Bioconversion of Biodiesel Derived Crude Glycerol by Immobilized *Clostridium pasteurianum*: Effect of Temperature

Swati Khanna, Arun Goyal, Vijayanand S. Moholkar[*]

Abstract—Batch fermentation of 5, 10 and 25 g/L biodiesel derived crude glycerol was carried out at 30, 37 and 45^0C by *Clostridium pasteurianum* cells immobilized on silica. Maximum yield of 1,3-propanediol (PDO) (0.60 mol/mol), and ethanol (0.26 mol/mol) were obtained from 10 g/L crude glycerol at 30 and 37^0C respectively. Maximum yield of butanol (0.28 mol/mol substrate added) was obtained at 37^0C with 25 g/L substrate. None of the three products were detected at 45^oC even after 10 days of fermentation. Only traces of ethanol (0.01 mol/mol) were detected at 45^0C with 5 g/L substrate. The results obtained for 25 g/L substrate utilization were fitted in first order rate equation to obtain the values of rate constant at three different temperatures for bioconversion of glycerol. First order rate constants for bioconversion of glycerol at 30, 37 and 45^oC were found to be 0.198, 0.294 and 0.029/day respectively. Activation energy (Ea) for crude glycerol bioconversion was calculated to be 57.62 kcal/mol.

Keywords—activation energy, *Clostridium pasteurianum*, crude glycerol, immobilization

I. INTRODUCTION

GLYCEROL, a major waste of biodiesel industry can be used as a feedstock for production of numerous commodity chemicals [1]-[4]. The conversion of glycerol to various types of compounds may be carried out by both chemical as well as biological means. The biological conversion of glycerol excels over chemical conversion with respect to higher yield and selectivity, normal reaction conditions, and use of cheaper biological catalysts. Many micro-organisms are known to convert glycerol to different value added products such as 1,3-PDO [5], dihydroxyacetone [6], butanol [7]-[10], single cell oils [11], polyhydroxyalkanoates [12], ethanol [13], succinic acid [14] etc. *Clostridium pasteurianum*, a gram positive anaerobe, can ferment glycerol to butanol, ethanol and 1,3-PDO.

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Butanol and ethanol are alternate fuels while 1,3-PDO is an important feedstock for synthesis of polymers such as polyesters and polyurethanes. Immobilized *Clostridium pasteurianum* cultures have also been used for bioconversion of biodiesel derived crude glycerol [15]. Immobilized cells provide various advantages over free cells viz. simpler downstream processing, decreased substrate and product inhibition, reusability and renewability. It is well known that biological systems show optimum activity at a particular range of temperature. This is due to the presence of enzymes which show best activity at a particular temperature or temperature range. The present work tries to investigate the effect of variation of temperature on product profile of crude glycerol fermenting immobilized *C. pasteurianum* cells. We have tried fitting first order rate equation for this immobilized system to obtain temperature specific rate constants which were finally used for calculating the activation energy required for bioconversion of crude glycerol to butanol, 1,3-PDO and ethanol by immobilized cells.

II. MATERIAL AND METHODS

A. Materials

Clostridium pasteurianum MTCC 116 (ATCC 6013) was procured from Microbial Type Culture Collection, Chandigarh, India. 60-120 mesh size silica (column chromatography grade, Merck, Germany) was used as an immobilization support and was procured from Merck, Germany. Crude glycerol was generated in homogenous alkali catalysis of soybean oil with a composition of glycerol (90-95% v/v), methanol (5-10% v/v) and Na_2SO_4 (3-5% w/v). The anaerobic assembly for growth of *Clostridium pasteurianum* on agar slants was procured from Himedia, India. The analytical standards for gas and high performance liquid chromatographic analysis were procured from Sigma Aldrich, USA and Merck, Germany. All other chemicals used were of analytical grade (Merck, Germany and Himedia, India).

B. Immobilization of Cells

The lyophilized cells were revived in Cooked Meat Media (CMM) and maintained on CMM agar slants under anaerobic condition.

The cells were immobilized in Reinforced Clostridial Media (RCM) broth of following composition (g/L distilled water),

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beef extract (10), yeast extract (3), peptone (10), glucose (5), soluble starch (1), sodium chloride (5), sodium acetate (3), agar (0.5), cysteine hydrochloride (0.5). The initial pH of the media was adjusted to 6.8±0.2. The silica particles were dried at 120°C for 24 h in a dry oven before use. The support was added to the media after approx. after 24 h (after the end of lag phase of the culture). The support was kept for 48 h with the media at 37°C with shaking at 200 rpm. The broth was centrifuged and the support with immobilized cells was shaken with phosphate buffer (50 mM, pH 7.5) for 10 min. It was washed thoroughly with distilled water and dried for 24 h at room temperature. The cross linking of immobilized cells was done by incubating them with 0.1% glutaraldehyde solution for 1 h, followed by washing with phosphate buffer (50 mM, pH 7.5), and then with water and were dried at room temperature for 24 h.

C. Batch Fermentation

Batch fermentation was carried out in 250 mL custom-built Erlenmeyer flasks containing 1 g/L yeast extract, 0.01 g/L $CaCl_2·2H_2O$, 0.1 g/L $MgSO_4·7H_2O$, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 and 5 mg/L $FeSO_4·7H_2O$ (pH = 6.8). Three different crude glycerol concentrations, 5, 10 and 25 g/L, were considered for the present study. Different concentrations of crude glycerol were added to each flask containing 3 g of immobilized support. Three different fermentation temperatures viz. 30, 37 and 45°C were considered for the present study. The flasks were kept in an incubator shaker with shaking at 200 rpm. The flasks were sparged with nitrogen gas before incubation as well as after every 24 h to maintain anaerobic conditions. The samples were withdrawn after every 2 days upto a period of 10 days. All the batch fermentation experiments were carried out in triplicate and the results presented are the mean of the three experimental runs.

D. Analysis and Calculations

The fermentation products and their quantity were determined using Gas Chromatograph (Varian, CP 3800) using a CP Wax 52 CB capillary column (250 mm×0.25 mm×0.39 mm, Varian). The oven temperature was programmed from 45°C to 100°C with an increment of 3°C/min and after 100°C, an increment of 5°C/min upto 200°C. The injector and detector temperatures were 230°C and 250°C, respectively. Nitrogen gas was used as a carrier at a flow rate of 2.0 mL/min. The utilization of crude glycerol was determined by HPLC analysis using Hi-Plex-H column (8µx300mmx7.7mm, Varian) with 100% HPLC grade water as the mobile phase. The HPLC apparatus comprised of a pump (Series 200, Perkin Elmer) operated at a flow rate of 0.5 mL/min, a refractive index detector (Series 200, Perkin Elmer), a vacuum degasser (Series 200, Perkin Elmer). The glycerol utilization results obtained with 25 g/L crude glycerol were fitted in first order rate equations to obtain rate constants at three different temperatures. These rate constants were then fitted in Arrhenius equation wherein the value of activation energy (Ea) was calculated from the plot of ln k vs 1/T.

III. RESULTS

A. Trends in Butanol Production

Max. yield of butanol (0.28 mol/mol) was formed with 25 g/L initial substrate concentration at 37°C (Fig. 1C). 5 g/L crude glycerol gave next highest butanol yield of 0.13 mol/mol at 37°C. 10 g/L substrate concentration was particularly inhibitory for butanol production. Fermentation temperature of 30°C produced negligible amount of butanol from 25 g/L and 5 g/L substrate while, no butanol was detected with 10 g/L substrate at the same temperature (Fig. 1). Higher temperature of 45°C did not produce any butanol, with all three substrate concentrations studied, to be detected by gas chromatograph. Thus, 37°C is the most suitable fermentation temperature for production of butanol from crude glycerol by immobilized *C. pasteurianum* cells. For, all three initial substrate concentrations considered, the yield of butanol was found to increase with time at both 30 and 37°C.
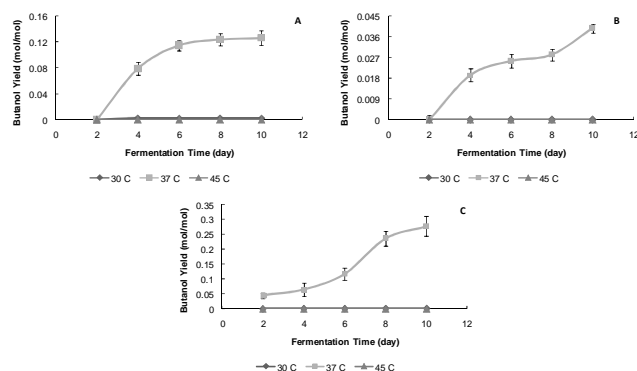

Fig. 1 Trends in production of butanol with initial crude glycerol concentration of (A) 5 g/L, (B) 10 g/L and (C) 25 g/L at varied temperatures. 10 g/L crude glycerol formed minimum amount of butanol at all temperatures as compared to other concentrations studied

B. Trends in 1,3-PDO production

Fig. 2 depicts 1,3-PDO production profile for three different substrate concentrations at three different temperatures. Maximum yield of 1,3-PDO (0.60 mol/mol) was obtained at 30°C with 10 g/L substrate. On further increasing the temperature to 37°C, 1,3-PDO yield decreased to 1/4th of that obtained at 30°C (0.15 mol/mol). An increase in substrate concentration from 10 g/L to 25 g/L led to a decrease in yield of 1,3-PDO at both 30 and 37°C. Both 10 and 25 g/L substrate yielded more product at the lowest fermentation temperature except 5 g/L, which exhibited a higher production of 1,3-PDO at 37°C. No 1,3-PDO was detected at 45°C with all three substrate concentrations studied. As was observed in case of butanol production, the production of 1,3-PDO also increased with an increase in fermentation time.

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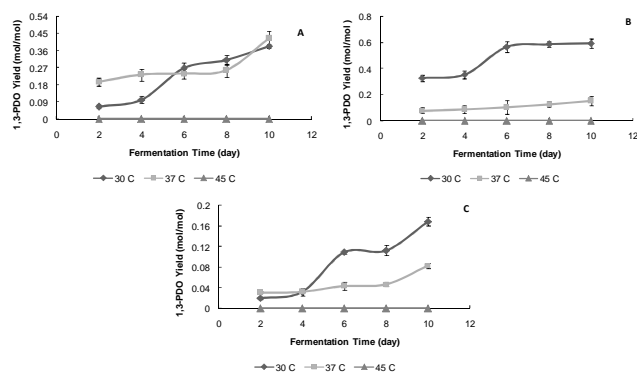International Journal of Chemical and Molecular Engineering
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Fig. 2 Trends in production of 1,3-PDO with initial crude glycerol concentration of (A) 5 g/L, (B) 10 g/L and (C) 25 g/L at three different fermentation temperatures

C. Trends in ethanol production

Fig. 3 depicts trends in ethanol production with change in temperature and initial substrate concentration. Maximum yield of ethanol (0.26 mol/mol) was obtained at 37^0C with 10 g/L substrate. The next higher ethanol yield (0.10 mol/mol) was obtained at 37^0C with 5 g/L crude glycerol. At 45^0C, traces of ethanol were detected with 5 g/L substrate but other two substrate concentrations did not yield any ethanol at the same temperature. Also, no ethanol was detected with 5 g/L substrate at fermentation temperature of 30^0C, and only negligible amount of ethanol was formed with 10 g/L substrate at the same temperature. As was observed with other two products, the yield of ethanol increased with time at 30 and 37^0C.
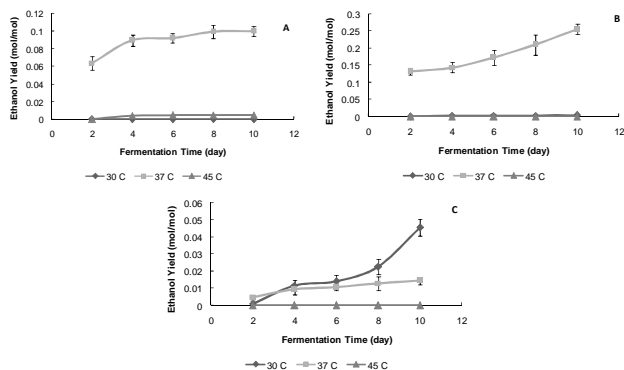

Fig. 3 Trends in ethanol production with (A) 5 g/L, (B) 10 g/L and (C) 25 g/L initial crude glycerol concentration at three fermentation temperatures

C. Rate Constants and Activation Energy

In order to understand the actual mechanism by which temperature exerts its effect on bioconversion of crude glycerol to solvents, one needs to look at the kinetic data as well as thermodynamic data. The substrate consumption data for 25 g/L initial substrate concentration was used further for calculating rate constants at three fermentation temperatures. The glycerol utilization data so obtained, fitted well in first order kinetic equation with values of R^2 ranging from 0.85 to

0.95. The values of first order rate constant (k) at 30, 37 and 45^0C were calculated to be 0.198, 0.294, 0.029 /day respectively. The k values depict clearly that the for 25 g/L initial glycerol concentration, the rate of glycerol bioconversion is highest at 37^0C and the lowest at 45^0C. A higher k value at 37 ^{0}C than at 30^0C gave a higher total product yield at 37^0C. Also, none of the desired products were detected at 45^0C due to the lowest k value. Activation energy (Ea) for bioconversion of crude glycerol was found to be 57.62 kcal/mol using Arrhenius equation (Fig. 4).
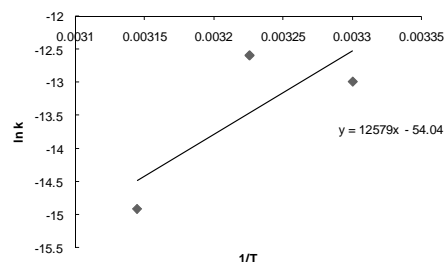

Fig. 4 ln k vs 1/T (Kelvin^{-1}) curve for calculating activation energy (Ea) of glycerol bioconversion to the three solvents

E. Utilization of 25 g/L crude glycerol

The utilization of 25 g/L substrate after 2 days of fermentation was similar at both 37 and 45^0C. The highest total product yield at 37^0C may be attributed to the highest glycerol utilization at this temperature. Although approx. 6 g/L substrate was consumed at 45^0C, but none of it was converted into detectable products. This may be due to conversion of glycerol to other undetectable compounds such as succinate, acetate, lactate, CO_2.

IV. DISCUSSION

Clostridium pasteurianum exhibits a biphasic fermentation pattern wherein the cells first go for acids formation (acetate, butyrate) known as acidogenic phase and the lowering of pH due to acids accumulation causes the production of solvents (ethanol, butanol) known as solventogenic phase [10]. The production of 1,3-PDO occurs before accumulation of acids. A comparison of results obtained from present study is rather difficult, as there is only limited literature on fermentation of crude glycerol by *Clostridium pasteurianum* cells. Also, the effect of temperature on fermentation profile of crude glycerol by immobilized *C .pasteurianum* has not been studied as yet. Comparing among the product profiles obtained at three temperatures studied, it can be seen that total yield of products is higher at 37^0C (0.66 and 0.37 mol/mol for 5 and 25 g/L respectively) than at 30^0C (0.38 and 0.22 mol/mol for 5 and 25 g/L respectively) for both 5 and 25 g/L substrate but the same is not true for 10 g/L substrate which formed a higher amount of product at 30^oC (0.60 mol/mol) than at 37^0C (0.45 mol/mol). A higher temperature of 45^0C is not at all suitable for production of solvents from crude glycerol.

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The trends observed in this study at 37^0C matched well with earlier results [10] from free cells of *C. pasteurianum* (growing on crude glycerol) in many aspects.

- 10 g/L substrate formed negligible amounts of butanol while, highest amount of butanol was obtained with 25 g/L substrate.
- Highest ethanol yield obtained with free cells was obtained with both 5 and 10 g/L substrate. Highest ethanol yield with immobilized cell was obtained with 10 g/L substrate.

It is known that high concentrations of glycerol are inhibitory to *C. pasteurianum* cells [10], [15]. The same effect is quite evident here as there is a decrease in total solvent yield at 25 g/L crude glycerol concentration. 37^0C was the most suitable temperature for obtaining maximum yield of butanol while 30^0C gave the highest yield of both 1,3-PDO and ethanol. Thus, production of these three fermentation products is temperature specific. This may be due to different temperature optima for enzymes involved in ethanol, butanol and 1,3-PDO production pathways. The future work may aim at varying the type of substrate for solvent production at different fermentation temperatures for understanding their effect on product profile.

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