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Improved bioconversion of crude glycerol to hydrogen by statistical optimization of media components

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Bioconversion of crude glycerol to hydrogen has gained importance as it addresses both sustainable energy production and waste disposal issues. Until recently, statistical optimizations of crude glycerol bioconversion to hydrogen have been greatly focused on pure strains. In this study, biohydrogen production from crude glycerol by an enriched microbial culture (predominated with *Clostridium* species) was improved by statistical optimization of media components. Plackett–Burman design identified $MgCl_2.6H_2O$ and KCl with negative effect on hydrogen production and selected NH_4Cl, K_2HPO_4 and KH_2PO_4 as significant variables. Box–Behnken design indicated the optimal region beyond design area and studies were continued by ridge analysis. Central composite face centered design envisaged a maximal hydrogen yield of 1.41 mol-H_2/mol-glycerol$_{consumed}$ at concentrations 4.40 g/L and 2.27 g/L for NH_4Cl and KH_2PO_4 respectively. Confirmation experiment with the optimized media (NH_4Cl, 4.40 g/L; K_2HPO_4, 1.6 g/L; KH_2PO_4, 2.27 g/L; $MgCl_2.6H_2O$, 1.0 g/L; KCl, 1.0 g/L; Na-acetate.$3H_2O$, 1.0 g/L and tryptone, 2.0 g/L) revealed an excellent correlation between predicted and experimental hydrogen yield. Optimization of media components by design of experiments enhanced hydrogen yield by 29%.

1. Introduction

Concern over increasing environmental pollution and the need for a sustainable energy source have led to an increased global biodiesel production for its use as a diesel substitute. This increased biodiesel production has resulted in severe waste disposal crisis due to the production of proportionally equivalent amounts of crude glycerol as the by-product [1]. Since crude glycerol is an excellent carbon source, bioconversion of crude glycerol has been an interesting area of study. Moreover, the process also offers a sustainable method for the disposal of biodiesel waste. Bioconversion of crude glycerol to hydrogen (H_2) [2,3], 1,3-propanediol [4], ethanol [5], and lactic acid [6] have been extensively studied.

Fermentative H_2 production from crude glycerol has gained much importance due to high H_2 content and is a promising substrate for sustainable energy production with high energy content [7]. Selembo et al. [8] studied H_2 production, in batch type fermentation, from crude glycerol using mixed inoculum, reporting a yield of 0.31 mol-H_2/mol-glycerol. Efficient bioconversion of crude glycerol to H_2 was reported by Ngo et al. [9], wherein pretreated crude glycerol was used as the substrate, assisted with N_2 sparging and pH control, using *Thermotoga neapolitana*, reporting a yield of 2.73 mol-H_2/mol-glycerol$_{consumed}$. In a recent publication, Jitrwung et al. [10] investigated the effect of optimizing media optimization on bioconversion of crude glycerol to H_2 by *Enterobacter aerogenes*, reporting a yield of 0.84 mol-H_2/mol-glycerol. Eco-biotechnological application for improved bioconversion of crude glycerol has been recently studied. Comparison of crude glycerol utilization from different bio-diesel plants and its bioconversion to H_2 by a functional mesophilic microbial consortium enriched from activated sludge was investigated by Varrone et al. [11]. The authors reported a H_2 yield of 0.90 mol-H_2/mol-glycerol from minimal media supplement with 15 g/L crude glycerol and a 97% degradation efficiency of the sole carbon source. Implementing statistical optimization for improved biohydrogen production from crude glycerol by mixed microbial inoculum has gained importance. Varrone et al. [12] have reported on improved H_2 production from crude glycerol using statistical approach in optimizing the culture conditions. They investigated the biohydrogen production efficiency of mesophilic mixed microbial culture on crude glycerol as the carbon source, reporting a yield of 0.96 mol-H_2/mol-glycerol. Apart from culture conditions such as

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temperature, pH, inoculums size and optimal substrate concentration, microorganisms require appropriate concentrations of media components for optimal metabolism during fermentation process [13,14]. N_2 is an important media component, assisting in the synthesis of proteins, nucleic acids and enzymes, significant for the microbial growth and hydrogenase enzyme activity [14,15]. Optimal phosphate concentration positively affects the H_2 production due to its buffering capacity. High phosphate and potassium ion concentrations are reported to cause an increased cytoplasmic osmotic pressure, thus negatively affecting the microbial growth [15,16]. Magnesium, an important component for cell wall and cell membrane synthesis and an enzyme cofactor, also plays a pivotal role in microbial growth and metabolism [17].

Our previous study focused on enriching H_2 producing mixed microbial culture. This study aims in improving the earlier reported H_2 yield by optimizing the current media composition, without altering the original media components, using design of experiments (DoE) and response surface methodology (RSM). DoE and RSM are efficient approaches for such optimization tasks, as shown by previous studies [12–14]. In this study, Plackett–Burman design was used to screen the significant media components [18]. The path of steepest ascent [19], Box–Behnken [20], ridge analysis [21–24] and Central Composite Face Centered Design (CCD) [25] models were used to optimize the concentrations of screened media components. Finally, the validation of the model was performed at the predicted optimal concentrations.

2. Materials and methods

2.1. Glycerol source and culture conditions

Industrial glycerol was kindly provided by Savon Siemen Oy (Iisalmi, Finland). The crude glycerol contained 45% (v/v) glycerol and 30% (v/v) methanol with an alkaline pH (~12). The inoculum used for this study was an enriched microbial consortium (mainly comprised of *Clostridium sporogenes* strain CL3 (Accession no: JF836014.1), *Clostridium subterminale* isolate DSM 758 (Accession no: EU857637) and uncultured bacterium clone (Accession no: FJ512181.1)) [3] from activated sludge enriched in modified HM100 medium (NH_4Cl, 1.0 g/L; K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.3 g/L; $MgCl_2.6H_2O$, 2.0 g/L; KCl, 4.0 g/L; Na-acetate.$3H_2O$, 1.0 g/L; tryptone, 2.0 g/L; Na-dithionite, 0.5 g/L and resazurin, 0.002 g/L) amended with crude glycerol [3]. The pre-inoculum was prepared by inoculating 10% of the enriched microbial community in 120 ml serum bottles with working volume of 50 ml HM100 medium containing pure glycerol (5 g/L). The pre-inoculum was grown at 150 rpm with an initial pH of 6.5 and cultivation temperature at 40 °C.

For optimization studies, ammonium chloride (NH_4Cl), dipotassium phosphate (K_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), magnesium chloride hexa-hydrate ($MgCl_2.6H_2O$) and potassium chloride (KCl) were chosen. The media was prepared in similar way, varying the selected variables and keeping the remaining media components in concentrations as of the original media composition. The DoE studies were conducted in batch type fermentation with 120 ml serum bottles with a working volume of 50 ml sterile anoxic crude glycerol (1 g/L) amended enrichment medium. The investigations were performed as triplicate experiments at 40 °C and 150 rpm with a growth period of 72 h.

2.2. Analytical methods

Crude glycerol, volatile fatty acids and alcohols were analyzed by high performance liquid chromatography (HPLC; LC-20AD, Shimadzu, Japan). The HPLC was equipped with a 300 mm × 8 mm Shodex SUGAR (SH1011) column and a refractive index detector (RID-10A). The HPLC samples were prepared as described previously [18]. The proportions of H_2 gas were measured using gas chromatograph (GC-2014, Shimadzu GC), fitted with a thermal conductivity detector and a 2 m × 2 mm PORAPAK column. N_2 was used as the carrier gas with a flow rate of 20 ml/min. The temperatures of column, detector and oven were maintained at 80 °C, 110 °C and 80 °C respectively. Measurements for each sample were repeated twice and averaged.

Substrate blank (i.e. cultivation without the crude glycerol) was included in all experiment sets to deduct H_2 produced from tryptone in the growth media. The H_2 yield values were calculated as described previously [26]. The presence of methanol in crude glycerol and its utilization by the microbial community was tested. From growth-curve test and end-metabolite distribution it was observed that the functional community did not utilize methanol (data not shown). Hence, methanol was excluded from the H_2 yield calculations.

2.3. Optimization procedure

2.3.1. Plackett–Burman design

Plackett–Burman design [18] was chosen to screen and identify variables that had significant influence on the bioconversion of crude glycerol to H_2. Plackett–Burman design is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \tag{1}$$

where Y is the response, β_0 is the model intercept and β_i is the linear coefficient, and X_i is the level of independent variable.

Five media components, NH_4Cl, K_2HPO_4, KH_2PO_4, $MgCl_2.6H_2O$ and KCl, were selected to investigate the trivial components influencing H_2 production. Based on Plackett–Burman design, each factor was prepared in two levels: −1 for low level and +1 for high level (Table 1). The main effect of each variable to the response was then calculated as the difference between the averages of the high level (+1) and the low level (−1) response measurements of that variable. In this study, five assigned variables were screened in eight experimental designs. The level of each factor used in the experimental design along with the response and details of the linear model constructed using the Plackett–Burman design is reported in Tables 1 and 2 respectively. The analysis of variance (ANOVA) was performed to select the variable with significant effect (p value > 0.05) on the response. The results presented were average H_2 produced from triplicate experiments with standard deviations.

The Design-Expert (version 8.0.7.1, Stat-Ease Inc., MN, USA) was used for statistical analysis.

2.3.2. Path of steepest ascent

The identified significant variables from Plackett–Burman design were subjected to steepest ascent approach. This optimization step helps to move the experimental region from the design center towards the direction of an optimal response. The path of steepest ascent is based on the first order polynomial model (Eq. (1)) obtained from the Plackett–Burman design and provides information on the test range of selected variables for further optimization steps [19]. Table 3 reports the concentration range of the selected variables to move the design area towards optimal response. The average yield, with standard deviations, was calculated from triplicate experiments for each design points.

2.3.3. Box–Behnken design

For further optimization of the selected media components for enhanced H_2 production, a three-variable Box–Behnken design