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Ethanol Fuel Production from Glycerol using *Bacillus cereus.*

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ABSTRACT

Bioethanol for use as a biofuel can be produced from several starting materials. Glycerol, one of the byproducts produced during biodiesel production, can also be considered as a potential carbon source for bioethanol production. In the present work, *Bacillus cereus* is used for ethanol production from pure glycerol using shake flask studies. In addition, fermentation parameters such as, glycerol concentration, broth pH, incubation time & aeration condition were varied using one-factor variation at a time. It was noted that the presence of oxygen during fermentation has a significant positive effect on ethanol biosynthesis. But, in the shake flask, mixing intensity was insufficient to overcome oxygen diffusion due to higher broth viscosity. The broth pH of about 5 to 6 resulted in good yield of ethanol under aerobic conditions. The glycerol concentration beyond 0.6 M concentration did not yield a good amount of ethanol owing to the higher viscous conditions. A mini bioreactor was designed to improve aeration conditions in order to overcome problems associated with aeration in shake flask studies. Further, this bioreactor was used for the fermentation of purified raw glycerol to ethanol using *Bacillus cereus*. Under experimental conditions of73.6 g/L glycerol concentration, at a pH 5.0 and aeration rate of 100 mL/min, incubation of 36 hours yielded 0.30 kg-ethanol per kg-glycerol with ethanol conversion efficiency of 59.8% and broth ethanol titer 23.5 g/L.

Keywords: Bioenergy, Biofuels, Bioethanol, Glycerol, Bioreactor.

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INTRODUCTION

The variety of raw materials and production technologies have played a vital role in the past few years for the production of biofuels and is going to continue for several decades [1-2]. The major concern behind this is the cost of raw material, waste management, re-utilization and value addition to byproducts [3-4]. Most of the biological processes are energy conservative and also produce byproducts that are re-usable. In a similar context, when transesterification of oil is carried out to produce biodiesel glycerol is coproduced along with biodiesel. The amount of glycerol produced is approximately 10% (by weight) of biodiesel produced [5]. Due to the enormous cost involved in purification of glycerol, use of raw glycerol is limited to only a few applications. Instead, raw glycerol can be fermented to produce another fuel bioethanol using minimal energy consumption [6-8]. Fermentation of glycerol can be achieved using a suitable microbe that can assimilate the raw material and can sustain high ethanol concentration. Bacteria like *Escherichia coli, Enterobacter aerogenes* can ferment glycerol to ethanol and other value added products [8-11]. Since glycerol is not a preferential substrate for many microbes, their metabolic pathway needs to be activated by the use of suitable stress factor [8,10,12].In the present study, the bacterium *Bacillus cereus* was used to produce ethanol from glycerol. Parametric analysis in shake flask studies with pure glycerol showed that the oxygen supply is necessary for ethanol production. A mini-bioreactor was designed to provide air to the fermentation system and analyzed with the use of raw glycerol. The raw glycerol obtained from biodiesel industry was partially purified before it was used for fermentation. We are reporting a process with 95.6% glycerol conversion efficiency in this manuscript.

MATERIALS AND METHODS

Materials

A facultative aerobic bacterium *Bacillus cereus* (Identified equivalent to ATCC 14579T at IMTECH, Chandigarh), isolated from honey, was used as the microbe for fermentation. Analytical reagent grade chemicals were used for all analysis. The crude glycerol was obtained from Udupi district Biofuel Information & Demonstration Centre, Nitte.

Glycerol Fermentation by Shake Flask Studies

Pure glycerol broth was prepared using commercial glycerol and diluted to the required concentration with distilled water. The broth pH was adjusted to the required preset value using phosphoric acid and sodium carbonate. About40 ml fermentation samples in the shake flask were sterilized and inoculated with 3ml of the bacterium *Bacillus cereus*. This bacterium was previously grown on the glucose nutrient broth for 48 hours. The flask containing inoculated glycerol broth was kept on an orbital shaker maintained at 150 pm at room temperature. The fermentation was carried out for predetermined time interval of 24 hours. Anaerobic condition was maintained by purging sterile CO_2 gas soon after inoculation, but just before fermentation. Aerobic condition was maintained by keeping the flasks on an incubator shaker. The glycerol concentration in the fermentation broth was varied from 0.1M (9.2 g/L) to 1.0 M (92 g/L). The experiments were conducted at pH 5.0, 6.0, 7.0 and 8.0 for 24 and 48 hour time intervals. The agitation condition was kept constant at 150 rpm. The ethanol and glycerol concentration of the broth were analyzed using potassium dichromate method and sodium periodate method respectively [13-14].

Construction of Small scale Bioreactor and Fermentation of Pure Glycerol

A minibioreactor (40mL working volume) was constructed as shown in Figure 1. Aeration was provided with air pump connected to a 40micron membrane filter. The sterile air flow rate was controlled using a valve. The flow rate was measured using the method of downward displacement of water. The agitation condition was provided using stirrer assembly connected to an external 12V DC motor. Stirrer speed was fixed to 150 rpm as indicated by a digital torque meter. Pure glycerol was used as raw material to evaluate the performance of bioreactor. In order to evaluate the significance of forced aeration in the bioreactor, the process parameters in the bioreactor were maintained as follows: glycerol concentration – 0.6 M and 0.8 M; pH – 5.0; agitation speed – 150 rpm; air flow rate – 80 mL/min.

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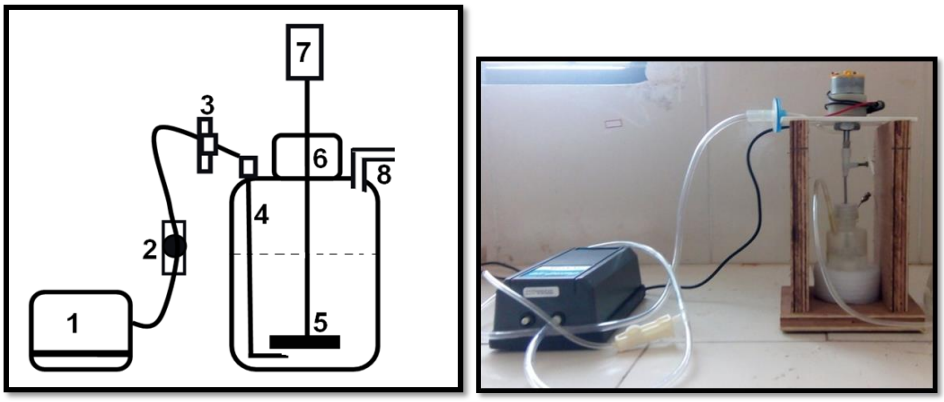

Figure 1: Small scale bioreactor – Schematic sketch and Fabricated bioreactor (1: Air pump, 2: Air flow controller, 3: Micro filter, 4: Needle Sparger, 5: Agitator, 6: Lid / cap, 7: Motor, 8: Air vent)

Purification of Raw Glycerol and Bioreactor Studies

Raw glycerol was obtained from Udupi district biofuel information and demonstration centre and purified using a 3 stage process. Glycerol is a byproduct of the transesterification of *Pongamia* oil during biodiesel production. The raw glycerol was first heated to evaporate the methanol completely. Then the product was treated with phosphoric acid followed by solvent extraction with isopropanol. The extract phase was decolorized using charcoal adsorption [15]. This glycerol was further diluted as per requirement and fermentation was carried out in the bioreactor as described in section 2.3. The fermentation experiments were carried out with conditions similar to that of shake flask as detailed in section 2.2. The bioethanol produced is measured in terms of g/L in fermentation broth; bioethanol yield is measured as gram ethanol produced per gram glycerol used. Ethanol production efficiency is calculated as actual ethanol yield to theoretical maximum yield (0.5 g ethanol/g glycerol) [3,9].An ethanol yield of 0.5 g ethanol/g glycerol corresponds to 100% ethanol production efficiency.

RESULTS AND DISCUSSION

Shake Flask Experiments

Effect of Glycerol Concentration and Broth pH

Shake flask studies with pure glycerol was performed with varying concentration of glycerol (0.1M to 1M), pH (5, 6, 7 & 8), aerobic and anaerobic condition for 24 and 48 hours with variation in one variable at a time approach. Bioethanol yield increased with glycerol concentration up to 0.6M under aerobic condition. As glycerol concentration increases beyond 0.8M, ethanol production dropped (Figure2). A 6.5% of maximum efficiency was achieved in this process. Keeping the glycerol concentration at a constant level of 0.6M, the broth pH was varied from 5 to 8 in increments of 1 unit. As shown in the Figure 3, bioethanol production decreased gradually with an increase in pH from 5 to 8. The highest ethanol production efficiency was found to be 8% at pH 5, at 0.6M glycerol concentration.

Dharmadi et al. (2006) have reported that in the case of bacterial fermentation of glycerol to ethanol and other products, at basic pH, the formate ions accumulate due to the inhibition of the enzyme formate hydrogen lyase. This in turn reduces the uptake of glycerol and production of ethanol and other byproducts [9]. Therefore, an acidic pH of 5 is favorable for higher production efficiency and yield of bioethanol.

Combined Effect of Oxygen and Broth Viscosity on Bioethanol Production

The Figure 2 and Figure 3 clearly indicate that oxygen (or aerobic condition) is very much necessary for bioethanol production. The bioethanol production efficiency is very high when oxygen is available as electron acceptor during fermentation. But, under aerobic condition, bioethanol yield (or efficiency) increases with an increase in glycerol concentration up to 0.6M. The viscosity of broth increases from 0.8 cP to 1.2 cP as

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the glycerol concentration increases from 0.1M to 0.6M. As a result, the diffusivity of oxygen (or air) present in overhead space decreases, hence the oxygen flux is retarded in the shake flask during fermentation. This creates virtual anaerobic pockets deep in the medium leading to anaerobic metabolic pathway. This essentially reduces the ethanol production and thus affects the process. Even if the agitation speed was increased to a higher rate, no significant improvement was observed. Raymond et al. (2013) have shown that *Enterobacter aerogenes* S012 fed with 50 g/L pure glycerol produced 24g/L bioethanol under aerobic condition where as under anaerobic condition, when incubated for 72 hour, yielded 18.5 g/Lethanol [10]. Further, it was concluded that some amount of oxygen is required for the better yield of ethanol. In the current study, results indicate that oxygen plays a vital role in ethanol production from glycerol by the bacterium *Bacillus cereus*.

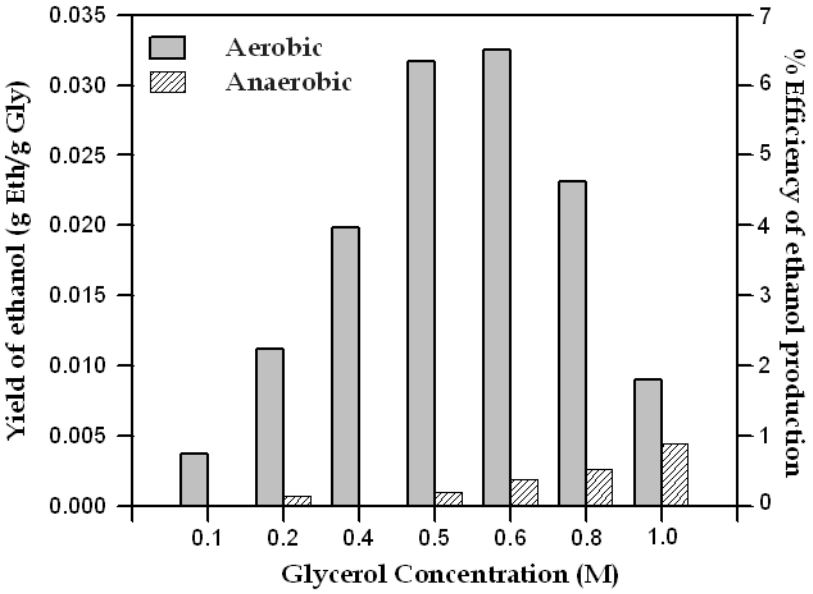

Figure 2: Effect of glycerol concentration on bioethanol production (at pH 5 & 24 hours)

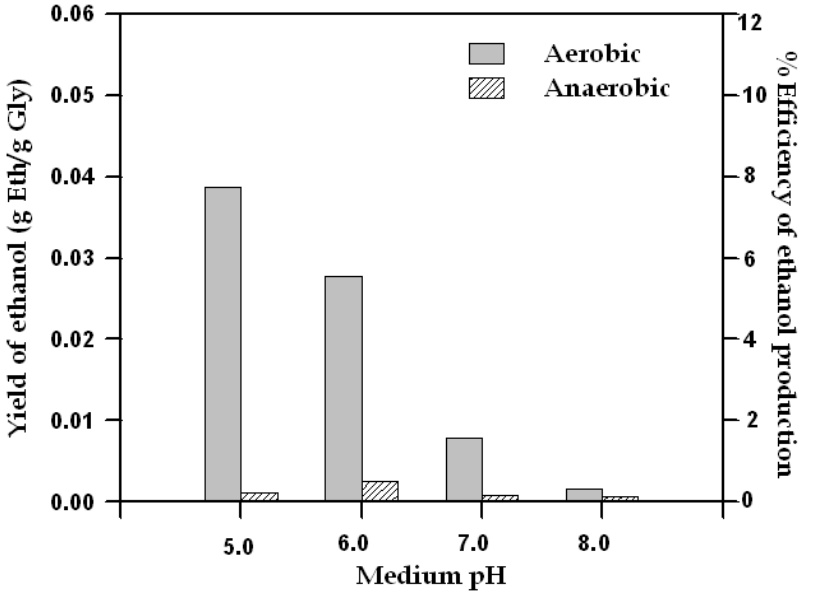

Figure 3: Effect of pH on bioethanol production (at 0.6M glycerol concentration & 24 hours)

Impact of Aeration Condition on Biomass Growth

The incubation period for the glycerol fermentation was increased from 24 hour to 48 hour, where fermentation of glycerol increases but not significantly. The biomass concentration in the fermentation broth

was measured using the gravimetric technique. The biomass was filtered through a wetted and pre-weighed Whatmann No. 1 filter paper. The wet biomass concentration was recorded as grams of wet cells per liter (g/L). In Figure 4 the biomass concentration are recorded from experiments conducted with 0.6M glycerol concentration and different broth pH under aerobic as well as anaerobic conditions. Under acidic condition also the trend was similar. Here, the biomass concentration was found to be less in aerobic fermented broth in comparison with anaerobic fermented broth. This trend remains same even after continuing the incubation period from 24 hour to 48 hour. It is evident that, the oxygen facilitates glycerol metabolism by *Bacillus cereus* to drive the metabolic pathway towards bioethanol synthesis. On the contrary, absence of the oxygen drives the metabolic pathway towards the increase in biomass. The ratio of the biomass growth rate at 48 hour to that at 24 hour under different broth pH conditions is shown in the Table 1. This ratio is higher in aerobic condition than that of anaerobic condition (at pH 5.0, 4.6 & 3.4 respectively). During the first 24 hour of fermentation, anaerobic condition favors biomass production whereas aerobic condition does not. But, the rate at which the biomass grows during next 24 hour span is less under anaerobic condition compared to that underaerobic condition. However, ethanol production efficiency drops during this period for aerobic condition. It could be due to diversion of metabolic path from ethanol production towards biomass production under increased ethanol concentration.

Table 1: Ratio of biomass growth rates under aerobic and anaerobic conditions

Broth pH	Ratio of Biomass growth rate, R (R = Rate at 48 hour / Rate at 24 hour)	
	Aerobic	Anaerobic
5.0	4.6	3.4
6.0	3.5	2.5
7.0	2.6	1.4
8.0	1.5	1.1

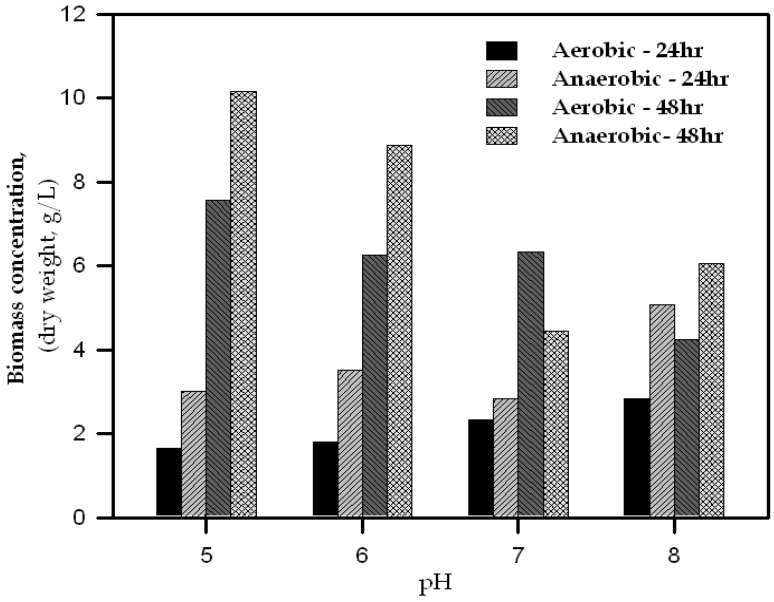

Figure 4: Biomass concentration in shake flask studies

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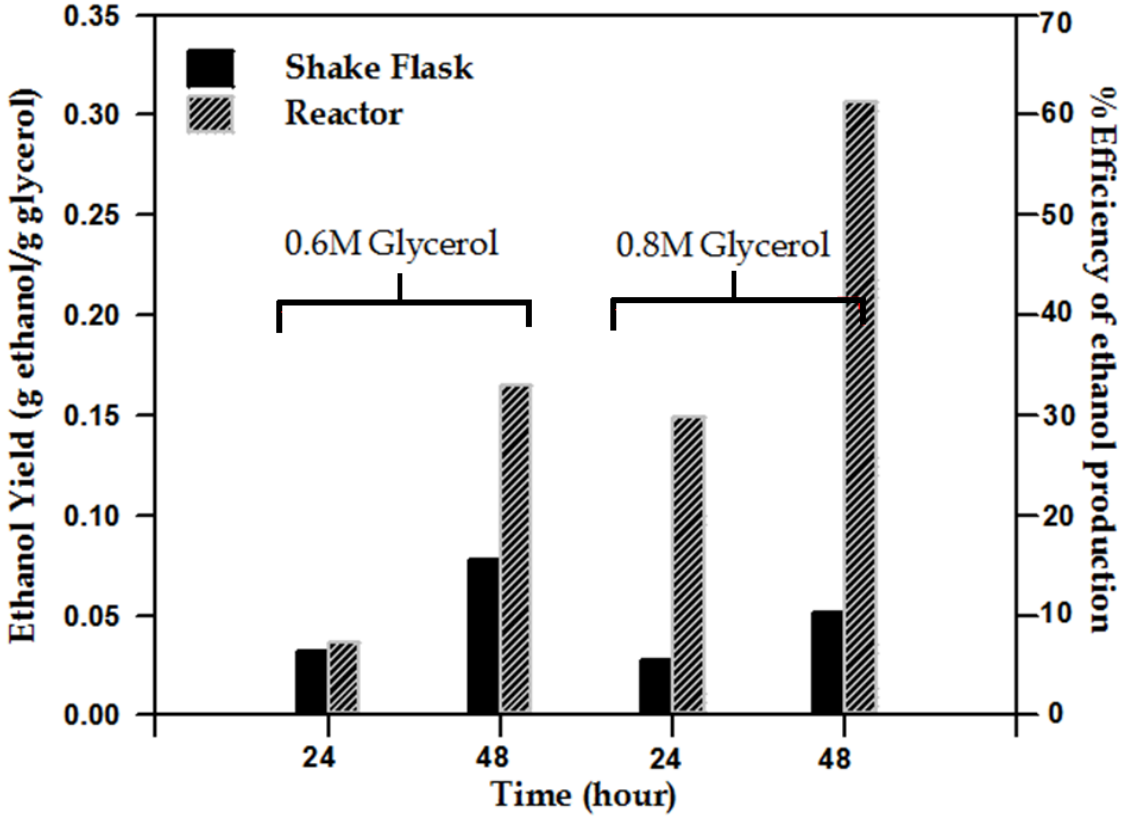

Figure 5: Reactor Performance in comparison with Shake flask studies

Bioethanol Production in Mini-Bioreactor

Performance Analysis of Mini Bioreactor

The shake flask studies revealed that poor aeration condition with viscous nature of broth was the key factor for lower efficiency of bioethanol production. A maximum of 16% bioethanol conversion efficiency could be achieved in shake flask studies for an incubation period of 48 hours at pH 5.0. To alleviate this, amini bioreactor was designed (section 2.3) providing aeration and agitation facilities mimicking a large scale fermenter. This mini bioreactor was tested for its performance with an aeration condition of 80mL/min (2VVM) and pure glycerol broth having 0.6M glycerol concentration and pH 5.0. Yield of ethanol in bioreactor was almost doubled in comparison with shake flask experiments(Figure 5).

The concentration of glycerol was further increased to 0.8M and the fermentation was carried out for 24 hours and 48 hours. At glycerol concentration of 0.8 M, the bioethanol production efficiency in the bioreactor was 60% while in the shake flask experiment the efficiency of ethanol production was only 10% at 48 hour. The higher concentration of glycerol, thus higher viscosity, did not contribute to the retardation in oxygen flux in case of the bioreactor. Hence, it is evident that the designed bioreactor is efficient enough to overcome the limitations of viscosity and oxygen flux as observed in case of shake flask studies.

Fermentation of Purified Glycerol

A 3 staged process was used to purify the raw glycerol obtained from the biodiesel industry. The purity of the glycerol was analyzed to be approximately 95 g/L corresponding to nearly 1M. Further it was diluted to a final broth concentration of 0.8M (73.5 g/L) and subjected to fermentation at a pH of 5.0, 150 rpm, aeration rate of 100 mL/min (2.5 VVM). The bioreactor was run for 24 hours, 36 hours and 48 hours. The bioethanol yield of 0.21, 0.30 and 0.33 was obtained respectively. Considering the productivity of the

bioethanol, a 36 hour run yields a better production efficiency of 59.8%. This production efficiency is similar to the efficiency obtained for pure glycerol, and in this case a broth ethanol titer of 23.5 g/L was obtained.

Chanthoom et al (2016) used *Enterobacter aerogenes* to produce ethanol from crude glycerol at a maximal glycerol concentration of 40 g/L [16]. They observed that, there was no effect of impurities in the production of ethanol. The maximum ethanol yield of 204 mM (or 9.4 g/L) was obtained at 25 g/L glycerol concentration. Maru et al. (2016) used mixed consortia of *Escherichia coli* along with *Enterobacter* sp. to produce hydrogen and ethanol from purified glycerol [17]. A maximum of 1.53 mol H_2/per mol glycerol and 1.21 mol ethanol/ mol glycerol (about 60% efficiency) was obtained. Thus, *Bacillus cereus* is a promising newly reported strain, which is capable of producing ethanol from purified glycerol with high conversion efficiency, where oxygen supply and controlled pH play an important role.

CONCLUSION

Glycerol, pure as well as purified (from the biodiesel stream), is a good carbon source for production of ethanol using a facultative aerobic bacterium *Bacillus cereus* under aerobic condition. The presence of oxygen is essential for the bioethanol synthesis. Increase in viscosity as the glycerol concentration increase poses mass transfer limitations to oxygen flux. Further, a small scale bioreactor designed for aerobic fermentation was designed to provide proper aeration and agitation conditions, where the mass transfer limitations were easily overcome by the designed bioreactor. Purified glycerol was converted to bioethanol by *Bacillus cereus* under the conditions such as 73.6 g/L glycerol concentration, pH 5.0, aeration rate 100 mL/min, incubation time 36 hours, yielded 0.30 kg-ethanol per kg-glycerol with ethanol conversion efficiency of 59.8% and broth ethanol titer of 23.5 g/L.

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