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Microbial Production of 1, 3-Propanediol using Newly Isolated *Enterobacter cloacae* GNTEW13-1, GNTEW13-V and its Enhancement Studies by UV Mutagenesis

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ABSTRACT

Keywords

Crude Glycerol, 1, 3-propanediol, *Enterobacter cloacae*, Mutagenesis.

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The crude glycerol has been converted to 1, 3-propanediol which is not only a useful final product but also a valuable starting compound for producing polymers, A newly isolated *Enterobacter cloacae GNTEW13-1 and GNTEW13-V* has shown a high produciblility of 1, 3-propanediol by using biofuel derived crude glycerol as a sole carbon and energy source. In the preliminary stage the *Enterobacter cloacae GNTEW13-1 and GNTEW13-V* has grown on the glycerol based medium to enhance the more utilization of glycerol for the high production 1, 3-propanediol. Total 10 mutants were screened after UV Treatment and among these mutants, 3 mutants showed increased production over parent strains.

Introduction

Worldwide the global interest is being directed towards research and commercialization of several microbial fermentation technologies for chemical production. 1, 3-propanediol is one of the oldest known fermentation products. It was reliably identified as early 1881 by August Freund, in glycerol fermenting mixed culture containing *Clostridium pasteurianum* as the active organism (Freund, 1881). Later in 1914, Viosenet described a wine spoiling *Bacillus* that produced the substance. Quantitative analysis of 1, 3-propanediol produced by different *Enterobacteria* had started at the microbiology school of Delf and was successfully continued at Ames, Iowa (Mickelson and Werkman, 1940) In the 1960s, Interest shifted to the Glycerol attacking enzymes, in particular to the glycerol and dioldehydratase, as these enzymes were peculiar in the 1, 3-propanediol synthesis pathway. 1, 3-propanediol forming clostridia were first described in 1983 as part of a process to obtain a special product from glycerol excreting algae (Nakas *et al*., 1983).

The need for sustainable resource supply, the rapid advances in biotechnology and microbial genetics and the strategic shift of major chemical companies into the area of

life sciences are some of the driving forces for renewed interest in producing bulk chemicals from renewable resources by biological process. The Biotechnological synthesis of 1, 3-propanediol appears to be an attractive alternative to chemical synthesis as it is carried out under milder operational conditions, and it does not generate toxic products. Considering the yield product recovery and environmental protection, much attention has been paid to microbial production of 1,3-propanediol (Xiu *et al*., 2007). Moreover the microbial process can use glycerol as substrate, which is low-cost renewable resource appearing in increasing quantities as the principal byproduct from fat saponification, alcoholic beverage manufacture and biodiesel production units. Glycerol is not only cheap and abundant, but its greater degree of reduction than sugars offers the opportunity to obtain reduced chemicals at higher yields than those obtained using sugars (Dharmadi *et al*., 2006).

1, 3-propanediol is a typical product of glycerol fermentation and has not been found in the fermentative conversion of other organic substrates. Only very few organisms, all of them bacteria, are able to form it (Biebl *et al*., 1999). A number of microorganisms can ferment sugars to glycerol but they cannot convert glycerol to 1, 3-propanediol. Others may have the ability to ferment glycerol to 1, 3-propanediol and still others can ferment mixtures of glycerol and sugars to 1, 3-PDO, however, none, can ferment sugars directly to 1, 3-PDO (Cameron *et al*., 1998). There are reports where new metabolic pathways have been designed in cells by combining the ability to ferment sugar to glycerol and subsequently glycerol to 1, 3-PDO (Forage and Lin 1982; Tong and Cameron, 1992).

The biotechnological production of 1, 3-PDO from glycerol has been demonstrated for several bacteria, such as *Klebsiella, Clostridia, Citrobacter, Enterobacter and Lactobacilli* (Sauer *et al*., 2008). Several *Clostridial* species suc as non pathogenic *Clostridium butyricum* (Papanikolaou *et al*., 2004), *C. pasteurianum* (Biebl, 2001) grow on glycerol and form 1, 3-PDO. Although it might be easier to handle facultative anaerobes, but since all these strains are classified as opportunistic pathogens, special safety precautions are required to grow them. Along with *K. pneumonia*, other species of same genera have also been exploited for 1,3-PDO production, such as *L.oxutoca* (Homann *et al.,* 1990; Yang *et al*., 2007) and *K.planticola* (Homann *et al*., 1990). *Lactobacillus brevis, L.buchneri* and *L. reuteri* (Peng *et al*., 2002) have been shown to use glycerol as an external hydrogen acceptor source during fermentation.

Materials and methods

Collection of Sample

The biofuel derived crude glycerol was obtained from the biofuel information and demonstration unit established by the Karnataka State Biofuel Development Board, at the department of Biotechnology Gulbarga University, Gulbarga, Karnataka India.

Microorganism

In the Process of converting crude glycerol to 1, 3-Propanediol the *Enterobacter Cloacae GNTEW-13-1 and GNTEW-13-V*, which are previously isolated by us in our laboratory (Waghmare and Naik, 2015), was stored in the department of Biotechnology Gulbarga University, Gulbarga, Karnataka, India. Later the isolated strain was grown on the different concentration (1%, 2%, 5%, 10%, 15% & 20% of glycerol based medium.

Culture medium

The strain was maintained in LB agar Medium containing 5% Crude Glycerol in culture tube at 4°C. Pre-cultures of pure culture inoculums were cultivated in Hungate test tubes in an appropriate 1, 3-Propanediol production medium (37°C, 18 h).

Fermentation medium

The composition of the fermentation medium was (%, w/v): glycerol-2.0, K_2HPO_4-.0069, KH_2PO4-0.025, $(NH_4)_2SO_4$-0.4, $MgSO_4.7H_2O$-0.02, yeast extract-0.15 and 1.0ml of trace elements solution. The composition of trace elements was (%, w/v): $MnSO_4.4H_2O$-0.01, $ZnCl_2$-0.007, $Na_2MoO_4.2H_2O$-0.003, H_3BO_3-0.006, $CoCl_2.6H_2O$-0.02, $CuSO_4.5H_2O$-0.002, $NiCl_2.5H_2O$-0.002, 0.2 ml $FeSO_4$ solution (Hao *et al*., 2008). The composition ferrous sulfate solution was (%w/v): $FeSO_4.7H_2O$-0.5.The fermentation medium was supplemented with biofuel derived crude glycerol at a concentration of 20.0 ± 1.0 g/L. The crude glycerol composition was (w/w) 90.5% glycerol, 7% NaCl, 10.11% moisture, and pH 6.5. The media were autoclaved (121°C, 20 min.).

Experimental Methodology

The strain was maintained at 4 °C on LB medium. For inoculums, cells were cultivated at 30 °C in a rotary shaker at 120 rpm using 100 ml anaerobic flasks containing 50 ml of 2 % glycerol based LB medium. 5ml of inoculums has been added in to the 50 ml production medium. Further these flasks are shaken generally by a gyratory shaker at 200-250 rpm. at 37^0C temperature. The sample has been collected at every 24 hours of time interval followed by pH measurement for the quantification of 1, 3-propanediol production and other byproducts. The experiment was performed in duplicate.

1, 3-PDO Production by UV mutants

A 8 ml of bacterial suspension taken in an aseptic Petri-plate without cover was exposed to UV light for 5 to 60 minutes at a distance of 40cm from UV lamp with a wavelength of 2537 A^o and a power of 30 W to get 96% lethality. Number of mutants obtained during mutagenesis was shown in Table .1. Total 10 mutants were screened after UV Treatment and among these mutants, 3 mutants showed increased production over parent strains. From the above three mutants, UV-2 and UV-4 Mutants were resulted from 20 min UV exposure. And the remaining one mutant UV-6 was obtained from 30 min UV exposure. 1, 3-PDO production by UV-Mutants in comparison with parent strain was shown in table 2.

UV Mutant 6 had shown the maximum 1, 3-PDO production of 16.8mg/ml over the parent strain (15.01mg/ml). The molar yield (0.21mol/mol) and productivity (0.15g/L/H).

Analytical Methods

Determination of bacterial growth

Growth was followed by optical density measured at 600 nm and O.D. values were converted to cell dry weight per volume (mg dw·mL-1)

Analysis of the fermented production medium

1, 3-PDO, 2, 3-butanediol and glycerol content were analyzed by high performance liquid chromatography (Waters ®). It was used column Aminex® HPX-87H, 300 x 7.8

mm (Bio-Rad Laboratories Ltd) and pre-column (Bio-Rad Laboratories Ltd), IR detector (Waters 2414), binary pump (Waters 1525), furnace and temperature controller module (Waters) chromatographic software: Breeze. 0.005 M H_2SO_4 act as a mobile phase with flow rate 0.5 ml/min and Injection volume was 20 µl, 65° C as working temperature.

Results and Discussion

Enterobacter cloacae GNTEW-13-1 & GNTEW 13-V was found to be a potent 1, 3-propanediol producer by utilizing a biofuel derived crude glycerol as a sole carbon source. It was evaluated that almost all biofuel derived crude glycerol was consumed with the final cell concentration observed to be approximately 1.8 g·L-1.

Table.1 Number of mutants obtained during UV Mutagenesis

Time duration of UV Mutagenesis	Number of Colonies (CFU/ml
Parent	300
5Min	35
10 min	27
20 min	18
30 min	2
40 min	1
50 Min	-
60 min	-

Table.2 1, 3-PDO production by UV-Mutants in comparison with parent strain

UV Mutants	1,3-Propanediol mg/ml
Parent strain	15.0
UV-1	11.5
UV-2	16.5
UV-3	14.7
UV-4	16.2
UV-5	9.0
UV-6	16.8
UV-7	10.0
UV-8	10.7
UV-9	11.1
UV-10	13.0

Fig.1 Metabolic pathways of glycerol metabolism (Zeng and Biebl, 2002)

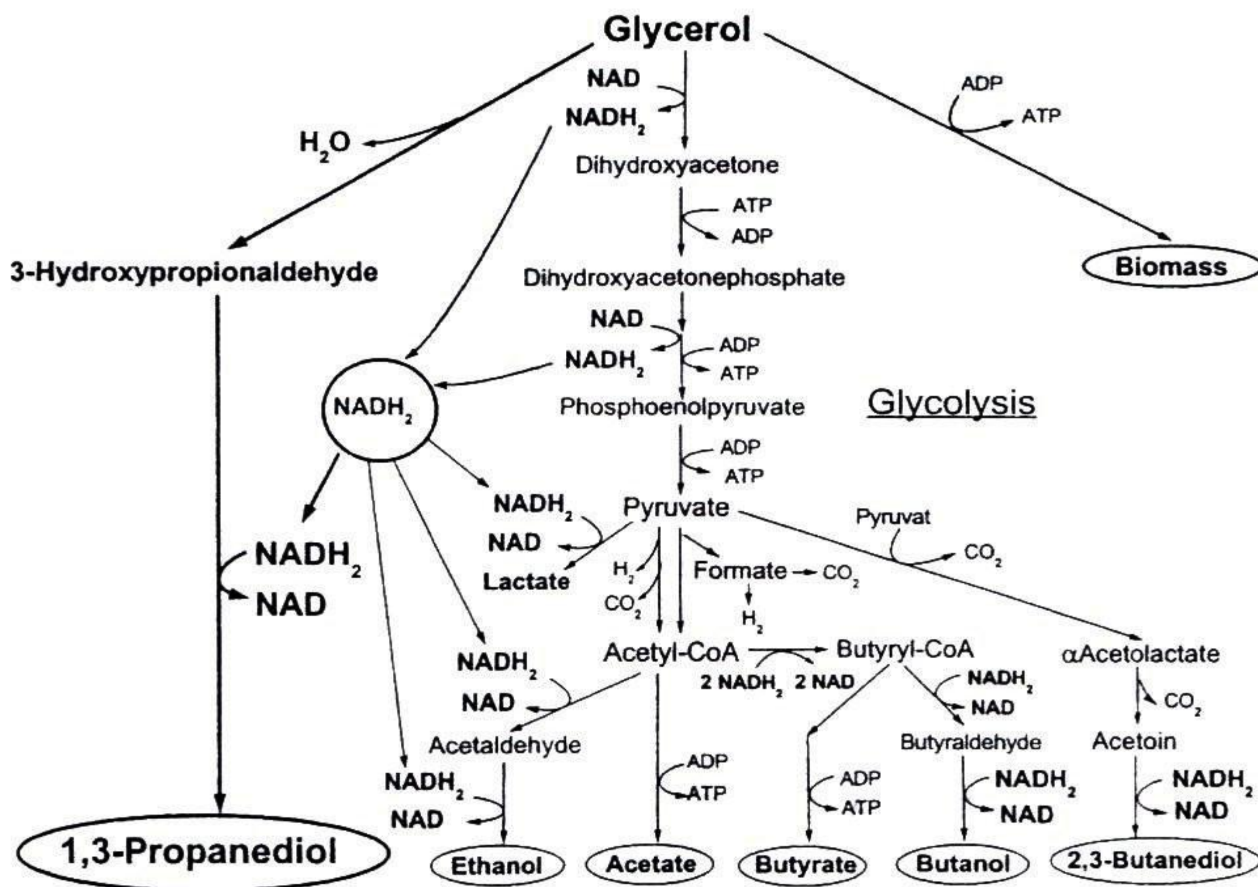


Fig.2 Bar chart showing the production of 1, 3-propanediol by UV-Mutants

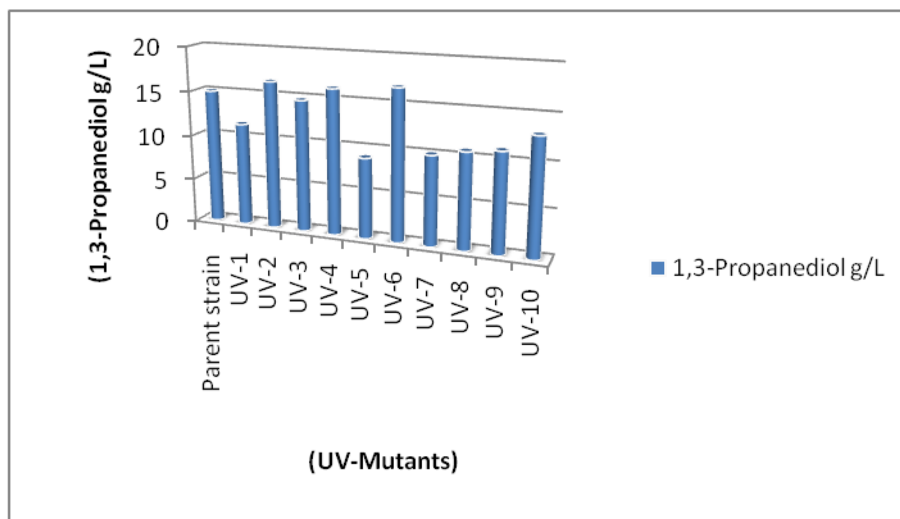


Fig.3 HPLC chromatographic peak showing the production of 1, 3-propanediol by *Enterobacter cloacae* GNTEW-13-1

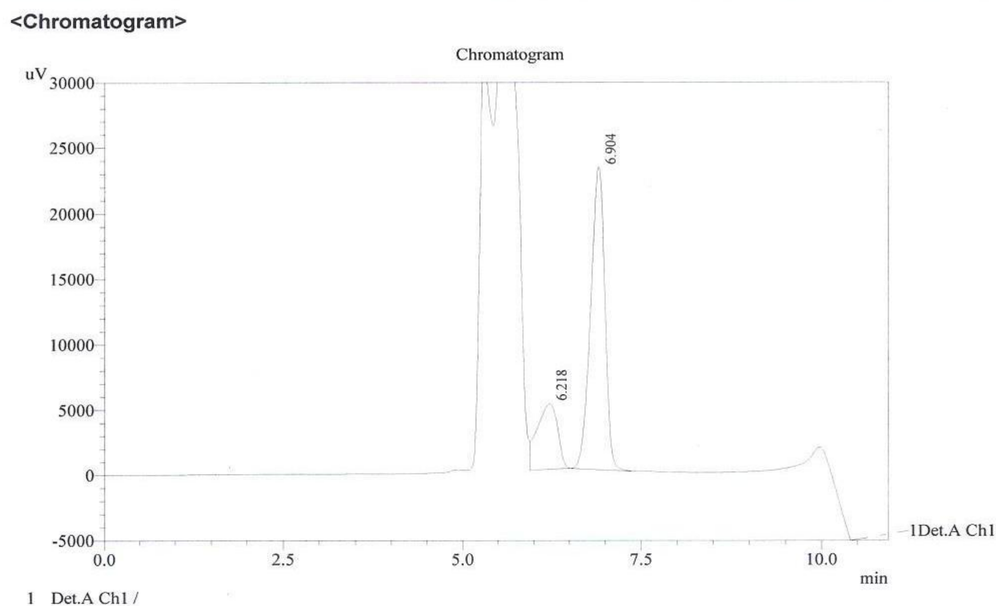
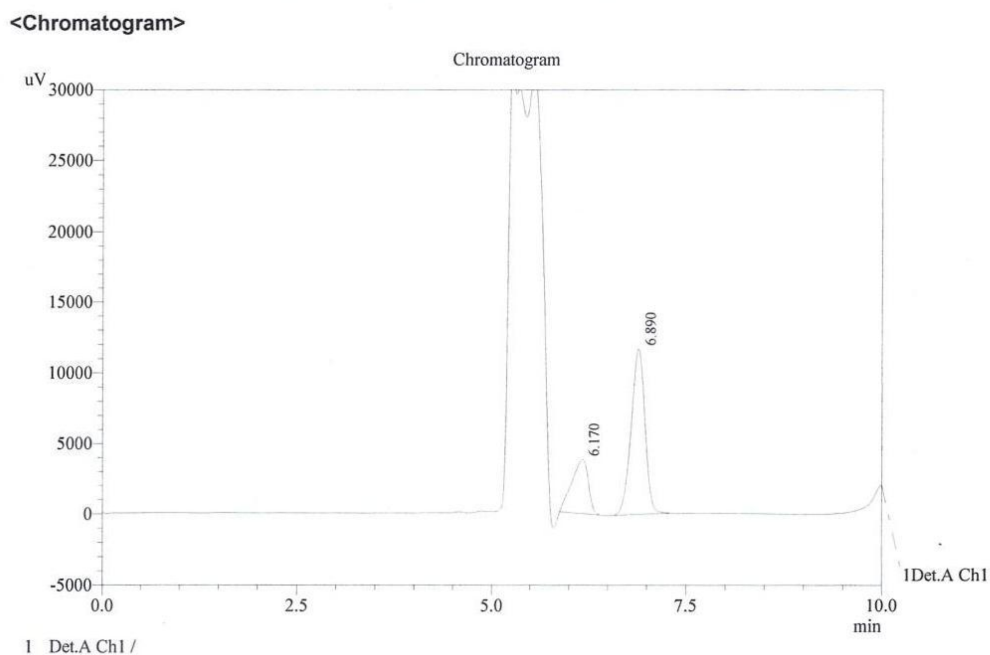


Fig.4 HPLC chromatographic peak showing the production of 1, 3-propanediol by *Enterobacter cloacae* GNTEW-13-V



The Newly isolated strain *Enterobacter Cloacae GNTEW-13-1 & GNTEW-13-V* has the good potentiality to produce 1, 3-propanediol, with the final concentration of 15.01 mg/ml and 14.99 mg/ml of 1, 3-PDO (Figure 1 & Figure 1). However, all

byproducts are associated with a loss in 1, 3-PDO relative to acetic acid (Zeng and Biebl, 2002). A very important parameter that can affect 1, 3-PDO yield is pH. The initial pH of culture medium was been found to be 7.0, but the pH considerably reduced after 24 h of fermentation experiment, reaching to 4.15. These changes may be probably due to the production of some organic acids during the fermentation time.

In the present study the newly isolated *Enterobacter cloacae GNTEW-13-1* and *Enterobacter cloacae GNTEW-13-V* has the potentiality to utilize the biofuel derived crude glycerol as a sole carbon source to produce 1, 3-propanediol with higher yield.

The final concentration of the 1, 3-propanediol produced by *Enterobacter cloacae GNTEW-13-1* and *Enterobacter cloacae GNTEW-13-V* is 15.01gm/ml and 14.99gm/ml respectively.

However it is very much important to carry out some of the new experiments by optimizing the strain in order to increase the yield and high productivity of 1, 3-propanediol than the present.

1, 3-propanediol production was enhanced when *Enterobacter cloacae GNTEW-13-1* and *Enterobacter cloacae GNTEW-13-V* was subjected to mutagenesis with UV mutagens at sub lethal concentrations. Table .2, summarizes the production of 1, 3-propanediol by UV mutants. Among which the UV Mutant 6 had shown the maximum 1, 3-PDO production of 16.8mg/ml over the parent strain (15.01mg/ml).

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