cDNA Cloning of Stearoylacp Desaturase (*sad*) Gene from Palm Oil (*Elaeis guineensis*)

Ira Djajanegara[1], Lollie A. Putri [2], Indra Eko Setiyo[3], Dwi Asmono[3]

[1] Center of Bioindustry BPPT
[2] Agriculture Faculty - North Sumatera University (USU)
[3] Center for Palm Oil Research (PPKS) Medan

Abstract

As one of the source of edible oil used in food, the quality of palm oil becomes an important parameter. Nowadays, consumers demand edible oil which is high in unsaturated fatty acid due to the link of saturated fatty acid with cholesterol. In order to overcome the slow process of breeding program due to the length of the life cycle of palm oil, transgenic palm oil with higher unsaturaed fatty acid is constructed. The first step to achieve this goal is cloning the target gene from the palm oil mesocarp using PCR method with cDNA as the template. In this experiment, cDNA was synthesized from total RNA isolated from palm oil mesocap and specific primer pairs was designed. At the end of this experment, a 541 bp DNA fragment was obtained and proved by sequencing as a fragment of gene encoding Stearoyl ACP Desaturase (sad) from palm oil (Elaeis guineensis) mesocarp.

Keywords: *sad*, cDNA cloning, palm oil, PCR

Introduction

Palm oil is the second largest source of edible oil in the world, which is produced in the tropical countries (Scowcroft, 1990). It is used as the most price competitive liquid cooking oil in many parts of the world. It is also used in the making of other food products like shortenings, margarines (Sudin et.al., 1993) and spreads (Pantzaris, 1993). The challenge that the oil palm industry will face in the next 21st century is the ability to mantain profitability in the face of labor shortage and limited land resources. At present, palm oil is contributing around 20% of world oil and fat production. It is predicted that the demand for oil will grow faster than the rise and supply (Oil World Annual, 2001). By the year 2020, it is expected that nearly 2% of the world's oil and fat demand will have to be met by palm oil and it will capture approximately 50% of the world's oil and fat trade (Rajanaidu and Jalani, 1995). Due to this projected demand, it is important to increase the yield of oil palm as well as to improve the palm oil quality at a better rate than that has been achieved by conventional breeding (Parveez, 1998).

Raw oil extracted from palm oil composed of 40% palmitic acid (C 16: 0), 5% stearic acid (C 18: 0); 38% oleic acid (C18:1) and 11% linoleic acid (C18:2). Linoleic acid (C18:3) usually accounts for less 1% (Aziz et. al., 1986). In addition, edible oil from palm oil also rich in â-karoten (500-700 ppm) and vitamin E composed of *tocopherol* and *tocotrienol* (600-700 ppm) (Darnoko, 2001). Differences in the amount of Carbon chain backbone of the fatty acid determines the type of the oil fraction produced. In general, edible oil extracted from palm oil contains 1:1 of liquid fraction and solid fraction. The amount of liquid fraction will increase concomitantly following the increase of unsaturated fatty acid. At the moment, genetic program for palm oil improvement is concentrated on inceasing the liquid fraction of the oil by increasing the oleic acid content. This effort hopefully will increase the nutrition value as well as the economic

value of the oil which is required by the *oleo-chemical* and *oleo-food* industries.

The effort of integrating gene(s) controlling oil quality in palm oil through conventional breeding is expensive and time consuming. This is due to the long geneaton time (10 – 13 years), open pollinated behaviour and requirement of large amounts of planting material which all contribute to he slowness of conventional genetic improvement. The appeareance of sterility in some of intespecific mating cases and the difficulty of selection process because based on morphology appeareances are also adding to the existing problems. All the above limitations make oil palm conventional breeding program does not develop as fast as expected. Therefore another approach is required to complement the conventional breeding program of the pam oil.

Developments of oil palm tissue culture techniques, combined with the ability to transfer genes of inetrest into elite germplasm are attractive tools techniques with which to overcome the slowness of genetic improvement in this perennial crop. Additionally, genetic engineering can source genes from any plant, animal, bacteria, fungus or virus (Gasser and Fraley, 1992). Approximately, four to five years are required to produce transgenic plantlets carrying a new trait from initial date of explants culture. Taking into account the requirement of back-crossing in conventional breeding, genetic engieering could save 80-90% of the time required for introducing a new gene/trait into oil palm (Parveez, 1998).

Effort to manipulate the fatty acid content is basically genetic modification on the biochemical pathway of the fatty acid biosynhesis (Fig. 1). Several key enzymes in fatty acid biosynthesis such as *palmitoyl ACP desaturase* (PET), *β-ketoacyl ACP synthase II* (KAS II) and *δ9 stearoyl ACP desaturase* (SAD) have been used as the target for genetic transformation to increase the essential fatty acid content of palm oil (Parveez *et.al.*, 1999). Gene encoding *δ9 stearoyl ACP desaturase* (*sad*) from Indonesian palm oil has not been cloned and used in palm oil genetic engineering.

The main goal for genetic engineering of oil palm is to change oil quality and in particular to increase oleic acid content (Cheah et.al., 1995) which can be used as feedstock for oleochemical industries (Pryde, 1983). The transformation of oil palm with antisense

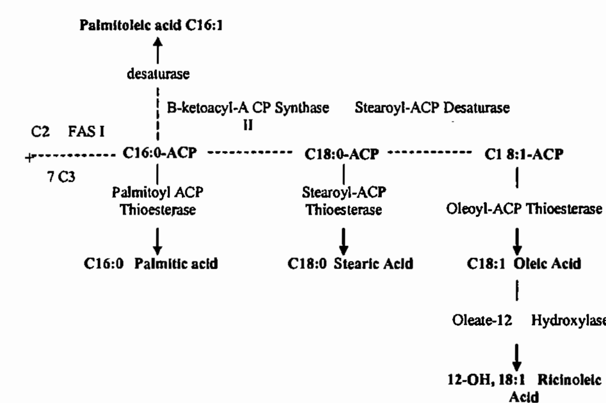

Figure 1. Fatty acid biosynthesis in palm oil

δ9 stearoyl ACP desaturase (SAD) is an important fatty acid biosynthetic enzyme responsible for the production of oleic acid. It is a soluble enzyme in the plastid which introduces a cis double bond into saturated stearoyl-ACP (18:0-ACP) at the Δ_9 to form saturated *mono-oleoyl-ACP* (18:1-ACP) (Fig. 1) (Abdullah *et.al.*, 2000). It has an important housekeeping role for producing unsaturated fatty acids for membrane lipid biosynthesis. At the oil accumulating tissues like anthers, seeds and mesocarp, it is involved in the developmentally regulated process of storage lipid biosynthesis. An important objective of the oil palm genetic engineering is to increae the level of oleic acid in the palm oil at the expense of palmitic acid (16:0). The strategy is to antisnese palmitoyl-ACP thioesterase (PET) and to increase expression of β-keto acyl –ACP-synthetase II (KAS II). Manipulation of the *δ9* stearoyl ACP desaturase (SAD) may also be required to cope with possible accumulation of stearic acid when palmitoyl-ACP thioesterase is

reduced and β-keto acyl –ACP-synthetase II activity is increased. In addition, there is also an interest in producing high stearate palm oil for use as a cocoa butter substitute. All of these may be achieved by down regulating expression of the δ9 stearoyl ACP desaturase gene.

The first step to achieve this goal is to clone the *sad* gene from palm oil cultivar that is widely cultivated in Indonesia. Since the purpose is for the expression either downregulating or upregulating, the most apropriate way is to clone the gene from cDNA to avoid obtaining fragment containing intron region. Once the fragment of *sad* gene was obtained, then antisense construct can be made. In this paper we present the process of cloning *sad* gene by PCR using cDNA made from mesocarp of Indonesian palm oil (*Elaeis guineensis*).

Materials and Methods

Plant materials used in this experiment is the palm oil germplasm collection planted at Bah Jambi Afdelling III plantation in 1993. Those plants are the result of backross between 107-22-32 T and 87-56-56 D that was identified before using random amplified polyorphic DNA (RAPD) technique to be the elite line producing high quality oil. Total RNA was extracted from mesocarp according to the Mayda (1990). The Quality of total RNA was determined quantitatively by specrophotometer and qualitatively by gel electrophoresis.

First stand cDNA was synthesized using *reverse transcriptase* with oligo-dT (TP, 5'-GTC GAC TCG AGA ATT TTT TTT TTT TTT TTT-3') as the primer (Invitrogen). The reaction for the first strand cDNA synthesis was 70°C for 10 min of total RNA and oligo dT primer followed by addition of 5x 1st strand buffer, DTT and dNTP mix incubated at 42°C for 2 min before addition of Superscript II (Invitrogen). The mixture was incubated at 42°C for 60 min to make the cDNA strand. Inactivation of the enzyme was done by incubation at 70°C for 15 min.

The cDNA was the used as the template for cloning sad gene using 2 specific primers (primer 2 R & F). Primer 2 R and primer 2 F were designed using the *Primer3* program. Primer 2 F design is 5'-CAG AGA ACA GCC CCT ACC-3' while primer 2 R is 5'-CTT CCC GGC CAT AGA TCC-3'. The primer pair was subjected to analysis using *Fast PCR* program for optimum amplificaton condition. PCR condition used for cloning is denaturation at 95°C for 1 min followed by 94°C for 1 min, 59°C for 1 min, 72°C for 2 min which is repeated 25 x and ended with extension at 72°C for 20 min. The PCR product was ranat DNA gel electrophoresis system (BioRad) using 1 Kb plus Ladder as marker (InVitrogen).

The target fragment was excised and purified using Qiagen Gel Purificatio Kit (Qiagen). The isolated fragment was then cloned into pCR2.1 (TOPO TA Cloning Kit, Invitrogen) and the ligation product was transformed into *Eschericia coli* TOP10F'. The transformants were screened using media containing kanamycin (50 µg/ml), X-gal and IPTG. The plasmid from positive transformants were isolated using standard procedure (Sambrook et.al., 1989). The plasmids were checked by double digestion with BamHI (5U/µl) and XhoI (2.5 U/µl). The digested plasmids were then ran at DNA gel electrophoresis system (BioRad), visualized using UV transilluminator (BioRad) and documented using Gel documentation system (KODAK).

The positive plasmid was then subjected for sequencing using M13 R and M13 F primer respectively. The sequences were analyzed and compared to the existing *sad* sequences at the GenBank using BlastN (www.ncbi.nih.gov) program (Robison, 2005).

Results and discussion

The total RNA was isolated either from young fruit (≤ 15 weeks after anthesis) and mature fruit (> 15 weeks after anthesis) to obtain the optimum source for RNA isolation from palm oil mesocarp. Based on

spectrophotometer reading at 260 nm and 280 nm, the method used was succesfully able to isolate RNA either from young or mature fruits (Table 1). This is reflected by the ratio of A260/A280 yield which is between 2.0 – 2.5. This yield indicates that there were no protein nor DNA contaminaton in the RNA yield. The method used was also able to obtain ample total RNA (1.1 µg/ml – 1.7 µg/ml) for cDNA synthesis. However, at a glance the yield and quality of RNA from young fruit is slightly better than the one from mature fruit (Table 1 and Figure 1). This is probably due to the less content of oils, alcaloids and phenolics present in the young fruit compared to the mature fruit.

In this experiment, total RNA obtained from mature fruit no. 10 was used to synhesize cDNA because the amount is ample enough (1.384 µg/µl) and the quality is good enough (A260/A280 = 2.33)

Table 1. Absorbance of RNA isolated from young and mature fruit

No.	Sample	A260	A280	A260 /A280	RNA (mg/ml)
1	Young fruit 1	0.995	0.423	2.35	1.592
2	Young fruit 2	0.873	0.329	2.65	1.397
3	Young fruit 3	1.005	0.431	2.33	1.608
4	Young fruit 4	0.758	0.309	2.45	1.213
5	Young fruit 5	0.765	0.380	2.01	1.224
6	Young fruit 6	0.751	0.311	2.41	1.202
7	Young fruit 7	1.087	0.510	2.13	1.740
8	Young fruit 8	0.931	0.396	2.35	1.490
9	Young fruit 9	0.852	0.357	2.38	1.363
10	Young fruit 10	0.897	0.386	2.32	1.435
11	Mature fruit 1	0.786	0.381	2.06	1.258
12	Mature fruit 2	0.754	0.352	2.14	1.210
13	Mature fruit 3	0.845	0.378	2.23	1.352
14	Mature fruit 4	0.987	0.456	2.16	1.579
15	Mature fruit 5	0.854	0.371	2.3	1.366
16	Mature fruit 6	0.689	0.336	2.05	1.102
17	Mature fruit 7	1.023	0.475	2.15	1.637
18	Mature fruit 8	0.691	0.303	2.28	1.106
19	Mature fruit 9	0.795	0.380	2.09	1.272
20	Mature fruit 10	0.865	0.371	2.33	1.384

Another consideration is the fact that lipid biosynthesis in palm oil occured actively at 17 weeks after anthesis at the time wich it's oil content incease dramatically to 23.07% at 19 weeks after anthesis (Budiani, 2004). Since δ9 stearoyl ACP desaturase (SAD) is one of the key enzyme in lipid biosynthesis, choosing RNA extracted from mature fruit (> 15 weeks after anthesis) was apropriate.

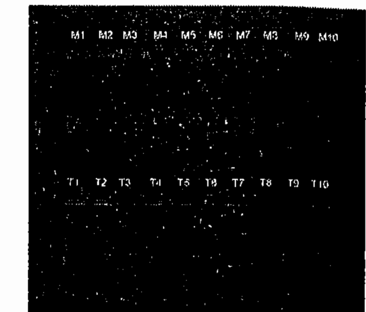

Figure 1. Total RNA isolated from young fruits (M) and mature fruits (T)

PCR amplification using the primer 2 R & F yielded a ± 541 bp fragment which is the predicted size using *FastPCR* program (Figure 2). The same PCR reaction was ran using genomic DNA extracted from palm oil mesocarp yielded 3 fragments (data not shown). One of the fragment has exactly the same size as the fragment obtained from PCR reaction using cDNA as the template. The same size fragment obtained either using cDNA or genomic DNA as the template probably due to the fact that the primer pair was designed to amplify only.the *intron* area. Usually, the fragment obtain from PCR reaction using genomic DNA as the template has a bigger size than the one from cDNA due to the presence of *intron* area in the genomic DNA sequence. In addition, the presence of more than one fragment in PCR reaction using geomic DNA as the template due to the fact that the primes were designed using sequences of *sad* gene from the *GenBank* and most of those sequences are cDNA sequences which when the primers applied to genomic DNA those primers were able to bind at several positions in the genomic DNA.

The fragment was then excised from the gel and purified using *Qiagen Gel Purification Kit* (Qiagen) and transformed into *Escherichia coli* TOP10F. Transformaton yielded 10 white colonies grown in LB plates supplied with kanamycin, X-gal and IPTG. Theoritically,

nonrecombinant colonies will synthesize â-galactoside due to the presence of X-gal as a substrate and IPTG as an inducer which will cause the blue color of the colonies. Therefore, those 10 white colonies are candicates of *E. coli* cells bearing the 541 bp fragment of putative *sad* gene.

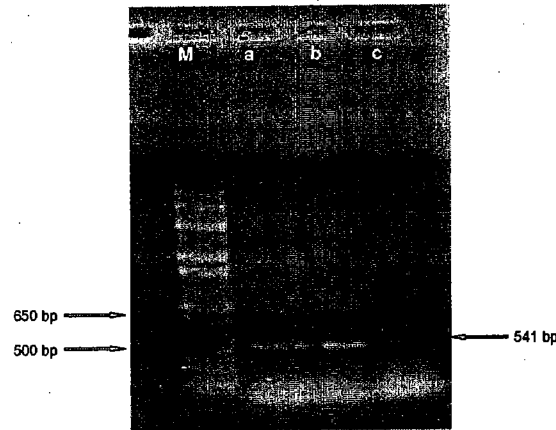

Figure 2. PCR amplification using cDNA as template and primer 2. M= 1 Kbplus DNA marker (InVitrogen), a= 30 cycles, b= 35 cycles, c= 25 cycles

In order to verify the presence of the 541 bp insert, plasmids from those colonies were isolated and subjected to double digestion using BamHI and XhoI to release the fragment. The results of digestion is the release of the 541 bp fragment from the vector pCR2.1 leaving the 3.9 Kbp as the vector backbone (Figure 3). The double digestion result in Fig. 3 showed that only one among the 10 candidate plasmids does not carry the inserted fragment. Double digestion result of the plasmid no. 2 showed a slightly higher weight of the vector backbone.

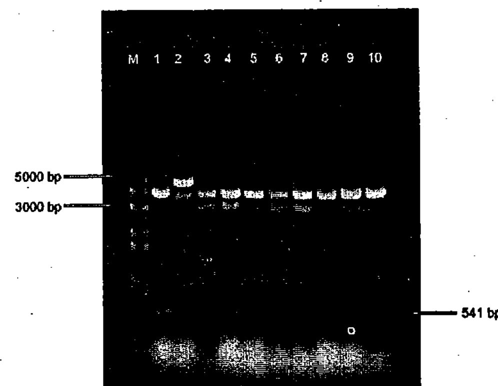

Figure 3. Double digestion of the candidate transformants with *Bam*HI dan *Xho*I. M= 1 Kbplus DNA marker (InVitrogen)

Furthermore, to confirm the result from the previous restriction digest, plasmids isolated from the 10 candidate transformants were used as PCR template using the same primer pairs for amplifying the 541 bp fragment. The result from the PCR screening is shown in Figure 4. Based on Figure 4, all the candidate plasmids carry the inserted fragment including plasmid no.2. Therefore, a slightly bigger vector backbone shown in Figure 3 is probably due to incomplete digest or partial digest occured in plasmid no.2.

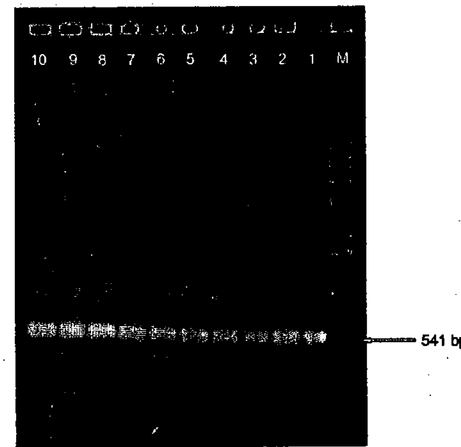

Figure 4. PCR screening of the putative plasmid bearing the 541 bp fragment

Results presented in figure 3 and figure 4 indicates that all the plasmid except plasmid no.2 are containng the inserted 541 bp fragment which is suspected to be fragment of *sad* gene. Therefore, plasmid no 1 (B1) was chosen to be ientified using sequencing method. The primer used for sequencing was M13 R which is recomended for sequencing of fragment inserted into pCR2.1 vector. The sequencng result is as follows :

>B1 M13R

GNTACTCATCTCAAGGACGNTTCCNNA
ATCCACTAGTAACGGCCGCCAGTTGCT
GGNAATTCGCCCTTCTTCCCGGCCAT
AGATCCAACTGNAAAGGTNAGCGTGGT

GCTTGCTTGGCCCTTCCCTGAGCTCTT
TCTTCAAGCCTCCTGATCCTGGGAGCAA
GAGTGCAGACAAAGTCCTGGGCCCTC
TTACCTTCCCCAGAGAGGCCAGTTAGC
TCCCCCACTTTCCACCTATCAACGAGGA
ACTCAAGTATGTCAGCATAGTCCTTGGC
CGTGTACACACCCAAACGCTGGGCCACT
GCTGAGAAGTGCTCGAAGAGGTTATCAT
CCTGACCATCGTACATCAGATGGGCAGG
CATCGAGATCTTCTTCTTCATCATGTCAG
CAAAGGCAAGAACAGTACCATCTGGGG
TCAATCTCAAACAGTTCTCCCTATCTTT
GTATAGGCNGCCTCAANGGNNGTTTCTC
ATCTGAGGCAATTNTACCNCNTATGTGA
CCNAANTTTAGGNCCCCNNNTCCTTG
GAATGCCNGGGAATNNCCCNNGGGAAAA
NAAAGGNCCCCCTCTTTTGAAAAGGGG
TGTNTACAAAACCNAGGGGGNGGGGGT
GTTNTTTNAGAGGGGAANTTTNGANN
NTATNATCAANNNNNNGGGGGNNGGCC
CTTCGNAGNNNGNTTTTNANGNGNCN
CCNANTCCCCCCTNNNGGGGGGGGC
GTATTANTTNTTTNGGGGGGCNGTTTTT
TTAAAANNGGGNNGGNGG

Sequence analysis using Blastn program (http://www.ncbi.nlm.nih.gov) showed 86% and 94% similarity with the sequence of gene encoding Stearoyl ACP-Desaturase (*sad*) from *Oryza sativa* (XM 463624, AP008207, AK103568, AP003437, AP006843, AK060087 & AK058979) and from *E.guineensis* var. tenera (AF 143501, AF507965 & U68756) deposited at the GenBank. Based on that analysis, it can be concluded that the 541 bp fragment is the fragment of *sad* gene from palm oil variety in Indonesia.

The fragment obtained can be used further to obtain the 5' region and the 3' region of the *sad* gene for obtaining the full length clone. Full length clone will be useful to construct *sense* orientation of the gene to create *overexpression* of this gene which will yield transgenic palm oil with higher oleic acid. Alternatively the fragment obtained can also be used to construct *antisense* orientation of the *sad* gene to make transgenic palm oil plants high in stearic acid for other oleo chemical industries for example as a substitute for cocoa butter.

References

Abdullah, S. N., Shah, F. B. and S. C. Cheah. 1995. Construction of oil palm mesocarp cDNA library and the isolation of mesocarp-specific DNA clones. Asia Pacific Journal of Molecular Biology and Biotechology 3(2):106-111.

Abdullah, S. N., Cheah, S.C., Othman, A. and Wahid, M. B. 2004. Stearoyl-ACP Desaturase Genes From Oil Palm. Malaysian Palm Oil Board Information Series 220:1- 4.

Azis, A., Rosnah, M.S., Mohamadiah, B., Wan Zailan, W.O. 1986. Proceeding of Malaysian Biochemical Society Conference 12:147-151.

Budiani, A. 2004. Ekspresi protein spesifik dalam biosntesis minyak dan kloning gen penyandi ht-ACCase subunit Biotin Karboksilase dan Enoil-ACP Reduktase dari mesokarp kelapa sawit (*Elaeis guineensis*). Disertasi S3. Sekolah Pasca Sarjana, Institut Pertanian Bogor. Bogor.

Cheah, S.C. Sambanthamurthi, R. Siti Nor Akmar, A., Abrizah, O., Manaf, M. A. A., Umi Salamah, R. And Parveez, G. K.A (1995). *In* J.C. Kader and P. Mazlak (*eds.*) Plant Lipid Metabolism. Netherlands:Kluwer Academic Publishers, pp. 570-572.

Darnoko. 2001. Jurnal Penelitian Kelapa Sawit 9(2-3): 63 – 77.

Gasser, C.S. and Fraley, R. T. 1992. Transgenic Crops. Scientific American. June:62-69.

Mayda, E. 1995. RNA Isolation. Biotechniques 19:34 – 737.

Oil World Annual. 2001. ISTA Meilke GmbH, Germany.

Pantzaris, T. P. 1993. Trends in yellow fats consumption in EEC. Palm oil Development 18:3 – 7.

Parveez, G. K. A. 1998. Optmization of parameters involved in the

tansformation of oil palm using the biolistic method. PhD thesis, Universiti Putra Malaysia.

Parveez, G. K. A., S. Ravigadevi, S. N. A., Abdullah, A., Othman, U. S., Ramli, O., Rasid, M. M. Masri and S. C. Cheah. 1999. Production of transgenic oil palm – curent success and future considerations. Proceedings of the 1999 PORIM International Palm Oil Congress, 1-6 September 1999, Kuala Lumpur.

Pryde, E. H. 1983. Utilization of commercial oilseed crops. Economical Botany 37:459 – 477.

Rajanaidu, N. and Jalani, B. S. 1995. World-wide performance of DXP planing material and future pospects. *In* Proceedings of the 1995 PORIM National Oil Palm Conference-Technologes in Plantation, The way forward. 11- 12 July 1995. Kuala Lumpur : Palm Oil Research Institute of Malaysia, pp. 1- 29.

Robison, K. 2005. BLAST. http://arep.med.harvard.edu/seqanal/blast.html.

Rozen, S. And H. Skaletsky. 2000. Primer 3 on the www for general users and for biologist programmers. Methods on Molecular Biology 132:365 – 386.

Sambrook J., E. F., Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. Book 1 and 2, 2nd ed. Cold Spring Harbor Lab. Press, New York.

Scowcroft, W. R. 1990. New fats and oils through biotechnology. INFORM 1:945 – 951.

Sudin, N., Sahri, M. M., Kun, T. Y., Oh, F. 1993. Modification of palm kernel oil and their fractions for margarine. Palm Oil Development 18:1-3.