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RESEARCH ARTICLE

OPTIMIZATION OF PRODUCTION MEDIA FOR HIGH YIELD PRODUCTION OF Γ- LINOLENIC ACID BY USING *CUNNINGHAMELLA SP.* RKC008 THROUGH SUBMERGED FERMENTATION

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ABSTRACT

Cunninghamella sp. was isolated from Leaf Litter samples and partial identification was done by using the morphological characteristics. Production medium optimization studies were carried out in submerged fermentation and 4 different media compositions were designed among these CP03 showed high yield. Different parameters like pH, Residual sugar estimation, Dry cell weight, Oil content and harvesting time were evaluated. CP03 media showed highest dry cell weight (17.3 g/L) and Oil content (1.77g/L at 96 Hrs) followed by CP01 with 12.36 g/L of dry cell weight and 1.38 g/L of Oil content at 96 Hrs. Qualitative estimation for the extracted γ- Linolenic acid was done by using Gas chromatography and the results showed 12.9894% for the γ- Linolenic acid extracted from CP03 media & 12.5145% for γ- Linolenic acid extracted from CP01 media.

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INTRODUCTION

Gamma Linolenic acid is a Poly unsaturated fatty acid (PUFA) which is considered as one of the essential fatty acids (EFA).γ- Linolenic acid/ cis 6,9,12- Octadecatrienoic acid (18:3, n-6) is an Omega – 6 fatty acid (ω – 6) and contains 3 double bonds in its structure. So, this has been grouped into Tri unsaturated acids/ Triens (Ali Choopani, 2016). Human body cannot synthesize EFA as it lacks some of the important enzymes required for synthesis (Aleksandra, 2014). The metabolic transformations of γ- Linolenic includes the conversion of Stearic acid (18:0) into Oleic acid by Delta - 9 desaturase which in turn converted to Linoleic acid by Delta – 12 desaturase. In presence of Delta - 6 desaturase enzyme, γ- Linolenic acid is synthesized from Linoleic acid (Yao Zhang *et al*., 2007)**.** γ- Linolenic acid is more effective n – 6, essential fatty acid when compared to Linoleic acid (Hung-Chang Chen, 1997). γ- Linolenic acid is suggested as an essential requirement for the patients suffering Cancer, Diabetes, Aging, Hypertension *etc*, as it is Anti - atherosclerotic, Anti – thrombotic, Anti – Inflammatory, Anti – cancer and Hypocholesterolemic (Hung-Chang Chen, 1997; Sâmia, 2009). γ- Linolenic acid is conventionally extracted from the plant seeds of Evening Primrose (*Oenothera biennis* L.), Borage (*Borago officinalis* L.) and Blackcurrant (*Ribes nigrum* L.).

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Extraction of γ- Linolenic acid from the plant seeds has several disadvantages like variation in quality and quantity based on the region and climatic conditions. The genetically modified Safflower seeds which yield about 70% of γ- Linolenic acid are not accepted due to the controversies of using genetically modified compounds as Neutraceuticals. Due to these disadvantages, Oleaginous microbes have drawn much attention in the production of γ- Linolenic acid (3)**.**Molds have the ability to accumulate high concentration of Poly unsaturated fatty acids. Similarly, some of the bacteria also can incorporate certain lipids *viz,* Polyhydroxyalkanoic acid and triacyl glycerols. Some of the commonly used fungi for γ- Linolenic acid production are *Mortierella spp*, *Mucor spp*, *Rhizopus spp*, *Zygorhynchus spp and Cunninghamella spp* (Gema *et al*., 2002). As per the previous research, *Cunninghamella spp* can incorporate large amounts of lipids (Marjan Ganjali Dashti, 2016). In the current research, *Cunninghamella sp.* was used for the production of γ- Linolenic acid, medium optimization studies were carried out for achieving high yields and characterization of γ- Linolenic acid was done by using Gas chromatographyanalysis.

MATERIALS AND METHODS

Isolation and partial identification of fungal strain

Leaf litter samples were collected from Sadasivapet, Telanagana.

Samples were serially diluted and plated on Potato Dextrose Agar and incubated at 26 ^{0}C for 7 days. Culture morphology of the fungal strain was observed and microscopic observation was performed. Fungal isolates were screened in selective media for assessing the potent lipid producing fungal strain. Media composition (in g/L):Glucose- 30, Ammonium tartrate- 1.0, KH_2PO_4- 7.0, Na_2HPO_4 -2.0, $MgSO_4.7H_2O$-1.5, $CaCl_2.2H_2O$-0.1, $FeCl_3.6H_2O$-0.008,$ZnSO_4.7H_2O$-0.0001, $CuSO_4.5H_2O$-0.001; $Co(NO_3)_2.6H_2O$- 0.0001 and $MnSO_4.5H_2O$-0.0001, pH- 6.0 (Marjan Ganjali Dashti, 2016)**.** Based on the morphological characters the fungal strain was partially identified and the pure culture was maintained in 30% Glycerol stocks at -80^{0}C

Inoculum preparation

250 ml of Potato Dextrose broth was prepared and transferred to 500 ml of Erlenmeyer flask. The fungal strain was inoculated and kept on orbital shaker for 48 Hrs at 200 rpm.

Optimization of production medium

To achieve high yield of lipid, different media compositions were selected and medium optimization was carried out with variations in the C: N ratio. Inoculum was added and kept on rotator shaker for 48-120 Hrs at 200 rpm. Medium showing high γ- Linolenic acid content was used for the further studies. Different parameters such as pH, Residual sugar estimation by using DNSA method (Miller, 1959)**,** Harvesting time optimization and Dry cell weight were checked at regular intervals of time. The batch culturing was repeated for 3 times to draw the perfect conclusion (Marjan Ganjali Dashti, 2016)**.**

Extraction of lipid and qualitative estimation by using Gas chromatography

Collected mycelial mass was crushed by using mortar and thistle. Grinded mycelial mass was treated with Chloroform – Methanol in the ratio 2:1 for 3 Hr. using Soxhlet apparatus. Extracted lipids were concentrated and Sodium sulphate was added in order to remove the moisture (Somashekar *et al*., 2002). Lipid extraction was done for all the mycelia mass collected from different media compositions and the lipid content results were compared. Extracted lipids were checked for the presence of γ- Linolenic acid by using Gas chromatography (GC). The instrument used was YL6500GC of YL Instrument (30m x 0.25 mm column) (Yao Zhang, 2017).

RESULTS AND DISCUSSION

Isolation and partial identification of fungal strain

26 fungal isolates were isolated from 10 leaf litter samples and labeled as RKC001 to RKC026. Fungal isolates were grown in selective media and 9 isolates were screened based on the growth pattern. Morphology of all the fungal strains were observed under Phase contrast Microscope (Nikon Eclipse E 200). Based on the morphology and sporulation, RKC008 was identified as *Cunninghamella sp.* The *Cunninghamella sp.* colonies were white to off white in colour and the sporangiophores were straight and erect. Pure culture was maintained in Ultra low freezer at -80^{0}C. As per the previous research reports on *Cunninghamella sp.*, it has proved to be high lipid producing Oligeanous fungi (Marjan Ganjali Dashti, 2016; Gema *et al*., 2002; Hung-Chang Chen and Tse-Ming Liu, 1997).

Optimization of Production

Production medium optimization studies were carried out for achieving high lipid production. 4 media compositions were designed and labeled as CP01, CP02, CP03 and CP04. The media compositions are listed in All 4 media compositions designed were used for further experiments. Results of different parameters like pH, residual sugar, dry cell weight, harvesting time optimization and oil content were given in Table 2. When dry cell weights were compared CP03 (17.30 g/L) showed highest cell biomass weight followed by CP01 (12.36 g/L) where as CP02 and CP04 showed less dry mycelia weight *i.e.* 3.23 g/L and 5.70 g/L.Fig.1.As per the research reports, carbon and nitrogen plays a major role in achieving high biomass (Stanbury, 1984). Addition of Molasses as an extra additive showed beneficial effect in getting good mycelia weight and oil content.

Oil content of all different media were compared, among which CP03 showed highest oil content *i.e.* 1.77 g/L followed by CP01(1.38 g/L) at 96 Hrs. Whereas results of CP02 and CP04 showed less oil content i.e. 0.76 g/L and 1.01 g/L. Fig 2.0. Results taken at 120 Hrs showed decrease in the oil content in all the media compositions.

Table 1. Optimized media compositions

Ingredients	Media composition in percentage			
	CP01	CP02	CP03	CP04
Glucose	1.0	0	0.5	0.5
Sucrose	0	0.5	0	0
Corn Steep Liquor	0	0	0	1
Molasses	0	0	1	0
Yeast extract	0.5	0.5	0.1	0.2
Malt extract	0.3	0.3	0.2	0
Peptone	0.4	0.4	0.2	0.2
Ammonium tartarate	0.05	0.1	0.2	0.3
KH_2PO_4	0.5	0.7	0.8	0.5
$MgSO_4$	0.5	0.1	0.2	0.15
Calcium chloride	0.01	0.01	0.01	0.01
$FeCl_3$	0.0002	0.0002	0.0002	0.0002
$ZnSO_4$	0.0001	0.0001	0.0001	0.0001
$MnSO_4$	0.0001	0.0001	0.0001	0.0001
$CuSO_4$	0.0001	0.0001	0.0001	0.0001
$Co(NO_3)_2$	0.0001	0.0001	0.0001	0.0001
pH	6.0	6.0	6.0	6.0

Table 2. Data on different parameters tested at 48 Hrs

Media	pH	Residual sugar (g/L)	Dry cell weight (g/L)
CP01	9.53	2.90	12.36
CP02	4.61	1.15	3.23
CP03	9.35	1.64	17.30
CP04	4.90	0.04	5.70

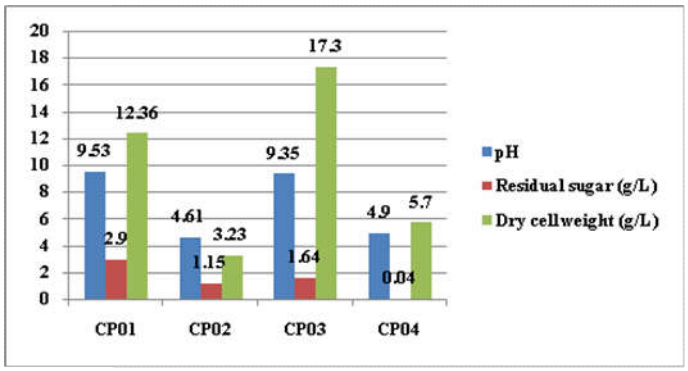

Fig.1. Graph showing different parameters

Media	Oil content (g/L)				
	At 24 Hrs	At 48 Hrs	At 72 Hrs	At 96 Hrs	At 120 Hrs
CP01	0.31	0.62	0.87	1.38	1.03
CP02	0.18	0.35	0.59	0.76	0.43
CP03	0.25	0.92	1.31	1.77	1.50
CP04	0.29	0.74	1.00	1.01	0.55

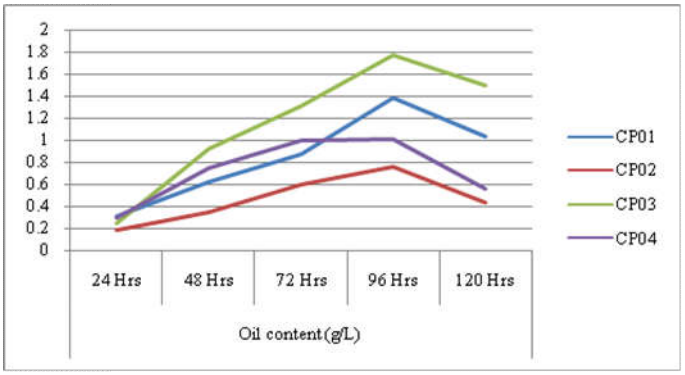

Fig. 2. Graph showing the Oil content checked at different intervals of time

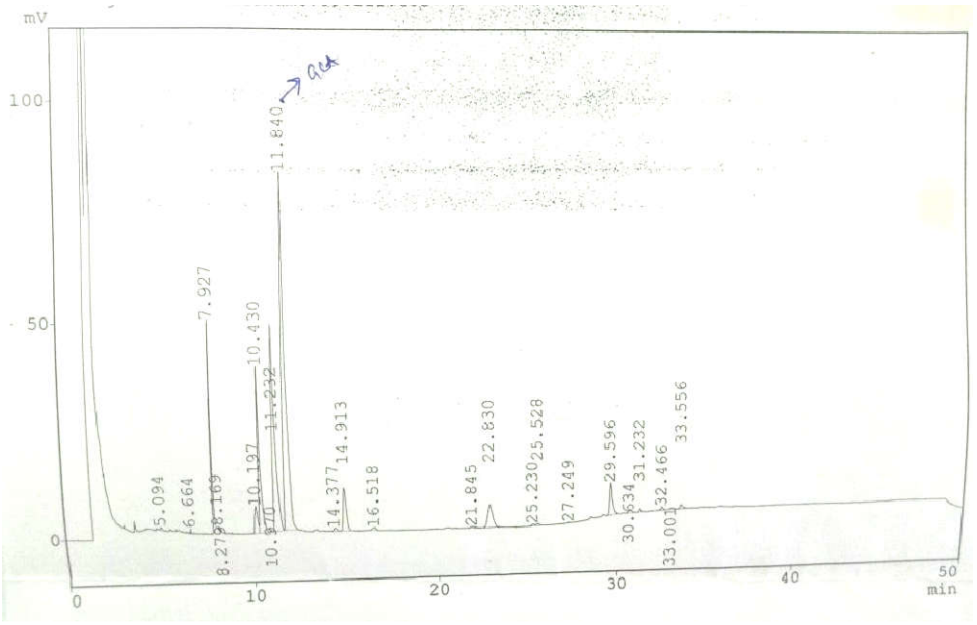

Fig.3a. Chromatogram of γ- Linolenic acid standard

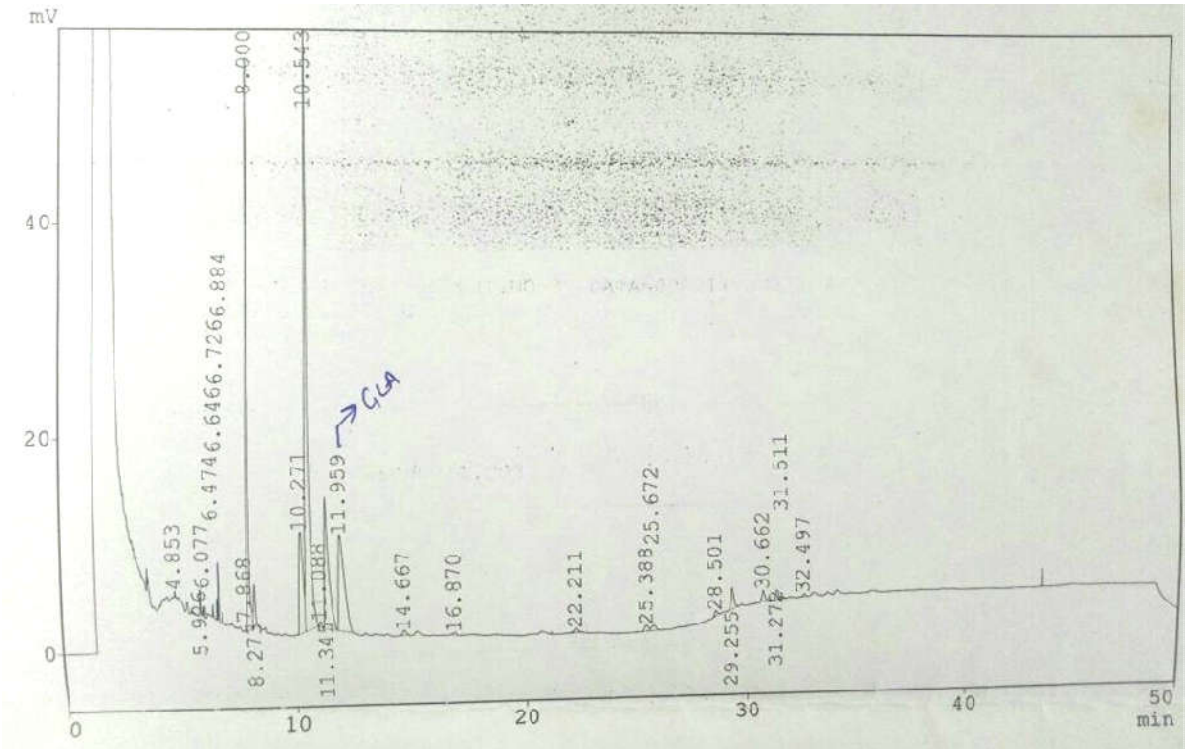

Fig.3b. Chromatogram of CP03

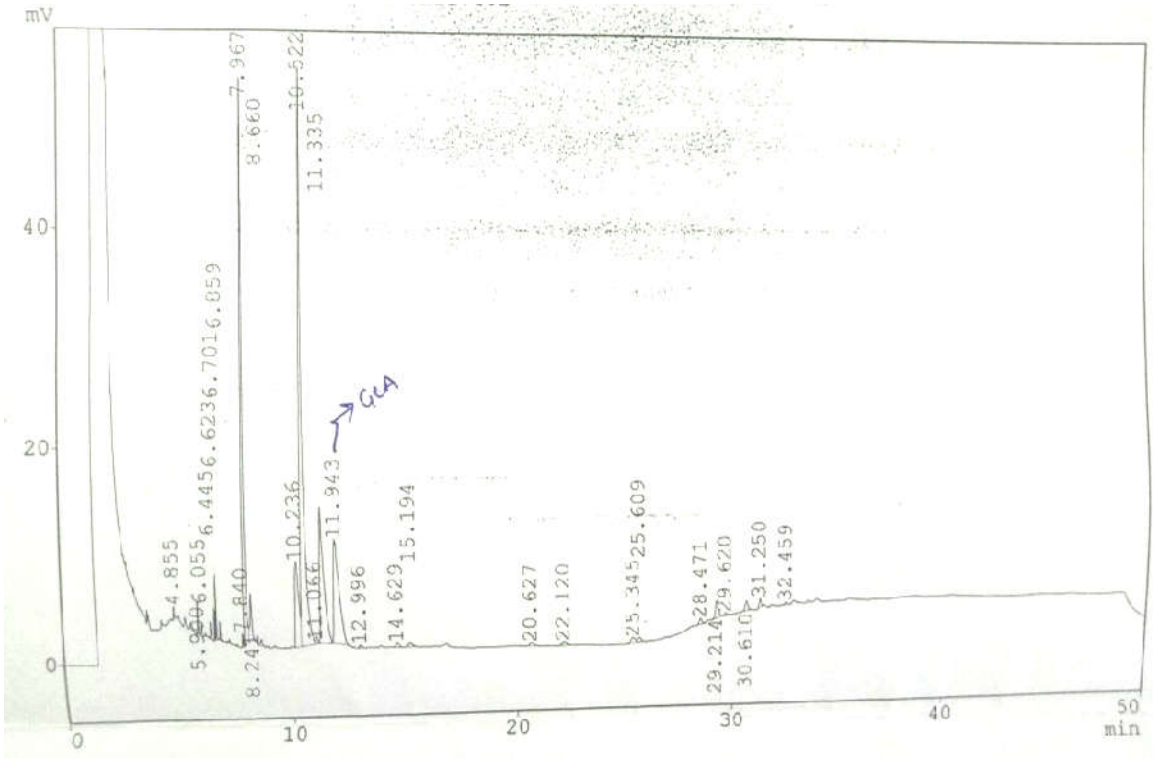

Fig.3c. Chromatogram of CP01

Therefore, 96 Hrs was taken as the optimum harvesting time Table 3. Previous research studies indicated increase in oil content when Ammonium tartrate and metal ions were added in the media composition (Farhila Muhid, 2008).The oil content collected from CP03 and CP01 were qualitatively estimated by using Gas Chromatography (YL6500GC). The testing samples (CP03 and CP01) were tested against γ-Linolenic acid standard (41.5938%) Fig 3a. Results showed that sample CP03 has 12.9894% & CP01 showed 12.5145% Fig 3 b & c.

Conclusion

Medium optimization for high yield production of γ- Linolenic acid showed that CP03 showed high cell dry weight with 17.3 g/L and 1.77 g/L of oil content. As per the results, 96 Hrs is considered as the optimum harvesting time for achieving high γ- Linolenic acid. Similarly, GC analysis showed 12.9894% of γ- Linolenic acid which was extracted from CP03. So, CP03 media can be used for γ- Linolenic acid production by using *Cunninghamella sp*.

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