NOVEL STRUCTURED LIPID SYNTHESIS WITH DESIRED N-6:N-3 RATIO USING RICE BRAN LIPASE

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Abstract. In this research, attempts were made to use rice bran lipase (RBL) from bran to optimize the fatty acid profile of existing commercial sunflower oil by either replacing or incorporating n-3 fatty acids from marine source that can be a value addition to the superior usability of rice bran. Preliminary assay of crude lipase with the substrate 4-nitrophenyl palmitate (4-NPP) showed specific activity of 0.04 U/mg. Subsequently a 9.45-fold purification (specific activity of 0.37 U/mg) was achieved through ammonium sulfate precipitation followed by fast protein liquid chromatography (FPLC) and reverse-phase high performance liquid chromatography (RP-HPLC) method of purifications. The SDS-PAGE and in-gel overlay staining of purified RBL with 4-methylumbelliferyl (4-MU) butyrate showed a single band of 33 kDa. Purified RBL showed a specificity for natural oils having long chain unsaturated fatty acids with

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a maximum specific activity (0.452 U/mg) in case of rice bran oil. Immobilized RBL was used to blend fish oil free fatty acids (FFA) with sunflower oil (SO) at varying ratios (v/v). Clinically desirable novel structured lipid (SL) were obtained with n-6:n-3 ratios (v/v) of 5.6:1 and 3.6:1 Thus, the current study offers better nutritional desirability to the existing nutrition profile of commercial sunflower oil and further can be exploited to modify other triglycerides to optimize the n-6:n-3 ratio.
Keywords: *4-nitrophenyl palmitate, 4-MU butyrate, structured lipid, acidolysis, n-6:n-3 ratio*

Introduction

Lipases are ubiquitous water-soluble enzymes with high substrate specificity (Ramakrishnan et al., 2013). The evolutionary pathway of lipases suggests that they form a multigene family and have common aspect like sequence homology based on a shared organization of intron-exon boundaries which led to the postulation that these lipases emerged from a common ancestral gene (Wong and Schotz, 2002). Extensive investigation in past few decades on functional differences among them gained focus to deduce the true characteristics of lipase and their involvement in a wide array of metabolic pathways. Lipases or triacylglycerol acyl hydrolases (EC 3.1.1.3) are the biocatalysts for different mechanical procedures, because of their multifaceted properties (Kanmani et al., 2015). Reports concerning the extraction of plant lipases have been going on to decrease the cost of enzyme mediated commercial processing and to increase the usage of plant lipases (Mishra and Kandali, 2019). Plant originated lipases has provided a great alternative to microbial lipase for potential commercial exploitation as industrial enzymes due to their specificity, low cost, availability and ease of purification (Villeneuve et al., 2003; Enujiugha et al., 2004; Paques and Macedo, 2006; Polizeli et al., 2008). Most of the work published on rice bran lipase (RBL) deals with its deactivation to stabilize the bran or its structural and biochemical properties. Although few efforts have been made to extract lipase from rice bran for commercial use, due to the low yield of the enzyme or the deficiency of information on the enzyme content of different rice varieties, the failure of these studies to date may be explained by a simple reason: only one or two varieties of rice have been examined. The enzyme, however, was discovered to be particularly susceptible to deactivation, even under ordinary conditions. A commercial enzyme must be stable to work well. It was therefore necessary to ascertain conditions under which the enzyme would be reasonably stable as such or by means of immobilization. In the present investigation, attempts were made towards value addition of rice bran by using immobilized RBL to synthesize a novel health product aiming at industrial application of the purified RBL. Different research reports suggested that an optimum ratio of n-6:n-3 fatty acid ratio in the diet is essential to avoid various ailments. Nowadays, it is known that excesses of either n-6 or n-3 polyunsaturated fatty acid (PUFA) showed immunosuppressive effects, and that maintenance of the immune response could be possible with n-6:n-3 ratios between 2:1 and 4:1 (Palombo et al., 1999; Fan et al., 2003).

A ratio of 4:1, 2.5:1, 2–3:1, 5:1 of n-6:n-3 fatty acids were correlated with prevention of cardiovascular disease, reduced colorectal cancer, suppressed rheumatoid arthritis, reduced asthma (Weber et al., 1989; Broughton et al., 1997). Marine oils are considered the major commercial source of n-3 fatty acids (Gamez et al., 1999). Fish oil is preferentially used as raw material to prepare n-3 PUFA concentrates after chemical or enzymatic hydrolysis. Based on experimental and clinical studies, the most favorable n-6/n-3 ratio for human nutrition is proposed to range between 2:1 and 4:1 (Candela et al., 2011). In the present study, immobilized RBL was used to synthesize structured lipids

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(SLs) having optimized and clinically desirable n-6:n-3 fatty acid ratio through acidolysis reaction between sunflower oil and fish oil.

Materials and methods

The present investigation was conducted in the laboratory of Department of Biochemistry and Agricultural Chemistry, Assam Agricultural University, Jorhat (26.7247° N, 94.1936° E). Ranjit, a major high yielding rice variety of Assam was procured from the "Instructional cum Research (ICR) farm" of Assam Agricultural University, Jorhat (26.7247° N, 94.1936° E). The sample was air-dried and sun-dried followed by cleaning. The rice variety was milled and polished to obtain bran portion. The rice bran sample obtained was collected in polyethylene bags and carried immediately to the laboratory in the icebox to keep it under stable condition.

Defatting and extraction of rice bran proteins

Defatting of rice bran was done manually using n-hexane with small modifications (Prabhu et al., 1999). Rice bran was defatted with five times the volume of n-hexane by manual stirring for 40 min each for 3 times at room temperature. After defatting, the rice bran sample was air-dried in a fume hood until the hexane was completely removed and prepared for protein extraction. Alkaline extraction of protein from defatted rice bran was performed according to the method described by Bhardwaj et al. (2001), with some modifications. The defatted rice bran was stirred with 40 times volume of extraction buffer (pH 7.5) containing 10 mM Tris-HCl containing 1.0 mM ethylenediaminetetraacetic acid (EDTA) at 40 °C for 12 h. The extract was passed through a cheese cloth and then centrifuged at 13000 rpm for 15 min at 40 °C. The supernatant was collected carefully and used for total soluble protein estimation 660 nm (Lowry et al., 1951).

Purification of rice bran lipase (RBL)

The obtained rice bran protein extract from defatted bran was precipitated by saturated ammonium sulfate fractionation (0-25, 25-50, 50-75, 75-100% w/v) with constant stirring at 4 °C according to Pabai et al. (1995) with little modification. The precipitates in each fraction were harvested by centrifugation at 13000 rpm for 10 min and then re-suspended in 10 mM Tris-HCl buffer at pH 7.5. The salt precipitated fractions were concentrated using Pierce™ Protein Concentrator PES (10-kD cut-off) which helped in the removal of excess ammonium sulfate, if any, during precipitation step. The concentrated protein sample was then applied to the Shodex PROTEIN KW-803 (Gel-filtration FPLC column, dimension 8 mm × 300 mm) fitted with Bio-Rad Biologic Dou Flow low-pressure (5 Mpa) chromatography system with a buffer consisting of 10 mM Tris-HCl, pH 7.4. Elution was carried out with the same buffer at a flow rate of 1 ml/min. Fractions were collected in 1 min intervals over a total elution time of 20 min. FPLC eluted fraction was loaded onto a C18 reverse-phase column (Acclaim TM 300, particle size 3 µm, pore size 300 Å, diameter 4.6 mm, length 150 mm) attached to RP-HPLC system. Before loading, the column was pre-equilibrated with water plus 0.1% (v/v) trifluoroacetic acid. Protein was eluted from the column using a linear gradient of 30% to 100% (v/v) acetonitrile (HPLC grade) plus 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/ min. The elution profile was

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monitored by peptide-specific absorbance of 215 nm. Fractions were collected at 1 min intervals and each fraction was evaluated for the presence of lipase activity after dialyzing the samples extensively.

Determination of RBL activity

Lipase activity was determined by performing both spectrophotometric and titrimetric assay by following Vijayakumar and Gowda (2013), with little adjustments. For spectrophotometric method of lipolytic assay, 4-nitrophenyl esters with varying degrees of chain lengths were used as substrates. The substrate stock solution was 2 mM 4-nitrophenyl ester in dimethyl sulfoxide (DMSO) and was stored at -18 °C. The substrate working solution with a final concentration of 0.2 mM 4-nitrophenyl ester was prepared freshly just before the assay each time. The hydrolysis of 4-nitrophenyl ester was carried out at 30 ± 2 °C for 10 min in 1 mL of 10 mM Tris-HCl buffer pH 7.5 containing 0.2 mM substrate after which the reaction was quenched by adding 100 µl of 10 mM citrate buffer (pH 4.0). A blank was prepared without the enzyme addition. The released 4-nitrophenol was determined by measuring the absorbance at 405 nm using a spectrophotometer (Jasco V-630 Bio spectrophotometer, Jasco Corporation, Tokyo, Japan). One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of the product (4-nitrophenol) per minute under the said assay conditions. Enzyme activity was calculated by using *Equation 1:*

$$\frac{(A_{405nm}\ \text{Test sample} - A_{405nm}\ \text{Blank}) \times \text{total assay volume (ml)} \times \text{dilution factor}}{\text{Molar extinction coefficient} \times \text{incubation time (min)} \times \text{volume (ml) of enzyme aliquot}} \quad \text{(Eq.1)}$$

Similarly, a titrimetric method of enzyme assay was performed using triglycerides as substrates. An aqueous emulsion of tributyrin and tripalmitin (5% w/v) and 0.1% Triton X-100 were prepared in 10 mM Tris-HCl buffer of pH 7.5. The mixture was stirred for 20 min to obtain a stable emulsion. The reaction mixture comprising of 2 mL of the appropriately diluted enzyme (0.5-1.0 mg/ml), 4 ml emulsion and 0.01 mL of 0.1 M $CaCl_2$ was incubated with shaking at 150 rpm for 1 h at 30 ± 2 °C. The reaction was quenched with the addition of 4 ml of distilled ethanol. In blank ethanol was added before the addition of the substrate. The liberated acid was titrated against 0.05 N NaOH with phenolphthalein as an indicator. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of the product (fatty acids) per minute under the said assay conditions which was calculated by using *Equation 2:*

$$\frac{(\text{Volume of Test solution} - \text{volume of Blank}) \times \text{Normality of NaOH} \times 1000}{\text{Volume (ml) of enzyme aliquot} \times \text{incubation duration (min)}} \quad \text{(Eq.2)}$$

RBL hydrolytic activity was also assayed by reaction using various oil (coconut, palm, olive, sunflower, and rice bran oil) as substrates followed by alkali titration method described by Pierozan et al. (2011), with some modifications. Oil (10%, w/v) was emulsified with gum Arabic (5%, w/v) in 10 mM Tris-HCl buffer, pH 7.5, for approximately 2 min and 3,000 rpm using a domestic blender. A 2 mL sample of the enzyme was added to 18 mL of this emulsion. After incubation in a shaker for 15 min at 37 °C and 150 rpm, the reaction was stopped by the addition of 20 mL of distilled ethanol. The amounts of fatty acids liberated was then titrated with 0.05 M NaOH until pH 11. Reaction blanks were run in the same way but adding the sample after the

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addition of stopping solution. Enzyme hydrolytic activity measurements were carried out in triplicate. One unit of enzyme hydrolytic activity was defined as the amount of enzyme that yields 1 μmol of fatty acids per minute in the assay conditions.

SDS-PAGE analysis of RBL

The protein sample after quantification was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The purified bran enzyme fraction was resolved on 12.5% acrylamide in the Bio-Rad Mini-PROTEAN® Tetra Handcast System. 10-20 μg of protein sample was loaded to each lane followed by electrophoresis at 80 V for 3 h. A more sensitive silver staining was done by Sambrook et al. (1989), to visualize the resolved band. Further an in-gel overlay activity assay in the same gel was performed using 4-MU butyrate as the substrate with some modifications to the original protocols described by Prim et al. (2003) and Diaz et al. (1999). Briefly, after electrophoresis, SDS was removed by washing the gel with 25% isopropanol for 15 min, after which the gel was immersed in distilled water for 15 min. The gel was rinsed several times in 10 mM Tris-HCl buffer at a pH of 7.5 and then the substrate 4-methylumbelliferyl (4-MU) butyrate (0.25 mM at a final concentration) was added. The blue fluorescence of the product 4-MU chromophore, detected under ultraviolet illumination, was used to visualize the lipolytic activity present in the same gel itself.

Immobilization of RBL

RBL was immobilized through calcium alginate entrapment with little modification to the protocol described by Kanmani et al. (2015). Purified RBL (in 10 mM Tris-HCl; pH 7.5) was mixed with 3% (w/v) sodium alginate and the lipase-alginate mixture was added using a syringe into 1% calcium chloride solution from a constant distance. The beads thus formed were allowed to harden in calcium chloride solution for 1 h. The beads were kept in 10 mM Tris-HCl buffer of pH 7.5 at 4 °C for further use.

Novel triglyceride synthesis using immobilized RBL

In our present experiment, we have used the pure immobilized lipase from rice bran as a catalyst due to its ease of production, mild reaction conditions and specificity for synthesizing structured lipids (SLs) to attain improved nutritional or functional properties. For this alkaline hydrolysis (saponification) of marine fish oil and its subsequent blending with commercially available sunflower oil at different proportions (*Table 1*) through a transesterification reaction has resulted in SLs. Further purification of SLs and their compositional analysis by gas chromatography-mass spectrometry (GC-MS) were performed to reveal the altered n-6:n-3 ratio compared to the original sunflower oil without acidolysis.

Alkaline hydrolysis of commercially available fish oil was performed by following the method of Litwack (1960), with a little modification. To 10 g of fish oil in a 500 mL round-bottom flask, 0.02% butylated hydroxytoluene (BHT), 100 mL of 95% ethanol and 10 mL of 40% NaOH were added and mixed. The mixture was refluxed for 90 min in a boiling water bath by attaching the flask with a cold-water reflux condenser. The alcohol remains were evaporated and a thick viscous product was obtained which was dissolved in 300 ml of hot water followed by acidification of the solution to a pH of 3.0 by adding 12% HCl. Further heating was continued over a hotplate until a sharp

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separation of an upper fatty acid layer occurs. The aqueous layer was decanted off by using a separatory funnel. The upper fatty acid layer was poured off into a clear separatory funnel and 150 mL of hot water was added followed by vigorous shaking to remove excess glycerol, salts, and HCl if any. This process was repeated for 4 times after which the fatty acid layer was finally removed from the separatory funnel and stored in a tight screw cap container at -20 °C.

Table 1. *Experimental model and levels of the variables used for optimizing the acidolysis of sunflower oil (SO) with free fatty acid (FFA) obtained from commercially available fish oil*

Treatments	Variables	
	Reaction time (h)	SO: fish oil FFA (mole ratio)
T_1	12	1:1
T_2	12	1:4
T_3	12	4:1
T_4	24	1:1
T_5	24	1:4
T_6	24	4:1
T_7	36	1:1
T_8	36	1:4
T_9	36	4:1

T represents the treatments

Acidolysis reaction between sunflower oil (SO) and free fatty acid (FFA) from fish oil was carried out following slight modifications to the original method described by de Araujo et al. (2011). 2.5 ml of mixture containing sunflower oil and fish oil FFA @ 1:1 (v/v), 1:4 (v/v) and 4:1 (v/v) were kept for 12 h, 24 h and 36 h each separately for acidolysis reaction. Immobilized enzyme (2%, w/w) of the total reaction volume i.e., 50 mg free RBL (or 1.75 g of immobilized RBL) with n-hexane @ 2 ml/g of substrate were used per a reaction volume of 2.5 ml. Pre-incubation conditioning without the enzyme was given by only taking sunflower oil, fish oil FFA, n-hexane, 0.001% BHT at 37 °C for 10 min. Reactions were carried out in airtight screw-capped falcon tubes at 48 °C for 12 h, 24 h, and 36 h in a temperature-controlled shaking incubator with 150 rpm. Reaction was stopped by adding 2 ml of acetone into each of the reaction mixture placed in their respective tubes. The molar ratios between sunflower oil and fish oil FFA and the duration of the respective reaction mixtures for acidolysis are given in *Table 1*.

Purification of structured lipids (SLs)

The products of the acidolysis reaction (SLs and FFAs) were separated with some modifications to the original method described by Hita et al. (2007). The reaction mixture was dissolved in n-hexane to a total volume of 200 mL and 150 mL of 0.8 N KOH (hydro-alcoholic solution with 30% ethanol) added. This mixture was agitated and the hydro-alcoholic phase (containing the FFAs as their potassium salts) along with the hexane phase containing the novel triacylglycerols (TAGs) decanted. The

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hydroalcoholic phase was extracted twice more with 20 mL of n-hexane and both n-hexane solutions mixed and the hexane was evaporated off.

Fatty acid profiling of the structured lipids (SLs)

For fatty acid methyl ester (FAME) preparation the acid-catalyzed transesterification of the SLs through methanolic sulfuric acid method was conducted (Antolin et al., 2008). The obtained SLs (*Table 2*) after acidolysis were placed into 50 mL.

Table 2. *Amount of structured lipids (SLs) used for fatty acid methyl ester (FAME) preparation*

Treatments	Reaction time (h)	SO:Fish oil FFA (mole ratio)	Amount of SLs obtained after acidolysis (weight in g)	Amount of SLs used for FAME preparation and GC-MS analysis (weight in g)
T1	12	1:1	1.061	0.053
T2	12	1:4	0.483	0.024
T3	12	4:1	1.916	0.096
T4	24	1:1	1.138	0.057
T5	24	1:4	0.522	0.026
T6	24	4:1	2.011	0.100
T7	36	1:1	1.337	0.066
T8	36	1:4	0.547	0.027
T9	36	4:1	1.942	0.097

FAME: Fatty acid methyl ester, T represents the different treatments used

Falcon tubes to which 10 M KOH (17.5× of the added SL amount) and methanol (132.5× of the added SL amount) were mixed. The mixture was kept for incubation at 55 °C for 90 min with occasional stirring for 5 sec at each 20 min interval. 10 M H_2SO_4 (14.5× of the added SL amount) was added to the mixture after the previous solution was cooled down to room temperature. The incubation step was repeated as before for 90 min followed by which n-hexane (75× of the added SL amount) was added with thorough mixing for 10 min at RT. A quick centrifugation at 5000 rpm for 5 min was done to separate the hexane layer containing the FAME. Excess hexane was evaporated off to concentrate the sample for GC analysis. FAME of respective samples (SLs) were injected to an analytical gas chromatography instrument (Perkin Elmer CLARUS 680 GC) equipped with an FID detector and column of diphenyl dimethyl polysiloxane; model: Elite-35MS (Dimension: 60 m×0.25 mm, 0.25 µm) with a working range of 40-360 °C.

Helium (He) was used as carrier gas @ 1 mL/min. Inlet and detector temperature was kept at 280 °C and the oven temperature was programmed as follows: Initial Temp: 60 °C; Initial Hold: 2.00 min (Ramp 1: 5 °C/min to 140 °C, hold for 5.00 min; Ramp 2: 5 °C/min to 300 °C; hold for 5.00 min. with a maximum temperature of 350 °C. Peaks were identified and the fatty acids were quantified from relative peak area and internal library data.

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Statistical analysis

The data were reported as (mean ± standard deviation (SD) for triplet replication performed by OPSTAT developed by O.P. Sheoran, CCS, HAU, Hisar (India). The Tukey's Honestly Significant Difference (HSD) test with a confidence of 95% are done by using SPSS 19.0 statistical analysis (IBM, New York, USA).

Results

Extraction, purification and determination of RBL activity

The 10 g of defatted rice bran was used to obtain crude lipase/esterase extract through the alkaline extraction method. Lowry method of total soluble protein estimation showed the presence of 417 mg of protein in 10 g of defatted rice bran sample. The 0-25% ammonium sulfate precipitated fraction was shown to have the maximum specific activity (*Table 3*) out of all the ammonium sulfate precipitated fractions.

Table 3. *Specific activity of crude rice bran lipase at different levels of percent ammonium sulfate saturation*

Ammonium sulfate fractions (% saturation)	Specific activity (U/mg)
0-25	0.007
25-50	0.003
50-75	ND
75-100	ND

ND: Not detected

Five sharp peaks obtained from the FPLC chromatogram over an elution period of 15 min, out of which the peak number-1 (6^{th} to 7^{th} min) showed the presence of highest lipolytic activity (*Table 4; Fig. A1*).

Table 4. *Purification table showing rice bran lipase (RBL) activity by spectrophotometric method using 4-nitrophenyl palmitate (4-NPP) as substrate*

Fraction	Protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Fold purification	Yield of activity (%)
Crude extract	417	18.87	0.04	1	100
0-25% ammonium sulfate precipitation	122.9	9.32	0.07	1.75	49.39
FPLC	14.81	2.08	0.14	3.5	11.02
RP-HPLC	0.88	0.33	0.37	9.45	1.74

FPLC: Fast protein liquid chromatography, RP-HPLC: Reverse phase high performance liquid chromatography

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Further a gradient elution (30-100%) using acetonitrile as eluent through RP-HPLC showed a maximal lipolytic activity in the eluate representing peak number 4 in the RP-HPLC chromatogram (*Fig. 1; Table 4*).

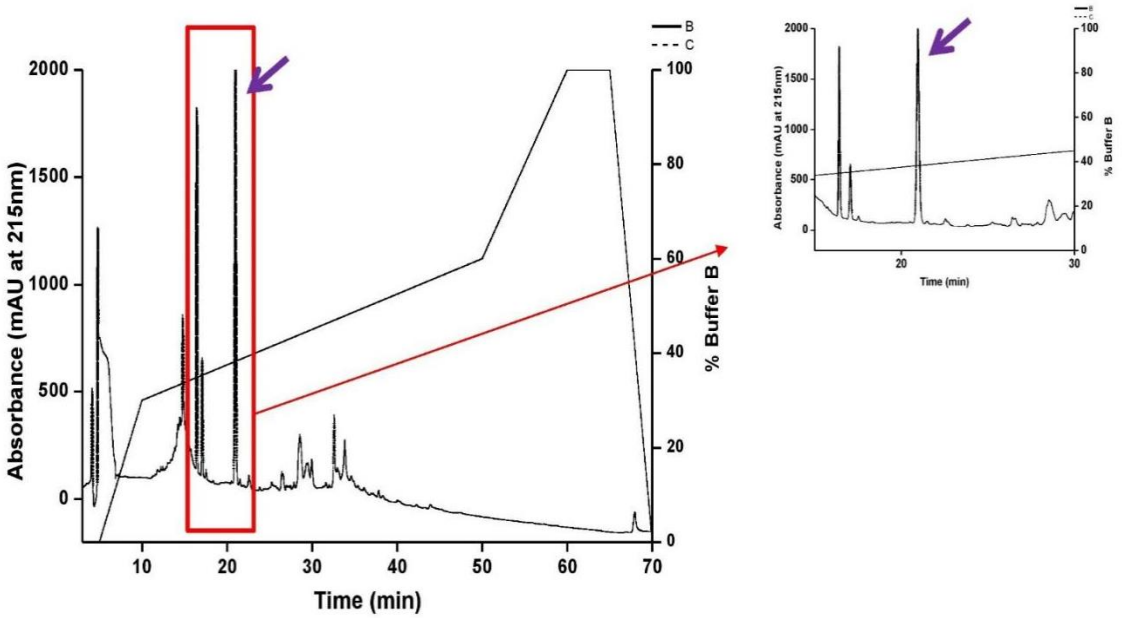

Figure 1. *RP-HPLC chromatogram obtained by loading the lipase fraction (peak number-1) eluted from FPLC column. Several peaks retained (except arrow indicated) on RP-HPLC column depict retention of non-specific hydrophobic proteins having no lipase activity. Arrow mark indicates the chromatogram representing the eluted lipase fraction with highest activity*

The RP-HPLC purified RBL fraction showed a 9.45-fold purification compared to the crude bran enzyme preparation with the highest specific activity of 0.37 µmol min^{-1} mg^{-1} (*Table 4*; *Fig. 2*).

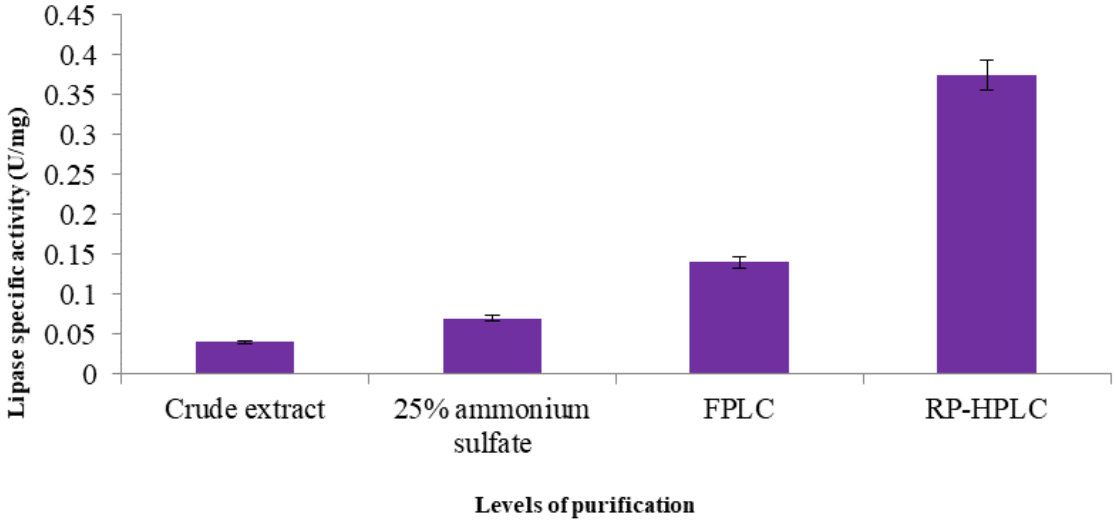

Figure 2. *Histogram showing pure lipase specific activity at different levels purification using 4-nitrophenyl palmitate (4-NPP) as substrate*

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Activity comparison between crude rice bran lipase and wheat germ lipase

The crude rice bran lipase was evaluated for the lipolytic activity using substrates of similar fatty acyl chain length such as tributyrin in titrimetric assay and 4-nitrophenyl butyrate in spectrophotometric assay. The specific activities of the crude rice bran lipase were 0.012 U/mg and 0.022 U/mg in titrimetric and spectrophotometric assay respectively (*Table 5*). A comparison between the activity of crude rice bran lipase preparation and pure wheat germ lipase (type I, cat. no. L3001, Sigma-Aldrich) has been shown in *Table 5*. The pure wheat germ lipase showed a specific activity of 5.83 U/mg in contrast to crude rice bran lipase specific activity of 0.012 U/mg using tributyrin as the substrate in titrimetric assay method. On the other hand, using 4-nitrophenyl butyrate (4-NPB) as substrate through spectrophotometric assay resulted in specific activities of 0.67 U/mg and 0.022 U/mg in case of pure wheat germ lipase and crude rice bran lipase respectively (*Table 5*).

Table 5. *Comparison of crude rice bran lipase with pure wheat germ lipase using two different assay methods*

Method of assay	Substrate used	Specific activity of different enzyme preparation (U/mg)	
		Crude rice bran lipase	Pure wheat germ lipase
Titrimetric	Tributyrin	0.012 ± 0.002	5.83 ± 0.40
Spectrophotometric	4-nitrohenyl butyrate	0.022 ± 0.002	0.67 ± 0.04

Data in table showed the mean ± standard deviation (SD)

Activity of purified enzyme from rice bran using synthetic substrates

The fold purification of RBL with their respective specific activities at different levels of purification has been given in *Table 4* and *Figure 2*. After all the purification steps the effect of different substrates (butyric acid, palmitic acid) with varying degrees of chain length (C4, C16) on the specific activity of pure RBL is studied and presented in *Table 6* and *Figure 3*.

Table 6. *Pure rice bran lipase (RBL) activity using synthetic substrates*

Method of assay	Type of substrate	Specific activity (U/mg)
Titrimetric	Tributyrin	0.223 ± 0.010
	Tripalmitin	0.278 ± 0.006
Spectrophotometric	4-nitrophenyl butyrate (4-NPB)	0.015 ± 0.002
	4-nitrophenyl palmitate (4-NPP)	0.378 ± 0.003

Data in table showed the mean ± standard deviation (SD)

Activity of purified enzyme from rice bran using oil natural substrates

The hydrolytic activity assay of RBL using various natural substrates such as (coconut, palm, olive, sunflower and rice bran oil) was evaluated and presented in *Table 7* and *Figure 4*. The enzyme activity assays using synthetic and natural substrates

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were conducted to determine the nature of the purified enzyme. From the experiments, this could be concluded that the purified enzyme was lipase rather than esterase.

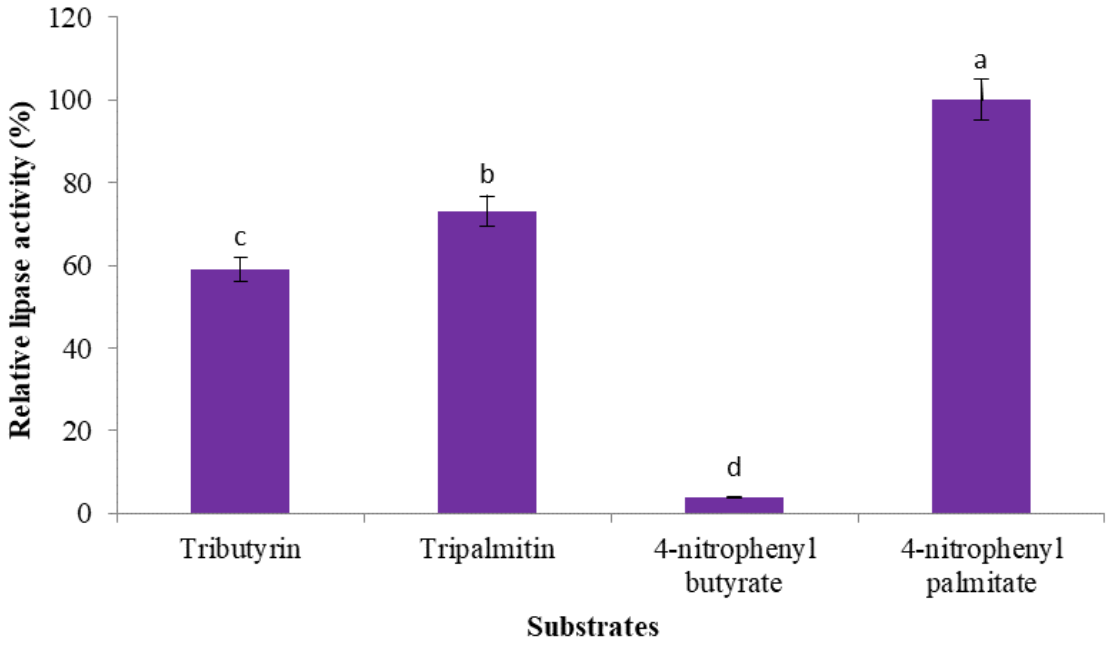

Figure 3. *Histogram showing relative RBL activity in the presence of different substrates*

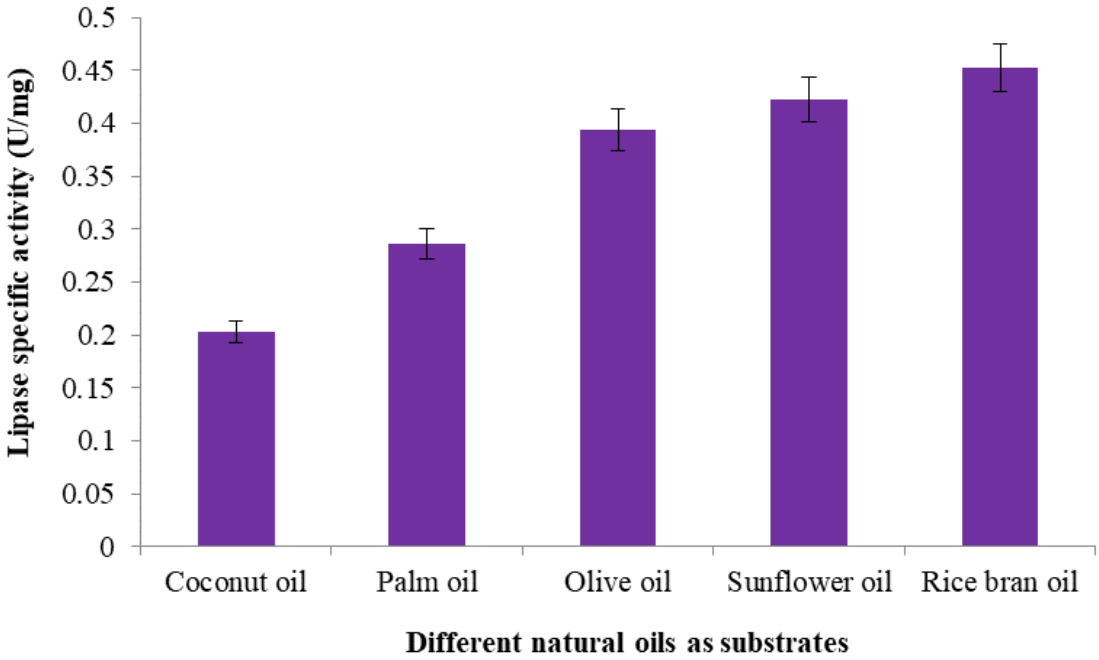

Figure 4. *Histogram showing pure lipase activity on natural oils as substrates*

SDS-PAGE analysis of RBL

The silver staining of RBL fractions obtained from different purification steps showed retention of a band at about 33 kDa (*Fig. 5*) against a broad range (11-245 kDa)

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pre-stained protein ladder (HIMEDIA; product code: MBT092). Further a distinct fluorescent band at 33 kDa resulted from in-gel overlay staining under UV illumination supports the band positioning of pure RBL (*Fig. 5*). The in-gel specific activity staining of the RBL and a fluorescent band (lane-6; *Fig. 5*) with a lipase specific fluorescence substrate (4-MU butyrate) shows the purified rice bran enzyme to be a lipase enzyme. Further, the enzymatic activity (*Tables 5* and *6*) with respect to two assay methods (titrimetric and spectrophotometric) confirm the purified bran enzyme to be a lipase enzyme which is clear from the substrate preference (of varying chain length; *Tables 5* and *6*) at different level of enzyme preparation (crude and purified; discussion section can be referred in this regard).

Table 7. *Rice bran lipase (RBL) activity on natural substrate using titrimetric method*

Substrates	Specific activity (U/mg)
Coconut oil	0.203 ± 0.010
Palm oil	0.286 ± 0.011
Olive oil	0.394 ± 0.028
Sunflower oil	0.422 ± 0.012
Rice bran oil	0.452 ± 0.019

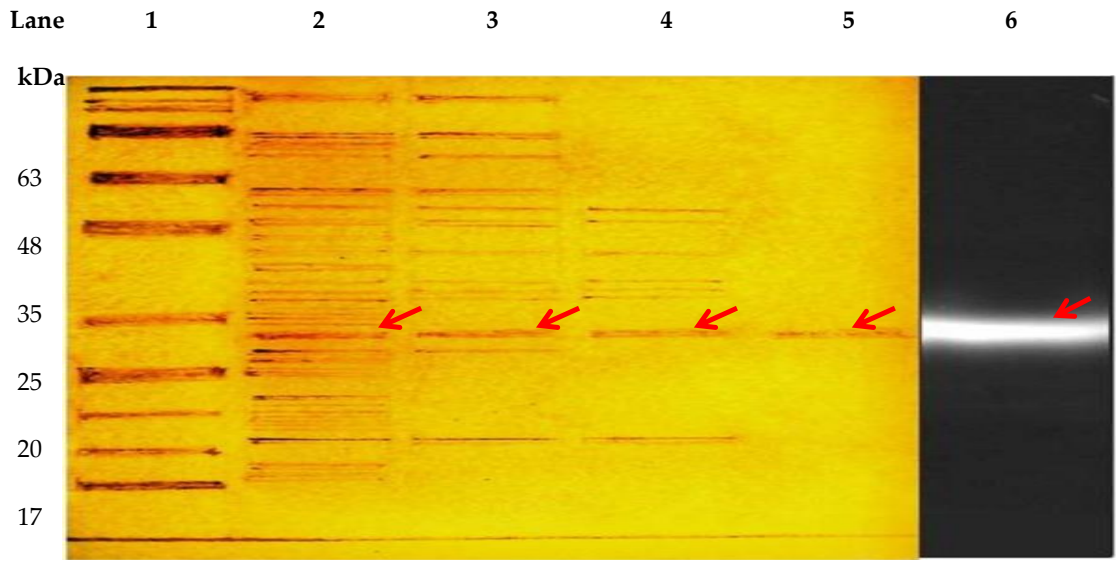

Figure 5. *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of rice bran lipase at different levels of purification. Lane 1: protein ladder (range 11-245 kDa); lane 2: crude bran enzyme; lane 3: 25% ammonium sulfate precipitated fraction; lane 4: FPLC fraction; lane 5: RP-HPLC fraction; lane 6: In-gel overlay assay with fluorescent substrate 4-MU butyrate*

Novel structured lipid synthesis using immobilized RBL

The pure RBL was immobilized through calcium alginate mediated entrapment method. Out of all the treatments mentioned in *Table 1*, only two treatments T1 and T8

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had the products with the desired level of n-6:n-3 fatty acid ratio compared to the sunflower oil without acidolysis (control). The n-6:n-3 fatty acid ratio in commercial fish oil was found out to be 1.2:1 (*Table 8; Fig. A2*) before acidolysis reaction.

Table 8. *Fatty acid content and profile*

	Sunflower oil (control)	**Fish oil (control)**	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	**T_7**	**T_8**	**T_9**
SFA	11.2	1.3	8.0	5.7	8.0	6.6	7.2	7.0	6.3	9.2	7.6
MUFA (n-9)	30.1	19.9	8.3	0.3	15.3	18.3	16.3	ND	20.3	15.3	ND
n-6	58.5	42.6	68.1	63.6	74.7	73.3	76.7	91.2	71.7	57.4	91.0
n-3	0.2	34.6	12.1	ND	ND	ND	ND	ND	ND	15.6	ND
n-6/n-3	292.5	1.2	5.6	63.6	74.7	73.3	76.7	91.2	71.7	3.6	91.0

MUFA: Mono-unsaturated fatty acid, SFA: Saturated Fatty Acid, ND: Not Detected, T1-T9: Treatments of acidolysis reaction (also see *Tables 1* and *2*); Numerical values presented under each fatty acid category depicts the % area occupied on the GC-MS chromatogram by the same fatty acid

The unfavorable n-6:n-3 fatty acid ratio of 292.5:1 in commercially available sunflower oil (*Table 8; Fig. A3*) was brought down to 5.6:1 and 3.6:1 in case of T1 (*Table 8; Fig. A4*) and T8 (*Table 8; Fig. A5*) respectively in our current experiment. The obtained products of acidolysis (SLs) with a desired n-6:n-3 fatty acid ratio as mentioned above are shown in *Figure 6*. The fatty acid profiling for other treatments (T2, T3, T4, T5, T6, T7, T9) are presented in *Table 8*, which could not attend the desirable n-6:n-3 fatty acid ratio but they had a considerably lower n-6:n-3 fatty acid ratio compared to the control.

Discussion

Protocol optimization for rice bran lipase extraction was carried out starting with the method of Bharadwaj et al. (2001) as the base method. A little modification of this method resulted in a higher yield of the bran lipase of about 417 mg of protein/10 g of defatted bran. The extraction buffer consisted of 10 mM Tris-HCl and 1 mM EDTA with a pH of 7.5. This extraction buffer was used to extract more specifically lipase which is present as major enzyme in the bran of rice variety 'Ranjit'. For activity assay of the enzyme crude extract, initially a short chain fatty acyl substrate i.e., butyric acid was used as substrate. Tributyrin for titrimetric and 4-nitrophenyl butyrate for spectrophotometric assay were used to check for the presence of lipase activity in the crude rice bran protein concentrate as lipase has the easiness in hydrolyzing the short fatty acyl chain in emulsion as compared to long chain triglycerides (Rosenstein et al., 2000; Fojan et al., 2000; Carriere et al., 2001; Chahiniana et al., 2009). Lipases belong to the α/β hydrolase superfamily and are known to function better in the lipid-water interface through 'interfacial activation' with an ultimatum of increase in lipase activity at the interface (Ericsson et al., 2008), which is why our experiment with emulsion of short fatty acyl chain showed high lipolytic activity.

Around 1.8 times more lipase activity in rice bran enzyme extract was observed in case of spectrophotometric assay as compared to titrimetric method of assay (*Table 5*). Differential lipase activity was observed between crude RBL and pure wheat germ lipase even with the substrates having similar fatty-acyl chain length (*Table 5*).

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Figure 6. *Products obtained before and after acidolysis. A: Sunflower oil before acidolysis (control-1), B: Fish oil free fatty acids obtained from saponification of commercial fish oil before acidolysis (control-2), C: Structured lipids (SLs) obtained from T1, D: Structured lipids (SLs) obtained from T8*

Lipases display high activity against emulsions of slightly water-soluble short-chain TAG (Aizono et al., 1976; de los Angeles et al., 2015). For this reason, an emulsified triglyceride (tributyrin) was used as a substrate for the titrimetric method (*Table 5*) and hence 8.7-fold higher lipase activity can be seen in case of pure wheat germ lipase through titration method (*Table 5*). Brunschwiler et al. (2013) showed that crude and pure form of lipase had higher activity in spectrophotometric and titrimetric assay method respectively. This is due to possible presence of esterase in crude preparation and limitations of non-specific esterase hydrolysis in spectrophotometric method (Brunschwiler et al., 2013; Mishra and Kandali, 2019).

In the present research crude rice bran lipase was subjected to ammonium sulfate precipitation with different percent saturation followed by FPLC and RP-HPLC to get the pure lipolytic enzyme. Our findings showed 0-25% ammonium sulfate precipitated fraction is having the maximum specific activity (*Table 3*) out of all the precipitated fractions (0-25, 25-50, 50-75, 75-100% w/v). This 0-25% ammonium sulfate precipitated fraction was subjected to FPLC. Peaks obtained from the FPLC chromatogram showed the presence of highest lipase activity in the peak number-1 (6^{th} to 7^{th} min) (*Fig. A1*) which was then pooled into a single followed by RP-HPLC and the highest specific activity was observed in 4th peak shown in the RP-HPLC chromatogram (*Fig. 1*).

The maximum activity was shown by 0-25% ammonium sulfate fraction and a similar observation was also reported by Rajeshwara and Prakash (1995), where 25% ammonium sulfate precipitated fraction from rice bran showed maximum lipolytic activity. In another experiment by Borkar et al. (2009), an extracellular lipase from Pseudomonas aeruginosa SRT 9 had shown maximum lipolytic activity in the 30% ammonium sulfate fraction. In fact, the optimal range of maximum lipase specific activity is between 20-40% ammonium sulfate fractionated elution (Pabai et al., 1995).

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SDS-PAGE (12%) was carried out using crude and purified enzyme extract obtained at different levels of purification which was followed by in-gel activity staining with fluorescent substrate. The silver staining of SDS-PAGE and in-gel overlay staining of the same gel with a fluorescence substrate (*Fig. 5*) revealed retention of a common band for all the different stages of purification of rice bran lipase. The molecular weight of the common band was determined with the help of a reference protein ladder of known molecular weight which showed the molecular weight of the rice bran lipase to be approximately 33 kDa. The distinct fluorescence activity observed with lipase specific fluorescent assay using 4-methylumbelliferone detected under UV illumination confirmed the purified lipolytic enzyme to be lipase rather than esterase.

Antecedently a rice bran lipase was detected and found out to be 33 kDa molecular weight after successive chromatography of DEAE-cellulose, Sephadex G-75 and CM-Sephadex C-SO by Aizono et al. (1976). Another report of purified lipase from rice bran by Rajeshswara and Prakash (1995) through ion exchange chromatography using DEAE-Sepharose CL-6B followed by column chromatography using sephadex G-75 was found out to be of molecular weight 30 kDa. Vijayakumar and Gowda (2013), also reported a 35 kDa lipase from rice bran lipase. Lipase specific fluorescent assay using 4-MU butyrate as substrate was performed earlier by Roberts (1985). International Union of Biochemists claimed lipase as carboxylic acid esterase (EC 3.1.1) due to their specificity for ester of carboxyl groups and such esterase from rice bran were found out to be 25 to 27 kDa (Chuang et al., 2011; Hamada et al., 2012).

The fold purification of rice bran lipase with the respective specific activities at different levels of purification is given in *Table 4* which showed 1.75, 3.50 and 9.45-fold increase in the specific activities of 0-25% ammonium sulfate precipitated, FPLC and RP-HPLC fractions, respectively. After all the purification steps the effect of different substrates (butyric acid, palmitic acid) with varying degrees of chain length (C4, C16) on the specific activity of pure enzyme was studied and presented (*Table 6*).

In titration method use of tributyrin as substrate showed closer activity to tripalmitin despite of the fact that lipase enzyme preferably acts on long chain fatty acid because in titration, emulsion of tributyrin was used and lipase display high activity against emulsions of slightly water-soluble short-chain TAG such as tributyrin (Hita et al., 2007). In spectrophotometric assay almost 96% less activity was recorded in case of the substrate 4-NPB as compared to the substrate 4-NPP because the 4-NPB is not a lipase specific substrate (Chuang et al., 2011). The 26% reduction in the lipase activity in case of titrimetric method from spectrophotometric method even after using the same long chain fatty acyl substrate, palmitate might be due to the less sensitivity of titration method to small acidity change. Similar results were also observed by Brunchwiller et al. (2013). In our present investigation, putting together the results of activity assay (*Table 4*) and band detection in SDS-PAGE followed by fluorogenic gel overlay assay of the RP-HPLC fraction (*Fig. 5*) provide the evidence about the purified fraction to be a lipase enzyme having an approximate molecular weight of 33 kDa.

A hydrolytic activity assay of pure RBL on different oil (coconut, palm, olive, sunflower and rice bran oil) as substrates was performed and the data obtained are presented in *Table 7* which showed highest and lowest specific activities of RBL towards rice bran oil and coconut oil respectively. The hydrolytic activity of RBL was maximal with natural oils containing long unsaturated fatty acids (C18:1, C18:2) like rice bran, sunflower, olive and palm oil. The hydrolysis of coconut oil, which is known

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to be rich in saturated fatty acids, was low. Similar outcomes were also reported by Vijayakumar and Gowda (2013) and Kanmani et al. (2015), where coconut oil hydrolysis showed low lipase activity compared to other natural oils like rice bran, sunflower, olive and palm oil.

The extreme conditions could lead to enzyme degradation or loss of activity, so immobilization (physical entrapment) may be appropriate for sensitive and labile biomolecules. Enzymes with the ability to do this will have better operational performance (Idris et al., 2012; Gupta et al., 2013). In order to bring down the unfavorable n-6:n-3 ratio in commercially available sunflower oil we standardized a method of acidolysis which included blending of fish oil free fatty acids with sunflower oil at different ratios and with 12 h, 24 h and 36 h of incubation period during the acidolysis reaction (*Table 1*).

The concept of SLs implies modification of the fatty acid composition and/or fatty acids location on the glycerol backbone, and improvement of the physical and/or physiological properties of dietary lipids. Studies have also shown that enzymatically or chemically modified TAGs exhibit similar or, in some cases, greater benefits compared to blended oils with similar fatty acid compositions (Nagaraju and Lokesh, 2007). We obtained a n-6:n-3 fatty acid ratio of 3.6:1 and 5.6:1 after 12 h and 36 h of acidolysis with a ratio of 1:1 and 1:4 between sunflower oil and free fatty acid from fish oil respectively (*Table 8*), which showed that the immobilized lipase could be used to optimize the fatty acid profile of existing commercial sunflower oil by either replacing or incorporating n-3 fatty acids from marine source. Torres et al. (2003) synthesized designer acylglycerols rich in residues of eicosapentaenoic, docosahexaenoic, conjugated linoleic, and/or stearic acids by using immobilized microbial lipase.

Pacheco et al. (2010) performed sunflower oil interesterification with palmitic-stearic acids blend by using Lipozyme RM IM to produce SLs. Another experiment performed by Li and Ward (1993) showed sunflower oil modification with n-3 PUFA concentrates obtained from cod liver fish oil using an immobilized fungal lipase. Subroto et al. (2019) had synthesized a SL through acidolysis of fish oil and milk fat fatty acids resulting in the incorporation of fish oil PUFAs. Similarly, in the current study an unfavorable ratio of 292.5:1 (n-6/n-3) (*Table 8; Fig. A3*) in sunflower oil was brought down to the desired level of 3.6-5.6:1 (n-6/n-3) by incorporating fish oil free fatty acids (*Table 8; Figs. A4* and *A5*).

Conclusion

From the findings it could be concluded that the purified RBL had a preference for triglycerides with long chain fatty acids. Maximal hydrolytic activity of RBL towards both synthetic and natural substrates with long chain triglycerides provided plausible reason to elucidate the rice bran enzyme to be a lipase. A process of enriching sunflower oil with n-3 fatty acids modifying the n-6:n-3 fatty acid ratio to the desired level by incorporating fish oil n-3 fatty acids utilizing immobilized lipase from rice bran was optimized. This approach was helpful in value addition to the rice bran which is otherwise considered as waste by-product. Further research strategy to look into the enzyme active site structure and specific mechanism of catalysis needs to be formulated in future. The cloning and expression might provide a platform for explaining the large diversity of reactions and substrates handled by plant lipases in nature which are poorly

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understood. Identification of phenolic class and the compound responsible for lipase inhibition might open up a new insight for lipase specific inhibition just after milling rather than to go for bran stabilization which unnecessarily results in affecting non-targeted nutritional parameter of rice bran. Commercial oil other than sunflower oil can be tested in order to modify the existing fatty acid profile which can impart a better nutritional paradigm.

Conflict of interests. The authors declare no conflict of interests.

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APPENDIX

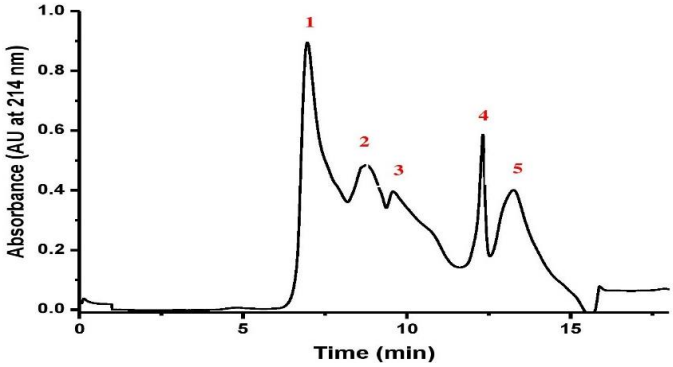

Figure A1. *FPLC chromatogram of 25% ammonium sulfate fractionated rice bran lipase. 1, 2, 3, 4, 5 indicate the serial number in which the respective peaks eluted out of fast protein liquid chromatography column. Peak-1 showed the highest lipase activity with undetected lipolytic activity in rest of the eluted peaks*

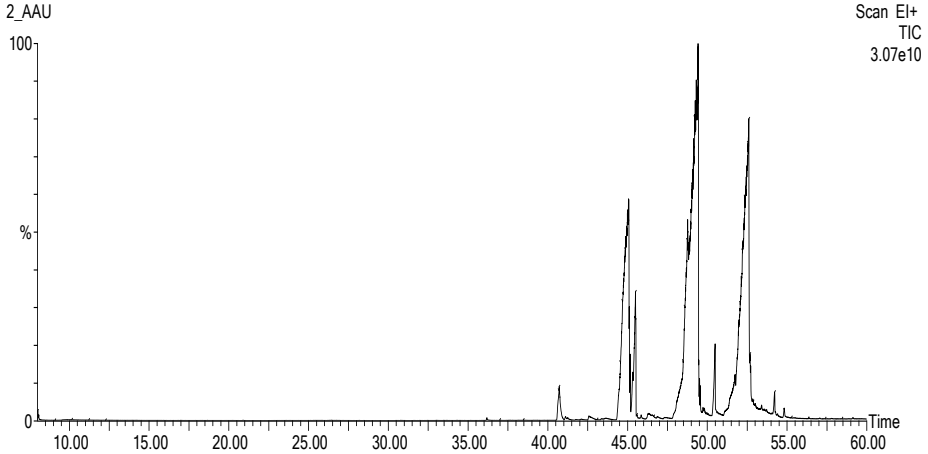

Figure A2. *GC-MS chromatogram for marine fish oil (before acidolysis)*

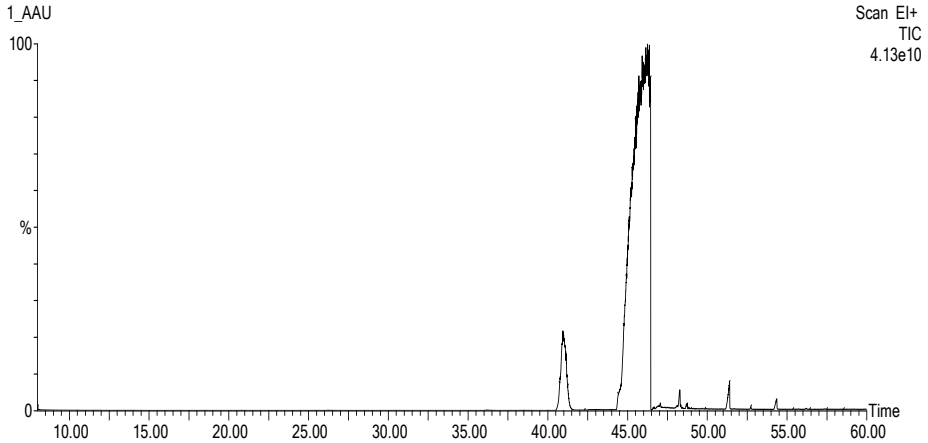

Figure A3. *GC-MS chromatogram for sunflower oil (control; before acidolysis)*

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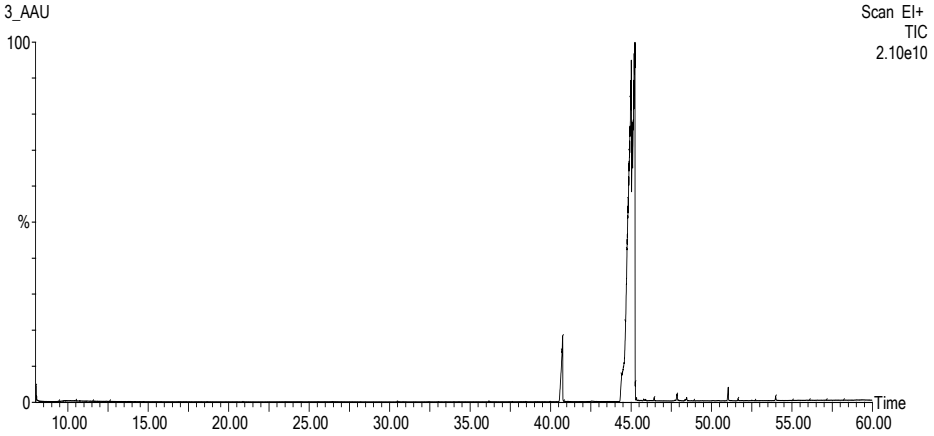

Figure A4. *GC-MS chromatogram for T1*

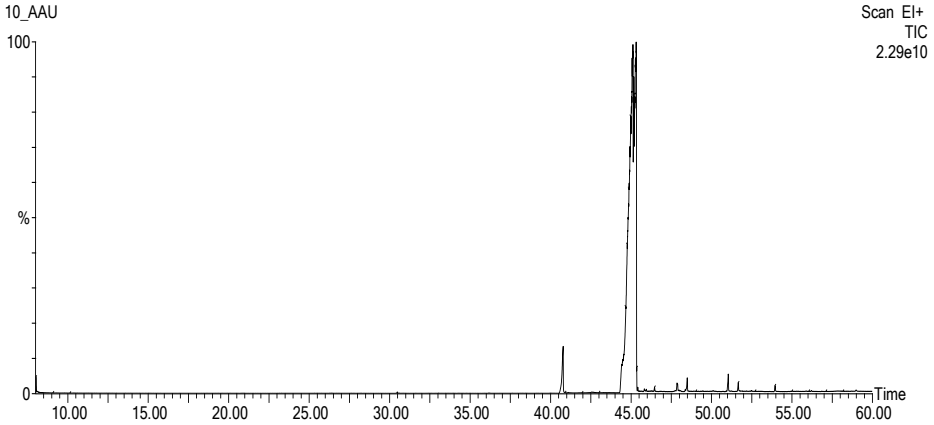

Figure A5. *GC-MS chromatogram for T8. T8 (treatment no.8): Sunflower oil: FFA of fish oil = 1:4, Reaction time = 36 h*

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