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Isolation of Squalene from Rice Bran Oil Fatty Acid Distillate Using Bioprocess Technology

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Abstract*: *Squalene has been isolated from rice bran oil fatty acid distillate (RBO FAD) by a combination of biohydrolysis, bioesterification and molecular distillation processes. RBO FAD contains higher amount of (~15%) unsaponifiable matters which mainly contains sterols, tocopherols and hydrocarbons. The major component of hydrocarbons is squalene which is an important natural antioxidant and utilized in various functional food applications. In the present study, the neutral glycerides present in RBO FAD are hydrolyzed using lipase Amano 30 (Candida rugosa) in the presence of water. The hydrolysed FAD is then esterified with butanol using non-specific enzyme NS40013 (Candida antarctica) for 8 h. The esterified product is then distilled by molecular distillation in two stages. Distillation temperature is maintained at 175±2^{0}C and 185±2^{0}C in the first and second stages respectively at 2-3 Pascal. Final distillate contains 88.2% squalene which is further concentrated by boiling with alcoholic KOH and extracted through hexane. The final product contains 96.5% squalene as determined by HPLC technique.
Keywords*: *Squalene, Molecular Distillation, Bioesterification, Biohydrolysis, Fatty acid distillate.

I. INTRODUCTION

Squalene is a natural triterpenic hydrocarbon with molecular formula $C_{30}H_{50.}$ It is widely present in animal and vegetable kingdom and is important due to its antioxidant nature. Squalene gets attention of the scientific world due to the beneficial effects of some natural products containing it which is observed in the human health. Squalene is recognized as biochemical intermediate for the synthesis of cholesterol and other steroids.

Squalene has several beneficial properties [1] and is utilized in cosmetics, medicine, fine chemicals and functional food applications as bactericidal and fungicidal agent, antistatic and emollient or moisturizing agent [2,3,4]. It is also used as an immunologic adjuvant in vaccines. Recently, squalene has been proposed to be an important part of the Mediterranean diet as it may be a chemo preventive substance that protects people from cancer [5].

Squalene is synthesized in our body by the liver and is secreted in large quantities by the sebaceous glands. It is transported in the blood by low density lipoproteins [6]. Squalene represents 12% of the lipids secreted by the sebaceous glands and it is not transformed in cholesterol [7,8].

The highest squalene concentration in human body is found in skin lipids (500 μg/g) and in the adipose tissue (300 μg/g) [9] but the concentration is much low where the active biosynthesis takes place as in liver (75 μg/g) or small intestine (42 μg/g).

Squalene has been isolated from various sources by different methods. One of the main sources of squalene is deodorizer distillate or fatty acid distillate obtained from fats and oils refinery industries as by product. Norhidayah et al. [10] recovered squalene from palm fatty acid distillate using super critical fluid extraction technology and optimized the process. Akgun [11] used olive oil deodorizer distillate where squalene has been concentrated from an initial amount of 24.10% to 66.57% (w/w). Olive oil deodorizer distillate was also used by Xynos et al. [12] for squalene isolation using centrifugal partition chromatography. Gunawan et al. [13] isolated and purified squalene from soybean oil deodorizer distillate by a modified soxhlet extraction and silica gel column chromatography. Rice bran oil deodorizer distillate was used for isolation of squalene by Sugihara et al. [14] through a novel fractionation method.

Recently, bioprocess technology has been adopted in various fields for the extraction and isolation of value added products from cheap raw materials. With this idea, the present investigation has been made for the isolation of squalene from RBOFAD through a combination of biohydrolysis, bioesterification and molecular distillation methods. Here, initially for the hydrolysis of neutral glycerides in RBO FAD, lipase Amano-30 (*Candida rugosa*) is used in the presence of water for a period of 8 h. After that, the hydrolysed FAD is esterified with excess butanol using non-specific enzyme NS40013 (*Candida antarctica*). Two stage distillation processes has been applied by taking butyl ester in molecular distillation apparatus. The final distillate is treated with alcoholic KOH and extracted through hexane which ultimately gives a product with 96.5% squalene.

II. EXPERIMENTAL

A. Material

RBO FAD was obtained from M/s. Sethia Oils Ltd., Burdwan, West Bengal, India. The enzyme used in the following studies was Novozyme 40013, immobilized lipase from *Candida antarctica*, a kind gift of Novozyme South Asia Pvt. Ltd. Bangalore, India. Lipase AY 'Amano' 30 was a kind gift of Amano Enzyme Inc., Nagoya, Japan. Squalene for HPLC analysis was obtained from Sigma-Aldrich Co., St. Louis, MO, USA. HPLC grade solvents were obtained from Spectrochem Pvt. Ltd. Mumbai, India. Except otherwise specified, all other chemicals used were A.R. Grade.

B. Enzymatic hydrolysis of RBOFAD and Bioesterification of hydrolysed RBOFAD

RBO FAD (100 g) was taken in a 500 ml Stoppard Erlenmeyer flask and water (60% by weight of neutral glycerides) containing lipase powder (5.0% 'Amano' 30) was added. The reaction mixture was magnetically stirred with a 1 inch Teflon coated stir bar at 35 ± 2^0C in a controlled bath for 8 h. After 8 h of reaction, the hydrolysis was complete and the oil layer and the water layer containing enzyme and glycerol were separated by centrifugation. After that, the oil layer i.e. hydrolysed RBOFAD was taken in a round bottom flask along with butanol (100% excess) for further esterification. The mixture was then stirred by a magnetic stirrer at 60 ± 2^0C for 8 h using 5% (by weight of substrates) lipase catalyst (NS 40013). In both the cases, reaction was monitored by estimating the free fatty acids in the reaction mixture withdrawn periodically. After 8 h of reaction, the product mixture was filtered for removing the enzyme and the product butyl ester was kept for molecular distillation.

C. Molecular distillation of butyl ester and determination of squalene by HPLC

The butyl ester of RBOFAD was distilled 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. Distillation process was carried out in two stages, initially at 175 ± 2^0C and 2-3 Pascal for 1 h to get distillate I. Residue I was again distilled at 185 ± 2^0C and 2-3 Pascal for 1 h to get distillate II. Squalene rich distillate II was further boiled with NaOH and finally extracted with hexane to get purified squalene which was identified by HPLC technique.

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. 10 µl of the sample 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 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.

III.RESULTS AND DISCUSSIONS

Table 1 shows the compositions of fatty acids, neutral glycerides and unsaponifiable matters present in RBOFAD. It contains higher amount (15.11±0.16%) of unsaponifiable matters of which sterols, tocopherols and squalene are present at 13.13±0.33, 35.24±0.74 and 49.38± 0.77% respectively. Before enzymatic hydrolysis, RBOFAD was thoroughly bleached to remove peroxides.

Table I: Analytical characteristics of rbofad

Component	Amount (%, w/w)	Component	Amount (%, w/w)	Component	Amount (%, w/w)
FFA (Total)	74.22±2.03	Neu. Glycerides	9.73±0.4 1	Unsap. Matters	15.11±0.16
Palmitic acid	27.47±0.44	MAG	13.19±0.17	Sterols	13.13 ±0.33
Stearic Acid	2.05±0.003	DAG	32.21±0.21	Tocopherols	35.24±0.74
Oleic acid	36.24±1.24	TAG	54.33±0.81	Squalene	49.38± 0.77
Linoleic acid	33.2±0.78				

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

The neutral glycerides present in the RBOFAD were hydrolysed by 'Amano' 30 lipase as shown in Fig. 1. Initially, the degree of hydrolysis was very rapid and it was almost completed (99.13%) after 8 h of reaction as depicted in Fig. 1. Initial high rate of hydrolysis is due to the fact that the complex formation between glyceride molecules and active sites of enzymes is rapid and thus breakage rate is fast. So within 3-4 hours, maximum conversion has been achieved.

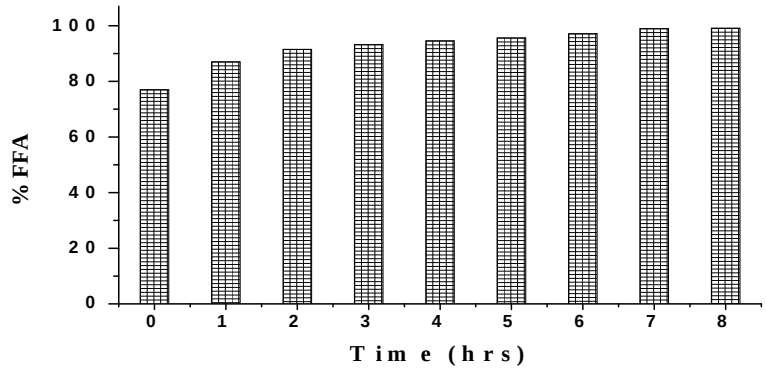

Fig. 1. Hydrolysis of RBOFAD

The hydrolyzed FAD was then esterified with butanol (100% excess) in presence of NS40013 enzyme. After 8 h, the esterification reaction reached equilibrium and the final esterified product contained about 2.1% FFA. Fig. 2 shows the sequence of bioesterification reaction between hydrolysed RBOFAD and butanol.

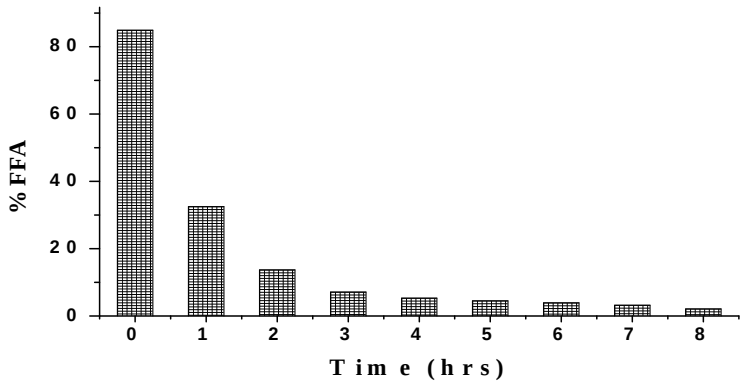

Fig. 2. Bioesterification of hydrolysed RBOFAD and butanol

To concentrate squalene in the final product, the butyl ester was distilled in Molecular Distillation apparatus in two stages. In the first stage most of the butyl ester was separated (98.1±0.74%). Distillation of residue I in the second stage gave distillate II (squalene rich) and residue II. Yield and composition of distillate and residue are given in Table 2.

Table 2: Composition Of Distillate And Residue After Molecular Distillation (%, W/W)

Component	Distillate I	Residue I	Distillate II	Residue II
Yield	83.2±0.88	16.8±0.11	52.38±0.48	47.54±0.24
FFA	1.85±0.002	----	----	----
Butyl ester	98.1±0.74	----	9.1±0.03	---
Unsap. Matters	---	---	90.9±0.65	76.8±0.67
Squalene	----	----	88.2±0.32	0.8±0.001
Tocopherols	---	---	---	35.3±0.12
Sterols	---	---	---	22.7±0.13
Others	----	---	2.7±0.005	17.9±0.23

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

Distillate II (squalene rich) contained 90.9±0.65% unsaponifiable matters along with some amount of butyl ester (9.1±0.03%). The main component of unsaponifiable matters in distillate II was squalene (88.2±0.32%) which was present in the crude RBO FAD. The yield of residue fraction was 47.54±0.24% which was also rich in sterols (22.7±0.13%) and tocopherols (35.3±0.12%). The distillate II was then boiled with NaOH and extracted with hexane to recover the final product. The final product contained 96.52±0.81% squalene as determined by HPLC technique. The density, refractive index and flash point of the final product were 0.852±0.008 g/mL, 1.478±0.006 and 110±0.87^{0}C respectively with very low acid value (<0.05±0.001), peroxide value (<0.1) and anisidine value (0.3±0.002).

IV. CONCLUSIONS

It can be concluded that microbial lipase technology with non specific lipase offers an excellent way to isolate squalene from a cheap raw material like rice bran oil fatty acid distillate. Enzymatic process may be preferred than chemical catalytic process in producing better quality squalene which can be utilized as commercial grade squalene and can be used in various personal care products. Recycling of enzyme can be done for cost minimization of bioprocess technology which helps future researchers to rethink about this technology. So biocatalytic process along with the use of molecular distillation in a novel technology for isolation of not only squalene but also other valuable products from by products of oil industries.

V. ACKNOWLEDGMENT

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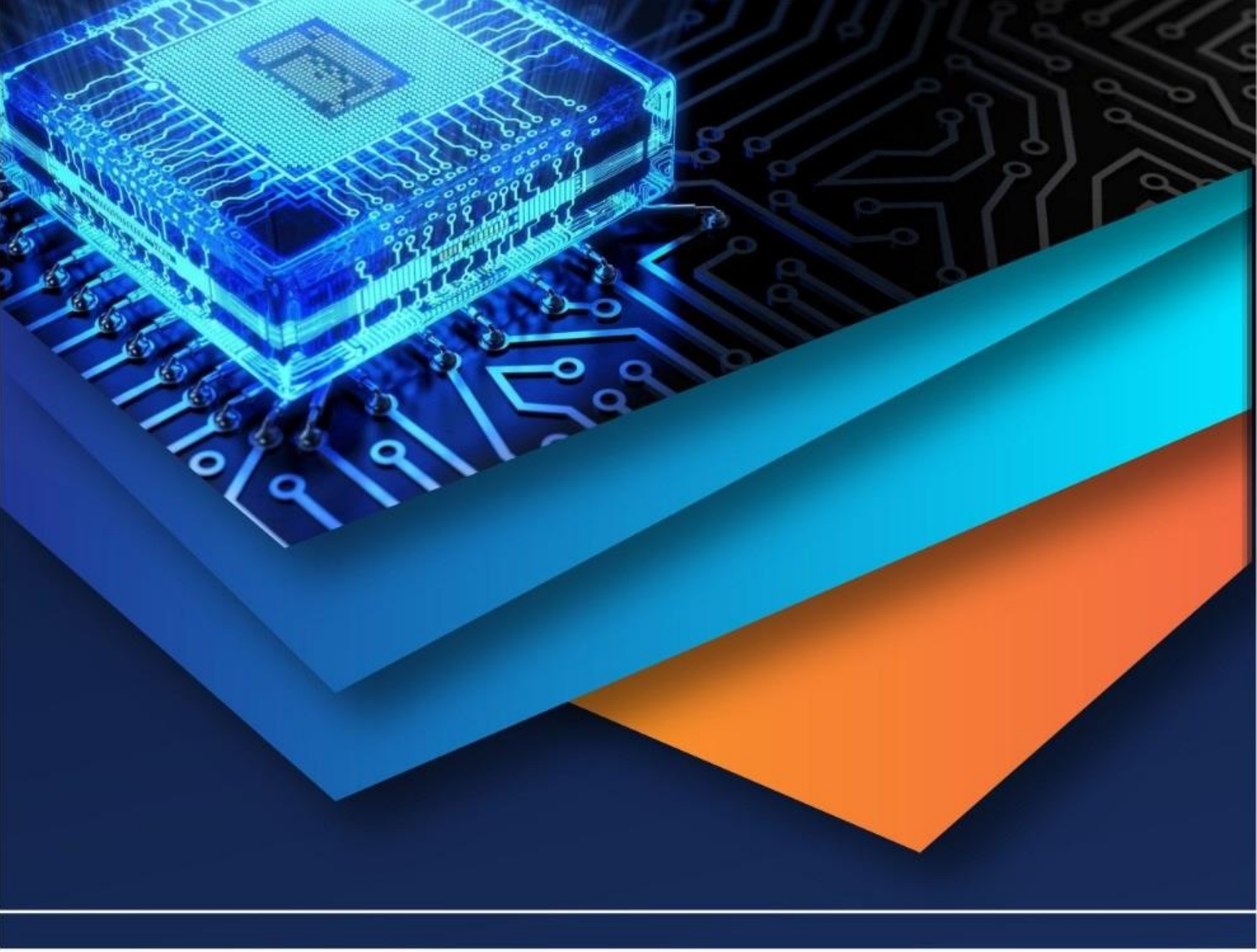

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