the final product contained 53.8% DAG along with TAG, MAG and other micronutrients [24]. But no study has been made for the preparation of higher amount of DAG in the oil with micronutrients like tocopherols, sterols and squalene from cheap raw materials. So, in the present investigation, an extended work has been made from our earlier work to produce DAG rich product (72.86%) from RBOFAD containing sterols, tocopherols and squalene using lipase catalyzed reaction in a solvent free system.

2. Materials and Methods

2.1 Materials

RBOFAD was collected from Sethia Oil Mill, Burdwan, West Bengal, India. Glycerol (A.R.) was purchased from E. Merck (India) Pvt. Limited. The lipase NS 40013 (*Candida antartica*, a non-specific immobilized lipase) was a kind gift of Novozymes, South Asia Pvt. Ltd., Bangalore, India. Except otherwise specified all other chemicals used were A.R. Grade.

2.2 Methods

2.2.1 Bleaching of RBOFAD

About 500 gm of RBOFAD was taken in a 1 L round bottom flask and heated under vacuum (2-4 mm of Hg pressure) in a boiling water batch for 15 min. After that 5% Tonsil earth (Sud Chemical Company, Germany) and 0.6% activated charcoal (E. Merck, India) were added and shaken vigorously for 20 min under vacuum. Then it was cooled to 45^0C and filtered under vacuum. The bleached FAD was stored in a refrigerator for further study.

2.2.2 Enzymatic esterification of bleached RBOFAD

Bleached RBOFAD and glycerol were taken in different molar ratios in a round bottom flask and stirred by a magnetic stirrer under vacuum of 4 mm Hg for 8 h using 5% (by weight of substrates) lipase catalyst (NS 40013). The temperature of the reaction was maintained at 65 ± 2^0C. The esterification reaction was monitored by estimating the free fatty acids in the reaction mixture periodically withdrawn. After 8 h of reaction, the product mixture was filtered for removing enzyme and kept for purification.

2.2.3 Purification of esterified products

The esterified product was purified in a molecular distillation unit (Model MS-300, SIBATA Scientific Co. Ltd., Japan). It was a falling film type apparatus and was provided with a rotating wiper that continuously rubbed the falling film on the evaporating surface. The temperature of the reaction product was maintained at 145 ± 2^0C and 12 pascal pressure to remove the residual fatty acids and volatile impurities. The amount of DAG in the final products was determined by standard column chromatographic method.

2.2.4 Determination of fatty acid composition by gas liquid chromatographic (GLC) method

Fatty acid composition was determined by gas liquid chromatographic method after converting the fatty acids into methyl esters. The HP-5890A GLC was connected with an HP-3390A data integrator. The GLC was fitted with a glass column (1.83 m X 3.173 mm i.d.) packed with 10% DEGS supported on Chromosorb-WHP (100/200 mesh) of HP make. The oven temperature was programmed from 100^0C to 190^0C at 5^0 / min. The injector and the detector block temperature were maintained at 230^0C and 240^0C respectively. IOLAR-2 nitrogen was used as the carrier gas with flow rate 30 ml/min. The fatty acid esters peak were identified and calibrated with standard methyl esters. Data were represented an average of three determinations.

2.2.5 Determination of tocopherols by colorimetric method (Emerie-Engel method)

Total tocopherol content was measured according to the standard IUPAC method of Emerie – Engel [25].

2.2.6 Determination of sterols and squalene by HPLC method

The HPLC instrument (Waters, USA) was provided with Binary HPLC pump 1525 and Waters Dual Absorbance UV detector 2487 and Refractive Index detector 2414. The column (4.6 X 155 mm) used was Novapak bonded C18 having micro particulate silica of particle size of about 5 µm. The isocratic flow rate was 0.5ml/min. The whole system was supported by Breeze 2000 software. For the determination of sterols approximately 1.0 mg of unsaponifiable matter was dissolved in HPLC grade hexane and was filtered through a Millipore filter. 10 µl of the solution was injected and the materials were detected according to the retention time and quantified with reference to the standard sample. The mobile phase used was consisted of HPLC grade hexane, acetonitrile and isopropyl alcohol in the ratio of 75:15:10 v/v. The UV detector was used at 210 nm for squalene and 230 nm for sterols.

3. Results and discussion

Table 1 shows the compositions of fatty acids, unsaponifiable matters and neutral glycerides present in RBOFAD. It contains 75.19% free fatty acids and 9.7% neutral glycerides of which 5.2% TAG, 3.1% DAG and 1.4% MAG. RBOFAD contains higher amount (14.23%) of unsaponifiable matters of which sterols, tocopherols and squalene are present at 13.3, 37.4 and 49.3% respectively. Before enzymatic esterification process, RBOFAD was thoroughly bleached to remove peroxides. Bleached RBOFAD was esterified with glycerol in different proportions using non-specific NS 40013 lipase. Here three sets of experiments namely P-I, P-II and P-III were done to study the enzymatic esterification reaction. FAD and glycerol concentration were maintained at 2:1 and 2:1.25 and 2:1.50 for P-I, P-II and P-III respectively.

Table 1: Analytical characteristics of RBOFAD

Characteristics	*Amount (%, w/w)*
Free fatty acid	75.19±2.03
i) Palmitic acid	28.47±0.44
ii) Stearic acid	2.05±0.003
iii) Oleic acid	37.24±1.24
iv) Linoleic acid	33.2±0.78
Neutral glycerides	9.7±0.4
i) Monoacylglycerol	1.4±0.007

ii) Diacylglycerol	3.1±0.01
iii) Triacylglycerol	5.2±0.009
Unsaponifiable matters	14.23±0.16
i) Sterols	13.3 ±0.33
ii) Tocopherols	37.4±0.74
iii) Squalene	49.3± 0.77

Values are represented as mean ± S.D. n=3

Figure 1 demonstrates that the rate of enzymatic esterification reaction in each case for 8 h and it shows that increasing concentration of glycerol decreases the concentration of FFA during the course of reaction. After 8 h of reaction, the product mixture contains 5.4, 5.1 and 4.8% FFA in products I, II and III respectively. The esterified products were then purified in molecular distillation apparatus at 145±2^{0}C and 12 Pascal pressure to remove the residual fatty acids and volatile impurities. The composition of the molecular distilled esterified products is shown in Table 2.

From Table 2 it can be seen that the products, P-I, P-II and P-III contained 25.59, 7.27 and 11.11% TAG, 47.54, 72.86 and 61.16%DAG and 13.24, 6.99 and 15.36% MAG respectively. Also P-I, P-II and P-III contained 13.63, 12.88 and 12.37% unsapofiable matters respectively. Increasing amount of TAG and MAG are formed in the bio esterification reaction mainly due to the random nature of the lipase. It also reveals from Table 2 that among the three products, P-II contained maximum amount of DAG (72.86%) than the other products. So it can be said that the ratio of RBOFAD and glycerol concentration 2:1.25 is the ideal ratio for getting the higher conversion DAG and the product P-II can be considered as DAG rich product with a good amount of unsaponifiable matters.

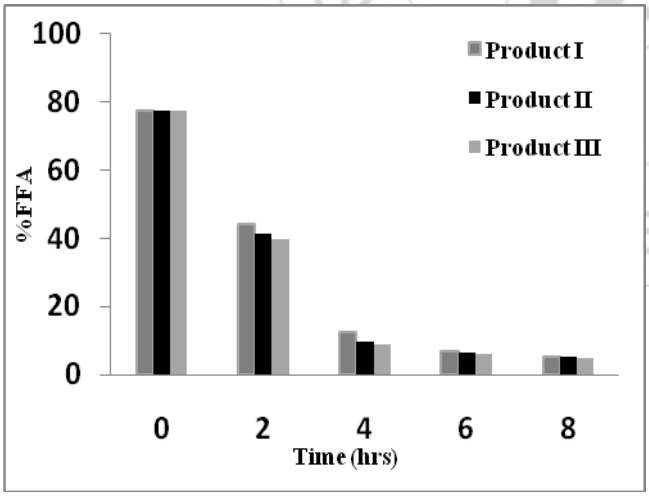

Figure 1: Enzymatic esterification of RBOFAD with glycerol in different proportions.

Table 2: Composition of DAG rich products (%w/w)

Product	*TAG*	*DAG*	*MAG*	*Unsap. matters*
P-I	25.59±0.37	47.54±0.34	13.24±0.13	13.63±0.24
P-II	7.27±0.04	72.86±0.58	6.99±0.05	12.88±0.22
P-III	11.11±0.11	61.16±0.21	15.36±0.09	12.37±0.13

Values are represented as mean ± S.D. n=3

Experimental studies also shows that increasing concentration of glycerol enhances the rate of formation of DAG but higher amount of glycerol further enhances the formation of TAG and MAG than DAG. From Table 2, we can see that when the ratio of RBOFAD to glycerol changes from 2:1.25 to 2:1.50 (enhancement of glycerol) in P-III, conversion of TAG increases from 7.27 to 11.11% and conversion of MAG increases from 13.24 to 15.36% but conversion of DAG decreases from 72.86 to 61.16%. Thus enhancement of concentration of glycerol is not permitted in this experimental study for the higher conversion of DAG. So, DAG rich product P-II with sufficient amount of unsaponifiable matters can be regarded a product of commercial importance.

P-I, P-II and P-III contain reasonable amounts of unsaponifiable matters which consisted tocopherols, sterols and squalene. Table 3 shows the composition of the unsaponifiable matters of DAG rich products where the desirable product P-II contains 29.31% tocopherols, 13.86% sterols and 51.93% squalene which is sufficiently quite good. Other products P-I and P-III contain nearly same amount of tocopherols, sterols and squalene. So P-II along with important micronutrients may be useful for functional food applications.

The qualities of DAG rich products are evaluated on the basis of acid value, peroxide value, anisidine value and colour. Table 4 shows the features of the DAG rich products P-I, P-II and P-III. It has been observed from Table 4 that all the products namely P-I, P-II and P-III confront good qualities with regard to above parameters. So considering maximum conversion of DAG from RBOFAD with micronutrients and on the basis of quality parameters of finished product, product P-II can be considered for further use as structured lipid or functional food ingredients.

Table 3 Composition of unsaponifiable matters of DAG rich products (%w/w)

Product	*Tocopherols (Total)*	*Sterols (Total)*	*Squalene*	*Others*
P-I	30.74± 0.33	13.11±0.16	52.46±0.37	3.69±0.12
P-II	29.31±0.32	13.86±0.17	51.93±0.31	4.9±0.14
P-III	28.78±0.17	14.63± 0.09	52.49±0.29	4.1±0.13

Values are represented as mean ± S.D. n=3

Table 4 Quality parameters of DAG rich products

Product	*Acid value*	*Peroxide value(meq/kg)*	*Anisidine value*	*Colour (Lovibond 1″cell)*
P-I	<0.1	<1	0.4±0.01	2.2Y+1.2R
P-II	<0.1	<1	0.6±0.01	1.9Y+0.9R
P-III	<0.1	<1	0.3±0.01	1.8Y+1.3R

Values are represented as mean ± S.D. n=3

4. Conclusion

DAG rich product can be produced from the relatively inferior grade raw material RBOFAD with the help of microbial lipase technology. The product contains considerable amount of micronutrients like sterols, tocopherols and squalene. The product is useful for

functional food applications, specialty products and has commercial importance. Further experimental study is needed for the product with much higher DAG along with the micronutrients.

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