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Biochemical and molecular studies of *Jatropha curcas* L. - Biofuel species

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ABSTRACT

Fossil fuels plays vital role in the development of the nation. The limitation of the Non-renewable energy required alternative energy sources such as solar, wind energy, hydro energy and biomass energy etc., and these renewable energies are eco-friendly in nature. The study has conducted for the determination of fatty acid profile in *Jatropha curcas* oil, determination of restriction sites in genomic DNA of *Jatropha curcas*, comparison of DGAT gene sequence alignment of different plants using Clustal W software. and PCR amplification of DGAT gene. The results confirmed that the solvent extraction produced high quality oil. From the thin layer chromatography plate, the saturated fatty acid like Linoleinic acid (0.14), Palmatic acid (0.38), Stearic acid (0.72), and unsaturated fatty acid like and Oleic acid (0.90) were identified. The genomic DNA was restriction digested with EcoRI, BamHI, PstI, HindIII. The observed that restriction bands for EcoRI was 3 sites and for Hind III was 1 site respectively. The amplification of gene encoding DGAT gene from the genomic DNA was carried out by polymerase chain reaction and also by bioinformatics software and was found to be approximately 2 kb size. Our study makes initial step for altering of the *Jatropha* seed oil for enhancing the oil content level.

Keywords: *Jatropha curcas,* Biofuel, DGAT, biodiesel, genomic DNA and Triglycerides

1. INTRODUCTION

Jatropha curcas has been considered as bioenergy crop and is also known as renewable energy source with many promising benefits. With the growing interest in biofuel in most of the countries, there is need for the National governments to develop mechanisms for harnessing the potential of the fast growing industry and benefit from the growing international trade in biofuels. The *J. curcas* plant is traditionally used for the medicinal purposes, is a strong shrub that can grow even on the poor soils and areas of very low rainfall (from 250 mm a year) hence it is being promoted as an ideal plant for the small farmers (Sarin et al., 2007; Foidl et al., 1996; Gressel, 2008). In view of the fact that, the *Jatropha* can be grown relatively well in marginal areas as compared to other traditional crops, it may help to reclaim the degraded land and protecting soil from the soil erosion. The trees are easy to establish (from seeds or cuttings), grow relatively quickly (producing seed after their second year) and are hardy to drought. On an average, each mature plant produces about 4 kgs of seed per year when the plant cultivated under optimal conditions. *J. curcas* has a long productive period of about 30-50 years (Banapurmath et al., 2008; Tamalampundi et al., 2008).

Scientific Classification

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Malpighiales*
Family: *Euphorbiaceae*
Genus: *Jatropha*
Species: curcas

Diacyl Glycerol Acyl transferase (DGAT)

Diacylgylcerol acyltransferase (DGAT: EC 2.3.1.20) is the most important enzyme in Kennedy pathway that is exclusively involved in the synthesis of storage oil in plants. In this study, identification and amplification of DGAT gene from *Jatropha curcas*, an important oil seed crop is reported. Metabolic pathway engineering in oil seed crop is burgeoning and promising technique to obtain a desirable oil quality and more yield for biodiesel production and to further applications. Fatty acid biosynthesis and assembly into triacylglycerol (TGA) are highly regulated at the biochemical level. Thus, identification and amplification of respective enzyme in this pathway is the major importance for the genetic manipulation. In plants, the fatty acid biosynthesis has been originated localized in the plastids and then exported to endoplasmic reticulum for the synthesis of triacylglycerides (TAG) through the enzymes of Kennedy pathway.

Triacyl glycerol Biosynthesis

The main storage lipids in plants are the triacylglycerides (TAG). TAG is the synthesized via the so called Kennedy pathway. This pathway operates in the Endoplasmic reticulum and TAG accumulates in structure known as "oil bodies" which are the surrounded by the phospholipid membrane.

The biosynthesis of triacylglycerides (TAGs) in oilseed plants involves three stages. The first is synthesis in the plastids, followed by modification of the fats catalyzed by enzymes in the endoplasmic reticulum. In the final stage the newly synthesized TAGs are stored in oil bodies (oleosomes) derived from the endoplasmic reticulum (Quettier and Eastmond, 2009).
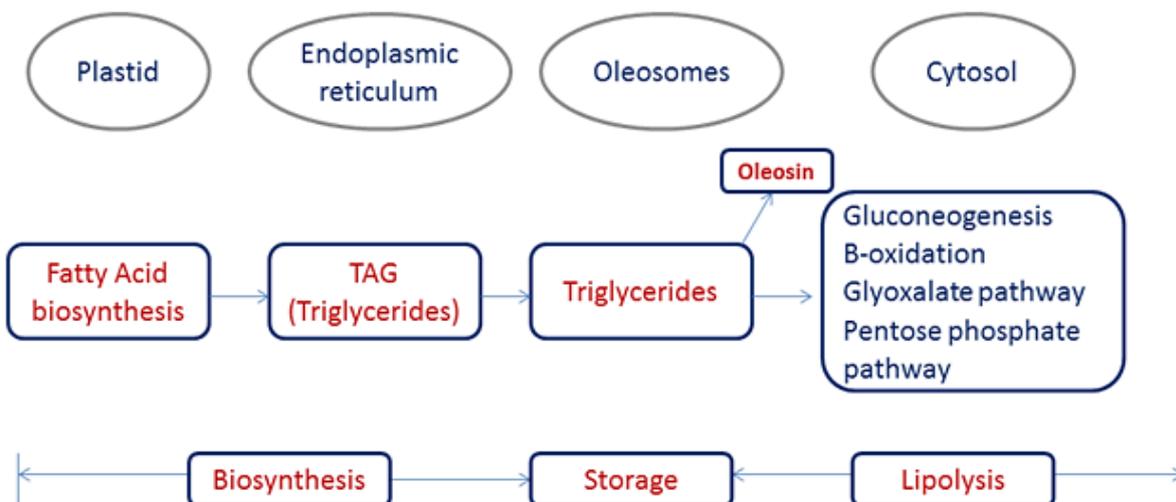

**Fig. 1.** The TAG Synthesis and Breakdown cycle

Kennedy Pathway

Biosynthesis of TAGs in developing seeds follows the Kennedy pathway in a reaction catalyzed by acyl CoA: diacylglycerol acyltransferase - DGAT (Baud and Lepiniec, 2009). A second pathway mediated by phospholipid: diacylglycerol acyltransferase (PDAT) has been described. This pathway can also be catalyzed by DGAT using two molecules of DAG to produce TAG and monoglycerol.

Various factors mediate fatty acid synthesis at the transcriptional, translational and enzyme activity level (Baud and Lepiniec, 2009). Lipid profiles in developing *J. curcas* seed have been studied. Lipid biosynthesis starts during the early stages of development, immediately after fertilization, and proceeds until maturation of the plant. It involves early development (till 22 days after fertilization) and maturation stages (Baud and Lepiniec, 2009).

DGAT plays vital role in a) encoding polynucleotide was useful for increasing TAG synthesis, b)Seed oil content, c) Oil quality in plants, d) Potential target in the genetic modification of plant lipid biosynthesis, e) Increased DGAT activity in plant oil seeds could lead to increased seed oil content and improvements in the fatty acid composition of the oil and f)DGAT activity may potentially effect cellular signal transduction.
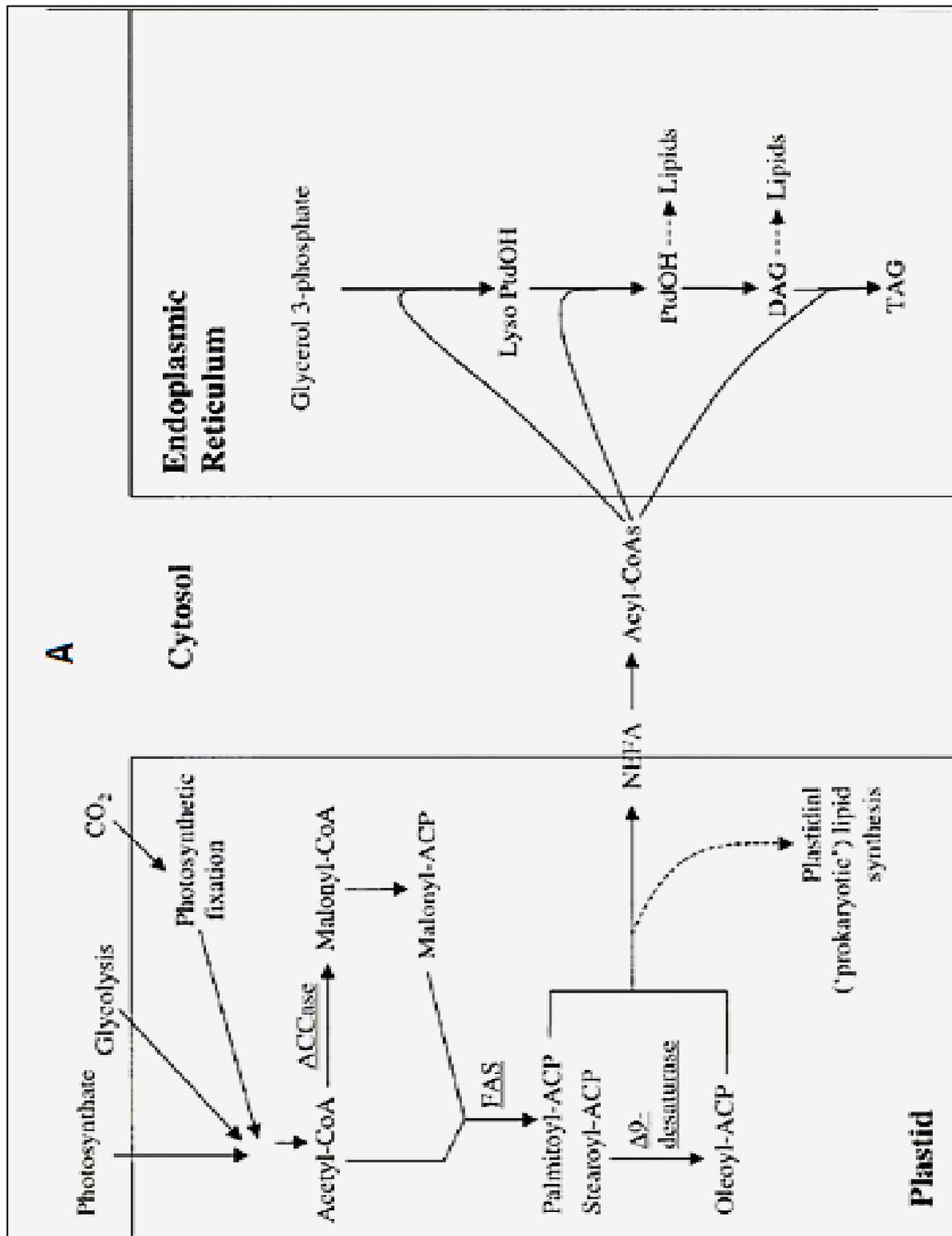
Fig. 2A. Kennedy pathway
(Source: Cagliari A *et al.,* 2011)
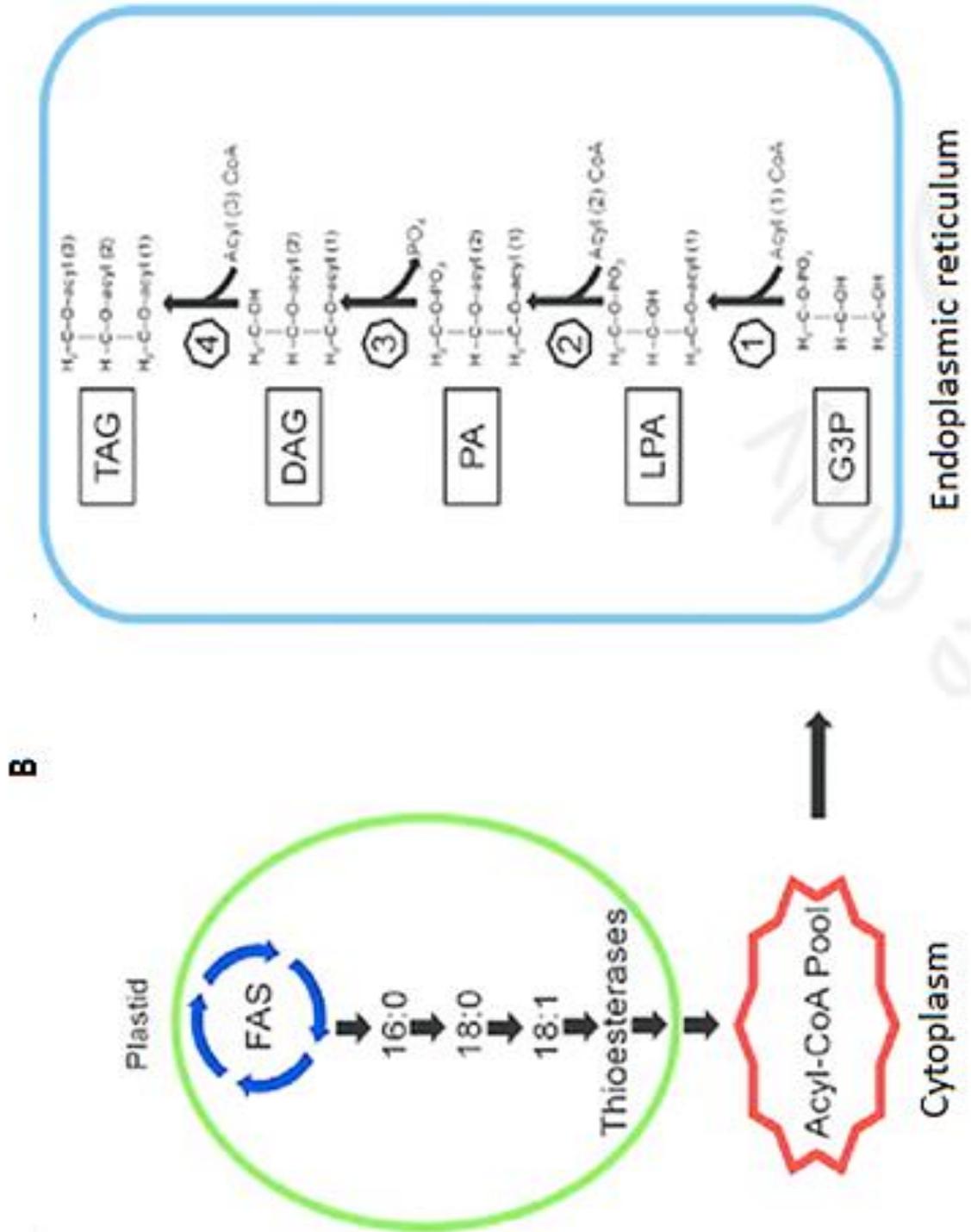
Fig. 2B. Kennedy pathway
(Source: Cagliari A *et al.*, 2011)

2. MATERIALS AND METHODS

Plant materials

Jatropha curcas seeds were collected from the Biodiesel Technology park, Gulbarga University, Gulbarga-Karnataka-India.

Soxhlet extraction

The method described by the Soxhlet (1879) method applied to extraction of lipids. According to the Soxhlet method, oil and fat from the solid material is extracted by the repeated washing with the organic solvent. Normally, hexane or petroleum ether under reflux in a special glassware.

Oil Extraction

The 500 g kernels of *Jatropha curcas* were dried, ground into small particles and placed in a porous cellulose thimble. The thimble was placed in an Soxhlet extraction chamber, which was suspended above a flask containing the solvent petroleum ether (Boiling point 40-60 °C) below the condenser. The flask was heated till the solvent evaporates and moves up into condenser where it was converted into a liquid that trickles into extraction chamber containing the sample. The solvent in the flask was evaporated and the mass of the remaining lipid (oil) was measured. The percentage of lipid in the initial sample was recorded.

Fig. 3. Soxhlet Unit

Thin layer chromatography (TLC)

Slurry of silica gel prepared in 0.02M sodium acetate buffer and spread 250 µm thick on glass plate. After spreading, were activated by heating at 105 °C for 30 minutes. 5 µl of sample is spotted as in the plate divided in a solvent containing Petroleum: Diethyl ether: Acetone (90:10:1). After development, that is the solvent as the top, plate is dried in a stream of warm air and sprayed with developing reagent; the plate is heated in the incubator and calculates the R_f values of the spots.

Fatty acid determination by High Performance liquid Chromatography (HPLC)

Fatty acid determination was carried out with HPLC system and the sample was exposed to high temperature during this process decomposition or isomerization of fatty acid derivatives were analyzed. Areas of the peak forms corresponding component amounts, peak forms and reduction times were studied.

Oil: *Jatropha* oil was extracted by soxhlet method was usedanalysis.

Chemicals:

Mobile Phase: Methanol

Solvent for the sample: Methanol

Column used: C18

Plant genomic DNA extraction by CTAB method.

The plant genomic DNA from the *Jatropha* leaves sample was extracted using CTAB method (Doyle and Doyle, 1990) with little modification in the extraction buffer (Tris HCl-100 mM (pH-8.0); NaCl (1.5M); EDTA-25 mM (pH-8.0); CTAB (3%); activated charcoal (2%); 1%-PVP and β-mercaptoethanol-0.2%).

200 mg leaf sample was crushed in 2 ml of extraction buffer by the pre-sterilized mortar and pestle; later the extract was transferred to 2.0 ml eppendrof tubes and incubated in a water bath at 65 °C for 50-60 min with intermittent mixing. The eppendrof tubes were centrifuged for at 10,000 rpm 10 min at 4 °C.

The supernatant was transferred to fresh eppendrof tubes and equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) added slowly and vortex. Later, the tubes were centrifuged at 10,000 rpm for 10 min at 4 °C.

The supernatant was taken in another fresh tube and mixed up with the equal volume chloroform: isoamyl alcohol (24:1) and again centrifuged at 10,000 rpm for 10 min at 4 °C; repeated the same step twice and to then later upper layer was transferred to a new tube. 1/10th of the 3M sodium acetate and two to three volumes of chilled 80% ethanol were added for precipitation of genomic DNA.

The eppendrof tubes were centrifuged at 5000 rpm for 10 minutes at 4 °C. Lastly, the pellet was air dried and dissolved in 200 µl of sterile milliQ water. The purity of the DNA was visualized by agarose gel electrophoresis with 1 X TAE buffer and purity checked by spectrophotometric method at 260 nm/280 nm.

Purification of Genomic DNA

RNase Treatment

The isolated genomic DNA was further purified by treating with RNase to remove RNA. To 100 µl of DNA isolated sample 5µl of RNase (5 mg/ml) was added and incubated at 37 °C for 1 hour.

Proteinase K Treatment

After incubation with RNase, Proteinase K treatment was done to make the DNA free from proteins. To the RNase treated 100 µl DNA, 0.25 µl of Proteinase K (20 mg/ml) was added and incubated at 37 °C for 30 min. The reaction mixture was made up to 500 µl with TE buffer and extracted with saturated phenol, chloroform, isoamyl alcohol treatment.

Agarose gel electrophoresis:

Agarose 0.8% (w/v) was prepared in 1X TAE buffer pH 8.0 (for 50X TAE buffer, 242 g Tris-base, 37.2 g EDTA, pH is adjusted with glacial acetic acid) by boiling for homogeneity. The platform of electrophoresis was sealed on open side by leucoplast. The comb was adjusted to 1mm above the gel slab and 1cm from one sealed side. The molten agarose was poured on to the platform and allowed to set at room temperature. After setting the comb, leucoplast were carefully removed. The gel slab was placed in electrophoretic chamber and 1X TAE buffer was poured into the chamber till the gel was completely immersed in the buffer. DNA sample (10 µl) was mixed with 8 µl tracking dye bromo phenol blue (BPB) (50% v/v glycerol,1mM EDTA pH 8.0, 0.25% v/v xylene cyanol and distilled water) and the sample was loaded into the slots of agarose gel. The loaded sample was electrophorised at constant voltage (50 V/cm^2). After electrophoresis the gel was observed under attached gel documentation system.

Restriction Digestion

Table 1. Components of Restriction Digestion

Sl. No.	Components	Quantity (µl)
1.	Template DNA	2.0
2.	Distilled water	15.7
3.	10X assay Buffer (1X)	2.0
4.	Restriction enzymes	0.3
5.	**Total Reaction Mixture**	**20.0**

2 µl of template DNA was added into a microcentrifuge tube followed by 0.3 µl of Restriction enzymes – Eco RI, BamH I, Pst I and Hind III in respective tubes; 2 µl of 10X

Assay buffer and 15.7 µl of deionised water. The contents were mixed by gentle flicking and incubate at 37 °C for 2 hrs. The reaction was terminated by increasing temperature to 50 °C for 1 minute. The tubes were removed from water both and then tubes were centrifuged at 12,000 rpm for 2 min to obtain the pellet. The reaction mixture was run by Electrophoresis unit at 50–100Vollts and bands were observed under UV- Transilluminator.

Agarose gel electrophoresis

Agarose 1 % (w/v) was prepared in 1X TAE buffer pH 8.0 (for 50 X TAE buffers, 242 g Tris-base, 37.2 g EDTA, pH is adjusted with glacial acetic acid) by boiling for homogeneity. The platform of electrophoresis was sealed on open side by leucoplast. The comb was adjusted to 1mm above the gel slab and 1cm from one sealed side. The molten agarose was poured on to the platform and allowed to set at room temperature. After setting the comb, leucoplast were carefully removed.

The gel slab was placed in electrophoretic chamber and 1X TAE buffer was poured into the chamber till the gel was completely immersed in the buffer. Digested DNA sample with respective enzymes (10 µl) was mixed with 8µl tracking dye bromo phenol blue (BPB) (50% v/v glycerol, 1 mM EDTA pH8.0, 0.25% v/v xylene cyanol and distilled water) and the sample was loaded into the slots of agarose gel. The loaded sample was electrophorised at constant voltage (50 V/cm^2). After electrophoresis the gel was observed under attached gel documentation system.

Primer Designing

The nucleotide sequences of the DGAT gene was collected from the database NCBI (Gen bank) for plants namely cDNA sequences of *Brassica napus* (DGAT1), *Nicotiana tabaccum* and *Arabidopsis thaliana* (DGAT1) were used and pasted all the sequences in FASTA format (starting with ' > ' symbol) in notepad and save it for further usage. Open the Clustal W software and then load the saved sequences. Select the complete alignment option of a window and the output files were saved. Clustal W will start analyzing the sequences; it compares all the sequences by keeping one sequence as a reference sequence and search for the homology with other sequence and also to identify conserved sequence in the gene with the help of CLUSTAL W Multiple sequence alignment tool (http://www.genome.jp).

The output result file will be generated and saved as (.aln) file. The (.aln) file is used for viewing alignment of the sequences for conserved regions in CHROMA. The conserved sequences were analyzed and primer designing was done with the help of softwares **Primer3 or Oligo4,** which is Windows and DOS based operating software for primer designing respectively.

Based on this, a set of gene specific primers were designed taking care that no intron interrupted the primers after observing their location on the *A. thaliana* DGAT. The primers were custom synthesized from Bioserve Pvt. Ltd, Hyderabad. The sequences of forward and reverse primer were:

DGATf1: 5' atggcgattttggattct 3', Tm 53 °C and
DGATr1: 5' accaatctttgtagaattc 3' Tm 51 °C respectively.

The following parameters were considered while designing a primer:

➢ Primer should be 18-20 bp in long.

➢ The melting temperature (T_m) difference between the two primers should be less than 1°C

➢ The GC content should be 45-60% for a stable primer and length of the amplified product were also analyzed.

➢ No primer pair complementarities.

➢ There should not be any dimer formation.

➢ Get down of three or more Cs or Gs at 3'ends of primers may promote mispairing at G or C rich sequence (because of which stable annealing could be avoided).

PCR amplification

The amplification of DGAT gene in genomic DNA was carried out by polymerase chain reaction (PCR) by using 18 bp forward and 19 bp reverse oligo primers. The oligo nucleotide primer pair was synthesized at Bioserve Biotechnologies Pvt. Ltd. Hyderabad, India.

Forward primer: 5' ATGGCGATTTTGGATTCT 3'   Tm 53 °C
Reverse primer: 5' ACCAATCTTTGTAGAATTC 3' Tm 51 °C.

The reaction mixture was standardized by several trials and final reaction mixture.

Table 2. Components of PCR reaction mixture

Component	Quantity (µl)	Final concentration
Taq assay buffer	2.0	1X
Genomic DNA (template)	2.0	20 ng/µl
Primers (Forward& Reverse)	1.0 (each)	10 picomole/µl
dNTP's	1.0	200 µM/µl
Taq DNA Polymerase	0.3	1 unit
Distilled water (HPLC grade)	12.7	
Total (Reaction mixture)	20.0	

The contents in the tube were mixed thoroughly. The Palm cycler (Corrbett life science) was programmed for 35 cycles. Each cycle involved the following steps in which the steps 2-4 were repeated 35 times.

Table 3. PCR amplification Step

SL. No	Steps	Temperature (°C)	Time
1.	Step 1 (initial denaturation)	94.0	2 min
2.	Step 2 (denaturation)	94.0	1 min
3.	Step 3 (annealing)	53.0	1 min
4.	Step 4 (Extension)	72.0	2 min
5.	Step 5 (final extension)	72.0	8 min
6.	Step 6 (End)	4.0	4 hrs

The amplified product was electrophorised on 2% agarose gel with 100bp DNA marker and electrophoretic profile was documented using photo gel documentation system (Vilber Lourmat, France) and photograph was printed using thermal printer (Sony Corporation, Japan).

Purification of PCR product

The amplified PCR product was purified by HipurA PCR product purification spin kit. 500 µl of PCR binding solution was added to 100 µl of PCR sample and mixed well. This mixture was then applied to Hi Elute Miniprep spin column and centrifuged for 1 minute at 10,000 x g (13,000 rpm). The flow was discarded and the column was replaced in same collection tube. 700 µl of wash solution was added to the column and centrifuged for 1 min at 10,000 x g (13,000 rpm). The flow through was discarded and column was replaced in a clean collection tube. Now, 50 µl of Elution buffer was added to the center of Hi Elute Miniprep spin column and centrifuged for 1 min at 10,000 x g (13,000 rpm). The presence of PCR amplification product in elute was confirmed by Agarose Gel electrophoresis. The PCR product was immediately used as the template for the amplification of the coding region of the DGAT gene.

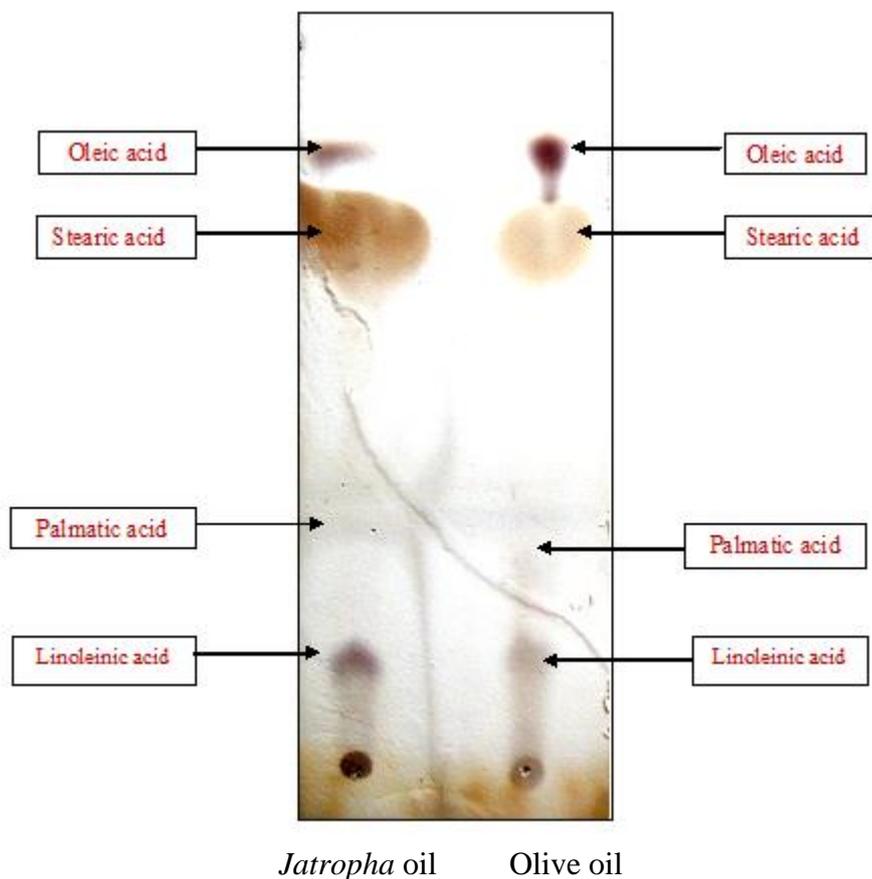## 3. RESULTS

Soxhlet Extraction

Using the petroleum ether as solvent in soxhlet extraction unit after 12 hrs we were extracted 100 ml of Jatropha oil and further we sent that oil sample for fatty acid analysis.

Thin Layer Chromatography

The fatty acid profile present in the crude *J. curcas* oil sample was identified by TLC comparision with standard Olive oil. With reference to the R_f value of the spots identified on the TLC plate saturated fatty acid like Linoleinic acid (0.14) Palmatic acid (0.38), Stearic acid (0.72), and unsaturated fatty acid like and Oleic acid (0.90) were identified (Table 4).

Table 4. TLC Plate showing lipid components

Spot No.	R_f of Jatropha oil sample	R_f of Olive oil sample	Fatty acids
1	0.14	0.16	Linoleinic acid
2	0.38	0.44	Palmatic acid
3	0.72	0.73	Stearic acid
4	0.90	0.92	Oleic acid

Jatropha oil   Olive oil

Fig. 4. TLC plate showing lipid components

Fatty acid determination by HPLC

REPORT

Sample Name: Plant extract   Data File: ...Venkatesh03.DAT
Method File: C:\Indtech\WinchromEx\User1\GK.Met
Detector: UV-VIS.   System: HPLC
Date: 16 Sep 2010   Time: 15:18:4
Run: ch1: 6
Type of Analysis : Percent On Area and Height
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	30.0

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	1.94	491	138336	0.7290	0.2294	BV	0.192
2	2.21	851	275982	1.2636	0.4577	VP	0.221
3	2.69	0	8767	0.0000	0.0145	PB	0.136
4	3.07	411	124676	0.6103	0.2068	BV	0.207
5	3.36	193	44805	0.2866	0.0743	VB	0.158
6	4.69	0	8167	0.0000	0.0135	BB	0.199
7	6.41	0	9621	0.0000	0.0160	BB	0.299
8	6.82	319	121242	0.4737	0.2011	BV	0.259
9	7.49	9353	6139542	13.8874	10.1814	VB	0.448

Fig. 5. Determination of fatty acids by HPLC

Isolation of Genomic DNA

Genomic DNA was isolated by Doyle and Doyle (1990) method and purified by using RNase and Proteinase K treatment. Purified genomic DNA was analyzed with 0.8 % Agarose gel and thick clear band was observed under UV transilluminator after staining with ethidium bromide (Fig. 6).

Fig. 6. Genomic DNA

Restriction digestion

The purified genomic DNA was restriction digested with Eco RI, BamH I, Pst I and Hind III and restriction digested was run on 0.8 % agarose gel and clear band was observed under UV transilluminator after staining with ethidium bromide and we observed Restricted bands for Eco R1 is 3 sites and for Hind III 1site (Fig)and these results are satisfactory with *Brassica jucea* (Ilaiyaraja N, et al., 2008).

Lane 1- lambda digested DNA
Lane 2- Bam H1
Lane 3- Hind III
Lane 4- Pst I
Lane 5- ECoRI
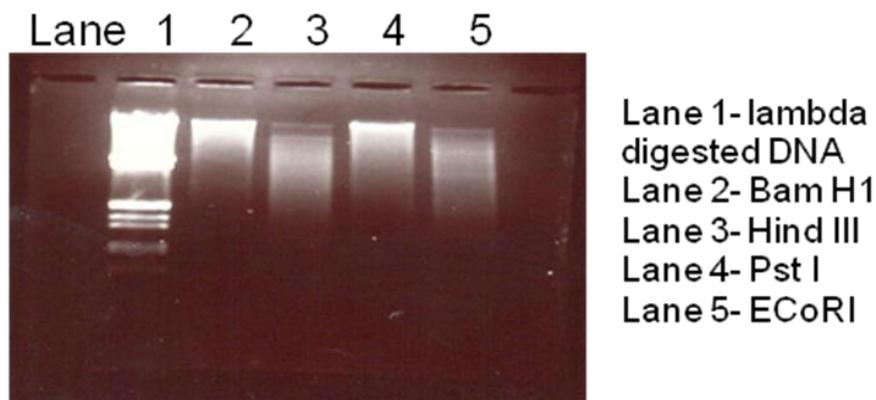
Fig. 7. Restriction digests using Eco R1 and Hind III

Primer Designing

Primer specificity is an important parameter in PCR primer designing. To amplify only the intended fragment, the primer should bind to target sequence only and not anywhere else. The multiple alignment of retrieved sequences revealed a highly conserved sequence in the DGAT genes of plants viz, *Brasicca napus*, *Arabidopsis thaliana* and *Nicotiana tabaccum*. The presence of highly conserved regions helped further in designing of primers. The successful primer was selected with each oligo having 18 and 19 nucleotides and sequences were:

Forward primer was 5' ATGGCGATTTTGGATTCT 3'
Reverse primer was 5' ACCAATCTTTGTAGAATTC 3'

The primers are having annealing temperature of 53 °C; as the primer length and GC content increases, the annealing temperature also increases because of more hydrogen bonds (3 hydrogen bonds between G-C and 2 hydrogen bond between A-T). Primer length not only affects the Tm but also uniqueness of the sequences in the template.
Primer paired is having 50% GC content with 1 °C of melting temperature difference (Fig. 2). If the percentage of GC content is less than 45 %, then non specific primer annealing takes place. The primer dimmer formation was not observed for above pair of primers. Dimer acts as competitor to amplification of target DNA.

PCR amplification

The amplification of gene encoding DGAT gene from genomic DNA was carried out by Polymerase Chain Reaction using 18 and 19 bp primers. The PCR amplified product obtained after 35 cycles was run on 2% agarose gel and observed under UV transilluminator.
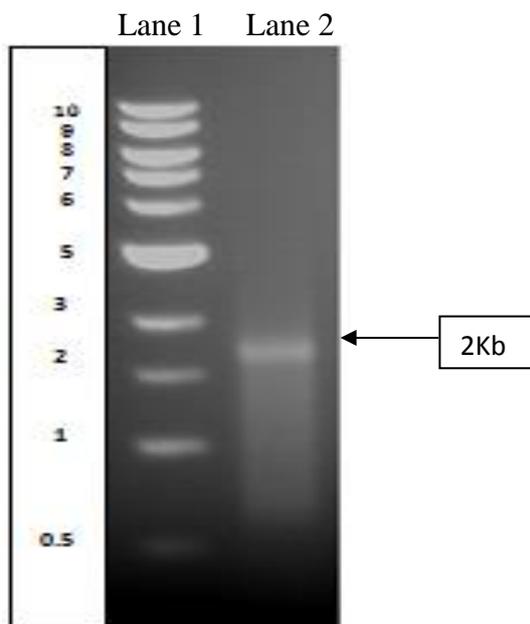
Fig. 8. PCR Product with 10Kb Ladder,
Lane 1- DNA ladder 10kb**,** Lane 2- Amplified gene of DGAT

4. DISSCUSION

The utility of any vegetable oil largely depends upon its chemical properties and fatty acid composition. High content of poly unsaturated fatty acids in jatropha oil meets its acceptability for biodiesel purpose. Fatty acid composition of Jatropha seeds very among its population. The variation is due to the variations of the enzymes related to the fatty acid synthesis. Molecular studies on Diacyl glycerol acyl transferase (DGAT) has been studied in many plants. Zou et al, (1999) reported that *Arabidopsis thaliana* with reduced DGAT activity showed the fatty acid composition rich in short chain unsaturated fatty acid and pour in long chain unsaturated fatty acid. A better understanding of DGAT modulators may provide new opportunities to enhance TAG accumulation in seed plants.

DGAT transcripts were reported earlier from micropsore derived cell suspension culture of *Brassica napus napus* (Nykifornk et al., 1999) and in olive drupe tissues (Giannoulia et al., 2000). Role of DGAT enzymes with localization in mustard tissues remaining to be determined. DGAT sequence vary among different plant species clustal W alignment for DGAT gene revealed that divergence between DGATs was concentrated in the N-terminal while many conserved regions were present in the middle and towards the C-terminal region.

Although the regulatory role of DGAT in oil synthesis has been suggested (Hobbs et al., 1999), complete understanding of the regulation of Kennedy pathway which is vital for the national engineering transgenic oil crops is still lacking. Our study makes initial step for altering of seed oil profile and in enhancing the oil levels

5. CONCLUSIONS

The funeral feeling is that first generation biofuels are not far from reaching saturation because of the limited availability of usable land. India still has some additional Doom for Jatropha, and promptly its plantation in exclusive waste lands. Jatropha biodiesel seems to be generally positive. Clearly *Jatropha curcas* L has huge potential has an biodiesel crop. At present lot of research is moving forward in different countries towards commercial production in landing research on cultivation based improvement management techniques and production technology.

However, the productivity curve for seeds is unclear and in many cases cultivation is going ahead even while the information on yields ,suitable land for cultivation and other matters is in accurate moreover hasty investment activities could ultimately hinder the steady expansion of the *Jatropha curcas* market, hence the properties of *Jatropha curcas* oil, processing technologies and downstream application technologies. Right now the most important challenges are to first of all to obtain high oil content in Jatropha seeds. Increase in high oil content in jatropha seed will make it to stand first in biodiesel producing species. Some crops such as palm oil, dubber, tea, soyabeans, corns are best example for the high oil content seed bearing plants.

As a new oil crops *Jatropha curcas* still in the initial stage and needs a slight modifications in oil content of seeds. Characterization of DGAT gene will develop a initial investment for making *Jatropha curcas* as a major raw material on fuel market.

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