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BIOCONVERSION OF GLYCEROL TO FREE FATTY ACIDS USING A NOVEL ISOLATED *Bacillus aryabhattai* IS07

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***Abstract*-Bioconverting glycerol into various valuable products is one of its promising applications due to its high availability at low cost. The objectives of this study were to isolate microbes from crude glycerol , evaluate the isolates for optimal glycerol utilization and characterize the bio-converted product. A Gram positive bacterium (Strain IS07) was found to be best among the isolates with reference to utilizing glycerol in the fermentation media. The 16S rRNA gene sequence of this isolate exhibited 98.5% similarity to *Bacillus aryabhattai* hence the strain was designated as *Bacillus aryabhattai* IS07. The media was optimised for maximal glycerol utilization using different growth factors, pH and nitrogen sources. The strain IS07 completely utilized 40mMoles of glycerol at the end of 24th hour and effectively produced 5.67mMoles of free fatty acids from 108mMoles of glycerol. 104 mMoles of pre treated glycerol from Jatropha oil biodiesel plant was also utilized and converted to free fatty acids within 24 hours by IS07. These microbial synthesised fatty acids are logical precursors to diesel like hydrocarbons and offer the flexibility to exploit a variety of biomass derived carbon sources along with glycerol. This is to the best of our knowledge first report of a glycerol utilizing bacterium capable of bioconverting 40mMoles of glycerol within 24 hours. This study opens up avenues for economically viable options for managing an abundant byproduct of biodiesel plants.**

***Keywords:*-Bacillus aryabhattai, Glycerol, Biodiesel, 16S rRNA**

I. INTRODUCTION

Biodiesel from plant oils is environmentally safe and renewable alternative to fossil fuels however their economic viability is a major concern. As biodiesel production by trans esterification of plant seed oil with methanol yields glycerol as the main by-product (10% by weight), offering an opportunity for bioconversion of crude glycerol to useful products using microbes. It has been recorded in recent past that every year approximately 1.6 million tons of glycerol is produced as obligatory by-product of biodiesel industry. This glycerol can be an attractive feedstock for producing useful chemicals [2]. There are reported data on various methods of utilizing crude glycerol by direct burning for energy, feed stock and chemical conversion to various products like 1,3-propanediol, hydrogen gas etc. Biological conversion of the crude glycerol to products like alcohols, fatty acids, various organic acids, dihydroxyacetone, glyceric and hydroxylpyruvic acids , glycerol carbonate and pigment production, such as prodigiosin and astaxantin [3] has been studied. PHAs have also been produced from *Pseudomonas* species using the co-product stream of biodiesel containing glycerol [4].

Glycerol utilization has been well studied in *Escherichia coli* (old 34, 36). Glycerol transport is facilitated by aquaglyceroporin (GlpF), and under aerobic conditions glycerol is then phosphorylated by glycerokinase (GlpK) to yield glycerol 3-phosphate, which is oxidized by glycerol 3-phosphate dehydrogenase (GlpD) to yield the glycolytic intermediate dihydroxyacetone 3-phosphate (old 34, 36). Rhamnolipids, a biosurfactant is also produced by *Pseudomonas aeuroginosa* using glycerol as the sole carbon source [5]. Besides these, several *Clostridium* species also form 1,3-propanediol along with butyric and acetic acid. The production of major fatty acids as stearic acid ,oleic acid by *Yarrowa lipolytica* and *Cryptococcu scurvatus* using glycerol as sole carbon source has also been reported [6, 7].

The present study was taken up in pursuit of isolating potential microbes from crude glycerol obtained from a biodiesel plant capable of utilizing glycerol as the sole carbon source and bio converting it to free fatty acids. Here, we describe identification and shake flask fermentations of a bacterium utilizing glycerol as sole source of crabon.

II. MATERIALS AND METHODS

Isolation of Microorganism: Curde glycerol from a biodiesel processing unit, GKVK, Bangalore, India was collected in a sterile container and stored at 4°C for further work. The glycerol-based agar medium was using the following composition: K_2HPO_4, 0.7g; Na_2HPO_4, 0.9g; $MgSO_4.7H_2O$, 0.4g; and $NaNO_{3,\ 2g}$; agar; pH 7.0. 10g of pure glycerol was added as the sole carbon source in the media. The samples were diluted 10 times with distilled and sterilized water prior to being spread on an agar plate and incubated at 37°C for 24 hours. Morphologically different colonies were selected and further sub culturing was carried out in the above-mentioned glycerol agar medium to obtain single colonies. Morphological characteristics were determined by microscopy, and Gram staining was performed. The isolated strains were stored at –80°C in 20% sterile glycerol for further studies [8].

Genomic DNA Isolation: Genomic DNA was extracted from the IS07 grown on LB broth at 37°C using standard CTAB method. 12-18 hr incubated bacterial culture was taken in microfuge tube and centrifuged at 10000 rpm for 2 min at 4°C. The pellet obtained was re-suspended in sterile water and washed and again centrifuged at 10000 rpm for 2 min at 4°C. To the pellet, 675 µl of extraction buffer was added and incubated at 37°C for 30 min. To the micro-centrifuge tubes, 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. Sample was centrifuged at 11000 rpm for 10 min at 4°C and clear solution was collected in a sterile microfuge tube. Equal volume of phenol: Chloroform: isoamylalcahol (25:24:1) was added and centrifuged at 10000rpm for 10 min at 4°C. Top aqueous layer was transferred to fresh microfuge and equal volume of Chloroform: Isoamyl alcohol (24:1) was added. Sample was centrifuged at 10000 rpm for 10 min. at 4°C and aqueous phase was taken in a sterile microfuge tube. 0.6 volumes of ice cold Isopropanol was added incubated at room temperature for 1 hr. Samples were centrifuged at 10000 rpm for 10 min and then pellet is washed in 500µl of 70% ice cold ethanol and centrifuged at 10000 rpm for 10 min at room temperature. Dried pellet was dissolved in sterile distilled water and stored in -20°C. Quality of DNA was checked after running on a 1% agarose gel in 1X TAE buffer and ethidium bromide staining. The DNA was quantified by measuring absorbance at 260nm using a Shimadzu 1800 spectrophotometer.

16S r DNA PCR Amplification: The extracted DNA was then used as template for 16S rDNA PCR amplification with universal 16S primers-forward 5' AGA GTT TGA TCC TGG CTC AG 3' and reverse 5' AAG GAG GTG ATC CAG CCG CA 3'. PCR sample volume was 25µl per sample with the reaction mixture comprising of 0.5 µl genomic DNA, 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.6), 0.3 µl *Taq* DNA polymerase(USB corporation, Cleveland, Ohio, USA), 2.5 µl dNTP mix, 2.5 µl reverse and forward primer each. PCR was performed on Master cycler (Eppendorf, Germany) with initial denaturation for 2 min at 92°C. First cycle had denaturation for 1 min at 92°C, primer annealing for 30 sec at 48°C and primer extension at 72°C for 2 min 10 sec. The cycle was repeated for 34 times and the process ends by final polymerization step of 6 min 10 sec at the temperature of 72°C. PCR product were resolved using electrophoresis in 1.2% agarose gel stained with ethidium bromide (EtBr), documented under UV light on UVIpro platinum gel documentation system.

16S rDNA Sequencing: 16S PCR products were purified using AxyPrep™ DNA Gel Extraction Kit obtained from AXYGEN Biosciences. DNA sequencing was performed on ABI PRISM big dye terminator cycle sequence reading reaction kit at Chromous Biotech (Bangalore, India) and the sequences obtained were compared with databases at National Centre for Biotechnology Information (NCBI) using the BLAST Program.

Glycerol Utilization optimization studies

The isolated strain IS07 was used in the study. A single colony of IS07 was inoculated in 100ml of Leuria Bertaini broth (Himedia, miller India) and

grown at 37 °C until it reached OD 1 at 600nm before it was used as an inoculum for fermentation.

Varying inoculum size: Glycerol broth (150ml each) was prepared in 500ml flask in triplicates. The media was sterilized at 121°C for 15min in an autoclave(Tomy ES-315, Japan). 1, 2, 5, 7 and 10%(v/v) of seed culture were inoculated into glycerol based broth in triplicates. An un-inoculated media was considered as control. The flasks were incubated at 37°C with shaking at 150rpm for 24h. sample (5ml) was collected in a sterile screw cap vials for every 6h. The growth curve was recorded by measuring the cell density at 600nm using spectrophotometer (Shimadzu,UV-1800). All samples collected were stored in -20°C for further analysis.

Varying glycerol concentration : Glycerol broth (150ml) with different glycerol concentrations (54, 108 & 217mM) was prepared in triplicates. Media was sterilized at 121°C for 15min in an autoclave (Tommy, Germany). Seed culture (5%, V/V) actively growing seed culture was inoculated under aseptic condition. An un-inoculated media was considered as control. The flasks were incubated at 37°C with shaking at 150rpm for 24h. Samples (5ml) were collected in a sterile screw cap vials at 6h intervals. All samples collected were stored in -20°C for further analysis.

Alternate nitrogen source and growth factors **:** Glycerol broth (150ml) with 217mM glycerol and supplemented with different combinations of Tryptone and yeast extract (Table 1), each was prepared in triplicates. The media was sterilized at 121°C for 15min in an autoclave (Tommy, Germany). Seed culture (5%, V/V) actively growing seed culture was inoculated under aseptic condition. An un-inoculated media was considered as control. The flasks were incubated at 37°C with shaking at 150rpm for 24h. Sample (5ml) was collected in a sterile screw cap vials for every 6h. All samples collected were stored in -20°C for further analysis

	Glycerol broth					
Component	A	B	C	D	E	F
Yeast extract (%,w/v)	-	-	1	1.5	1	1.5
Tryptone (%, w/v)	0.5	-	-	-	0.5	-

Table 1: Glycerol based broth with different combinations of Tryptone and yeast extract concentration

Crude glycerol utilization

Crude glycerol from *Pongamia* and *Jatropha* biodiesel industry was used in shake flask fermentation with IS07.

Pre-treatment of crude glycerol:

Crude glycerol (100g) in four different aliquots was acidified by the addition of H3PO4 to the pH of 7,5,3,1 using a precalibrated pH meter (Eutech), and shook at constant rate of 200rpm for 1h. Then it was left for 12 h until the solution had phase separated into the three distinct layers of a top FFA-rich layer, the middle glycerol-rich layer and the bottom inorganic salt rich layer. All layers were separated from each other by separating funnel. Subsequently the middle glycerol- rich layer was neutralised by the addition of 5M NaOH (Merck) to pH 7.0, left for a while and then filtered to eliminate the precipitated salt. Glycerol estimation and TLC was performed for all the samples.One-dimensional chromatography on pre-activated TLC silica gel 60 plates (Merck, Germany) was carried out in chloroform :methanol (80:20) saturated tanks. Samples were spotted 1.5 cm from the lower edge of the plate and at least 1.0 cm from the lateral border. The chromatograms were developed by the ascending technique with the desired mobile phase. The solvent front was drawn 5.0 cm from the application line. Air-dried plates were sprayed with vanillin sulfuric acid reagent. The plates were heated at 100°C for 20 min to develop the spots of the compounds.

Pretreated glycerol shake flask fermentation

Glycerol broth (150ml) with pre-treated glycerol (108mM) from different sources (*Pongamia* and *Jatropha*) were supplemented with yeast extract was prepared in triplicates. The media was sterilized at 121°C at 15psi for 15 min in an autoclave. Actively growing Seed culture (5%,V/V) was inoculated under aspectic condition. An un-inoculated media was considered as control. The flasks were incubate at 37°C with shaking at 150rpm for 24h. Sample (5ml) was collected in sterile screw cap vials for every 6hr. All samples collected were stored in -20°C for further analysis.

Glycerol estimation

The stored samples were thawed on ice and centrifuged at 6000rpm for 5min at RT to remove

bacterial cells. The cell free supernatants were collected in a sterile containers. The amount of free glycerol in the sample was measured with a UV-Visible spectrophotometer using a two-step reaction process[9]. This results in the formation of a yellow complex proportional to the amount of free glycerol in the sample. The sample is first treated with sodium periodate. Sodium periodate reacts with free glycerol in the sample to generate formaldehyde. Reaction between this formaldehyde and acetyl acetone produces the yellow complex, 3,5-diacetyl- 1,4-dihydrolutidine. This yellow compound exhibits a maximum absorbance peak at 410 nm, where its concentration in the sample is measured.

Glycerol was estimated based on standard calibration method with minor modifications. A 0.1ml diluted cell free Supernatant was mixed with 2 ml of sodium periodate solution (3mM sod periodate, 1M ammonium acetate , 0.6M acetic acid). 5 ml of 1:1 acetyl acetone and 2-propanol mixture was added to each reaction mixture and incubated at 50^{o}C for 20 min in water bath. The colour change was read at 410nm using UV Visible Spectrophotometer (Shimadzu UV1800, Japan). A standard calibration curve was developed with different concentrations (10, 20, 30, 40 mM) of pure glycerol.

Estimation of methylglyoxal

Methylglyoxal is one major product of glycerol metabolism hence it was estimated in the cells, One ml of thawed culture was centrifuged at 6000rpm for 5min at 4^{o}C. The cell pellet was washed in sterile distilled water and resuspended in 100ul of sterile distilled water. The suspension was sonicated for 3min in an interval of 30sec each to lyse the cells. The complete lysis was confirmed by observation under the microscope. The lysed bacterial cells were centrifuged at 8000rpm for 5min at 4^{o}C. Traditionally, methylglyoxal was determined by reaction with 2,4-dinitrophenylhydrazine with or with out chromatographic separation of the resultant dihydrazone (Osazone) and spectrophotometric detection [10] Methylglyoxal was estimated as described by Yadav et al [11] with minor modifications. 10ul of the supernatant was mixed with 250 µl of 0.2mM 1, 2 diaminobenzene, 100 µl of 5M perchloric acid. The absorbance of the derivatized MG was read immediately at 330nm.

Fatty acid profile analysis

Estimation of Fatty acids: The fermented product was analyzed for presence of LCFAs(Long chain fatty acid) such as eladic and linoleic acids ; SCFAs (short chain fatty acid) such as Butyric acid (4:0) and MCFAs (medium chain fatty acid) *viz.* Caproic acid (6:0), Caprylic acid (8:0), Capric acid (10:0) and Lauric acid (C12:0). These fatty acid components in fermented product were extracted and methylated to form respective FAMEs before loading on GC-FID. The accuracy of the method was verified by means of recovery assay with pure fatty acid components (31). This was accomplished by analyzing fatty acid standard mixture (Supelco, Bellefonte, PA) and spiked samples. The average recovery was found to be 89.6% as compared to pure methyl esters and with the FAME mix C4-C24 (Supelco, Bellefonte, PA)

Preparation of fatty acid methyl esters: The FAMEs were prepared as described by Christie [12] after one step liquid-liquid extraction of fermented sample. The fermented broth was taken for the sample preparation. To 25 ml of the fermented broth, equal volume of chloroform:methanol (5:2.5) was added, extracted in the separating funnel and the organic layer was collected. The solvent fraction was removed in rotavapour (Buchii, R 210) at 35º C at 40 kPa pressure. The components collected in the round bottom flask were re suspended in 2 ml of 5% methanolic sulfuric acid and refluxed at 90° C for 30 min in water bath. Upon cooling the sample, desalting was done with 1 ml of saturated NaCl solution. This mixture was extracted with equal volume of hexane (Merck, India) and the organic layer was collected and concentrated by purging N_2 gas. The solvent fraction was used for gas chromatographic estimation.

GC-FID analysis was done using the hexane extract. Column configuration used was DB-23, length of 30 mm, 0.25 mm diameter and 0.25 µm of thickness (Agilent technologies Inc). Initial oven temperature during the analysis was 130 °C hold for 1 min with rate of heating as 6.5°C /min to 170 °C. Again heat to 215 at the rate of 2.75 hold for 4 min , heat to 230 °C at a rate of 40 °C hold for 3

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min. Injector temperature used was 270 °C while detector temperature was 280 °C and a split of 1:25 and sample volume injected being 1 µl. Fatty acid standard mix with fatty acid methyl esters ranging from C4-C24 supplied by Sigma Chemical company India was used for comparing the retention time of the sample peaks.

III. RESULTS

Seven different isolates from crude glycerol were selected on the glycerol media and will be referred as IS01-IS07. The isolates were morphologically characterized and colony characteristics are shown in Table 2. All the isolates were Gram negative except IS07. All the isolates were grown in 54mM glycerol broth for 48hrs. Six isolates, IS01 to IS06 did not multiply beyond 0.2 OD after 48 hours hence were omitted for further optimization studies. The isolate, IS07 a Gram positive rod shaped bacterium was affiliated to *Bacillus aryabhattai* [13] as identified by 16S rRNA gene sequence.

Isolate	Source	Colony characteristics				
	Crude glycerol	Shape	Margin	Colour	Consistency	Gram character
IS01		Irregular	Wrinkled	Off white	Slimy	Gram-positive rods
IS02		Circular	Entire	Yellow	Slimy	Gram-negative rods
IS03		Circular	Entire	Off white	Slimy	Gram-positive cocci
IS04		Circular	Entire	White	Rough	Gram positive shortrods
IS05		Irregular	Irregular	White	Rough	Gram-positive cocci
IS06		Irregular	Irregular	White	Rough	Gram positive cocci
IS07		Circular	Entire	Off white	Slimy	Gram positive rods

Table 2: Colony characteristics of isolates

Optimization of inoculum size

The optimization of the fermentation process was taken up by modifying various parameters like starting inoculum size, media composition, fermentation pH and fermentation length. The shake flask fermentation was carried out with IS07 for different starting inoculum sizes like 1%,2%,5%,7% and 10%(v/v) Among the different Inoculum size 5 %(v/v) was best suited for optimum glycerol utilization. With 5% starting inoculum of IS07 we observed complete utilization of 40mM glycerol, in media after 24hr and the culture OD reached to 3.7 at 600nm which corresponds to $0.3X10^8$ CFU/ml. Whereas with 7% starting incolum it was 35.79mM glycerol utilization after 24hr(Fig.1). Further increase in the inoculum size to 10% had no effect on the glycerol utilization and growth declined after 24hr (data not shown).

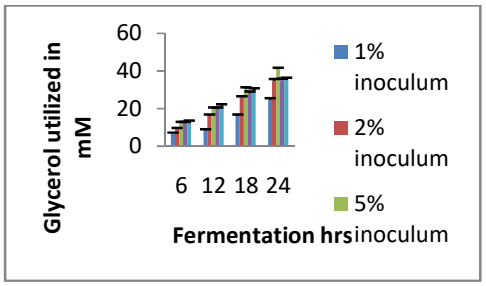

Figure 1: Optimization of inoculum size in the glycerol fermentation

Effect of glycerol concentration

Initially the IS07 was grown in media containing 54, 108 & 217mM glycerol with out any organic nitrogen sources/growth promoters. The isolate IS07 completely utilizes 40mM of glycerol at the end of 24^{th} hour and the effective uptake of glycerol was 40-45mM. In all the three different glycerol concentrations the number of cells at 18^{th} h were in the range of $0.3X10^8$-$0.32X10^8$ CFU/ml. Methyl glyoxal is an important product of the glycerol metabolism pathway hence the concentration of methyl glyoxal was monitored at various log hours during fermentation. A gradual increase in the production of methylgloxal from 8.3nM to 16.7nM was observed that declined after 24hr of incubation with highest concentration noted at 18 hour. (Fig.2)

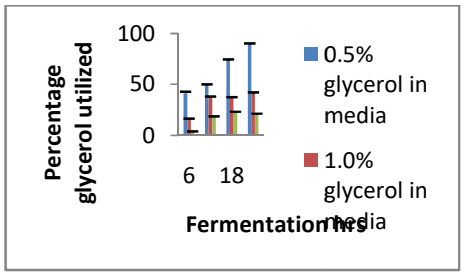

Figure 2: Glycerol utilization in media containing 0.5% (54 mM), 1.0%(108mM), 2.0%(217 mM) glycerol.

Effect of Tryptone, peptone and yeast extract on glycerol utilization

Glycerol utilization was improved with incorporation of growth factors like Tryptone and yeast extract in different combinations. 1.0%YE showed OD at 600 increased from 3.3 (with out yeast extract/tryptone) to 5.0 (with yeast extract and tryptone) and the media containing yeast extract and tryptone were found to be the best concentration for the complete utilization of glycerol ie. 108mM (10g). The utilization of glycerol was found to be growth dependent as the growth increased the glycerol utilization was also increased. The media containing only glycerol with out the nitrogen source or yeast extract showed only 40mM utilization of glycerol in 24hrs. Addition of 1.0% YE increased the glycerol utilization to 108mM in 24hrs. Incorporation of 1.5% tryptone and 1.5% YE did not further increasethe utilization (104mM) of glycerol by the isolate IS07(Fig.3). The presence of tryptone and yeast extract in the fermentation medium led to higher cell number 0.5X10^{8}CFU/ml as compared to 0.3X10^8 CFU/ml in tryptone and yeast extract free medium (Fig.4) .

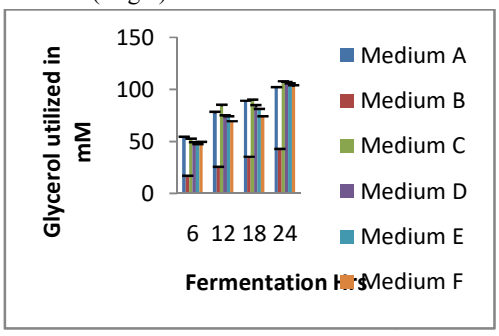

Figure 3: Glycerol utilization in media containing 108mM glycerol with Yeast extract and tryptone.(Medium A: 0.5% tryptone+Glycerol broth; Medium B: Glycerol broth only; Medium C: 1.0%yeast extract + Glycerol broth; Medium D: 1.5%yesat extract + Glycerol broth; Medium E: 1.0% Yeast Extract + 0.5% tryptone + Glycerol broth; Medium F: 1.5% Yeast Extract + 0.5% Tryptone + Glycerol broth.)

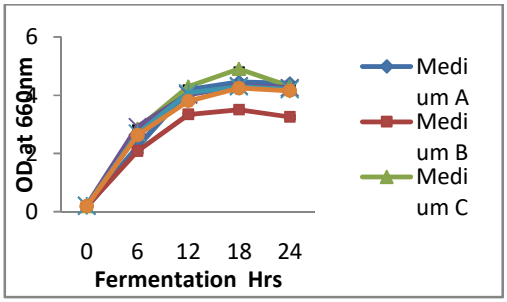

Figure 4: The IS07growth curve in media containing 1.0% glycerol with Tryptone and Yeast extract

Crude glycerol shake flask fermentation

The glycerol estimation was performed for the samples extracted at different pH where the sample at pH ≤3 contained 48% of glycerol. The IS07 growth was observed in pre-treated glycerol (extracted with different pH) for 24h. The crude glycerol pre-treated at different pH was inoculated with IS07 and incubated at 37°C for 24h. A linear growth was observed in the media supplemented with glycerol from pH3.0 and pH1.0. Whereas the media supplemented with glycerol from pH 9.0 and pH 7.0 showed growth inhibition of IS07.

The glycerol utilization was studied with two different sources. The pretreated glycerol obtained from two different sources showed 105mM pre-treated glycerol was utilized at the end of 24hr. The utilization was similar to the pure glycerol utilization explained in the previous graphs. The removal of contaminants has proved that the pretreated glycerol can be used as a source of glycerol.

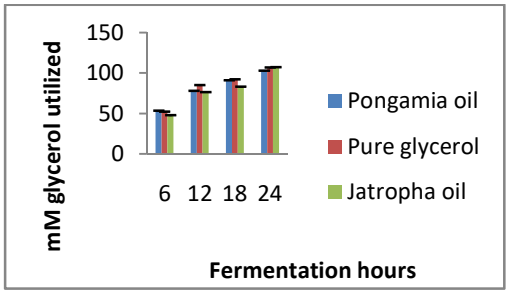

Figure 5: Glycerol utilization in media containing 108mM glycerol with pretreated glycerol from different sources like pongamia oil and jatropha oil.

Effect of Methylglyoxal on IS07 growth

Methylglyoxal toxicity to isolate IS07 was evaluated by incubating the cells with 0.1mM and 0.5mM concentration of pure MG in the glycerol fermentation broth and the growth was observed upto 24h. The presence of methylglyoxal in growth medium at the concentration of 0.1mM and 0.5mM inhibited the growth of IS07 (0.1OD at 600nm) where as linear growth was observed in control flask($0.28X10^8$CFU/ml). This indicates to the adverse effect of MG on growth of microorganism. We noticed that the MG production significantly decreased in all the combinations of yeast extract and tryptone in glycerol broth media as compared to the MG produced in yeast and tryptone free glycerol broth media. The MG production at 18hr was 16.7nM in glycerol broth and thereafter it decreased to 6.64nM at 24hr of fermentation. The media containing yeast extract exhibited 0.664nM of MG at 18h and there was negligible (non detectable) amount of MG at 24h of fermentation (Fig.6). Interestingly the MG concentration decreased upto 8-10folds in the presence of yeast extract and tryptone.

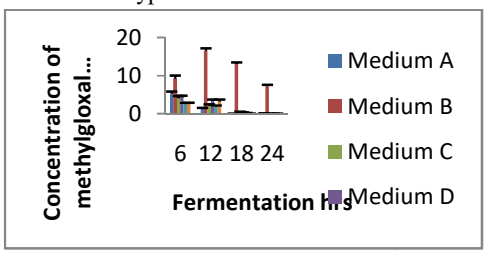

Figure 6: Levels of MG in media with different nitrogen sources and yeast extract

Fatty acid analysis:

Gas chromatography was used for Fatty acid analysis IS07 product characterization after fermentation. . The strain produced cis-10-Pentadecenoic Acid(15:1), Elaidic Acid (18:1), Linolelaidic Acid (18:2), G-Linolenic Acid (18:3n-6), Tridecanoic Acid(13:0), cis-10-Heptadecenoic Acid (17:1), cis-11-Eicosenoic Acid (20:1n9)4. the fatty acids when it was grown in glycerol broth with yeast extract. The long chain fatty acids(LCFA) like 0.5mM cis-10-pentadecanoic acid(15:1), 0.302mM of cis-10-heptadecanoic acid(18:1), 0.549mM Elaidic acid(18:1), 0.764mM linolelaidic acid (18:2n6t) was major among all the above. Collectively there were 15% short chain fatty acids and 79% short chain fatty acids. 108mM of glycerol made 5.765mM of fatty acids. The glycerol in the medium is utilized for the production of C16:0,C18:0(0.007mM), at the end of fermentation which is very less compared to C10:0 and C12:0. The decrease in the long chain fatty acid and increased in the short chain would have increased the permeability and fluidity of of the cell membrane at the end of fermentation which resulted in the good growth [14].

IV. DISCUSSION

Glycerol is abundant in nature, since it is the structural component of many lipids. It is also one of the principal compatible solutes, being widely produced in response to decreased extracellular water activity during osmoregulation in yeast [15]. Due to its ample occurrence in nature, many known microorganisms can naturally utilize glycerol as a sole carbon and energy source. These microorganisms have attracted attention to the potential use in bioconversion of abundant glycerol produced from biodiesel. Glycerol may substitute traditional carbohydrates, such as sucrose, glucose and starch, in some industrial fermentation processes [16]. Since glycerol is a non fermentable sugar. Generally bacillus species uses glucose as the preferred carbon source and energy [17].

In the similar research work by Murarka et al 2008 [18] stated that 8g utilization of glycerol in 96h of fermentation by *E.coli*. According to Rittmann et al,2008[19] glycerol utilization genes were cloned and expressed in *Cornybacterium glutamicum* where it has shown similar glycerol utilization after 33 hours . The isolate IS07 has tremendous potential to be exploited commercially as it bioconverts 108mM of glycerol in 24 hours. The pH of the medium , concentration of nitrogen source and growth factors like yeast extract are obligate requirements to reach higher cell densities. The wild type strains available till date require approximately 96 hours or more for bioconversion of the same amount of glycerol.

Crude glycerol is a by product produced from biodiesel plants via conventional trans-estrification process. Typically 10-12 (%/W) of crude glycerine is produced for every tonne of biodiesel produced. Due to the presence of organic and inorganic

contaminants in the crude glycerol such as soap and alcohol, traces of glycerides, vegetable colours, fatty acid esters of glycerol, mineral salt, water and residual FAME, it was first pre-treated. According to previous reports, the addition of a mineral acid such as H2SO4 or H3PO4 to crude glycerol can cause automatic phase separation into two or three distinct layers comprised of a free fatty acid (FFA) layer on the top, a glycerol-rich layer in the middle and an inorganic salt layer at the bottom. This is because the H+ from the mineral acid can protonate the soap bulk to insoluble FFAs which consequently separate out as a top layer due to their low density and low polarity compared to the polar glycerol. The untreated crude glycerol has about 30 (%/W) glycerol. The contaminant content was decreased resulting in an increase in the glycerol content to 48.43% in pre-treated glycerol which was used in the fermentation process. Purity of the pre-treated glycerol was confirmed by density and total lipid estimation. The 1.4 folds increase in the density and 10 fold decrease in the lipid content reflects the purification of crude glycerol. Further purification of crude glycerol has been reported by Mali Hunsom et al (2013) where they have stated that a combined process of solvent extraction with propyl alcohol followed by adsorption gives a high glycerol purity (99%) and reduction in the colour (99.1%). However we have used a single step treated crude glycerol as our main interest was to develop a time and cost effective fermentation process for crude glycerol utilization. The total utilization of crude and pure glycerol at the end of 24 hours of fermentation is 104 and 108 mMoles, a comparable value. Isolate IS07 is a highly efficient microbe for crude glycerol uptake and utilization.

Methylglyoxal (2-oxypropanal) is a 2-oxoaldehyde synthesized both enzymatically and non-enzymatically in cells and is a major intermediate of glycerol metabolism. Methylgloxal is generated as a by-product of glycolysis from triose phosphates and also by the spontaneous decomposition of glyceraldehydes-3-phosphate during growth on glycerol. Once formed, methylglyoxal irreversibly modifies macromolecules, forming advanced glycation end-products. Methylglyoxal reacts with guanyl nucleotides in nucleic acids to form imidazopurinone adducts and reacts with lysine and arginine residues in proteins forming crosslinks and imidazole derivatives and leads to inhibition of protein synthesis [20]. It has been reported in *E.coli* that a concentration of 1mM methylglyoxal leads to reversible inhibition of cell growth [21] We quantified the methylglyoxal production in IS07 and its effect on the growth. Methylglyoxal production was highest between 6-12 hours of fermentation and declined during 16-24 hours of fermenetation indicating a very effective functional glyoxylase in the cells responsible for metabolizing the accumulated methylglyoxal in the cells. The microbial strains wither extreme growth habitats due to the vast portfolio of enzymes they possess. The isolate IS07 is probably able to effectively uptake 108mM glycerol within 24 hours because the growth inhibiting methylglyoxal is metabolized by 16th hour and the cells continue to multiply in reduced MG environment.

The fermentation product characterization using Gas chromatography shows that IS07 preferentially makes free fatty acids like palmitic, stearic ,cis-10-pentadecanoic acid(15:1), 0cis-10-heptadecanoic acid(18:1), Elaidic acid(18:1), linolelaidic acid (18:2) and capric acid. These free fatty acids are important feedstock for a range of chemical reactions and this fermentation process describes a method for bioconversion of an ecologically harmful byproduct glycerol economically important compounds. The free fatty acids can also be integrated back to biodiesel as the fatty acids can be converted to useful liquid fuels by chemical catalytic or enzymatic esterification [22].

Concluding, IS07 isolate can be a potential microbe for bio conversion of biodiesel generated glycerol to important free fatty acids in a time effective fermentation process. Further studies using crude glycerol from biodiesel plant are underway to optimise the strain for industrial uses.

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