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Research article

ISOLATION AND SCREENING OF PUFA PRODUCING MARINE BACTERIA USING BIODIESEL-DERIVED CRUDE GLYCEROL AS CARBON SOURCE

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ABSTRACT: Biodiesel as an alternative fuel has gained major importance in the present years, usually produced by the alkali catalysed transesterification process yielding crude glycerol as a primary by-product which is of low economic value and has problems associated with its disposal, purifying this requires much higher investment. Present work focuses on using this crude glycerol as a carbon source to isolate marine bacteria and further screening for their ability to produce polyunsaturated fatty acids (PUFA). The approach is made to add up market value for the biodiesel derived crude glycerol, in solving the surplus problems and also finding an alternative for the PUFA production which are conventionally produced from the fish oil. By standard microbial procedures 29 isolates were obtained, out of which, two isolates GK12 & GK29 proved potential in producing PUFAs Linoleic acid (C 18:2) & Linolenic acid (C 18:3) considerably in a higher amount, confirmed by the gas chromatographic analysis. The microscopic studies revealed, isolates belonging to *Staphylococcus* family.

Key Words: Biodiesel derived crude glycerol, gas chromatography, lipid analysis, PUFA

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INTRODUCTION

The crude glycerol obtained from biodiesel production process contains much higher amount of impurities such as methanol, ash/salts, and residual fatty acids, which has limited application as compared to pure glycerol. Significantly, the price of crude glycerol has been decreased because of the large supply and less demand; this has resulted in glycerol glut (Johnson D and Taconi K 2007). As a consequence the by-product crude glycerol has become a waste product in biodiesel industries. The purification of crude glycerol requires thrice investment then the cost of the product, leaving purification of crude glycerol as a non-economically feasible solution (Yazdani S and Gonzalez R 2007). The economic challenge of disposing of the waste crude glycerol has caused biodiesel companies to shut down their plants (Pagliaro M et al 2007). To sustain the biodiesel industry, an alternative to utilize the crude glycerol have to be critically considered.

Bioconversion of crude glycerol to value added products turns to be a most economically feasible alternative (Keerthi P et al 2012). Crude glycerol can serve as carbon source in fermentation processes to produce various products such as 1, 3 propanediol (Gonzalez P et al 2006; Zheng P et al 2006), lipid and pigment (Narayan M et al 2005). Anaerobic fermentation of glycerol by *E. coli* generates a mixture of products like ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi Y et al 2006; Zhanyou C et al 2007). Under heterotrophic culture condition, *Schizochytrium limacinum*, algal species an prolific docosahexaenoic acid (DHA) producer can utilize this crude glycerol as a carbon source (Nakahara T et al 1996; Yaguchi T et al 1997; Yokochi T et al 1998). A variety of polyunsaturated fatty acids (PUFA) have been detected in microorganisms including bacteria, yeasts & fungi (Ratledge C and Boulton 1985; Kuswanto K and Sudarmadji S 1989; Ignatius S et al 2010).

MATERIAL & METHODS

Sample Collection

The biodiesel derived crude glycerol was procured from Biofuel Information and Demonstration Centre, Gulbarga University, Kalaburagi. The marine soil samples were collected from various coastal areas of Mumbai, Goa & Karnataka states, India.

Pretreatment of Crude Glycerol

The major amount of unreacted sodium hydroxide & methanol present in the crude glycerol are detrimental to the microbial growth. The pretreatment is crude glycerol is essential which is done by vaporizing the methanol at 75 ºC, further neutralized with 0.1 N HCl, followed with filtering. About 70% Glycerol was obtained by this pretreatment, which is used as carbon source for isolation and screening of marine organisms for conversion to value added products i.e. PUFA such as lenoleic acid & lenolenic acids.

Isolation of Crude Glycerol Utilising Marine bacteria:

The biodiesel derived crude glycerol utilizing marine bacteria were isolated by serially diluting marine soil sample under aseptic conditions and cultured on Sea water agar (SWA) medium composed of Peptone- 0.5%, yeast extract- 0.5%, Beef extract- 0.3%, Agar- 1.5% dissolved in Synthetic Sea water. The constituents of synthetic sea water are NaCl- 2.4%, $MgSo_4.7H_2O$- 0.7%, $MgCl_2.6H_2O$- 0.53%, KCl- 0.07%, $CaCl_2$- 0.01%; supplemented with 1% (v/v) crude glycerol. The axenic cultures were maintained on the same medium used for isolation of marine organisms.

Screening of PUFA Producing Marine Bacteria:

Primary Screening: H_2O_2 Plate assay:

The primary screening was carried out by H_2O_2 plate assay method (Tilay A and Annapure U 2012). This was performed in three different sets: Set 1: cultured on standard sea water agar (SWA) without glycerol supplement (control). Set 2: cultured on sea water agar supplemented with 1% (v/v) pure glycerol (SWA+1% PG) & Set 3: cultured on sea water agar supplemented with 1% (v/v) crude glycerol (SWA+1% CG), incubated for 24 hrs at 37±2ºC. Zone of inhibition was observed and further studied for PUFA production by gas chromatography.

Secondary Screening by Gas Chromatography:

Secondary screening experiment was performed in sets, for 24 hrs at 37±2ºC on orbital shaker maintained at 140 rpm; as per the primary screening experiment. The biomass was collected by centrifugation & followed with freeze drying overnight.

FAME Conversion & Gas Chromatography Analysis:

Fatty acid methyl ester conversion was performed as per (Watanabe K et al 1996). Gas chromatography analysis was performed using Agilent 6890 N system with split mode injector at 230ºC with a split ratio of 50:1, FID temperature 270ºC, controlled by ChemStation software. 1µl sample in hexane was injected; separation was performed on DB225 capillary column (30m X 0.25mm X 0.25mm) coated with 0.25µm, 50% cyanopropylphenylmethylpolysiloxane phase. Oven temperatures were programmed for 2 min at 160 °C, raised at 5°C min^{-1} to 230°C, and finally hold for 20 min at 230°C, with nitrogen as carrier gas, (flow rate at 1.0 ml/min). The injector temperature and detector temperature was maintained at 230 and 250°C respectively. Peaks were identified after comparison of the retention time with those of standard SUPELCO 37 component FAME mix from Supelco.

RESULTS

Isolation of Crude Glycerol Utilising Marine bacteria:

Based on the morphological characteristics, 29 isolates were selected and maintained on SWA supplemented with 1% (v/v) crude glycerol. The isolates were coded as GK1 to GK 29 (Fig no.1).

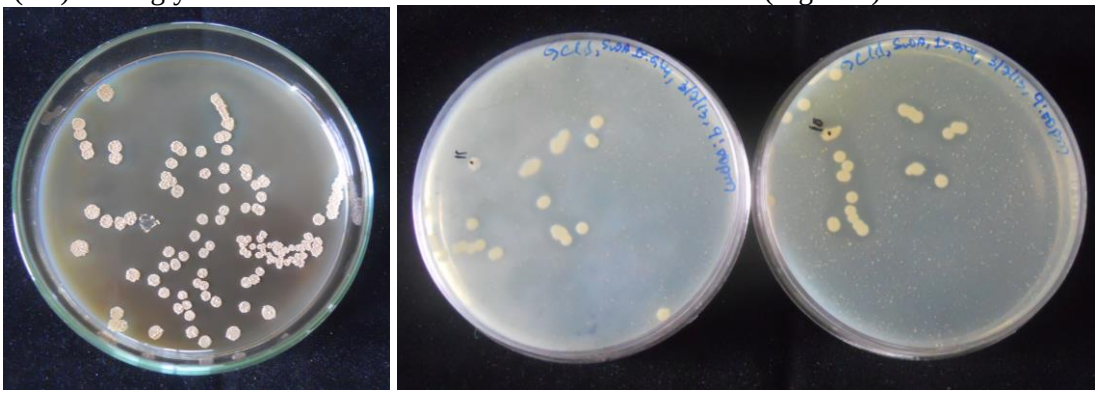

Fig no. 1. Marine organisms Isolated on SWA Supplemented 1% (V/V) Crude Glycerol Screening of PUFA Producing Marine Bacteria

Primary Screening: H_2O_2 Plate assay

Among 29 obtained isolates; 10 isolates showing resistance to H_2O_2 at a concentration of 0.5% were selected as the PUFA producers. The Results of the H_2O_2 Plate assay are as shown in Table no. 1 & Fig no. 2.

Table no. 1: Response of isolates to H_2O_2

Isolates	Set 1				Set 2				Set 3				Inference
	0.01 %	0.10 %	0.50 %	1 %	0.01 %	0.10 %	0.50 %	1 %	0.01 %	0.10 %	0.50 %	1 %	
GK1	+	+	-	-	+	-	-	-	+	-	-	-	-
GK2	+	+	+	+	+	+	+	-	+	+	+	+	+
GK3	+	+	-	-	+	+	-	-	+	-	-	-	-
GK4	+	+	+	-	+	+	+	-	+	+	+	-	+
GK5	+	+	+	-	+	+	+	-	+	+	+	-	+
GK6	+	-	-	-	+	-	-	-	-	-	-	-	-
GK7	-	-	-	-	-	-	-	-	-	-	-	-	-
GK8	+	+	-	-	+	-	-	-	+	-	-	-	-
GK9	+	+	+	+	-	-	-	-	-	-	-	-	-
GK10	+	+	-	-	+	-	-	-	+	-	-	-	-
GK11	+	+	+	+	+	+	-	-	-	-	-	-	-
GK12	+	+	+	+	+	+	+	+	+	+	+	+	+
GK13	-	-	-	-	-	-	-	-	-	-	-	-	-
GK14	+	+	+	-	+	+	+	-	+	+	+	-	+
GK15	+	+	-	-	+	-	-	-	+	-	-	-	-
GK16	+	+	-	-	+	-	-	-	+	-	-	-	-
GK17	+	+	+	-	+	+	+	-	+	+	+	-	+
GK18	-	-	-	-	+	+	-	-	+	+	+	+	-
GK19	+	-	-	-	+	-	-	-	-	-	-	-	-
GK20	+	+	+	-	+	-	-	-	-	-	-	-	-
GK21	-	-	-	-	-	-	-	-	-	-	-	-	-
GK22	+	-	-	-	-	-	-	-	+	-	-	-	-
GK23	+	+	+	+	-	-	-	-	-	-	-	-	-
GK24	+	+	+	-	+	+	+	-	+	+	+	-	+
GK25	+	-	-	-	+	-	-	-	-	-	-	-	-
GK26	+	+	+	-	+	+	+	-	+	+	+	-	+
GK27	-	-	-	-	-	-	-	-	+	-	-	-	-
GK28	+	+	+	-	+	+	+	-	+	+	+	-	+
GK29	+	+	+	+	+	+	+	+	+	+	+	+	+

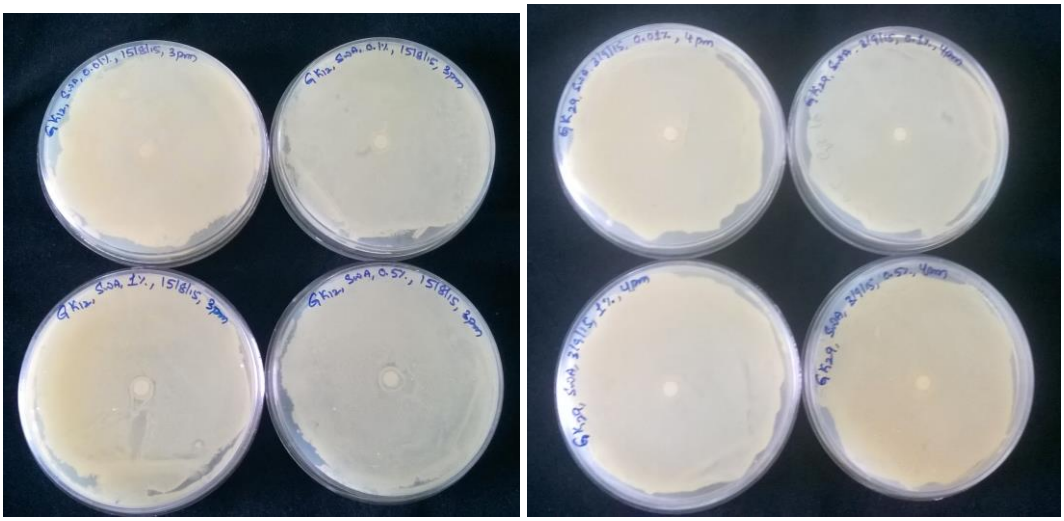

Fig no. 2. PUFA producers showing no zone of inhibition

Secondary Screening by Gas Chromatography:

The gas chromatographic analysis confirmed the production of Linoleic acid (C 18:2) & Linolenic acid (C 18:3), considerably high in percentage compared to other fatty acids produced by the screened isolates. GK12 & GK29 isolates proved to be potent in PUFA production. The percentage concentration of these PUFA's produced by positive isolates is shown in Chromatograms (Fig no. 3 & 4).

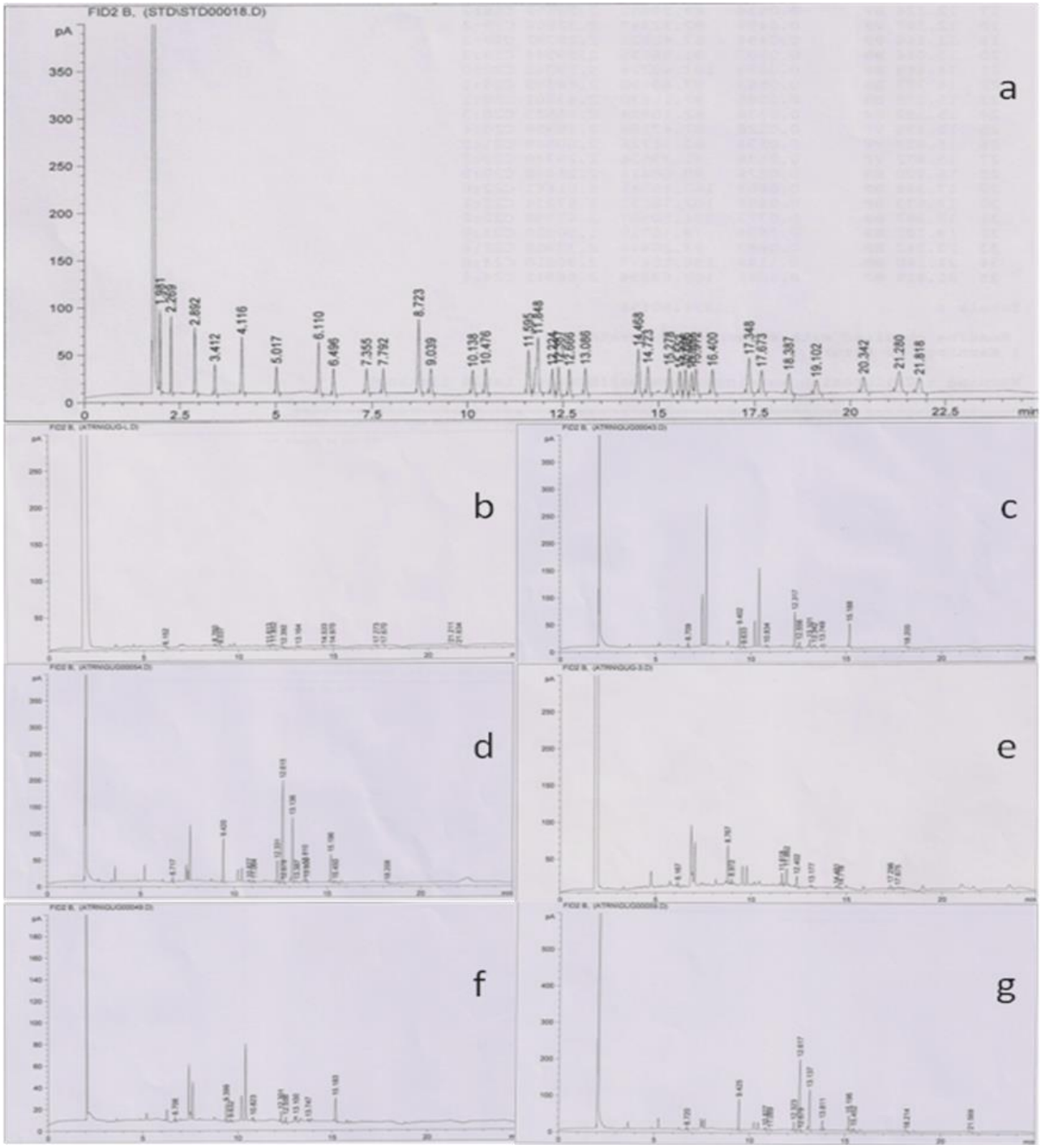

Fig no. 3. Chromatograms Showing Detection of C18:2 & C18:3

a. Standard fatty acid mix (C4- C24:1), b. GK12 on SWM, c. GK12 on SWM+ 1% PG, d. GK12 on SWM+ 1% CG, e. GK29 on SWM, f. GK29 on SWM+1% PG, g. GK29 on SWM+1% CG.

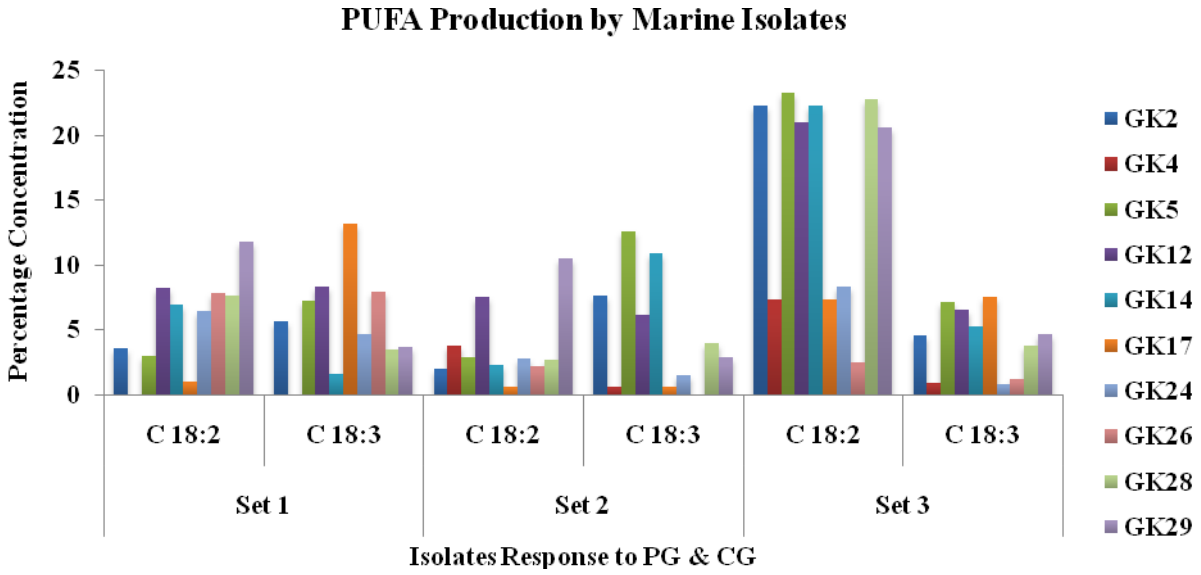

Fig no. 4. Percentage Concentration of C18:2 & C18:3

DISCUSSION

Naresh and Brian (2006); Gervasio et al (2009) reported biodiesel derived crude glycerol can be used as carbon source by microorganisms and converting it to various value added products like 1, 3- propanediol, dihydroxyacetone, succinic acid, polyglycerols, polyhydroxyalkonates, propionic acid, ethanol, citric acid, pigments, biosurfactants & can also be converted to value added lipids like PUFA which has high therapeutic applications (Narayan M et al 2005; Tilay A and Annapure U 2012; Nicol R et al 2012; Ahmed A et al 2014).

Jantima et al (2010) used mineral salt medium with 5% (w/v) crude glycerol as carbon source for screening of polyhydroxyalkonate producing bacteria; Ahmed et al (2014) used artificial sea water medium for screening of PUFA producing marine bacteria by using 2, 3, 5- triphenyltetrazolium chloride (TTC) dye. Tilay and Annapure (2012) used nutrient agar for isolation of marine bacteria and developed a simplified and rapid method for screening PUFA producers using hydrogen peroxide. In our present investigation, we have used sea water medium with 1% (v/v) Crude glycerol supplement as carbon source for the isolation of marine microorganisms & further screened for PUFA production were performed and similar results were obtained as to that of Tilay and Annapure (2012).

Watanabe et al (1996) reported the protocol for lipid extraction & FAME conversion directly from wet cells; this was also employed by Ahmed et al (2014) and as well in the present investigation.

Twenty nine different isolates were isolated on SWA supplemented with 1% (v/v) crude glycerol. In hydrogen peroxide plate assay, 10 isolates showed their ability to convert crude glycerol to PUFA. Among the 10 positive isolates, GK12 & GK29 isolates confirmed the production of Linoleic acid (C 18:2) and Linolenic acid (C 18:3); 8.3%, 8.4% on SWM, 7.6%, 6.2% on SWM+1% PG, 21.0%, 6.6% on SWM+1% CG & 11.8%, 3.7% on SWM, 10.5%, 2.9% on SWM+1% PG, 20.6%, 4.7% on SWM+1% CG respectively, by gas chromatographic analysis. This clearly states that the biodiesel derived crude glycerol has a promising effect on increased production of Linoleic acid & Linolenic acids by GK12 & GK29 isolates. Thus, the biodiesel derived crude glycerol can be used to produce PUFA, having high therapeutic applications with higher market value.

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