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Research Article

Enzymatic Deacidification of Rice Bran Oil Containing High Free Fatty Acids with Recycling

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Abstract

The enzymatic deacidification process for high free fatty acid (FFA) rice bran oils (RBO) has been investigated with special emphasis on the reusing of the enzyme. This process involves the suitability of the process as a viable alternative to the physical refining based on yield and quality of the refined RBO. Two samples of RBO, I and II containing different amounts of FFA and other constituents have been studied for this purpose by using two enzymes, non specific Novozyme 435 (*Candida antarctica)* and specific TL-IM (Immobilized lipase from *Thermomyces lanuginosus)*. Studies showed that Novozyme 435 is more effective for the reduction of FFA using 40% excess of stoichiometric amount of glycerol. So, further study has been conducted for recycling with Novozyme 435 enzyme in case of sample II. This enzyme has been recycled 60 times and it has been observed that the enzyme is still active. The bioesterified oil containing 3-4% residual FFA is then steam stripped to reduce its FFA content to 0.4%. The final product contains required amount of antioxidant like oryzanol.

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Introduction

India is producing a significant amount of high FFA rice bran oil. The refining of high FFA rice bran oil is a big challenge. Conventional alkali refining and physical refining processes are associated with huge amount of loss of neutral oil. Lipase catalysed bio refining method may be suitable for production of refined RBO of high quality. Rice bran (*Oryza sativa* L) oil may contain, as much as 30-40% free fatty acids, if the bran is not processed properly prior to the extraction of oil. High FFA content is one of the main drawbacks in refining RBO, as it is associated with greater oil loss and darkening of colour during alkali refining. Conventionally, RBO can be refined by degumming, dewaxing, alkali refining, bleaching and deodorization. However, the method involves substantial loss of oil. Therefore, some author proposed physical refining by spurging steam under vacuum with removal of FFA and odoriferous compounds [1, 2, 3]. This results in huge loss of FFA and oil especially if the oil contains high FFA. In general, the method of deacidification of a vegetable oil consists of conversion of its free fatty acids into neutral glycerides by re esterification with the free hydroxyl groups remaining in the oil or with added hydroxyl group through glycerol at high temperature with or without catalyst system. Re esterification is expected to increase the yield of neutral oil. Bhattacharyya et al refined high FFA (20.5%) RBO to food grade level. Alkali refining can further deacidify the re esterified oil containing some amount of FFA. Bhattacharyya and Bhattacharyya [4] have deacidified RBO containing 15-30% FFA to 2-3% levels by re esterification with glycerol with or without catalyst. The re esterified oil was further neutralized and bleached to produce light coloured edible oil.

Various authors have reported deacidification by enzymatic esterification. According to them, FFA content of oil can be reduced to varying level (2-3%) depending on concentration of triglycerides (TG), diglycerides (DG), monoglycerides (MG) in the oil, temperature, nature and concentration of enzymes. Sengupta and Bhattacharyya [5, 6] biorefined high FFA RBO with the help of 1, 3-specific *Mucor miehei* lipase followed by alkali refining, bleaching

and deodorization. They concluded that the high FFA RBO could be refined with a high degree of economy by a combination of enzymatic deacidification and alkali neutralization. Kosugi et al [7] utilized RBO containing 30-50% FFA to an oil containing more than 75% TG by means of immobilized lipase. The reaction was carried out at 60°C for 24 h with dehydration and reactant mixture by dry nitrogen flow under a positive nitrogen atmosphere. Rodrigues et al [8] deacidified vegetable oils by solvent extraction and they showed that by this process, no waste products are generated and can preserve nutraceuticals in the refined oils.

In the last few years, several workers have utilized the lipase catalysed esterification reaction to refine oils [9, 10, 11]. Kurashige [11] used diacylglycerol for the esterification of crude palm olein by using a lipase from *Pseudomonoas fluorescens*. The extent of esterification was high due to better solubility of diacylglycerol in oil. It was previously reported by Noris [12] that monoglyceride too can esterify FFA. Using this concept, Sengupta and Bhattacharyya [6] bio refined rice bran oil with the help of monoglyceride. They reported that FFA can be reduced to 2-4% depending on the amount of MG used. They concluded that MG could be effectively utilized instead of glycerol to reduce FFA in oils, producing oil of better quality with respect to triglyceride content. Using monoglyceride, some researchers [13, 14] successfully reduced FFA content in RBO from 35% to 3.5% followed by alkali refining, bleaching and deodorization to produce food quality RBO. Enzymatic deacidification of high FFA rice bran oil has a vital role for retaining oryzanol in the refined oil. Oryzanol has been proved to be a good antioxidant for human health. Cost of enzyme can be reduced by recycling it as its deacidifying property retains for a long time. Recycling of enzyme for different reactions has been done by several authors. Lu et al [15] reused enzyme during hydrolysis of steam exploded softwood residues for bioconversion process. Hartree et al [16] recycled enzyme and substrate after enzymatic hydrolysis of steam pre-treated aspen wood. Ramos et al [17] recycled enzyme during fed batch hydrolysis of cellulose derived from steam exploded Eucalyptus viminalis. So recycling of enzyme is useful and efficiency of productivity of the enzymatic process can be increased only by recycling it as much time as possible. The present study investigated recycling of a non-specific enzyme for deacidification of high FFA RBO up to 60 times in laboratory scale. It has been found that level of FFA is reduced to about 3% by this approach so that high cost of enzyme is utilised optimally in making enzymatic deacidification process commercially viable.

Experimental

Materials and Methods

High free fatty acid rice bran oil was provided by M/s. Sethia Oils Ltd., Burdwan, West Bengal, India. The enzymes used in the following studies are: TL IM: (Immobilized lipase from *Thermomyces lanuginosus* with catalytic activity 75 interesterification unit NOVO/gm (IUN/gm) and Novozyme 435 **(***Candida antarctica)* Immobilised lipase. All the enzymes were kind gift of Novozyme South Asia Pvt. Ltd. Bangalore, India. Hexane (B.P. 65-70°C), diethyl ether (B.P. 35-40°C) and Silica gel G were purchased from S.D. Fine Chemicals (Mumbai, India). Except otherwise specified all other chemicals used were A.R. Grade.

Acid value, Peroxide value, p-anisidine value, unsaponifiables of RBO were determined according to standard method described in the official and tentative methods of American Oil Chemists' Society [18]. Phosphorous content was measured by the standard procedure of Chen et al [19].

Determination of Oryzanol

Oryzanol content was determined according to the method of Gopala Krishna et al [20]. Samples of accurately weighed RBO (about 10 mg each) in triplicate were dissolved in hexane and volume made up to 10 mL and mixed well in a vortex. The O.D.s was measured in 1 cm cell at 314 nm in a UV-VIS spectrophotometer (UV-1601, SHIMADZU, Japan). Solution having OD greater than 1.2 were further diluted before reading OD. The oryzanol content in the oil was calculated using the formula.

$$\text{Oryzanol, g\%} = \frac{\text{OD of hexane solution}}{\text{weight (g) of oil} \times 10} \times \frac{100}{358.9}$$

A blank was performed with 10 mL hexane only. The specific extinction coefficient of oryzanol is 358.9.

Degumming, dewaxing and bleaching

The high FFA rice bran oil was degummed, dewaxed and bleached before biorefining. The oil was degummed by mixing together with 0.25% phosphoric acid of 85% concentration and 2% water on the weight of the oil at 70 °C for 30 min with constant stirring. The gummy materials were separated by centrifugation at 7000 rpm and finally water washed to remove the phosphoric acid. The water washed oil was dried under vacuum at about 90°C. The oil was dewaxed by winterisation process at 10-12^{o}C for 7 h followed by centrifugation. The dewaxed oil was bleached with 2% by weight of bleaching earth and 0.5% activated carbon under vacuum at 235±5°C for 20 min at 6 mm Hg. The bleached oil was then obtained by filtration under vacuum in the present investigation.
Degummed, dewaxed and bleached RBO (100 gm) was placed into a 250 mL conical flask fitted with standard B 24 joint. A predetermined amount of glycerol (either 40 % and / or 100 % stoichiometric excess) was added to the oil. 5% (w/w of oil) immobilised lipase was added. The oil was slowly heated to 50-60°C (at 4 mm Hg) and stirred with magnetic stirrer for 8 h. At regular 2 h interval aliquot was taken out and FFA was measured.

Quantitative determination of MG, DG and TG

The MG, DG, and TG content of crude and biorefined RBO were estimated by preparative thin-layer chromatography (PTLC). 0.5 mm thick layer of silica gel G (110-120 mesh) was applied to a 20×20 cm glass plate using 14 gm silica gel G and 28 mL distilled water. Plate was activated by heating at 110°C for 60 min. 0.1 gm exactly weighed oil was applied to the plate using a capillary and the plate was developed in 100mL hexane/ diethyl ether (80:20 vol/vol). Bands corresponding to MG, DG and TG were detected by iodine absorption and by R_f values [21] specific for each component. Each of the bands was scrapped from plate and extracted with chloroform. Each fraction was gravimetrically quantified as weight percentage of oil by evaporating chloroform under 4 mm Hg vacuum at 90°C.

Deodorization

Deodorization was carried out by conventional steam stripping at 180°C at 4 mm Hg.

Statistical analysis of data

All experiments were completed in triplicate unless stated otherwise and the results are presented as mean ± standard deviation. Statistical differences of mean values were analyzed (for esterification reactions) using students's t-test in STATISTICA software.

Results and discussions

Analytical characteristics of samples

The analytical characteristics of the two samples are shown in Table 1. We have considered two different samples containing different amounts of FFA, MG, DG, TG, unsaponifiables and oryzanol.

Table 1 Analytical characteristics of high FFA rice bran oils before enzymatic esterification

Characteristics	Sample I	Sample II
FFA (%)	35.5 ± 0.234	16.5 ± 0.180
MG (%)	2.5 ± 0.036	4.2 ± 0.041
DG (%)	7.7 ± 0.103	9.9 ± 0.167
TG (%)	51.5 ± 0.372	66.0 ± 0.361
Unsaponifiables (%)	2.61 ± 0.078	3.2 ± 0.067
Oryzanol (%)	1.7 ± 0.015	1.7± 0.017

Values are reported as mean ± s.d., where n=3 (n=no of observations).

Enzymatic esterification

The esterification reactions have been carried out by using two enzymes Novozyme 435 and TL-IM. The time study of esterification reactions with two enzymes is shown in Table 2**.** Using 5% TL IM enzyme with 100% excess glycerol of the stoichiometric amount, the FFA content was reduced from 35.5±0.334% to 19±0.195% after 8 hours of esterification reaction. On the other hand, the FFA content has been reduced to 2.1±0.011% after 8 h reaction using enzyme Novozyme 435. By using 40% excess of stoichiometric amount of glycerol, the FFA content in Sample-I has been reduced to 3.6±0.084% after 8 h of reaction with Novozyme 435.

In case of Sample-II, using 40% excess glycerol, the FFA content has been reduced from 16.5±0.180% to 3±0.072% within 6 h. In comparison with these two enzymes it can be stated that Novozyme 435 enzyme is suitable for bio-esterification reaction. So, further study has been conducted with Novozyme 435 enzyme and with 40% excess of theoretical amount of glycerol as appropriate glycerol concentration for esterification reaction.

Enzymatic esterification reactions using two different enzymes have been carried out at temperature 45^0C and at atmospheric pressure. Temperature is kept below 50^0C as enzyme has a tendency to denature at higher temperature.

Table 2 Deacidification of rice bran oil by esterification with glycerol using specific enzyme (TL IM) and non specific enzyme (Novozyme 435)

Enzyme	Time	Sample I + 100% excess Glycerol	Sample I + 40% excess Glycerol	Sample II+ 100% excess Glycerol	Sample II+ 40% excess Glycerol
		FFA %			
TL IM (5%)	0 hr.	35.5± 0.334	-	-	-
	4 hr.	27.0± 0.193	-	-	-
	6 hr.	22.0 ± 0.176	-	-	-
	8 hr.	19.0± 0.195	-	-	-
Novozyme 435 (5%)	0 hr.	35.5 ± 0.334	35.5 ± 0.334	-	16.5 ± 0.180
	4 hr.	24.6± 0.132	15.0 ± 0.119	-	8.1 ± 0.185
	6 hr.	12.8± 0.201	10.2 ± 0.097	-	3.0 ± 0.072
	8 hr.	2.1± 0.011	3.6 ± 0.084	-	3.0 ± 0.074

Values are reported as mean ± s.d., where n=3 (n=no of observations).

Enzyme recycling

The success of the bio-refining process depends on the cost of the enzyme. The cost can only be reduced by recycling the enzyme several times. In the present study, the enzyme Novozyme 435 has been recycled 60 times. The FFA content in the esterified products from sixty consecutive batches is shown in Table 3. It has been observed that the enzyme is still active after 60 times recycling to reduce the FFA of the high FFA RBO by esterification reaction with glycerol. The varying values of final %FFA for sixty batches may be due to the certain loss of enzyme in the isolation process. Isolation process includes enzyme filtration and centrifugation, washing with solvent and drying for further use.

One of the most interesting features in the bio refining process is the weight gain by refining in comparison with the other refining processes. The bioesterified oil containing 3-4% residual FFA is steam stripped to reduce its FFA content to a significant level and the quality of the final refined oil is shown in Table 4. It is observed that after steam stripping, the monoglyceride and diglyceride content are reduced to 1.6±0.008% and 10.2±0.101% from 6.6±0.047% and 11.7±0.077% respectively. The FFA content is reduced to 0.4±0.002%. The proportionate amount of TG in the steam stripped product has increased to 84.5±0.376%. The oryzanol content in the biorefined oil and also in final steam stripped oil is fully retained, which is one of the important advantages of the bioesterification process in comparison with other processes.

Table 3 Recycling of enzyme Novozyme 435 (5%) in esterification of RBO (16.5% FFA) with 40% excess of stoichiometric amount of glycerol (6 hrs duration)

No of recycling	% FFA	No of recycling	% FFA	No of recycling	% FFA
1	3.0 ± 0.009	21	3.3 ± 0.012	41	3.8 ± 0.011
2	3.7 ± 0.011	22	4.0 ± 0.008	42	3.7 ± 0.004
3	3.2 ± 0.010	23	3.9 ± 0.004	43	3.8 ± 0.002
4	3.4 ± 0.008	24	3.2 ± 0.005	44	3.6 ± 0.007
5	3.3 ± 0.012	25	3.1 ± 0.011	45	3.8 ± 0.014
6	4.0 ± 0.005	26	4.0 ± 0.006	46	4.0 ± 0.008
7	4.0 ± 0.003	27	4.1 ± 0.004	47	4.0 ± 0.004
8	3.9 ± 0.009	28	3.1 ± 0.005	48	3.2 ± 0.003
9	4.0 ± 0.008	29	3.5 ± 0.012	49	3.6 ± 0.009
10	4.1 ± 0.001	30	3.9 ± 0.004	50	3.8 ± 0.001
11	3.3 ± 0.011	31	4.1 ± 0.003	51	3.3 ± 0.010
12	3.4 ± 0.013	32	3.4 ± 0.016	52	3.7 ± 0.008
13	3.2 ± 0.018	33	3.4 ± 0.018	53	3.6 ± 0.012
14	3.8 ± 0.016	34	3.4 ± 0.009	54	4.0 ± 0.012
15	3.8 ± 0.018	35	3.9 ± 0.002	55	3.9 ± 0.005
16	3.0 ± 0.012	36	3.8 ± 0.001	56	4.0 ± 0.009
17	3.4 ± 0.007	37	3.2 ± 0.013	57	4.0 ± 0.005
18	3.2 ± 0.009	38	3.9 ± 0.008	58	3.6 ± 0.007
19	3.0 ± 0.010	39	3.9 ± 0.001	59	3.8 ± 0.001
20	3.3 ± 0.001	40	3.5 ± 0.012	60	4.0 ± 0.011

Values are reported as mean ± s.d., where n = 3 (n = no of observations).

Table 4 Characteristics of crude, biorefined, and steam stripped RBO

Composition	Degummed, dewaxed and bleached oil	Biorefined oil (enzyme Novozyme 435)	Biorefined and steam stripped oil
Yield (%)	-	-	97 ± 0.482
FFA	16.5 ± 0.180	3.0 ± 0.036	0.4 ± 0.002
MG	4.2 ± 0.041	6.6 ± 0.047	1.6 ± 0.008
DG	9.9 ± 0.167	11.7 ± 0.077	10.2 ± 0.101
TG	66.0 ± 0.361	76.0 ± 0.381	84.5 ± 0.376
Oryzanol	1.7 ± 0.017	1.7 ± 0.019	1.6 ± 0.026

Values are reported as mean ± s.d., where n=3 (n=no of observations).

Conclusion

The present study revealed that enzymatic deacidification or esterification can be effectively utilized for high free fatty acids rice bran oils to produce quality oils. Nonspecific lipase Novozyme 435 is utilized for this purpose. This

type of esterification process produces no by product, so isolation of used enzyme is simple. Recycling of enzyme can be successfully done in our study and also be implemented in industrial scale. The enzyme can be recycled sixty times or more which ultimately reduces the process cost. Another advantage of using enzyme for deacidifying purpose is that amount of antioxidant like oryzanol in the refined oil is same as that of crude oil. It is quite beneficial for human health. Therefore, enzymatic deacidification process can be adopted in bench scale to obtain maximum productivity and is advantageous in deacidifying high FFA RBO compared to chemical and physical refining. Our results also help future researchers in better understanding of the process as well as good product for the use and recycling of enzymes in different chemical and biochemical field.

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