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Penicillium halotolerans ACR-D24: a Psychrotroph with Potential for PUFA Production

Richa Sharma[1, 2], Farnaz Yusuf[1], Ankita magotra[1, 2] and Asha Chaubey*[1,2]
[1]CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu(India)
[2]Academy of Scientific and Innovative Research, New Delhi(India)

ABSTRACT

Linoleic acid (LA) is an unsaturated omega-6 essential fatty acid, precursor in the biosynthesis of indispensable cellular molecules, Arachdionic acid and Eicosanoids. Alternatives to its procurement other than from plants are being the most sought out for, due to increasing demand and plants sacrificial aspect of it. A psychrotrophic fungus, *Penicillium halotolerans* ACR-D24 from the cold Shiwalik region has been explored as a potential source for LA production. Optimization of various process parameters of growth like media, temperature, pH, etc. was done for exploiting the microbe to its full potential. Dextrin Peptone broth has been identified as a suitable medium for LA production in 7 days fermentation time. Optimum pH and temperature for LA production was determined to be pH 7.5-8.0 and 20˚C respectively. The optimized conditions could produce ~75% LA in the methylated crude extract of fermentation broth. Overall yield of 2.4g/L LA was obtained in the fermented broth after 7 days cultivation. The psychrotrophic fungal isolate, strain ACR-D24 can be exploited as a potential candidate for the production of LA under optimized physical parameters. This may be used for the synthesis of the commercially valuable nutraceutical and pharmaceutical products.

KEYWORDS: ***Penicillium halotolerans*; PUFA; Linoleic acid; methyl ester; cold Shiwalik region**

INTRODUCTION

Polyunsaturated fatty acids (PUFA) are not only important as structural components of membrane phospholipids but also as precursors of the eicosanoids of signaling molecules. The Essential Fatty Acids (EFA) are nutrients that must be provided in the food because they cannot be manufactured within the body. EFA have been divided into two groups of polyunsaturated fatty acid families i.e. omega (n)-3 and omega (n)-6 PUFA. It has been reported that, n-3 EFA must be provided with n-6 EFA to express biological effects, whereas the n-6 EFA are biologically active even without n-3 EFA [1].

Linoleic acid (LA), a colorless liquid at room temperature is an unsaturated omega-6 fatty acid with a lipid number of 18:2 cis, cis-9, 12. LA is used in the biosynthesis of Arachdionic acid (AA) and thus some Eicosanoids such as Prostaglandins [2, 3]. It is reportedly found in the lipids of cell membranes and is abundant in many vegetable oils such as, poppy seed, safflower, sunflower, and corn oils [4, 5]. LA is the only true n-6 EFA on the grounds that it is the starting point of the n-6 EFA metabolism and that it is the most abundant n-6 EFA in the diet. Due to anti-inflammatory, acne reductive, and moisture retentive on the skin, LA is an important component in the beauty products [5, 6, 7]. Other important physiological and medicinal properties include development of brain & retina, regulation of blood pressure and immune response [8].

Since plants as sole source cannot be sufficient to meet the ever increasing global demand for LA, there has been a constant search for higher and better producers of the above said unsaturated fatty acid from alternative sources like microorganisms. LA has been gaining potential interest for its commercial production by fermentation, due to its increasing commercial demand. Over the past several years extensive research has been done for microbial production of PUFA [9, 10, 11, 12, 13, 14, 15].

Microbial sources were identified as the most attractive potential sources [16]. Oleaginous microorganisms are able to convert agro-industrial raw materials into valuable lipids as γ-Linoleic acid via fermentation either

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as a solid state or submerged process [17]. The most investigated oleaginous yeasts belong to the genera *Yarrowia, Candida, Rhodotorula, Rhodosporodium, Cryptococcus,* and *Lypomyces* [18, 19, 20]. Oleaginous Fungi belonging to genus *Mortreilla* are the best known producers of PUFAs, Linoleic acid [21]. Fungi such as *Aspergillus niger* and *Penicillium* species are known producers of PUFAs with Linoleic acid as the principal fatty acid in their lipid profile along with Palmitic acid and Oleic acid but with species having different relative concentrations [22, 23]. Efforts have also been made to maximize PUFA yield by mutations, genetic manipulations, isolating new strains as well as optimizing the media for cultivating more efficient strains like improvement of Arachdionic acid production by *Mortierella* sp. [24] and DHA production by thraustochytrids [25, 26]. Production of gamma-Linoleic acid in *Pichia pastoris* (yeast) has been reported by expression of a delta-6 desaturase gene from *Cunninghamella echinulata* [27]. Production of PUFA generally gets influenced by biochemical and physical parameters including media composition and cultivation conditions [28]. The aim of this work was to isolate and screen EFA producing microbes of extreme cold habitat, and to optimize various process parameters for the putative EFA production.

EXPERIMENTAL PROCEDURES

SCREENING OF OLEAGINOUS MICROORGANISM

Microbes isolated from soil sample of Dras, a high altitude (about 16,000 feet) and sub-zero temperature region in the Shiwalik Hills of the North Western Himalayas, India, were screened for oleaginous potential by preparing methyl esters of the ethyl acetate extracts of the isolates and analyzing them by GC-MS analysis.

STRAIN IDENTIFICATION

The fungal strain with oleaginous potential was examined morphologically, microscopically and identified at molecular level. Fungal DNA was extracted using the Raeder and Broda method with some modifications [29]. The ITS region were amplified via PCR reaction using 18SrRNA primers ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTATTGATATGC. Sequence analysis was done with the basic sequence alignment BLAST program run against the NCBI database and the determined sequences were aligned using Clustal X and the distance matrices calculated and phylogenetic trees were constructed using MEGA 5.0 software [30]. The nucleotide sequence so obtained was submitted to the GenBank and assigned an accession number **KF888644.**

MEDIA USED AND CULTIVATION CONDITIONS

Media components in the experiment were procured from Hi-media and Sigma; solvents used were of reagent grade (RG).

Three cultivation media i.e. (PDB, DPB and Mineral medium to be renamed as M1, M2 and M3) were used in this study. The culture was inoculated into 100ml cultivation medium in 500 ml Erlenmeyer flasks and cultivated at 28°C for 7 days, with continuous shaking at 200 rpm. Cultivation medium M1 (g/L): Potato Dextrose Broth (PDB)- 24. Cultivation medium M2 (g/L): Dextrin 15; Peptone 5; Yeast Extract 1; K_2HPO_4 1; NaH_2PO_4 0.6; MgSO4 0.5; pH -7.0. Cultivation medium M3 (g/L): Yeast Extract 10; Peptone 10; Glucose 80; NaCl 4; KH_2PO_4 2.5; $MgSO_4$ 0.5; KCl 0.1, $CaCl_2$ 0.5; pH-7.0.

LIPID EXTRACTION

After cultivation, biomass was harvested from the fermented medium by filtration through Whatman No. 1 filter paper and excess of water was removed. The harvested mycelium was washed twice with distilled water and the cells were dried in an oven at 40°C. Dry biomass was homogenized in the presence of methanol and extract was added to the fermentation broth. Lipid was extracted from the filtrate by extraction with ethyl acetate. Residual moisture of the solvent was removed by adding anhydrous sodium sulphate, filtered with Whatman filter paper and then the lipid was concentrated under vacuum drying (Buchi Rotavapor, Germany).

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METHYL ESTER PREPARATION AND ANALYSIS OF FATTY ACID COMPOSITION

10mg of the extracted lipid was taken and fatty acid methyl ester (FAME) was prepared in 2% Methanol in H_2SO_4 and was kept overnight for esterification at 37ºC in a tightly closed round bottom flask. Methyl esters were extracted twice by adding two volumes of hexane and washed well with distilled water to remove any free acids. Residual moisture in the hexane layer was removed by adding anhydrous sodium sulphate, FAME was concentrated by evaporating solvent on a water bath under vacuum for further characterization.

GAS CHROMATOGRAPHY/ MASS SPECTROSCOPY

GC-MS was performed on Varian 4000 MS/MS Series Gas Chromatograph equipped with a FID and the capillary column CP Sil 8 CB (30 m x 0.32 mm x 0.25 µm film thickness; J & W Scientific, USA). Injector temperature was maintained at 280°C. The Column oven was programmed for 5 min at 100°C, increased to 250°C at 10°C/min, maintained for 20 min. The carrier gas i.e. Helium was used at a flow rate of 1.0 ml/min. The injection volume was 1.00µl, with a split ratio of 1:50. For identification of Fatty acids, structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the NIST library.

EFFECT OF PROCESS PARAMETERS

Based on the results obtained from different cultivation medium, we used Medium M2 for further optimization studies.

EFFECT OF FERMENTATION TIME

Time profile studies were carried out to understand the optimum fermentation time for production of LA by isolate ACR-D24. 100ml production medium in 500ml Erlenmeyer flasks were inoculated with the culture and incubated under shaking conditions at 28˚C. One flask was terminated every alternate day from 4th day onwards. Dry Biomass (DBM), lipid and LA contents were monitored as described previously. Optimum fermentation time i.e. 7 days was used for further studies.

EFFECT OF % SEED INOCULUM

Optimum seed (%) as inoculum for the optimum production of LA in DPB medium by ACR-D24 was determined by inoculating a range of seed size i.e. 2%, 4%, 6%, 8% and 10% in 100ml medium each and then incubated under shaking conditions at 28˚C for 7 days. Each flask was terminated after 7 days and DBM, lipid and LA contents were monitored as described previously via GC and GC-MS.

EFFECT OF TEMPERATURE

In order to determine the optimum temperature for the production of LA, 5 Erlenmeyer flasks containing 100 ml production medium, were inoculated with the culture ACR-D24 and incubated under shaking conditions at various temperatures i.e. 4ºC to 50ºC for 7 days. The flasks were terminated after 7 days. DBM, lipid and LA contents were monitored as described previously via GC and GC-MS.

EFFECT OF PH

For determining the optimum pH for the production of LA, 100ml production medium was adjusted to different pH values i.e. pH 5.0 to 9.0. The Erlenmeyer flasks were inoculated with the culture ACR-D24 and cultivated under shaking conditions at 28ºC for 7 days. Flasks were terminated after 7 days to monitor DBM; lipid and LA contents were monitored as described previously via GC/GC-MS.

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EFFECT OF CARBON SOURCE

In order to determine best carbon source for the production of LA, by inoculating ACR-D24 in DPB medium with different carbon sources i.e. Glucose, Dextrose, Sucrose, Starch, Lactose and Arabinose. The culture was grown under shaking conditions at 28ºC for 7 days. All the flasks were terminated after 7 days by filtration and DBM, lipid and LA contents were monitored as described previously via GC/GC-MS.

EFFECT OF NITROGEN SOURCE

Various nitrogen sources and their combinations were employed in DPB medium to see the effect of nitrogen source for LA production. We used Yeast Extract, Peptone and their combination in the ratio of 1:1. The media were inoculated with ACR-D24 and incubated under shaking conditions at 28ºC for 7 days. The flasks were harvested after 7 days to determine DBM, lipid and LA contents as described previously via GC and GC-MS.

PRODUCTION OF LINOLEIC ACID IN FERMENTER

In order to study LA production at fermenter scale, fermentation experiment was carried out using 3 litres of M2 medium in a 5L fermenter (Scigenics India Pvt. Ltd). All the optimized conditions, i.e. temperature 20ºC, pH-7.5, seed inoculum (6%) and fermentation period of 7 days were employed during the fermentation with an air of 0.5vvm, pressure 2 LPM and agitation of 200 rpm. Samples were withdrawn daily for a period of 10 days and methylated lipid extracts were analyzed for LA production via GC-MS.

RESULTS

SCREENING FOR OLEAGINOUS CULTURE

About 20 isolates from the Shivalik regions of Jammu & Kashmir (India) were screened for their oil/lipid producing potential. All the cultures were grown in cultivation medium M2 initially and screened for lipid contents in the fermented broth as described before. Only one fungal isolate ACR-D24 (accession number **KF888644**) showed the prominent production of LA and Palmitic acid.

STRAIN IDENTIFICATION

MORPHOLOGICAL CHARACTERISTICS

Fungal strain ACR-D24 was observed as velvety greenish groovy colony with creamish grooved margins with smooth to finely roughened greenish conidia (Fig.1a). Yellow soluble pigments were produced. Reverse colony showed yellowish coloration.

MICROSCOPIC CHARACTERISTICS

Microscopically, strain ACR-D24 showed the presence of short ampulliform phialides with tertiary/quarterverticillate branched conidiophores (Fig. 1b).

MOLECULAR IDENTIFICATION

Fungal strain ACR-D24 was identified as a *Penicillium* sp. showing maximum homology with *Penicillium halotolerans* strain DTO 148H9 and its sequence submitted in NCBI database bearing Accession number **KF888644** (Fig.1c). The psychrotrophic fungus has been submitted to Sir R.N. Chopra, Microbial Resource Centre, Jammu, India with accession number MRCJ-145.

EFFECT OF PROCESS VARIABLES

CULTIVATION MEDIUM

Three different cultivation media were used to cultivate LA producing strain ACR-D24. As shown in Fig. 3, maximum DBM (~15g/L) was obtained in medium 3 followed by Medium 2 (~10g/L). Medium 1 however, presented minimum DBM. Maximal LA production (~72%) was obtained in Medium 2 with Dextrin as sole carbon source. GC-MS plot data (Fig.2) showed that there were three major products viz. Linoleic acid methyl

ester, Palmitic acid methyl ester and Oleic acid methyl ester were present. Medium 1 and medium 3 showed about 10% oleic acid methyl ester in the crude extract, whereas medium 2 contained about 5-6% of oleic acid. We therefore used Medium 2 as the preferred medium for the production of LA. Further optimization studies for LA production were therefore carried out in Medium 2.

EFFECT OF % SEED INOCULUM

Effect of seed inoculum on biomass and LA production was observed after 7 days of fermentation. Results shown in Fig. 4, demonstrate that maximum dry biomass of about 10 g/L was obtained with 8% inoculum, whereas maximum LA (~80% of crude lipid extract) was obtained with 6% inoculum size. This was quite high as compared to the previously grown culture and therefore all further experiments were carried out in Medium 2 with 6% inoculum.

EFFECT OF CULTIVATION TIME

In order to see the effect of cultivation time, we observed the dry biomass as well as LA content in the methylated crude extract (Fig.5). It was observed that the dry biomass (~10g/L) was optimal after 7 day cultivation and then decreased with time might be due to limitation of substrate in the medium. Optimal LA (~75%) was also obtained after 7 day cultivation and then the level of LA decreased with cultivation time.

EFFECT OF TEMPERATURE AND PH

Since, LA producing strain ACR-D24 was isolated from the extremely cold Shiwalik range, it was capable to grow at low temperatures ranging from 4˚C to higher temperatures at 50˚C (Fig. 6a). Optimal temperature for growth and LA production was observed to be 20˚C and might be supported by the fact that PUFAs are formed at low temperatures. Similarly, too acidic or too alkaline conditions didn't support LA production and optimal LA was between pH 7.5-8.0. It was inferred that pH 7.5 to 8.0 at low temperatures can provide principally LA as the major fatty acid in the methylated crude extract (Fig. 6b).

EFFECT OF DIFFERENT CARBON AND NITROGEN SOURCES

Lactose followed by Dextrin and Arabinose were found out to be better as a carbon source for LA production (Fig.7a). However, none of the carbon sources were as good as dextrin to provide good biomass in the culture broth. Thus overall yields were highest in our previous medium i.e. Medium 2. Three combinations of nitrogen source were used in this study and it was observed (Fig.7b) that yeast extract alone could neither supported premier growth nor LA production. Peptone showed LA as well as dry biomass similar to our control Medium i.e. M2. However, a combination of yeast extract with Peptone in the ratio of 1:1 produced almost pure LA in the culture broth.

PRODUCTION OF LINOLEIC ACID IN FERMENTER

Penicillium sp. ACR-D24 was grown under the optimized conditions in a 3L fermenter for LA production for 10 days. About 50 ml sample was withdrawn each day starting from the 2 days of fermentation. It was observed that optimum level of LA (~75% of lipid extract) was produced after 7 days of fermentation in DPB medium (Fig. 8). An overall yield of 2.4 g/L LA production was achieved in the fermented broth of ACR-D24 after 7 days cultivation.

DISCUSSION

The limitations associated with the current sources of ω-3 fatty acids and ω-6 fatty acids from animal and plant sources have led to increased interest in microbial production. The main strategy to enhance the participation of the microorganisms in the industrial production of PUFA is screening for high PUFA producers, the manipulation of culture conditions to optimize PUFA production and the improvement of downstream processes [31].

The above characterized psychrotrophic *Penicillium* sp. strain ACR-D24 isolated as soil microbiotium of the majorly unexplored coldest place, Dras, J&K, India is shown to have the oleaginous potential of producing PUFA i.e. Linoleic acid. Earlier, *Penicillium chrysogenum, Penicillium roqueforti*, *Penicillium camemberti*,

etc. were found to be the potent producers of PUFAs mainly Linoleic acid (C18:2) and Palmitic acid (C16:0) , which accounted for about 80% of total Fatty acid content [32]. The strain ACR-D24 gave maximum yield 2.4 g/L LA on 7th day of fermentation from about 12g/L of dry biomass when incubated under shaking at 20^{o}C. DPB media was found to be the best out of the three media used as it comprises of Dextrin as carbon source and also well balanced combination of nitrogen sources with mineral salts like K_2HPO_4, NaH_2PO_4 and $MgSO_4$. According to previous report, higher growth rates were observed on phosphate addition [33]. The synergistic effect of the nitrogen sources i.e., Yeast Extract and Peptone in combination resulted in the better growth and greater yield of Linoleic acid. The fermentation period of seven days was found to be optimal for both the biomass and LA production and seems LA production to be an integral part of growth of the strain. The cold region isolate, *Penicillium* sp. is able to grow at a wide pH range between pH 5.0 to pH 9.0 with optimal cell growth and LA production at pH 7.5-8.0. Arachdionic acid productivity and cell growth were better under higher and lower pH conditions, respectively [34, 35] and found the optimal initial pH range for AA production and growth was 6.0-6.7 [10].

ACR-D24, a psychrotroph grows at a temperature range between 4˚C to 50˚C, with its optimal growth and LA production at low temperatures mainly between 10˚C to 20˚C. The degree of unsaturation in the fatty acid skeleton is known to be influenced by temperature, i.e. lowering the temperature tend to increase the proportion of unsaturated acids. An earlier study with fungus has shown increased production of GLA at low growth temperature with a corresponding increment in the degree of unsaturation of total lipids. The results of this study showed that the lower growth temperatures stimulate the biosynthesis of highly unsaturated fatty acids, an observation for some mesophilic and psychrophilic Mucor species [36]. The effects of temperature on the degree of lipid unsaturation may be exerted through the influence of temperature on oxygen tension of the media as Oxygen is a necessary cofactor in enzymatic desaturation, resulting in lower levels of unsaturated fatty acids with increase in temperature [37]. Accumulation of unsaturated fatty acids at low temperature could be exploited as a resource for increased biosynthesis of LA by *Penicillium* sp. The strain, being a psychrotroph, can tolerate a range of temperature (i.e. 4˚C to 50˚C) and thus can be employed commercially.

From the results, it could be concluded that the psychrotrophic strain ACR-D24, *Penicillium* sp. isolated from extreme cold habitat could be a potential source of LA. Medium 2 i.e. Dextrin Peptone broth was better choice for growing ACR-D24 for LA production with 6% inoculum and 7 days fermentation time. Optimum pH and temperature for LA production was determined to be pH 7.5 - 8.0 and 20^{o}C respectively. Combination of Peptone and yeast extract in the ratio of 1:1 exerted more impact on fungal metabolism to produce LA, while using dextrin as carbon source. The optimized conditions could produce about 75% Linoleic acid in the methylated crude extract of fermentation broth. An overall yield of 2.4 g/l LA production was achieved in the fermented broth of ACR-D24, *Penicillium* sp. after 7 days cultivation, as compared to the 1.8 g/L in the shake flask experiments. The isolate could be exploited further for derivatization of Linoleic acid and can be used for enrichment as nutraceutical or for conversion into pharmacologically active molecules.

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COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST: The authors declared no competing interests.

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FIGURES

Fig 1a: *Penicillium* sp. strain ACR-D24 isolated from the cold Shivalik region

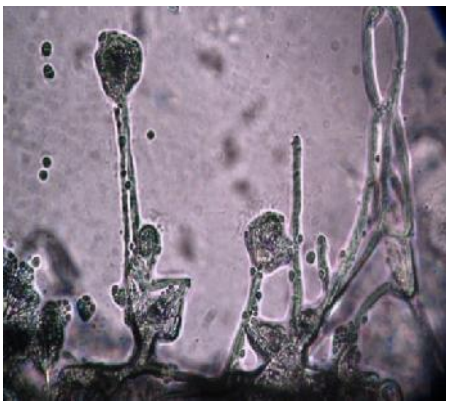

Fig 1b: Microscopic view of the strain *Penicillium* sp. strain ACR-D24

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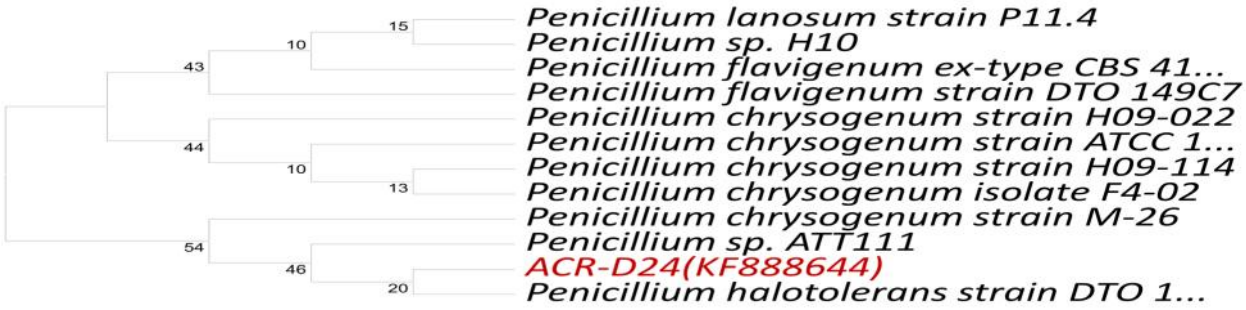

Fig 1c: Phylogenetic tree of strain ACR-D24. The evolutionary history was inferred using the Neighbor-Joining method [Saitou and Nei, 1987]. The evolutionary distances were computed using the Maximum Composite Likelihood method [Tamura et al., 2004] and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 4.0 [Tamura et al., 2007].

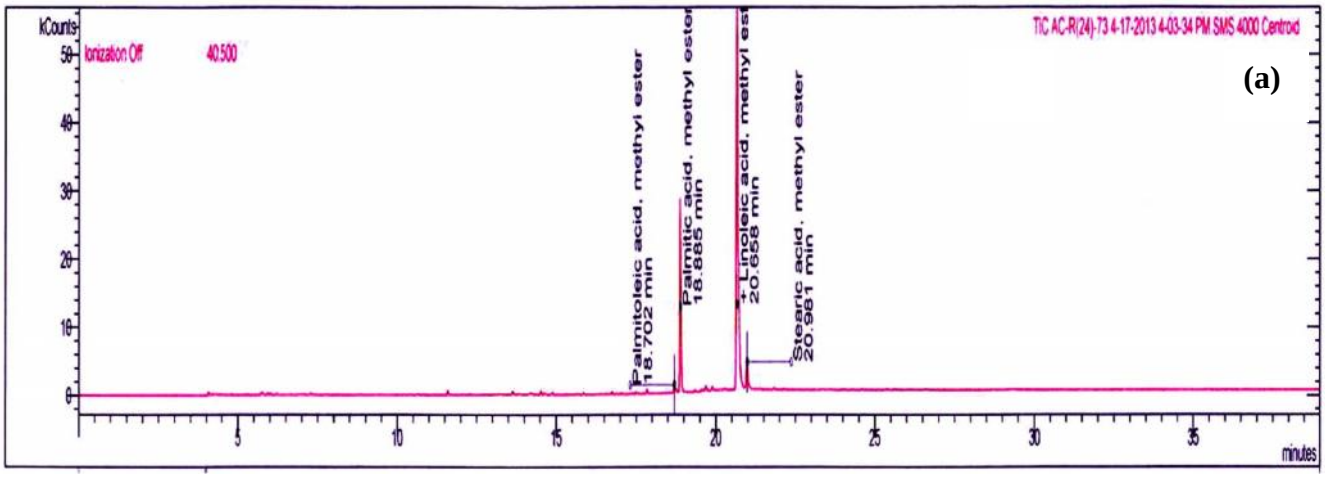

Fig 2a: GC-MS plot of methyl ester extract of fermentation broth of *Penicillium* sp. strain ACR-D24 grown in Medium 1

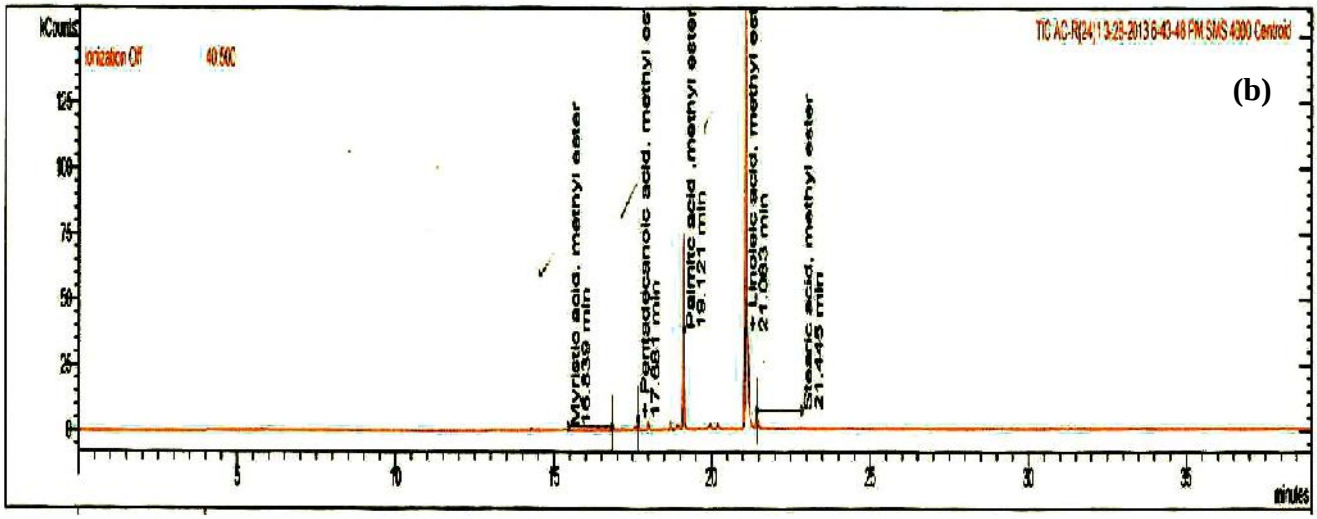

Fig 2b: GC-MS plot of methyl ester extract of fermentation broth of *Penicillium* sp. strain ACR-D24 grown in Medium 2

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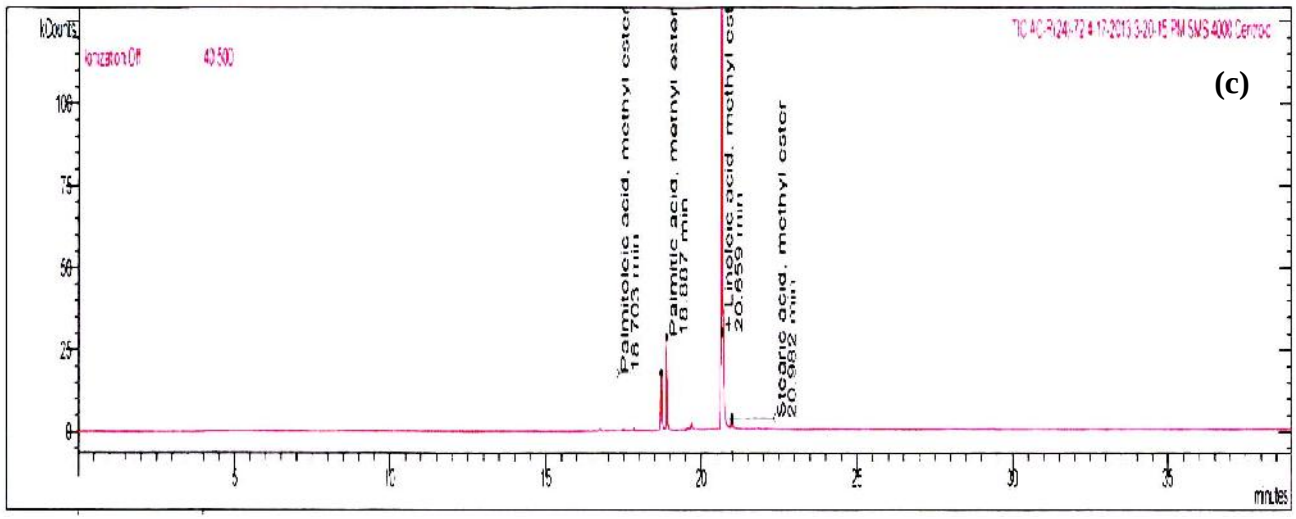

Fig 2c: GC-MS plot of methyl ester extract of fermentation broth of *Penicillium* sp. strain ACR-D24 grown in Medium 3

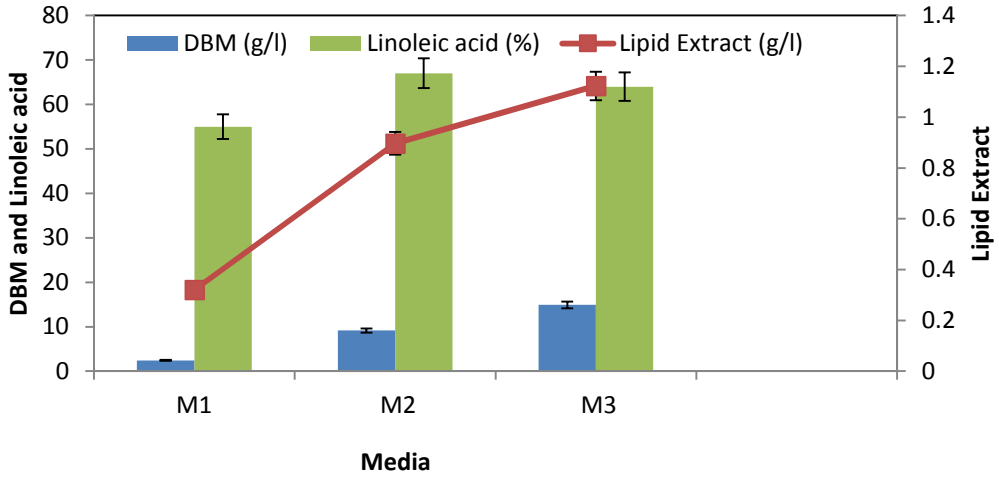

Fig 3: Effect of media on LA production from *Penicillium* sp. strain ACR-D24

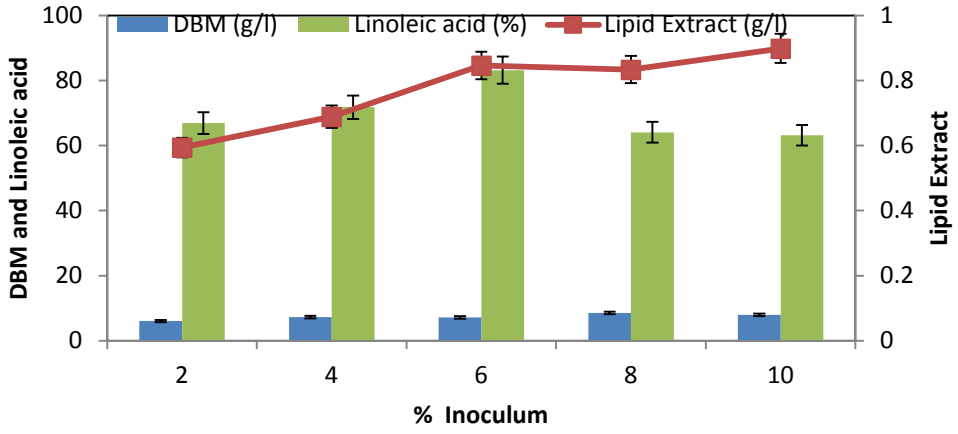

Fig 4: Effect of seed inoculum on LA production from *Penicillium* sp. strain ACR-D24

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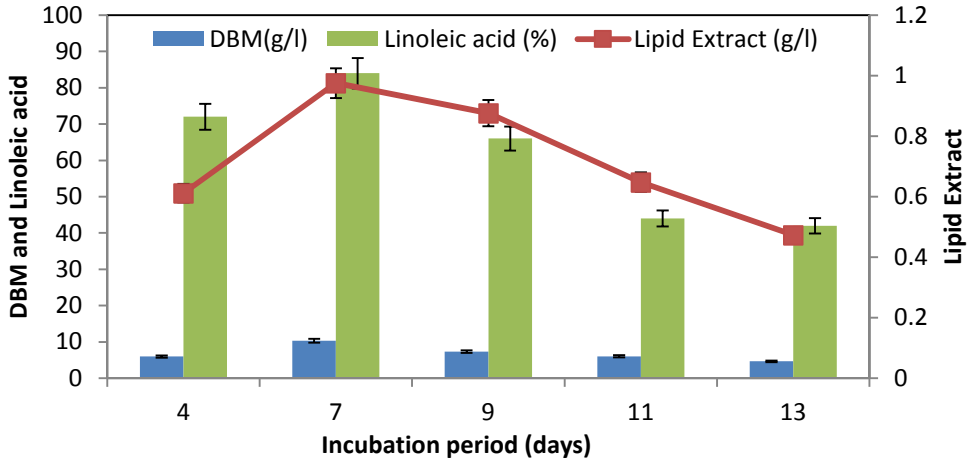

Fig 5: Effect of fermentation period on LA production from *Penicillium* sp. ACR-D24

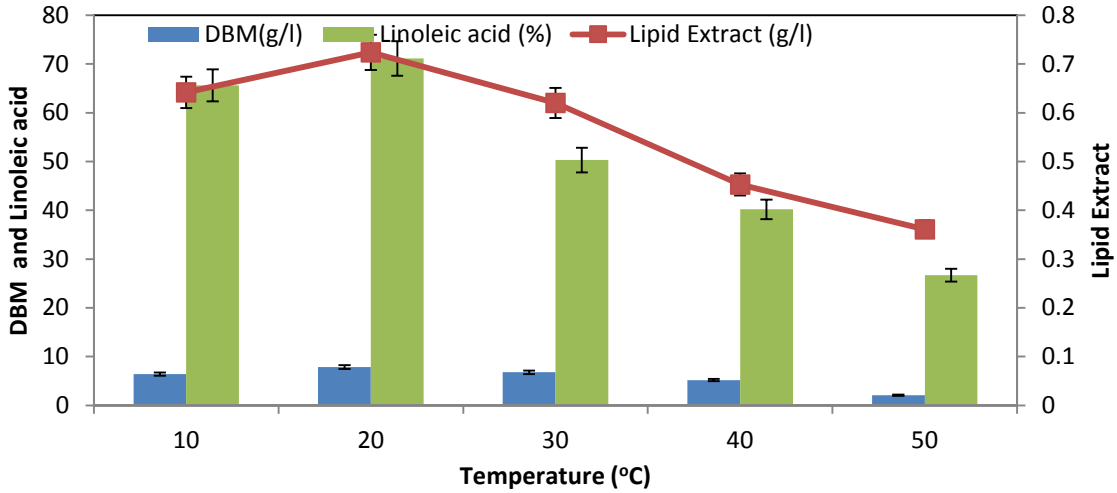

Fig 6a: Effect of temperature on LA production from *Penicillium* sp. strain ACR-D24

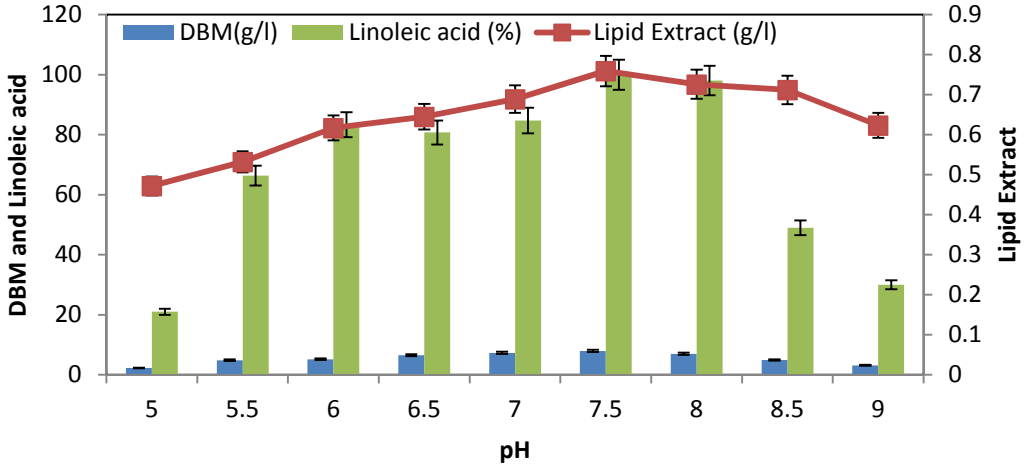

Fig 6b: Effect of initial pH on LA production from *Penicillium* sp. strain ACR-D24

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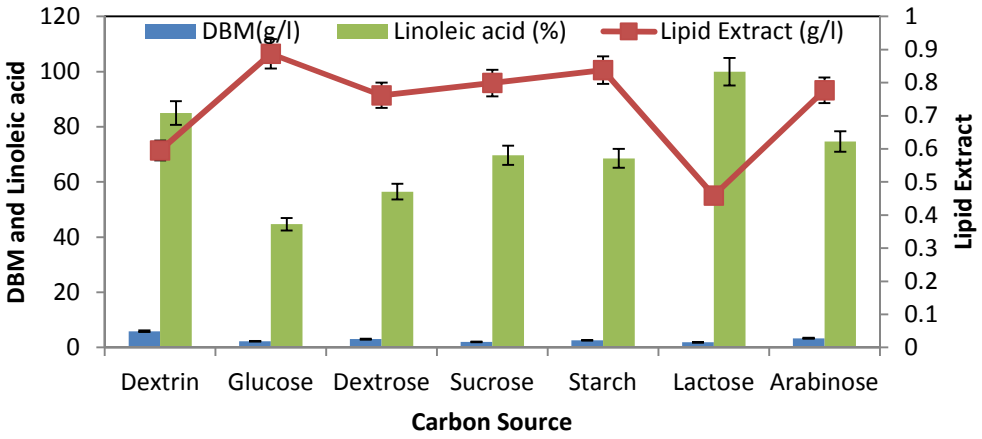

Fig 7a: Effect of carbon source on LA production from *Penicillium* sp. ACR-D24

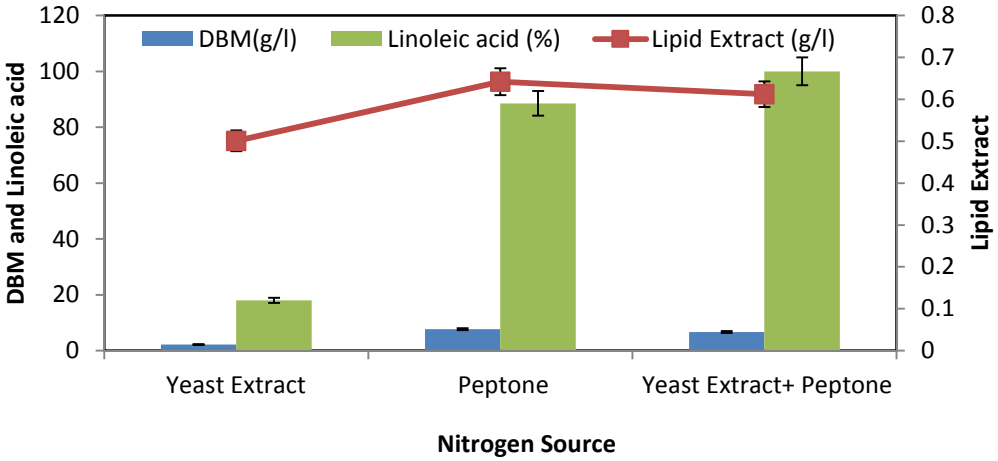

Fig 7b: Effect of nitrogen source on LA production from *Penicillium* sp. strain ACR-D24

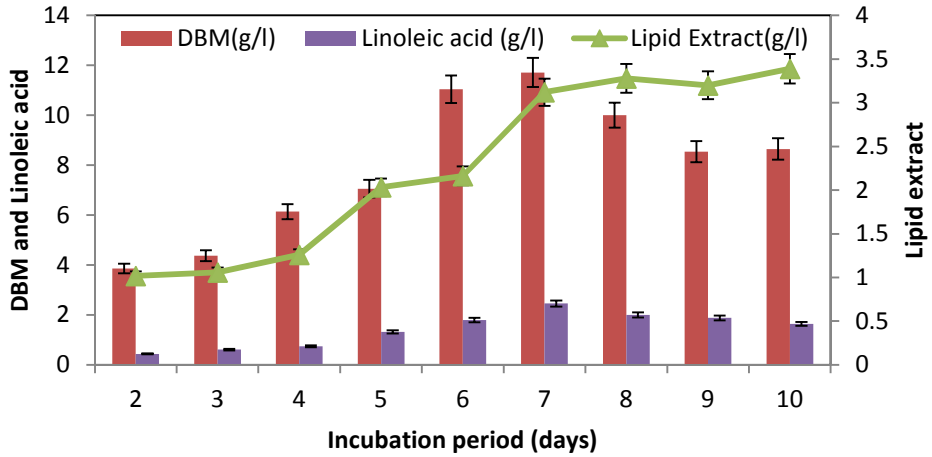

Fig 8: Fermenter studies for optimal production of LA from *Penicillium* sp. ACR-D24