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# Production of 1, 3 regiospecific lipase from *Bacillus* sp. RK-3: Its potential to synthesize Cocoa Butter Substitute

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## ABSTRACT

A *Bacillus* sp. RK-3 isolated from soil initially produced 3.28 IU/mL of 1, 3 regiospecific lipase in medium containing 1.0% olive oil. After process optimization, 10.56 IU/mL of lipase was produced in medium containing sunflower oil 1.5 %, tryptone 2 %, $Ca^{2+}$ 20 mM using 3 % inoculum in 250 mL Erlenmeyer flask containing 50 mL of the medium at pH 7.0, 250 rpm and 30 °C for 36 h. Scale up in 10 L bioreactor with 7.5 L of the optimized medium yielded 16.41 IU/mL in 30 h resulting in net 6.0 fold increase in enzyme units as against initial units of 3.28 IU/mL obtained under unoptimized conditions. The productivity in 10 L bioreactor is 0.547 IU/mL/h as against initial of 0.091 IU/mL/h. The lipase exhibited 95.12 % stability in hexane, followed by THF (75.83 %) and petroleum ether (73.85 %) after 24 h of incubation. Cocoa butter substitute (CBS) synthesis was attempted in a reaction containing 1.2 IU/mg of lipase using palm oil and methyl stearate in hexane. The reaction product being formed was analyzed qualitatively using Thin Layer Chromatography (TLC) and quantified by gas chromatography (GC) which showed 83.17 % conversion efficiency for CBS in 24 h.

*Keywords:* lipase, production, *Bacillus* RK-3, cocoa butter substitute

## INTRODUCTION

Lipase (triacylglycerol acyl hydrolase E.C. 3.1.1.3) catalyzes the hydrolysis and synthesis of long chain acylglycerols under aqueous, microaqueous and in organic solvents (Jaeger *et al*., 1999; Saxena *et al*., 1999; Hasan *et al*., 2006). This enables lipase to carry out a variety of different catalytic reactions like hydrolysis, esterification, transesterification, alcoholysis, acidolysis and ammonolysis which are important and are exploited in industries such as food and feed, detergents, pharmaceutical, leather, textile and paper (Saxena *et al*., 1999; Hasan *et al*., 2006). Though lipases are obtained from animal and plant sources too, the microbial lipases are of immense importance due to their regiospecific and fatty acid specificity. These properties are exploited for the retailoring of cheap vegetable oils to produce nutritionally important structured triacylglycerols like polyunsaturated fatty acids (PUFA), cocoa butter substitutes (CBS) etc (Sharma *et al*., 2001; Hasan *et al*., 2006).

Cocoa butter (CB) is used in confectionaries and cosmetics due to its flavour and aroma. It is characterized by sharp melting point, desirable physicochemical properties and fatty acid components (Shukla, 1996; Smith, 2001; Tchobo *et al*., 2009). The major components of CB are saturated-unsaturated-saturated (SUS) triacylglycerols which make it hard and brittle at room temperature but allow it to melt completely in the mouth (Lipp and Anklam, 1998; Wang *et al*., 2006; Liu *et al*., 2007). When used in a product, CB gives it a smooth texture, contractibility, flavour release and gloss (Liu *et al*., 2007).

However, due to the new directives of the European Union on chocolate along with the limited supply of CB, variability in quality, large demand and volatility in prices has made the search for alternatives a necessity (Tchobo *et al*., 2009). Several researchers report the use of 1, 3 regiospecific lipases to transesterify lower value fats and oils to synthesize CBS (Macrae and Hammond, 1985; Adlercrutz, 1994; Abigor *et al*., 2003. Though, lipases from plants like *Carica papaya* have been reported for CBS synthesis (Pinyaphong and Phutrakul, 2009), however, microbial lipases are preferred for enzyme mediated reactions due to the ease in their availability and production. 1, 3 regiospecific lipases have been reported from many fungi like *Aspergillus*, *Rhizopus*, *Candida* etc and bacteria like *Pseudomonas* and *Bacillus* (Saxena *et al*., 1999; Sharma *et al*., 2001).

Here, we report the production of a 1, 3 regiospecific lipase from newly isolated *Bacillus* sp. RK-3, its process optimization and scale up to a 10 L bioreactor. The lipase produced was partially purified and lyophilized. The dried enzyme was now used for synthesis of CBS.

## MATERIALS AND METHODS

### Chemicals and reagents

*p*-Nitro phenyl palmitate (*p*-NPP), methyl stearate, monolein, diolein, oleic acid were purchased from Sigma

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chemicals (St. Louis, USA) and silica gel 60 $F_{254}$ plates from Merck, Germany. Cocoa butter and palm oil were obtained from the local market. All other medium components and chemicals used were of analytical grade and were purchased locally (Himedia, Qualigenes and Sisco Research Laboratories Ltd., India).

**Microorganism**

A lipase producing bacterium *Bacillus* sp. RK-3, isolated from soil grows optimally at 37 °C and pH 7.0 in nutrient broth. This organism was maintained on nutrient agar slants containing 1 % sunflower oil in a BOD incubator at 10 °C (Yorco Sales Pvt. Ltd., New Delhi) with periodic sub-culturing.

**Enzyme production**

Lipase production was carried out in medium (50 mL in 250 mL Erlenmeyer flasks) containing (g/L): olive oil 1.0, glucose 2.0, peptone 5.0, beef extract 5.0, $Na_2SO_4$ 2.0, $KH_2PO_4$ 1.0, $K_2HPO_4$ 3.0, $MgSO_4 \cdot 7H_2O$ 0.1, pH 7.0. Gum acacia (1.0 %) was used as an emulsifier. Inoculum raised in nutrient broth (cell density at 600 nm = 0.6-0.8) was used at 2 % (v/v) concentration. Incubation was carried out at 37 °C and 200 rpm with regular sampling at 12 h intervals till 48 h. The samples were centrifuged at 8,000 rpm for 15 min at 4 °C. The cell free supernatant was used as lipase source.

**Lipase assay procedure**

The lipase assay procedure of Winkler and Stuckmann, (1979) was used. To 2.4 mL of freshly prepared *p*-NPP solution (30 mg of *p*-NPP was dissolved in 10 mL of iso-propanol and mixed with 90 mL of Sorenson buffer, pH 9.0), 0.1 mL of culture filtrate was added. The reaction mixture was incubated at 37 ±1 °C in a water bath shaker for 30 min. The reaction was terminated by the addition of 0.1 mL of 300 mM $CaCl_2$ (fused) solution. The reaction mixture was then centrifuged to clarify the solution. The absorbance of the supernatant was read at 410 nm (Shimadzu UV1700 Pharma Spec). Lipase activity was calculated from standard curve of *p*-nitro phenol prepared in the range of 10-100 µg/mL.

The international unit (IU) of lipase activity by *p*-NPP method is defined as the amount of enzyme required to release of 1.0 µmole of free phenol from the substrate per mL per min under the standard assay conditions.

**Evaluation of lipase for 1, 3 regiospecificity**

Regiospecificity of the nature of the lipase produced from this *Bacillus* sp. RK-3 was confirmed by the triglyceride hydrolysis. For this, 1.0 mL of triolein was incubated with 5.0 mL of enzyme sample at 30 °C; 100 rpm for 24 h. Aliquots of 200 Ll were withdrawn after 24 h and mixed with 5.0 mL of diethyl ether to terminate the reaction. The hydrolyzed products formed were analyzed on Thin Layer Chromatography (TLC) plates (Silica gel 60F, Merck, Germany) using a solvent system comprising of petroleum ether, diethyl ether and acetic acid in the ratio of 80:30:1. Spots were visualized by incubating the TLC plates in a saturated iodine chamber. The 1, 3 regiospecific lipase of *Rhizomucor miehei* obtained from Sigma (St.Louis, USA) was used as control.

**Process optimization for lipase production**

Different lipid sources (olive, sunflower, coconut, corn, mustard, linseed, groundnut, castor and soybean oils) were evaluated for lipase production at 1.0% concentration. The concentration of the optimal lipid source was further optimized between 0.5 to 3.0% v/v. Other physiological parameters like temperature (25-50 °C), agitation rate (100-300 rpm) and pH (4.0-11.0) were also examined for their effect on lipase production. Effect of different organic and inorganic nitrogen sources (1.0 % w/v) such as peptone, beef extract, yeast extract, tryptone, casein hydrolysate, urea, ammonium chloride, ammonium nitrate, diammonium dihydrogen phosphate and sodium nitrate were evaluated for lipase production. The effect of different carbon sources (fructose, lactose, sucrose, maltose, mannitol, sorbitol and glycerol) at 0.2 % w/v was investigated for lipase production. Various surfactants (0.02 %) and metal ions ($NH_4^+$, $Ba^{2+}$ $Fe^{3+}$, $Mg^{2+}$, $Mn^{2+}$, $K^+$, $Cu^{2+}$ and $Ca^{2+}$) at a concentration of 20 mM were evaluated for lipase production. Inoculum concentration (cell density at 600 nm = 0.6-0.8) was optimized in the range 1-5 % (v/v).

**Scale up of lipase production**

Large scale production of lipase was carried out in a 10 L bioreactor (Bioflo IV, New Brunswick Scientific Inc., USA) containing 7.5 L working volume of the optimized production medium. Sterilization was carried out at 121 °C for 15 min *in situ*. Fermentation was carried out at 30±1 °C, 3.0 % inoculum, aeration of 4.0 vvm and 250 rpm. Foaming was controlled by adding sterilized silicon oil during fermentation. The pH was maintained by the addition of 1N NaOH/1N HCl during fermentation. The dissolved Oxygen (DO) levels were maintained at 20-40 % saturation by agitation-aeration cascade system. Samples were withdrawn every six hours till 48 h and were estimated for lipase activity.

**Partial Purification**

The cell free supernatant containing lipase was precipitated overnight using ammonium sulphate at 80% saturation at 4 °C. The precipitate obtained after centrifugation was dissolved in 0.05 M glycine NaOH buffer (pH 9.0) and dialyzed against the same buffer with 3-4 changes every 4 h. The lipase rich dialysate was lyophilized (Vertis Genesis, 35 EL). This partially purified concentrated lyophilized lipase sample was examined for its stability in various solvents and was further evaluated for CBS synthesis.

### Effect of organic solvents on lipase activity and stability

Partially purified and lyophilized lipase (10 mg) was incubated with 1 mL of different organic solvents (benzene, methanol, chloroform, hexane, petroleum ether and propanol) at 37 °C, 250 rpm for 24 h in screw- tight vials. The samples were centrifuged and the organic solvents were decanted and evaporated at room temperature. Dried samples thus obtained were then dissolved in 1mL of glycine-NaOH buffer (0.05 M, pH 9.0) and the residual lipase activity was determined. The activity of enzyme was also assayed in buffer in the absence of the organic solvent which served as control.

All experiments were run in triplicate sets and the mean values are presented. The values were analyzed by Student's t-test.

### Synthesis of cocoa butter substitute

Palm oil (5.0 mL) was mixed with 4.0 g of methyl stearate in presence of 50 mL hexane. To this, 100 mg of partially purified lyophilized lipase and molecular sieves were added. The reaction mixture was incubated overnight at 37 °C. After incubation, contents were filtered by Whatman filter paper (No.1) and solvent was evaporated. The filtrate thus obtained was analyzed for cocoa butter substitute synthesis on TLC and subsequently by GC and Nuclear Magnetic Resonance (NMR) analysis.

The solvent system used for TLC analysis was hexane: diethyl ether (4:1). The standards of palm oil, methyl stearate and cocoa butter were compared with that of CBS synthesized by transesterification reaction by this lipase. Various concentrations of CBS formed were analyzed on GC. The analysis of standard cocoa butter substitute, control (without lipase) and sample (with reaction) was performed on a Shimadzu instrument using an RT ™-2330 column with split injection and FID detection. The injection temperature and the detector temperature was 250 °C. A gas flow of 10 cc/min (H) and a split ratio of 45:1 mL/min were applied. The temperature programme of the oven was: 200 °C to 250 °C @ 8 °C/min (hold 3 min). One micro liter of sample was injected with a 10 µL glass syringe (Hamilton). $^{1}$H NMR was carried out using JEOL 400,400 MHz.

## RESULTS AND DISCUSSION

A newly isolated bacterium identified as *Bacillus* sp. RK-3 produces 3.28 IU/mL of lipase under initial unoptimized conditions in 36 h. Process optimization of lipase production from this organism was carried out to increase lipase yield to effectively reduce the quantity used per reaction. The increased yield will improve the process economics for the different lipase catalyzed/mediated reactions including synthesis of cocoa butter substitute.

### Process optimization of lipase production

Since lipid sources significantly influence lipase production, various lipidic sources were evaluated. The data presented shows an increase in lipase titres when sunflower oil (3.68 IU/mL) was used instead of olive oil (3.28 IU/mL) which was the control. A similar preference for sunflower oil has been reported by Kulkarni and Gadre (2002) for *Pseudomonas fluorescens* lipase. On the other hand, olive oil is the preferred lipid source for *Bacillus mycoides* and *B. multivorans* (Thomas *et al*., 2003; Gupta *et al*., 2007).

When the temperature was lowered from 37 °C to 30 °C, nearly 1.6 fold increase in lipase yield to 5.16 IU/mL was recorded. Similarly, a lower optimal temperature of 30 °C was reported for lipase production from *Streptomyces rimosus* (Abramic *et al*., 1999), *P. aeruginosa* LST-03 (Rathi *et al*., 2002) and *P. fluorescens* NS2W (Kulkarni and Gadre, 2002). However, on the contrary, a much higher temperature of 45 °C has been reported by Rathi *et al.* (2002) from *Burkholderia cepacia*. Optimal lipase production from this organism was at pH 7.0 which is also the control. Similar was reported for lipase production from *Bacillus* sp. (Sugihara *et al*., 1991) and *Acinetobacter radioresistens* (Li *et al*., 2001).

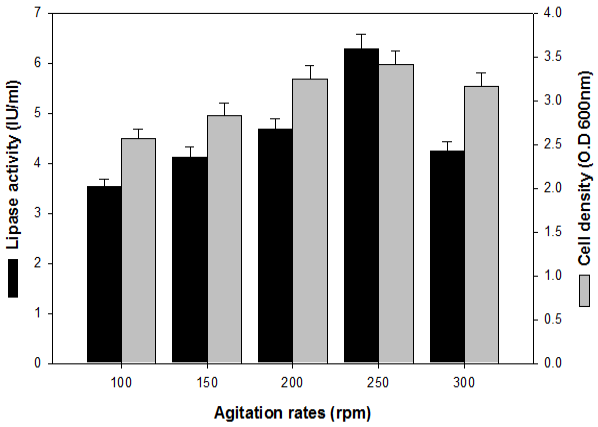



**Figure 1:** Effect of different agitation rates on lipase production by *Bacillus* sp. RK-3 in medium containing 1.5% sunflower oil (v/v)

Here, lipase production increased to 6.28 IU/mL when the agitation rate was increased to 250 rpm (Figure 1). This increase in lipase titers may be attributed to an increase in oxygen transfer rate to the organism for growth and lipase production (Lawrence *et al*., 1967). However, lower agitation rates of 150 rpm for *Pseudomonas* sp. (Gao *et al*., 2000), 160 rpm for *P. luteola* (Litthauer *et al*., 2002) and 200 rpm for *P. fluorescens* NS2W (Kulkarni and Gadre, 2002) has been reported while 250 rpm enhanced lipase production from *Pseudomonas aeruginosa* LST-03 (Ogino *et al*., 2000).

When different nitrogen sources were used for lipase production by replacing both peptone and beef extract

with tryptone (2%), lipase titres further increased to 7.10 IU/mL (Figure 2). Tryptone was reported for lipase production from *Bacillus circulans* MAS2 (Kademi *et al*., 2003) while yeast extract was used for *Bacillus* sp. (Thomas *et al*., 2003). Contrary to our results, a combination of tryptone (3.0%) with yeast extract (1.0%) was reported for lipase production from Ogino *et al.* (2000). Several researchers have reported the use of various carbon sources for influencing/increasing lipase production from both in bacteria and fungi (Pokorny *et al*., 1994; Mahadik *et al*., 2004). However, in this case, apart from glucose (0.2% control), any other carbon source like fructose, maltose, mannitol and sucrose did not support good lipase production. A supportive role of glucose in lipase production was reported by Khyami-Horani (1996) and Kulkarni *and* Gadre, (2002) for *B. licheniformis* and *P. fluorescens* NS2W respectively.

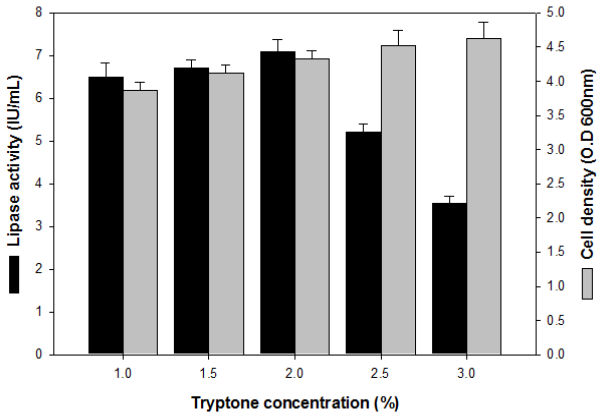



**Figure 2:** Effect of different concentrations of Tryptone (%) on lipase production by *Bacillus* sp. RK- 3 in medium containing 1.5% sunflower oil (v/v) at pH 7.0, 30 ℃ and 250 rpm

Since the addition of surfactants improves the dispersion of oil in water, it also increases the permeation of lipase through cell membrane (Tan *et al*., 2004). Therefore, evaluation of different surfactants showed that Tween 80 (0.02%) increased lipase titers to 8.10 IU/mL from this *Bacillus* sp. Sidhu *et al*. (1998) and Li *et al*. (2001) also reported Tween 80 for lipase production from *Bacillus* sp. RS-12 and *Acinetobacter radioresistens*. On the other hand, surfactants like Tween -20 and Tween -40 had an inhibitory effect on lipase production from *Rhizopus oligosporus* (Awan *et al*., 2003). Among different metal ions, $Ca^{2+}$ (20 mM) increased lipase production to 9.12 IU/mL. On the contrary, $Ca^{2+}$ inhibited lipase production from *Mucor hiemalis* (Akhtar *et al*., 1980). Other metal ions reported for increasing lipase yield are $Mg^{2+}$ for *Bacillus subtilis* 168 (Lin *et al*., 2006), *Serratia marsescens* mutant W1270 (Winkler and Stuckman, 1979) and *Bacillus* spp. RSJ1 (Sharma *et al*., 2002) and a combination of $Na^{+}$, $K^{+}$ and $Ca^{2+}$ for *B. mycoides* (Thomas *et al*., 2003).

When inoculum level was increased to 3.0% (v/v) as against 2.0% (v/v), final lipase yield of 10.56 IU/mL was obtained which is 3.2 fold higher as against the initial unoptimized yield (3.28 IU/mL). Any further increase in inoculum level lead to decreased lipase titers as higher inoculum concentrations may result in faster depletion of essential nutrients, thereby limiting further growth and reducing the lipase production.

**Scale up of lipase production**

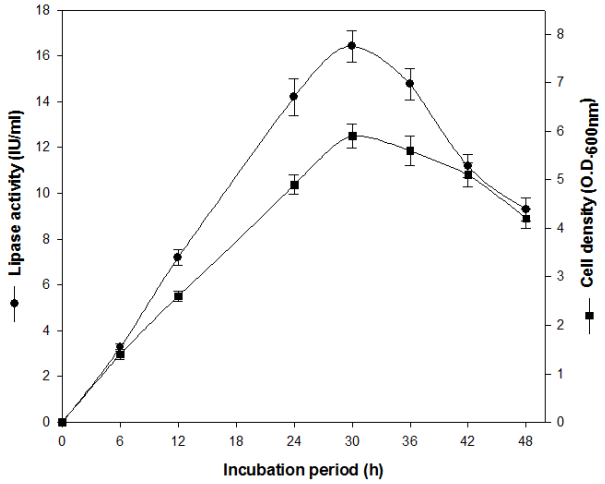



**Figure 3:** Scale up of lipase production in 10L fermentor

The enzyme production was carried out in a 10 L fermentor containing 7.5 L of the optimized production medium. The initial pH of the medium was kept at 7.0 and was only monitored. The fermentation was initiated with airflow of 5 lpm, 30 ℃ and initial agitation rate of 250 rpm. The dissolved oxygen was maintained at 20-40% constant saturation level. Use of these conditions for production resulted in final enhancement of lipase titers to 16.4 IU/mL in 30 h (Figure 3). This could be attributed to a better controlled environment in fermentor than in the flask. After 30 h, the substrate in the growth medium became a limiting factor, which resulted in no further growth and lipase production. The scale up finally resulted in an overall 6.0 fold increase in enzyme titers as against initial unoptimized production (3.28 IU/mL). The productivity also increased to 0.547 IU/mL/h as against initial unoptimized (0.091 IU/mL/h). Similarly, an increase in lipase titers was observed due to availability of nutrients, better agitation and aeration conditions by Rathi *et al*. (2002) for *B. cepacia*. However, in presence of higher dissolved oxygen concentrations, higher production titers were attained when *Thermos thermophilus* was grown in fermentor (Dominguez *et al*., 2005).

The lipase was partially purified by ammonium sulphate precipitation and lyophilized with final yield of 1.2 IU/mg and a fold purification of 4.46.

**Organic solvent stability**

Since, synthesis of CBS is carried out in presence of organic solvents, primarily hexane, the stability of this lipase was evaluated in different solvents. After 24 h of incubation, this lipase exhibited 95.12 % stability in hexane (Figure 4), followed by THF (75.83 %) and petroleum ether (73.85 %). Similarly, Lin *et al.* (2006) reported 100% residual activity in hexane by *Neurospora crassa* lipase. Contrary to our results, Sugihara *et al.* (1991) reported inhibitory effect of hexane on *Bacillus* sp. lipase. On the other hand, minimum stability with a loss of nearly 89 % was observed with propanol. However, more than 90 % lipase activity was reported from *B. cepacia* after 48 h of incubation in propanol (Yang *et al.*, 2007).

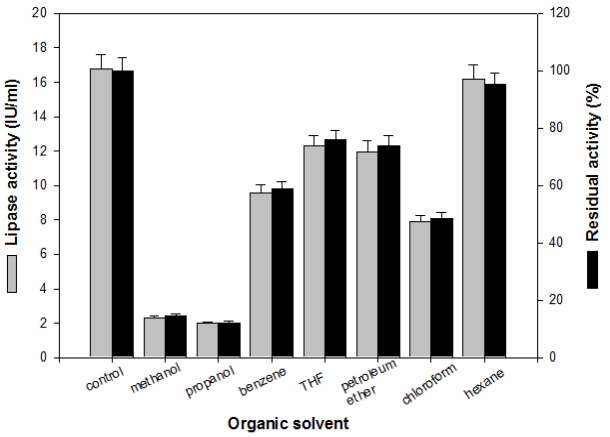
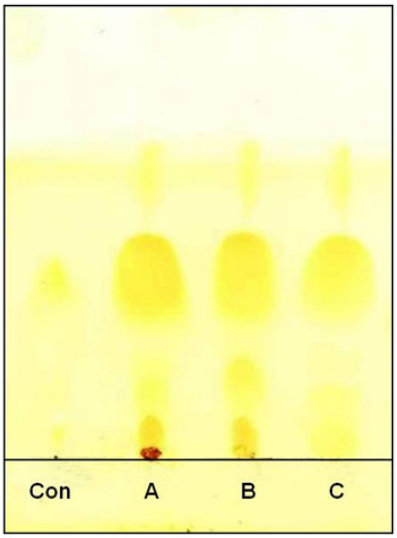



**Figure 4:** Effect of organic solvent on lipase production

**Synthesis of cocoa butter substitute**

Palm oil when transesterified by this *Bacillus* RK-3 lipase in presence of methyl stearate and hexane produces a thick, viscous and cream colored product. On qualitatively evaluating this product on TLC (Figure 5) using a solvent system of hexane: diethyl ether (4:1), showed that the TAG distribution in cocoa butter substitute obtained through Bacillus sp. RK-3 is similar to that of standard cocoa butter. These three compounds are also a major component of Cocoa Butter. Further quantification of the product by GC analysis (Figure 6) showed the presence of three types of triacylglycerols (TAGs) i.e. 1(3) palmitoyl-2-oleolyl-3(1) stearyl-sn-glycerol (POS), 1(3) stearoyl-2-oleoyl-3(1) stearoyl-sn-glycerl (SOS) and 1(3) palmitoyl-2-oleoyl-3(1) palmitoyl-s-glycerol (POP) which resulted in 38.28% synthesis of POS, 25.13% of SOS and 19.76% of POP indicating a Cocoa Butter Substitute synthesis efficiency of 83.17%.




**Con** : **Control (without lipase)**
**A** : **Reaction product (with *Bacillus* sp. RK-3)**
**B** : **Reaction product (with *Rhizomucor meihei* lipase, commercial lipase)**
**C** : **Commercial Cocoa butter (standard)**

**Figure 5:** Thin Layer Chromatography of the Cocoa Butter Substitute synthesized through transesterification of palm oil and methyl stearate in presence of *Bacillus* sp. RK-3 lipase in hexane

This distribution of the TAG components is similar to that of pure CB and results indicate that the product formed is a CBS. The CBS formed by transesterification of palm oil by this lipase solidified at room temperature with a sharp melting point of 33 °C. Pure CB also melts between 32 to 35 °C (Liu *et al.*, 2007) indicating similarity with the product formed by this lipase.

Furthermore, the CBS formed was reconfirmed by NMR (Figure 7). The results of the structural analysis of CBS carried out by $^1$H NMR ($CDCl_3$, 300 MHz) are δ 0.85-0.90 (9H, t, $CH_3$ palmitic C-16 and stearate ester C-18), 1.25 (48H, brs, 12x$(CH_2)_2$ at C-4 to 15 attached palmitic & 28H, brs, 14x $CH_2$ stearate ester) 1.61 (6H, m, $CH_2$ palmitic & stearate ester C-3), 2.30 (6H, t, $J$ = 6.0 Hz, $CH_2$, C-2, palmitic and stearate ester linkage), 4.26-4.32 (4H, m, $CH_2O$ palmitic ester), 5.34 (1H, m, CHO stearate ester).

Synthesis of CBS using palm oil has been reported by Bloomer *et al.* (1990), Undurraga *et al.* (2001), Abigor *et al.* (2006) and Pinyaphong and Phutrakul (2009). Palm oil is often a preferred source as it is a low cost fat and is readily available. Another reason is that the main component of palm oil mid fraction is the triglyceride POP which can be readily converted by transesterification reaction to POS and SOS, the major components in Cocoa Butter (Bloomer *et al.*, 1990).

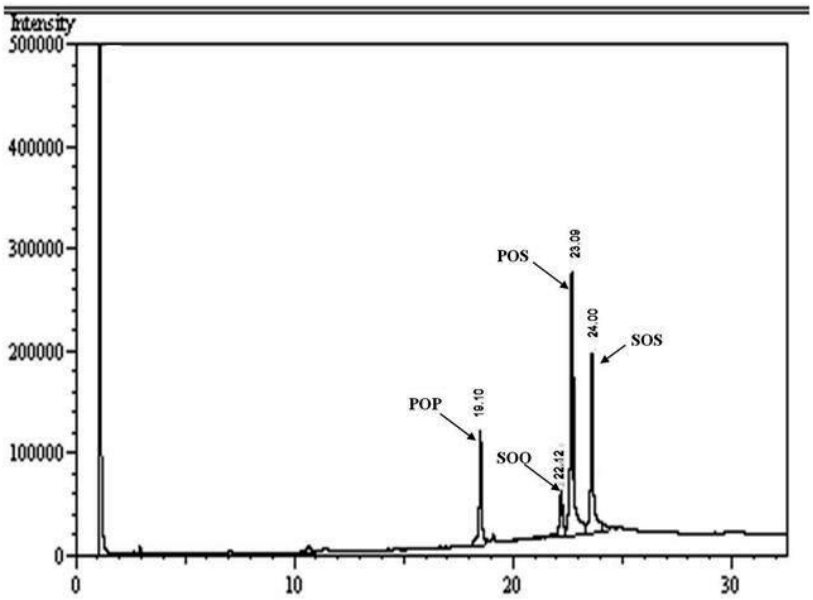



**Figure 6:** Gas Chromatography of CBS synthesized through transesterification of palm oil and methyl stearate in presence of *Bacillus* sp. RK-3 lipase in hexane. Rt. 19.10- POP; 22.12- SOO; 23.09- POS; 24.00- SOS

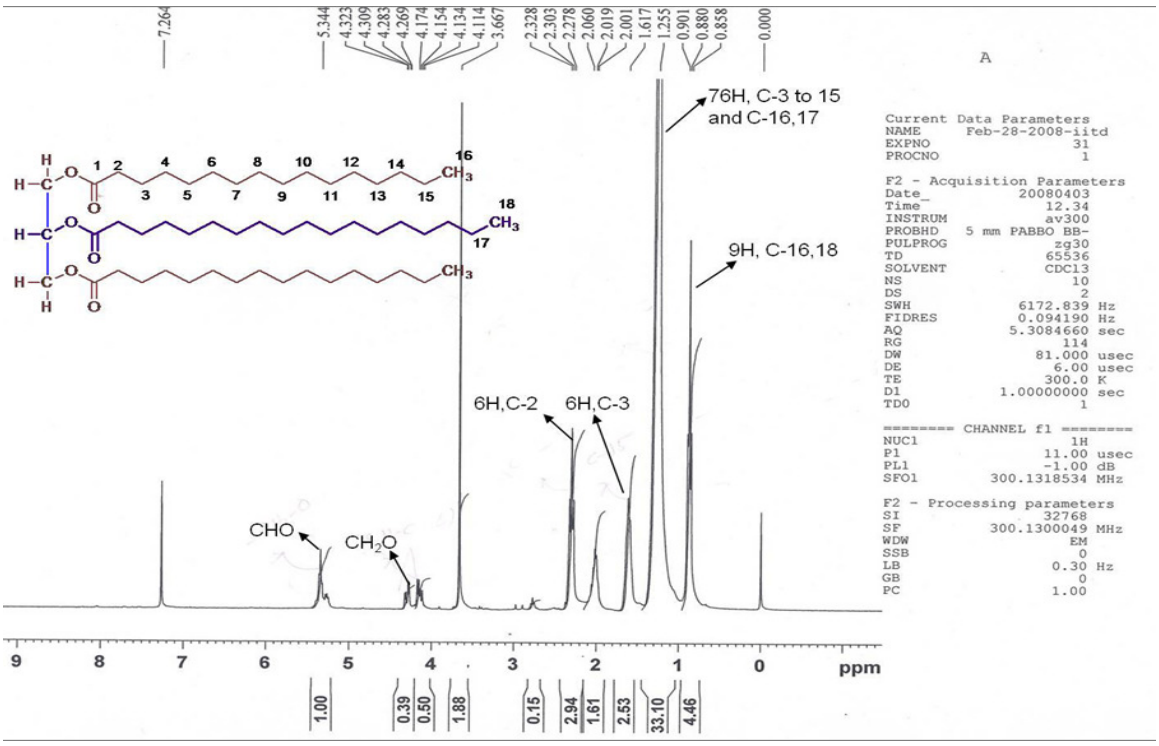



**Figure 7:** NMR of the CBS synthesized through transesterification of palm oil and methyl stearate in presence of *Bacillus* sp. RK-3 lipase in hexane

The important outcome from this study is that till date all the cocoa butter substitute synthesis has been carried out by Lipozymes, Amano, *C. papaya* lipase (Abigor *et al*., 2003; Liu *et al*., 2007; Pinyaphong and Phutrakul, 2009) However, on commercial scale it is worthwhile to have an enzyme supply which is not limiting. *Bacillus* sp. RK-3 is important since it produces indigenous lipase.

## CONCLUSION

This *Bacillus* sp. RK-3 produces a 1, 3 regiospecific lipase. This lipase could efficiently carry out synthesis of CBS from palm oil and methyl stearate in presence of hexane. Since, this lipase holds commercial importance, its production was process optimized initially in flasks resulting in 10.56 IU/mL. Further scale up to 10 L bioreactor resulted in 16.41 IU/mL of lipase titres in 30 h. The CBS synthesized had similar properties to that of commercially available cocoa butter.

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