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Production of Medium Chain Fatty Acid Rich Mustard Oil Using Packed Bed Bioreactor

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Abstract: A comparative study was done on the production of different medium chain fatty acid (MCFA) rich mustard oil using a stirred tank batchreactor (STBR) and packed bed bio reactor (PBBR) using three commercially available immobilised lipases viz. *Thermomyces lanuginosus*, *Candida antarctica* and *Rhizomucor meihe*. Three different MCFAs capric, caprylic and lauric acids were incorporated in the mustard oil. Reaction parameters, such as substrate molar ratio, reaction temperature and enzyme concentration were standardized in the STBR and maintained in the PBBR. To provide equal time of residence between the substrate and enzyme in both the reactors for the same amount of substrates, the substrate flow rate in the PBBR was maintainedat 0.27 ml/min. Gas liquid chromatography was used to monitor the incorporation of MCFA in mustard oil. The study showed that the PBBR was more efficient than the STBR in the synthesis of structured lipids with less migration of acyl groups. The physico-chemical parameters of the product along with fatty acid composition in all positions and sn-2 positions were also determined.

Key words: medium chain fatty acid (MCFA), mustard oil, packed bed bio reactor, lipase

1 Introduction

Medium chain fatty acids (MCFA) containing triglyceride, MCTs, are special food used as a supportive nutritional therapy. It increases calorific value of the food and improves palatability, digestibility and absorption of the food in comparison to long chain triglycerides (LCT). It reduces the risk of atherosclerosis and also helps in weight maintenance[1)] and these facts are well established.

Mustard oil is widely used edible oil in Eastern and Northern parts of India. It is rich in monounsaturated fatty acid (MUFA), erucic acid (EA, $C_{22:1}$, n-9) and some amounts of polyunsaturated fatty acids (PUFA). The minor component viz. tocopherol present in the oil may also provide beneficial effects to reduce risk factors for cardio vascular diseases (CVD). But erucic acid sometime leads to myocardial lipidosis and fibrosis and therefore low erucic mustard/rapeseed are now on demand. Presently the genetical modification of the crop is the only way to produce low erucic mustard/rapeseed oils.

But if we think in the other way of producing this kind of product along with desired fatty acids we could think about biocatalytic modification of the existing mustard/rapeseed oil. Medium chain fatty acids (MCFA) rich mustard oil was prepared in our previous study by enzymatic acidolysis reaction using *Thermomyces lanuginosus* lipase as biocatalyst[2)]. We thought about MCFA because triglycerides containing MCFA ie. MCTs, are special food which can be used as a supportive nutritional therapy. It increases calorific value of the food and improves palatability, digestibility and absorption of the food in comparison to long chain triglycerides (LCT). It reduces the risk of atherosclerosis and also helps in weight maintenance[1, 3)] and these facts are well established. Our further studies with the structured lipid prepared from mustard oil (MCTM) on animal model proved that capric acid rich mustard oil could act against different lifestyle diseases. The food intake, growth of animals and lipid content of mesentery showed that MCTM has anti-obese properties in comparison to native mustard oil. Again significant lowering of plasma and liver lipids which are high risk factors of cardiovascular diseases was also noticed. Consumption of MCTM improves the haematologi-

Abbreviations: MCFA - Medium Chain Fatty acid, MCT - Medium Chain Triglyceride, MO - Mustard Oil, C8MO- Caprylic acid rich Mustard Oil, C10 MO - Capric acid rich Mustard Oil, C12 MO - Lauric acid rich Mustard Oil, STBR - Stirred Tank Bio Reactor, PBBR - Packed Bed Bio Reactor

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cal and histological conditions which were disturbed due to hypercholesterolemia. It seems to produce an inhibitory effect on platelet aggregation thus reducing the chances of vascular diseases and finally atherosclerosis by reducing the interaction between platelets and vessel wall[4]. It was also observed that the deformity and fragility of erythrocyte membrane caused by cholesterol rich blood was partially reversed by capric acid rich mustard oil by virtue of their ability to lower the extent of hypercholesterolemia[5]. On the basis of some other data it can be proposed that dietary supplementation with capric acid might benefit humans leading to improved antioxidant defenses in individuals with hypercholesterolemia and thereby lowering risk of atherosclerosis[6].

The above studies proved the success of capric acid rich mustard oil for nutraceutical application. The product was prepared using packed bed bio reactor (PBBR) with *Thermomyces lanuginosus*, TLIM. Among the different MCFAs only capric acid was chosen to produce structured lipid with mustard oil in previous studies. Thus the aim of the present study was large scale production of three different MCFA (caprylic, capric and lauric acid) rich mustard oils using three different enzymes (*Thermomyces lanuginosus*, *Rhizomucor meihei* and *Candida antarctica*) in two different reactor systems, Stirred Tank Batch Reactor (STBR) and Packed Bed Bio Reactor (PBBR). The aim was also to investigate the utility of PBBR to synthesize various MCFA rich mustard oil by transesterification reaction using three different lipases and compare it with STBR regarding yield and composition. The scope of this study also included the analysis of physicochemical properties of the MCFA rich mustard oils to establish their suitability as edible oil.

2 Experimental

Fatty acids (capric acid, caprylic acid and lauric acid) were purchased from Merk, Mumbai, India and their purities were checked by gas-liquid chromatography (GLC). Mustard oil used was solvent extracted from authentic seeds, physically refined and bleached in the laboratory.

Three types of immobilized enzymes, e.g, *Thermomyces lanuginosus* (TLIM), *Rhizomuco rmeihei* (RMIM) and *Candida antarctica* (NS 435) used as biocatalysts, were generous gifts from Novozyme India Ltd., Bangalore, India. The original moisture content of the enzymes (2%, w/w) was kept intact to initiate the reaction successfully.

All other chemicals and solvents were of analytical grade and procured from SRL, Mumbai, India.

2.1 Production of MCFA rich mustard oil by Stirred Tank Batch Reactor (STBR)

MCFA (capric, caprylic and lauric) and mustard oil were taken in 3:1 molar ratio and stirred at 200 rpm at 60℃ for different time periods with 10% of three different lipozyme enzymes[2]. The total volume of the substrate was 100 ml. The synthesis of MCT rich mustard oils was first monitored by gas liquid chromatography (GLC) to study the amount of incorporation of fatty acids in mustard oil[2].

The variation in enzyme types (TLIM, RMIM and NS 435) was studied, keeping the other reaction parameters such as temperature (60℃) and substrate ratio (fatty acid: mustard oil :: 3:6) constant. The effect of fatty acid chain length to produce three different MCFA rich mustard oils was also studied.

2.2 Production of MCFA rich mustard oil by Packed Bed Bio Reactor (PBBR)

The reactor consisted of atubular glass column of 10 mm ID and 50cm long. It was also provided with a water jacket for temperature control. The immobilized enzyme packed into the reactor was retained in place by means of a sintered plate. The substrates were fed from the top of the bed and the products were collected at the bottom. The substrates were taken in optimized substrate ratio i.e. fatty acid:oil :: 6:3 and a minimum amount of hexane was added to bring fluidity to the reaction mixture. The substrates were previously blended and well-mixed at the reaction temperature before conducting the packed bed reaction and were poured into the enzyme bed maintaining a fixed sample head. Water from a constant temperature bath was circulated through the jacket by a peristaltic pump. A partial suction from a vacuum pump was given to maintain the constant flow rate (0.27 ml/min to get 100 mL product in 6 hr). 20 gm of enzyme was closely packed into the column by repeated tapping to avoid any air gap. Transesterification reactions were then carried out by passing the substrate through the column. The temperature was maintained at the desired value of 60℃ by passing water through the column jacket[7]. The product mixture was collected at the outlet (after each 100 mL product). The final product was steam stripped to remove the excess fatty acid and bleached. The triglyceride was then analyzed by gas chromatography to determine the incorporation of medium chain fatty acids in mustard oil.

2.3 Chromatographic analysis of oils

Fatty acid composition of MCT rich mustard oils were analysed by GC. Fatty acid methyl esters (FAME) were prepared[8] and the compositions were determined by GC analysis using an analytical gas chromatograph (Agilent 6890 Series) equipped with FID detector and capillary DB-Wax column (30m L, 0.32mm I.D, 0.25 μm FT). N_2, H_2 and airflow rate was maintained at 1ml/min, 30 ml/min and 300ml/min respectively. Inlet & detector temperature was kept at 250℃ and the oven temperature was programmed as 150-190-230℃ with increase rate of 15℃/min and 5 min hold up to 150℃ and 4℃/min with 10 min hold up to 230℃

respectively.

2.4 Analysis of 2-position

The prepared structured lipids were analysed for their sn – 2 position composition by the method of Luddy *et al.*[9].

2.5 Determination of different physical parameters

2.5.1 Viscosity

The viscosity of the formulations was determined using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE40 at 25 ± 0.5℃. The software used for the calculations was Rheocalc V2.6[10].

2.5.2 Refractive Index

Refractive index was determined using an Abbe type refractometer (Nirmal International, New Delhi, India) at 25 ± 0.5℃[11].

2.5.3 Slip Melting Point

Slip melting point was determined using slip melting apparatus by capillary tube method.

2.6 Statistical Analysis

All the data are presented as means with their standard errors. Statistical comparisons between groups were performed using one way ANOVA.

3 Result and Discussion

Three different lipases (TLIM, RMIM and NS 435) were screened in the reaction to assess the efficiency of the enzymes. Figure 1 indicates the comparison of incorporation of caprylic acid, capric acid and lauric acid in mustard oil using the above mentioned three different enzymes at different time intervals using STBR. The temperature was maintained at 60℃ and the enzyme amount was 10% (w/w) of the total reaction mixture. From Fig. 1 it is evident that NS 435 gave maximum incorporation of MCFAs in STBR. The amount of incorporation increased with time with the maximum incorporation at 24 hr.

Figure 2 depicts the incorporation of MCFAs in mustard oil using three different enzymes in PBBR after 6 hr of reaction. From the Fig. 2 it is evident that in this case also the incorporation was maximum in case of NS 435 enzyme. Here also the temperature was maintained at 60℃. This must be for non-selective nature of this particular enzyme which other two did not have.

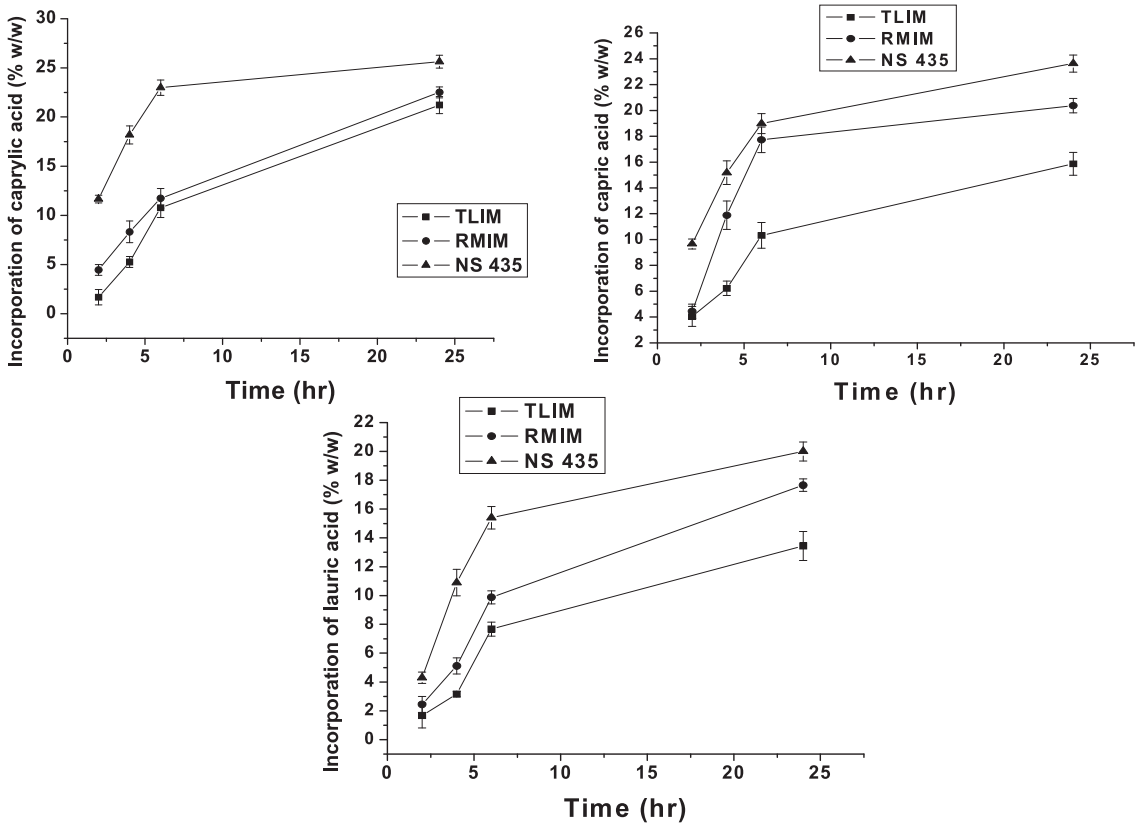

Fig. 1 Incorporation of different MCFA in presence of three different enzymes at different time intervals in STBR [Values are Mean±S.D.].

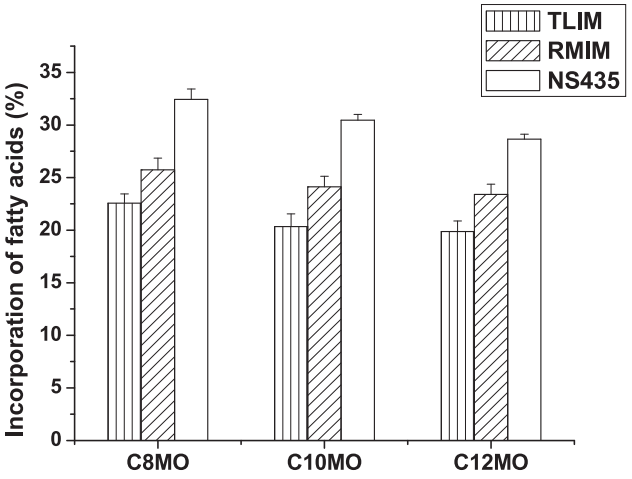

Fig. 2 Incorporation of different MCFA in presence of three different enzymes at different time intervals in PBBR [Values are Mean±S.D.].
(C8MO-caprylic acid rich mustard oil; C10MO-capric acid rich mustard oil; C12MO-lauric acid rich mustard oil).

The incorporation of MCFAs in the two reactors were compared after a definite time period and plotted in Fig 3. The flow rate of PBBR was so maintained that an equal amount of sample was processed in each reactor for a definite time period. The bar diagrams plotted in Fig 3 clearly indicates that the incorporation of MCFAs in PBBR was significantly faster than the STBR. The incorporation of caprylic acid was highest among the three fatty acids. The reason for the difference in incorporation may be the viscosity of caprylic acid. Though 60℃ temperature made the reaction mixtures sufficiently fluid, still higher viscosity of capric acid and lauric acid, which hinders mobility, consequently led to lesser reactivity of fatty acid and mustard oil. The partial suction given to the enzyme bed was effective to maintain the mass transfer throughout the bed at a constant rate.

Pancreatic lipase catalyzed hydrolysis of the caprylic acid rich mustard oil yielded 2-monoglyceride of the respective samples. Further analysis of fatty acid composition of those 2-monoglycerides determined the fatty acid present in the structured lipids prepared. Figure 4 shows the comparison of % incorporation of caprylic acid at sn-2 position of caprylic acid rich mustard oils prepared in two different reactors using three different enzymes. In both

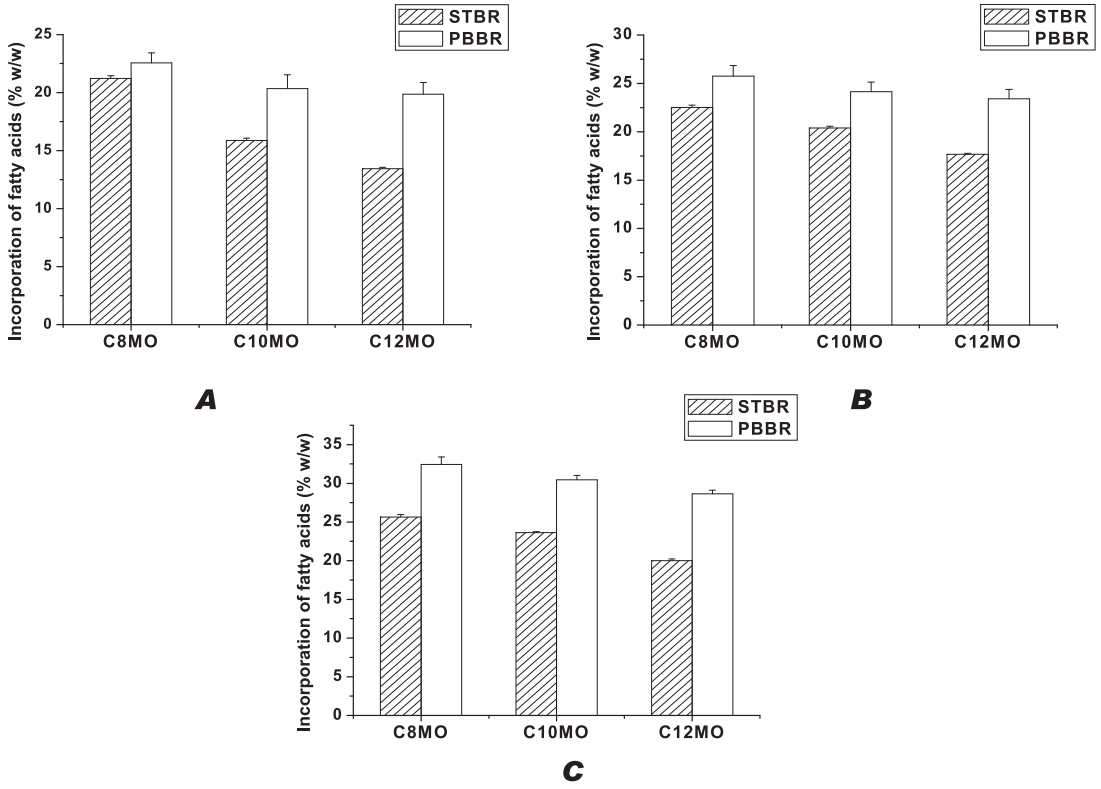

Fig. 3 Comparative effect of incorporation of different medium chain fatty acids in presence of three different enzymes in STBR and PBBR [Values are Mean±S.D.] after 6 hr of incubation.
(C8MO-caprylic acid rich mustard oil; C10MO-capric acid rich mustard oil; C12MO-lauric acid rich mustard oil; A-incorporation by TLIM; B-incorporation by RMIM; C-incorporation by NS 435).

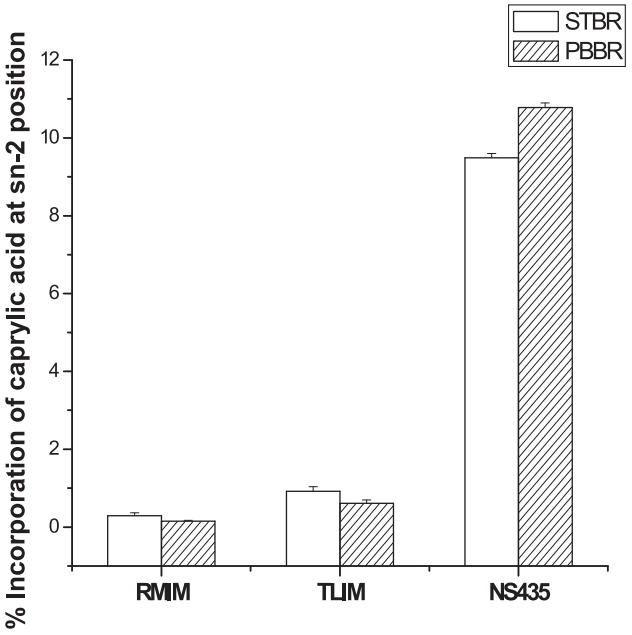

Fig. 4 Incorporation of caprylic acid at sn-2 position of mustard oil in two different reactors and with three different enzymes.

the reactors, amount of caprylic acid incorporated at sn-2 position was maximum in case of NS 435 enzyme which proves that NS 435 is non-specific in nature and RMIM and TLIM are 1,3 specific. The supremacy of the lipase NS435 among the three enzymes is just due to its non-regiospecificity. The presence of very small amounts of caprylic acid in case of reaction with RMIM and TLIM was probably due to acyl migration. However the acyl migration was very less in case of PBBR than STBR.

Fatty acid compositions of final product with highest incorporation of caprylic acid, capric acid and lauric acid in mustard oil after completion of 24 h with NS 435 enzyme which showed the highest activity are shown in Table 1. Similarly, fatty acid compositions of mustard oil incorporated with caprylic acid, capric acid and lauric acid produced by NS435 enzyme in PBBR for 6 hr are presented in Table 2. From the table it was clear that MCFAs were incorporated replacing erucic acid which was present in high amounts in mustard oil.

Changes of refractive index, viscosity and slip melting point of MCFA rich mustard oil produced in PBBR and STBR are shown in Table 3 and 4 respectively. The difference in the values with original MO proved the formation of new compounds and all the values were within the range of common edible oil[12]. It can be found that the for obvious reason as the amount of incorporation and chain length of incorporated fatty acid increased the slip melting point and viscosity of the product increased. Therefore it will be possible to produce a particular product with desired physical parameters following the described reaction parameters.

4 Conclusions

From this pilot study, it can be concluded that low erucic mustard oil can be prepared with desired MCFAs like caprylic acid, capric acid and lauric acid can be successfully by enzymatic acidolysis reaction in both STBR and PBBR. Different types of enzymes (TLIM, RMIM and NS 435) produced three different types of MCFA rich mustard oils with each MCFA in both the bio reactors. After performing a comparative study of the efficiency of the two reactors in

Table 1 Fatty acid composition of three different MCFA rich mustard oil in comparison with native mustard oil interesterified with NS 435 biocatalyst in STBR.

Fatty acid / Sample	Fatty Acid (% w/w)												
	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{22:1}$	$C_{24:0}$
Mustard Oil	–	–	–	2.24	1.15	9.42	17.94	10.75	0.80	5.15	2.00	48.55	1.30
Caprylic acid rich MO	25.64	–	–	1.86	1.12	11.19	14.02	8.21	0.70	3.54	1.99	30.43	1.30
Capric acid rich MO	–	23.64	–	1.86	1.12	11.09	14.15	8.25	0.71	4.00	1.99	31.89	1.30
Lauric acid rich MO	–	–	20.00	1.90	1.13	10.99	15.66	8.55	0.75	4.05	1.99	33.68	1.30

Table 2 Fatty acid composition of three different MCFA rich mustard oil in comparison with native mustard oilinteresterified with NS 435 biocatalyst in PBBR.

Fatty acid / Sample	Fatty Acid (% w/w)												
	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{22:1}$	$C_{24:0}$
Mustard Oil (MO)	–	–	–	2.24	1.15	9.42	17.94	10.75	0.80	5.15	2.00	48.55	1.30
Caprylic acid rich MO	32.43	–	–	1.87	1.12	9.92	13.03	8.55	0.70	4.00	1.99	25.09	1.30
Capric acid rich MO	–	30.45	–	1.28	1.15	8.90	14.13	9.69	0.70	4.02	1.99	26.39	1.30
Lauric acid rich MO	–	–	28.66	1.87	1.12	10.20	11.90	7.98	0.70	4.00	1.99	30.28	1.30

Table 3 Changes in refractive index, slip melting point and viscosity of different MCFA rich mustard oil produced in STBR.

Name of Samples	Enzyme	Refractive Index at 40℃	Slip melting point(℃)	Viscosity (cp) at 32℃
C8MO	NS435	Product	Product	Product
		1.436 ± 0.002	15.6 ± 0.03	19.40 ± 0.12
	RMIM	1.448 ± 0.012[a]	17.6 ± 0.05[a]	21.67 ± 0.09[a]
	TLIM	1.450 ± 0.011[a, b]	20.8 ± 0.02[a, b]	24.00 ± 0.14[a, b]
C10MO	NS435	1.455 ± 0.001	18.5 ± 0.14	22.30 ± 0.13
	RMIM	1.460 ± 0.011[a]	21.9 ± 0.09[a]	23.45 ± 0.11[a]
	TLIM	1.461 ± 0.006[a, b]	24.3 ± 0.08[a, b]	25.60 ± 0.10[a, b]
C12MO	NS435	1.450 ± 0.006	26.2 ± 0.08	27.80 ± 0.18
	RMIM	1.460 ± 0.007[a]	24.2 ± 0.10[a]	29.80 ± 0.19[a]
	TLIM	1.466 ± 0.01[a, b]	29.8 ± 0.11[a, b]	30.90 ± 0.10[a, b]

Refractive index of original mustard oil is 1.473 ± 0.003, Slip melting point is − 10 ± 0.04℃ and Viscosity is 40.67 ± 0.03 cp

Values are Mean ± S.D. (n=3)

[a] Comparison between NS435 group with the other two groups (p<0.05)

[b] Comparison between RMIM group and TLIM group (p<0.05)

Table 4 Changes in refractive index, slip melting point and viscosity of different MCFA rich mustard oil produced in PBBR.

Name of Samples	Enzyme used	Refractive Index at 30℃	Slip melting point(℃)	Viscosity (cp) at 32℃
C8MO	NS435	Product	Product	Product
		1.454 ± 0.004	11.5 ± 0.17	18.40 ± 0.12
	RMIM	1.461 ± 0.003[a]	15.8 ± 0.09[a]	20.45 ± 0.11[a]
	TLIM	1.461 ± 0.010[a]	18.6 ± 0.56[a]	23.67 ± 0.08[a]
C10MO	NS435	1.450 ± 0.012	9.8 ± 0.66	20.30 ± 0.15
	RMIM	1.452 ± 0.008[a]	12.3 ± 0.12[a]	21.67 ± 0.05[a]
	TLIM	1.455 ± 0.004[a]	15.4 ± 0.11[z, b]	24.56 ± 0.07[a, b]
C12MO	NS435	1.459 ± 0.006	15.5 ± 0.07	25.30 ± 0.14
	RMIM	1.460 ± 0.006[a]	23.9 ± 0.18[a]	27.67 ± 0.11[a]
	TLIM	1.465 ± 0.005[a]	24.1 ± 0.18[a]	29.81 ± 0.13[a, b]

Refractive index of original mustard oil is 1.473 ± 0.003, Slip melting point is − 10 ± 0.04℃ and Viscosity is 40.67 ± 0.03 cp

Values are Mean ± S.D. (n=3)

[a] Comparison between NS435 group with the other two groups (p<0.05)

[b] Comparison between RMIM group and TLIM group (p<0.05)

producing MCFA rich mustard oils indicate that the yield of MCFA rich mustard oil is much more with PBBR than STBR and among the three enzymes *Candida antarctica* (NS 435) showed the best results which may account for its non-specific nature. The incorporation of the desired fatty acids was much more with PBBR than with STBR.

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