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Recycling of enzyme in the deacidification of high free fatty acid rice bran oil in pilot scale

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Abstract: *Recycling of enzyme for the deacidification of high free fatty acids (FFA) rice bran oils (RBO) is a novel approach for the synthesis of triacylglycerols. In our study, enzymatic deacidification process has been carried out with special emphasis on the productivity of the process by recycling the enzyme. Two samples of RBO containing different amounts of FFA have been studied for this purpose using two enzymes, non specific Novozyme 435 (Candida antarctica) and specific TL-IM (Immobilized lipase from Thermomyces lanuginosus). Due to more effectiveness as revealed from our study, Novozyme 435 has been recycled successfully sixty times in laboratory scale and ten times in pilot scale for bio esterification of high FFA RBO. The bioesterified oil from pilot plant is stream stripped for the production of edible grade RBO.*

Keywords: *Bioesterification, Lipase, Novozyme 435, TL-IM.*

I. INTRODUCTION

Enzymatic deacidification for the production of edible grade triacylglycerols (TG) using high FFA RBO attracts considerable attention for simplicity of the process and no side products. Recycling of enzyme for bio refining of high FFA RBO in laboratory scale as well as in pilot scale is of considerable importance regarding cost effectiveness and time economization. Rice bran (*Oryza sativa* L) oil may contain 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 due to greater oil loss and darkening of colour [1]. Conventionally, RBO can be alkali refined or physically refined though both methods involve substantial loss of oil. Lipase catalyzed bio refining method may be suitable for RBO of high quality with minimum loss. Moreover, in this process, enzyme can be recycled many more times due to easy isolation of enzyme from the reaction mixture with same efficiency. Productivity of the process can only be obtained by recycling the enzyme which ultimately reduces process cost.

The method of deacidification of a vegetable oil consists of conversion of its free fatty acids into neutral glycerides by re esterification with or without the presence of catalyst [2]. Various researchers re esterified high FFA oils using different chemical and biological catalyst. Bhattacharyya and Bhattacharyya [3] deacidified RBO containing 15-30% FFA to 2-3% levels by re esterification with glycerol with or without catalyst. Enzymatic esterification process has been reported by various authors. According to them, FFA content of oil can be reduced to varying level (2-3%) depending on the composition of oil, temperature, nature and concentration of enzymes. Sengupta and Bhattacharyya [4] bio refined 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*. [5] utilized RBO containing 30-50% FFA to oil containing more than 75% TG by means of immobilized lipase at 60°C for 24 h with dehydration by dry nitrogen flow under a positive nitrogen atmosphere. Kurashige [6] used diacylglycerol (DG) 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 DG in oil. It was previously reported [7] that monoglyceride (MG) too can esterify FFA. Using this concept, Sengupta and Bhattacharyya [8] bio refined rice bran oil with the help of MG and concluded that MG could be effectively utilized instead of glycerol to reduce FFA producing oil of better quality. But no literatures are obtained regarding the recycling of enzyme for bio refining process of high FFA RBO.

Based on this situation, the present study investigated recycling of a non-specific enzyme (Novozyme 435) for deacidification of high FFA RBO up to sixty times in laboratory scale and ten times in pilot scale. By this approach, productivity of the bio refining process can be gained since reusing or recycling the enzyme can reduce process cost. So the efficiency of the process can be enhanced by this way after careful separation of the enzyme from the reaction mixture and by reuses it. This bio refining oil is then stream stripped for making it food grade and by this method, bio refining method can be utilised successfully in large industrial scale also.

Experimental

II. MATERIALS AND METHODS

High FFA RBO 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 and unsaponifiable of RBO were determined according to standard method described in the official and tentative methods of American Oil Chemists' Society (1991). Oryzanol content was determined according to the method of Gopala Krishna et al [9].

Degumming, de waxing and bleaching:

The high FFA RBO was degummed, de waxed and bleached before bio refining. 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 de waxed by winterisation process at 10-12°C for 7 h followed by centrifugation. The de waxed oil was bleached with 2% by weight of bleaching earth and 0.5% activated carbon under vacuum at 190±5°C for 20 min at 6 mm Hg. The bleached oil was then obtained by filtration under vacuum for further use.

Enzymatic esterification in laboratory scale and pilot plant:

For laboratory scale esterification, degummed, de waxed and bleached RBO 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 50^0 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. For recycling purpose, esterification process has been carried out for 6 hours with Novozyme 435 as catalyst for sample II.

In pilot plant, the bio esterification process was conducted in a 10 kg batch bioreactor with 4 kg of the high FFA degummed, de waxed and bleached oil. The enzyme (Novozyme 435) was recycled 10 times maintaining temperature 50^0 C at 4 mm Hg for six hours. Deodorization was carried out by conventional steam stripping at 180°C at 4 mm Hg.

Quantitative determination of MG, DG and TG:

The MG, DG, and TG content of crude and bio refined RBO were estimated by preparative thin-layer chromatography (PTLC) method. 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 [10] 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.

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 using student's t-test in Statistica software.

III. RESULTS AND DISCUSSIONS

Analytical characteristics of the sample.

The analytical characteristics of the two samples, considered here, are shown in Table 1. Two different samples contained different amounts of FFA, MG, DG, TG, unsaponifiable and oryzanol.

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

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

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

Enzymatic esterification and recycling in laboratory scale

The esterification reactions in laboratory scale 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. It is evident from Table 2 that Novozyme 435 is more efficient for esterification purpose. 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 whereas FFA content has been reduced to 2.1±0.011% using enzyme Novozyme 435. By using 40% excess of stoichiometric amount of glycerol, the FFA content has been reduced to 3.6±0.084% and 3±0.074% for sample I and sample II respectively after 8 h of reaction with Novozyme 435. Due to more effectiveness, further study has been conducted with Novozyme 435 enzyme and with 40% excess of theoretical amount of glycerol. Enzymatic esterification reactions using two different enzymes have been carried out at temperature 50^0C and at atmospheric pressure. Temperature is kept low 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→	TL IM (5%)				Novozyme 435 (5%)			
Hours→	0	4	6	8	0	4	6	8
Sample I+100% excess glycerol	35.5± 0.334	27.0± 0.193	22.0 ± 0.176	19.0± 0.195	35.5 ± 0.334	15.0 ± 0.119	12.8± 0.201	2.1± 0.011
Sample I+40% excess glycerol	-	-	-	-	35.5 ± 0.334	15.0 ± 0.119	10.2 ± 0.097	3.6 ± 0.084
Sample II+40% excess glycerol	-	-	-	-	16.5 ± 0.180	8.1 ± 0.185	3.0 ± 0.072	3.0 ± 0.074

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

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 in laboratory scale. The FFA content in the bioesterified products from sixty consecutive batches is shown in Table 3. 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.

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)

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

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

Enzymatic esterification and recycling in pilot plant

The analytical characteristics of the oils used for esterification reaction in the bioreactor are shown in Table 4. Here ten different batches are used for bioesterification purpose with same enzyme. The FFA, MG, DG and TG content in all the batches was varying from 16.8±0.274-22.2±0.261%, 2.1±0.003-3.8±0.004%, 9±0.164-11±0.121% and 61.1±0.361-66.6±0.272% respectively. Oryzanol content in all the batch oils was nearly similar (1.1±0.007-1.3±0.008%). After bioesterification, the analytical characteristics were also determined and tabulated in Table 5. FFA content was varying from 2.9±0.054-3.9±0.057%. There was very little increase in MG and DG content in the bioesterified oil.

Table 4 Analytical characteristics (%) of 10 batches of high FFA RBO samples used in bioreactor

Batch No	FFA	TG	DG	MG	Unsap	Oryzanol
1	22.1±0.228	61.1±0.361	10.6±0.072	3.5±0.002	2.6±0.003	1.2±0.002
2	22.9±0.301	61.8±0.402	9.80±0.119	3.5±0.017	2.6±0.003	1.2±0.001
3	16.8±0.274	66.6±0.272	10.7±0.093	3.3±0.007	2.6±0.006	1.1±0.007
4	18.9±0.299	64.0±0.401	11.0±0.121	3.2±0.011	2.9±0.007	1.2±0.001
5	21.6±0.201	65.0±0.296	9.0±0.164	3.3±0.005	3.0±0.001	1.3±0.008
6	22.2±0.261	61.3±0.215	9.8±0.046	3.5±0.009	3.1±0.001	1.2±0.001
7	20.0±0.098	64.3±0.169	9.5±0.086	3.3±0.002	2.8±0.007	1.1±0.002
8	20.0±0.172	63.9±0.189	9.1±0.037	3.8±0.004	2.9±0.011	1.2±0.004
9	19.9±0.302	63.8±0.423	10.1±0.103	3.1±0.012	3.0±0.018	1.3±0.008
10	20.9±0.285	63.0±0.248	11.0±0.118	2.1±0.003	2.9±0.009	1.3±0.002

Values are reported as mean ± s. d., where n=3

Table 5 Analytical characteristics (%) of 10 batches of bio refined RBO

Batch No	FFA	TG	DG	MG	Unsap	Oryzanol
1	2.9±0.054	78.0±0.519	11.1±0.111	4.9±0.024	2.9±0.022	1.2±0.004
2	3.3±0.018	77.9±0.616	12.5±0.163	3.9±0.071	2.5±0.042	1.1±0.005
3	3.0±0.039	77.6±0.482	11.6±0.284	4.0±0.101	3.7±0.067	1.1±0.001
4	3.5±0.027	74.9±0.581	13.8±0.079	4.2±0.077	3.1±0.078	1.2±0.003
5	3.6±0.033	78.5±0.878	10.8±0.055	3.6±0.089	3.5±0.038	1.3±0.006
6	2.9±0.009	76.9±0.673	12.9±0.072	4.2±0.054	3.0±0.092	1.2±0.008
7	3.9±0.057	78.0±0.461	11.9±0.117	4.2±0.026	2.9±0.105	1.2±0.011
8	3.1±0.041	78.3±0.682	10.6±0.284	4.0±0.016	3.9±0.039	1.1±0.002
9	3.4±0.082	79.1±0.510	10.8±0.111	4.0±0.029	3.0±0.063	1.0±0.018
10	3.5±0.059	78.5±0.429	11.9±0.255	3.3±0.064	3.0±0.073	1.0±0.006

Values are reported as mean ± s. d., where n=3

The bio refined oil upon further deodorization for the ten batches showed that FFA was reduced to 0.39±0.002-0.49±0.002%, TG increased to 83±0.692-85±0.511% with 10±0.289-11.5±0.218% DG, 1±0.008-2.1±0.001%MG (Table 6). Hence bio refining method reduced FFA to appropriate low level which could be effectively steam stripped to food grade quality.

Table 6 Analytical characteristics of 10 batches of bio refined and steam stripped RBO

Batch No	FFA	TG	DG	MG	Unsap	Oryzanol
1	0.44±0.001	84.0±0.552	11.1±0.174	1.3±0.004	3.0±0.183	1.2±0.007
2	0.43±0.003	84.1±0.581	11.5±0.218	1.2±0.009	2.6±0.067	1.2±0.002
3	0.47±0.001	83.9±0.428	11.5±0.149	1.2±0.003	2.9±0.095	1.1±0.004
4	0.49±0.002	83.0±0.692	11.0±0.151	1.9±0.002	3.2±0.111	1.2±0.006
5	0.41±0.003	83.2±0.582	10.6±0.211	2.1±0.001	3.6±0.119	1.3±0.001
6	0.40±0.002	85.0±0.493	10.0±0.289	1.3±0.003	2.9±0.115	1.2±0.001
7	0.41±0.007	84.3±0.585	10.8±0.166	1.4±0.003	3.0±0.138	1.1±0.007
8	0.43±0.001	83.1±0.555	11.0±0.149	1.5±0.007	3.1±0.171	1.1±0.009
9	0.39±0.002	84.0±0.672	10.8±0.154	1.8±0.003	3.5±0.149	1.0±0.002
10	0.40±0.001	85.0±0.511	10.6±0.138	1.0±0.008	3.1±0.139	1.0±0.008

Values are reported as mean ± s. d., where n=3

IV. CONCLUSION

Deacidification and recycling of enzyme for high FFA RBO in laboratory scale and in bioreactor can be effectively utilized to produce quality oils. Nonspecific lipase Novozyme 435 is utilized for this esterification process which produces no by product, so isolation of used enzyme is rather 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 nearly same as that of crude oil. It is quite beneficial for human health. Therefore, enzymatic deacidification process can be adopted in bench scale and is advantageous in deacidifying high FFA RBO compared to chemical and physical refining. The outcome

of our research work will also facilitate future researchers in better perceptive for the bioprocess to obtain quality product by using and reusing of enzymes in different chemical and biochemical sectors.

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