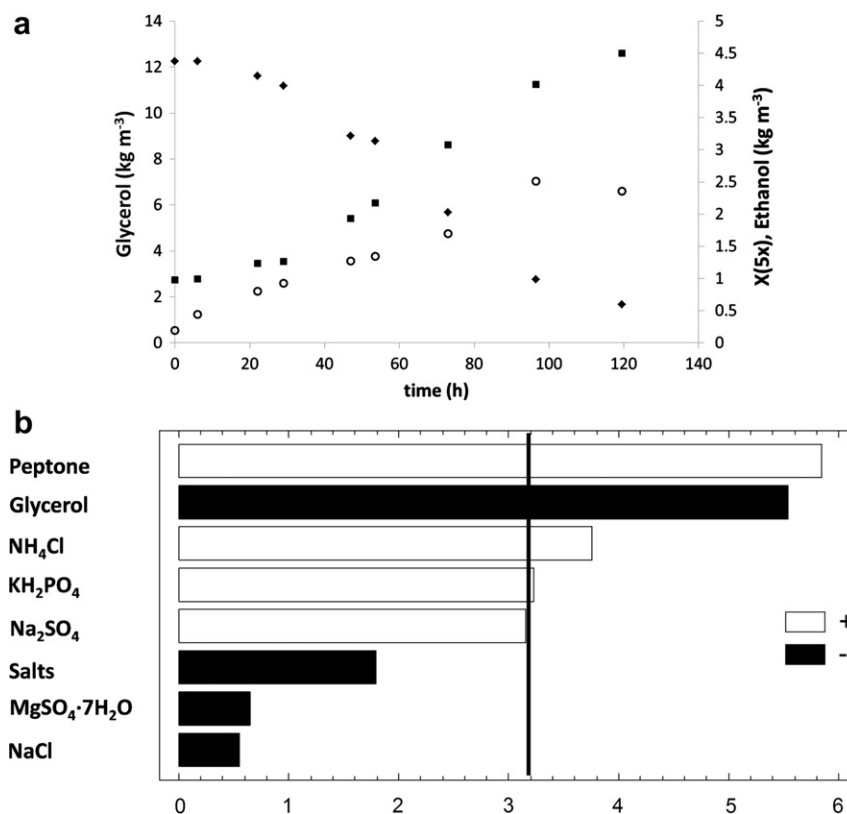
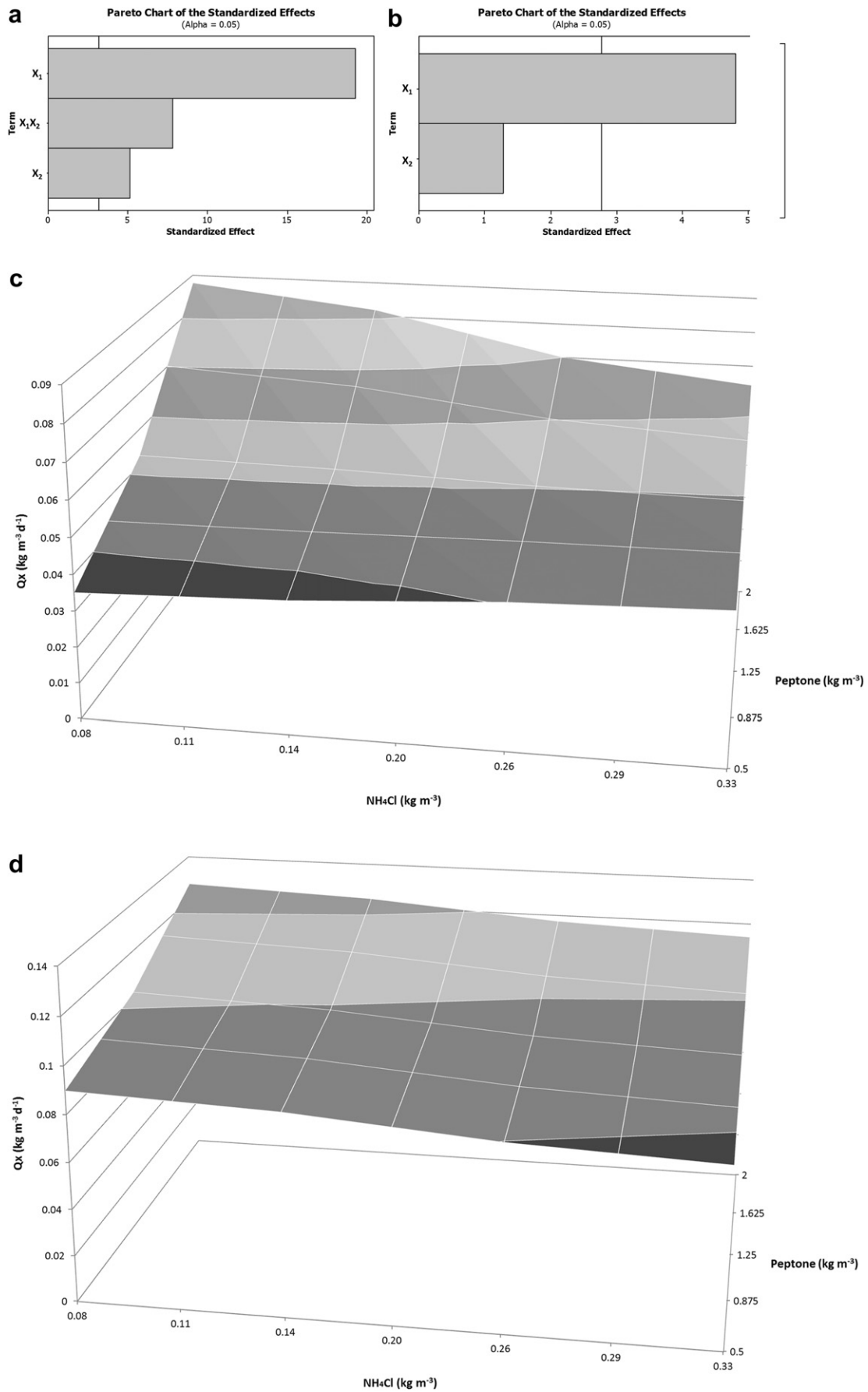


Fig. 2 – (a) A sample profile for Plackett-Burman screening design stage: biomass growth (○) (biomass level appears multiplied five folds), glycerol consumption (◆) and ethanol production (■). (b) Pareto diagram for screening design at 95% confidence interval (+: positive effect, -: negative effect).



**Fig. 3 – Pareto diagram for full factorial design with a 95% confidence interval ( $X_1$ : Peptone,  $X_2$ :  $\text{NH}_4\text{Cl}$ ): (a) interaction considered (b) interaction not considered; Response surface for uncoded terms, (c) interactions considered (d) interactions not considered.**

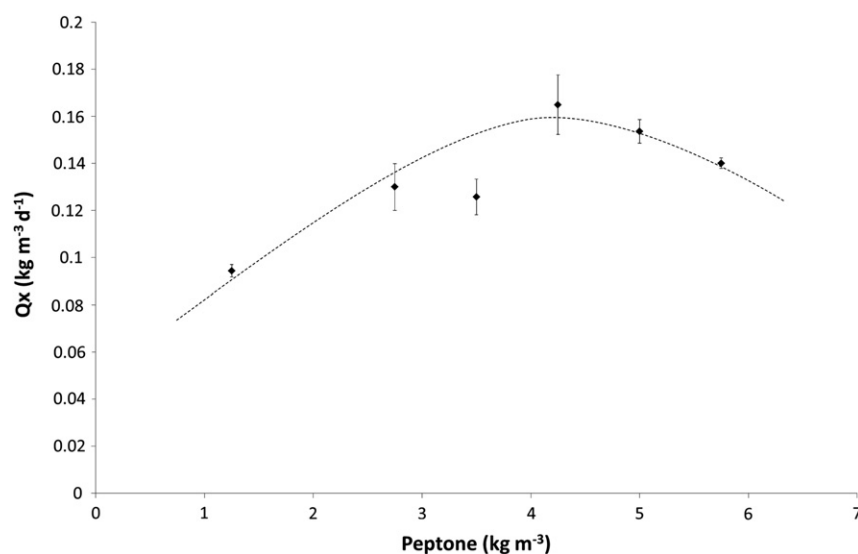


Fig. 4 – Biomass productivity ( $Q_x$ ) versus peptone concentration.

column (Bio-Rad, Hercules, CA). The operating conditions were 5 mM  $H_2SO_4$  in the mobile phase, at 0.6 mL  $min^{-1}$ , column temperature at 50 °C.

#### 2.4. Experimental design

A Plackett-Burman screening design (PB) for eight factors and twelve experiments was utilized [14]. The coded variables were defined according to:

$$x_i = \frac{2(C_i - \bar{C})}{(C_{max} - C_{min})} \quad (1)$$

where  $C_i$  represents each component studied, varying in the interval  $C_{min}$ – $C_{max}$ . Table 1 show the concentration of each component studied in the PB design. The screening design was used to determine which of the culture media components were significant with a 95% confidence interval. The design is presented in Table 2. Once the experiments were performed, the chosen response variable and factors were represented in a Pareto diagram.

In the next stage, a full factorial design  $2^2$ , shown in Table 3, and a response surface model were used. The design conditions were far from optimal. In this respect, a first-order model could be considered an adequate approximation to the true surface, and it would therefore be possible to find the optimal region using the method of the steepest ascent [18]. The data for Plackett-Burman and full factorial designs were analyzed using STATGRAPHICS Centurion XV, V15.2.05 (Stat Point, Inc.) software.

### 3. Results and discussion

For the PB design, twelve fermentations were performed, and the profiles obtained for biomass growth, glycerol consumption and ethanol production are shown in Fig. 2a. The maximum values obtained for ethanol yield ( $Y_{P/S}$ ) and specific growth rate ( $\mu$ ) were 330 g  $kg^{-1}$  of glycerol and 0.016  $h^{-1}$ , respectively.

Ethanol production is not associated with biomass growth. From Fig. 2a, it can be observed that ethanol is produced while biomass is in the growth stationary phase. These results suggest that an optimized formulation should potentiate a critical level of biomass growth to support ethanol production. Biomass productivity ( $Q_x$ , expressed as  $kgX m^{-3} h^{-1}$  where X represents biomass) was chosen as the response variable to be studied. After the PB design was performed for the eight factors, three factors were identified as positively significant and one factor was negatively significant at 95% confidence intervals [19]. Fig. 2b shows a Pareto graph of the effects of the PB screening design.

The Pareto diagram reveals that peptone was the most important component in the culture medium, for the response variable studied. The main positive influential factors were studied in a second step of the optimization process using a full factorial design and a response surface model. Glycerol and the other negative effect factors were fixed at low levels, as presented in Table 1, for this second step. Specifically, glycerol was fixed at 10  $kg m^{-3}$  as reported by Dharmadi et al. [20].  $KH_2PO_4$

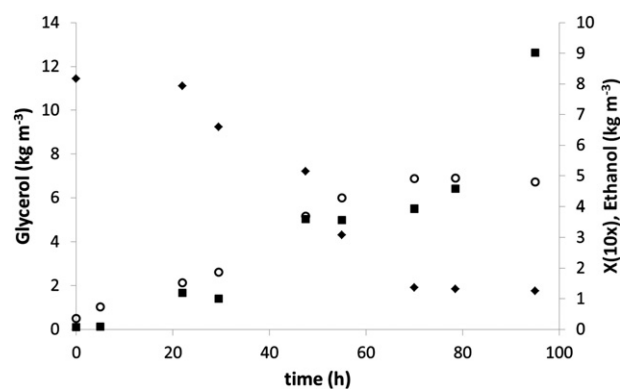


Fig. 5 – Concentration profile for optimized medium; biomass growth (○) (biomass level is multiplied ten-fold), glycerol consumption (◆) and ethanol production (■).

**Table 4 – A comparison of fermentation parameters obtained by Murarka et al. [17] and this work.**

Parameter	Units	This work	Murarka et al.	Strain
$\mu$	$\text{h}^{-1}$	$0.0313 \pm 0.0015$	$0.040 \pm 0.003$ ( $\mu_m$ )	MG1655
Mass fractional glycerol consumed	%	$84.46 \pm 0.023$	$\approx 80^a$	MG1655
$Y_{P/S}$	$\text{kg kg}^{-1}$	$0.906 \pm 0.023$	0.461	MG1655
$Y_{X/S}$	$\text{kg kg}^{-1}$	$0.059 \pm 0.019$	$0.0329 \pm 0.0029$	MG1655
$q_P$	$\text{kg kg}^{-1} \text{h}^{-1}$	0.212	$\approx 0.146^a$	MG1655
$Q_X$	$\text{kg m}^{-3} \text{d}^{-1}$	$0.165 \pm 0.0126$	$\approx 0.0752^a$	MG1655

a Calculated from reported data at Murarka et al.

was excluded for this second step because its significance is in the limit of the confidence interval and it is a component of the phosphate buffer. Therefore, peptone and  $\text{NH}_4\text{Cl}$  levels were studied. The results were adjusted to fit a first-order model, considering the scenarios with and without interactions between the factors. In both cases,  $X_2$  was less significant, as seen in Fig. 3. When no interaction was considered, the  $p$ -value for  $X_2$  was higher than 0.05 ( $p$ -value = 0.269) with a 95% confidence interval and determined to be negligible.

The first-order surface for both cases is shown in Fig. 3. To achieve a high value for  $Q_X$ , it is necessary to increase peptone and decrease  $\text{NH}_4\text{Cl}$  levels. The chosen condition used the no interaction model because it is impossible increase peptone and decrease  $\text{NH}_4\text{Cl}$  indefinitely along the steepest path. The coded model for the first-order surface with no interaction is represented in the following expression:

$$y = 0.101254 + 0.0249226x_1 - 0.0066453x_2 \quad (2)$$

where  $y$  is the biomass productivity ( $Q_X$ ) and  $x_1$ ,  $x_2$  represents peptone and  $\text{NH}_4\text{Cl}$  levels, respectively.

To find an optimum level for peptone, a new experiment, using only peptone, was conducted [15]. A screening of peptone levels between 1.25 and 5.75  $\text{kg m}^{-3}$  was performed, as shown in Fig. 4. A maximum value for  $Q_X$  was observed at 4.25  $\text{kg m}^{-3}$  of peptone.

With the optimum level for peptone determined, it was possible to formulate the optimum culture media for maximum biomass productivity: 10  $\text{kg m}^{-3}$  glycerol, 0.0806  $\text{kg m}^{-3}$   $\text{Na}_2\text{SO}_4$ , 0.0152  $\text{kg m}^{-3}$   $\text{NaCl}$ , 0.0310  $\text{kg m}^{-3}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 4.25  $\text{kg m}^{-3}$  peptone. A fermentation profile, using the optimized medium, can be observed in Fig. 5. The stationary phase for biomass growth was reached at 70 h of fermentation compared to 97 h in non-optimized medium (Fig. 2a). Moreover, ethanol production increased two-fold relative to the non-optimized medium (Fig. 2a).

Fermentation parameters were calculated for the experiments carried out with the optimized media culture presented in this work. As a control, these results were compared with previously reported data [17], as shown in Table 4. The media culture used by Murarka was a modified minimal medium (MM) designed by Neidhardt et al. [21]. Our results are significant considering that  $Q_X$ ,  $q_P$  and  $Y_{P/S}$  are 2.19, 1.45 and 1.96 times higher, respectively, than previously reported data for a similar process. The culture medium developed and optimized in this work can be considered a simple and attractive means to produce ethanol using glycerol as a carbon source and *E. coli* as a biocatalyst. A simple culture media is attractive

for future scale-up stages where low costs for medium formulation and preparation simplicity will be important.

## 4. Conclusions

A culture media for the biotransformation of glycerol to ethanol by *E. coli* was formulated and optimized. Thirteen compounds were initially included in the non-optimized media culture. This total was reduced to five components. The final components in media culture were glycerol,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaCl}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and peptone at concentrations of 10  $\text{kg m}^{-3}$ , 0.0806  $\text{kg m}^{-3}$ , 0.0152  $\text{kg m}^{-3}$ , 0.0310  $\text{kg m}^{-3}$  and 4.25  $\text{kg m}^{-3}$ , respectively. The calculated results for biomass productivity, ethanol specific productivity and glycerol to ethanol yield were 0.165  $\text{kg m}^{-3} \text{d}^{-1}$ , 212  $\text{g kg}^{-1} \text{h}^{-1}$  of cell mass and 59  $\text{g kg}^{-1}$  of glycerol, respectively.

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