J. Indian bot. Soc. (2022)
https://doi:10.5958/2455-7218.2022.00058.4

e-ISSN:2455-7218, ISSN:0019 - 4468
www.indianbotsoc.org

RESEARCH ARTICLE

Bacillus tequilensis lipase catalyzed synthesis of different esters for food industry

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Abstract: Lipase is a fat transforming enzyme with multiple applications in many industries including food and feed, dairy, oleo-chemicals, biodiesel production, pharmaceuticals, and others. They essentially show higher activity on long hydrocarbon chain fatty acid esters such as glycerides or sugar esters of lauric acid (C_{12} carbon fatty acid) and above. In this paper, applications of *Bacillus tequilensis* lipase in the synthesis of flavour and fragrance precursors and bio-surfactants have been described. The lipase successfully catalyzed the synthesis of these esters, wherein different fatty acids (C_4-C_{18}) were efficiently esterified with alcohols for the production of flavour and fragrance esters and with sugars for the synthesis of bio-surfactants. The lipase performed higher conversions with long-chain fatty acids such as plamitic and oleic acid (61–78%) as compared to shorter-chain fatty acids like butyric, caproic, caprylic, and capric acid (26–48%). These results suggest that this lipase is a candidate with potential for industrial applications with reference to the food, feed, and pharmaceutical sectors.

Keywords: Lipase, bio-surfactants, long hydrocarbon chain fatty acid esters, Alcohols, Sugars

Introduction

Lipases (Triacylglycerol acylhydrolases EC 3.1.1.3) are the most significant industrial hydrolases (Reis *et al*. 2009, Fickers *et al.* 2011) of the present century. This enzyme acts on fats and fatty acid esters and mediates their bioconversion under *in vivo* and *in vitro* conditions. Esterases preferentially hydrolyze "simple" esters, and typically only triglycerides made of fatty acids shorter than carbon number 6. Lipases are mainly active against water-insoluble substrates, such as triglycerides formed of long-chain fatty acids. (Helistö and Korpela 1998*;,Kulkarni et al.* 2002*)* (presented in the **Table 1)** Microorganisms including bacteria, yeast, and fungi, as well as their enzymes, are repeatedly utilized in a variety of food preparations to enhance flavour and texture, and they also provide enormous economic advantages to the food industry. Plants and animals favour microbial enzymes as a source because they are simple, affordable, and reliable to produce. Other lipases are primarily employed in the creation of cheddar cheese, the development of cheese flavours and other processes involving the preparation of fatty acids. Through their action on the milk fats to form free fatty acids following hydrolysis, they can enhance the distinctive flavour of cheese (Jooyandeh *et al.* 2009, Aravindan *et al.* 2007).*It* has been employed in many industries to catalyze different reactions for the production of food grade esters such as alcohol and sugar esters of fatty acids, synthesis of nutritional foods, structured glycerides, drugs, modified nucleotides, synthesis of cocoa butter substitutes from palm oil fraction and many more (Poonam *et al.* 2005, Meghwanshi *et al.* 2006, 2018, 2020). Unlike esterases, which act on short-chain fatty acids (C10 or less) (Meghwanshi and Vashishtha 2018). Using lipases as biocatalysts has many advantages, such as ambient reaction environment, fewer reaction steps, and no toxicity issues after processing of food, drugs, and other ingestibles Application of lipase in synthetic reactions requires that their water content be minimal as the presence of water directs the reaction towards hydrolysis. Dry lipases can be prepared by immobilizing them on different types of matrices such as Amberlite or, more easily they can be lyophilized to produce dry powders that can be used to carry out different synthetic reactions. Both immobilized and dried enzymes

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Received : 22 May 2022 Accepted : 22 June 2022 Published online : 03 October 2022

can be easily separated from the reaction product and can be reused Flavour and fragrance esters are aromatic compounds that have fruity odors and are used as artificial Flavour and fragrances (Bayout *et al.* 2020). Whereas, sugar-fatty acid esters have biosurfactant properties (Gumel *et al.* 2011). In this paper we describe the lipase catalyzed synthesis of Flavour and fragrance esters (esters of alcohol and fatty acids) and biosurfactants (esters of sugars and fatty acids). The *B. tequilensis* lipase was able to utilize fatty acids like Butyrica (C4), Caproic (C6), Caprylic (C8), Lauric(C12), Palmitic (C16), Stearic (C18), and Oleic(C18:1) for the production of these esters. However, the lipase showed lower conversions of small-chain fatty acids to corresponding esters as compared to long-chain and medium-chain fatty acids. Lipase's affinity for fatty acids increased as the carbon chain length increased (C4 C6C8C12C16C18). The lipase also showed preference for saturated fatty acids as compared to unsaturated fatty acids, which was evident from the higher conversion of stearic acid (C18) than oleic acid (C18:1).

Material and Methods

1. Chemical and reagents: - Fatty acids, primary, secondary and tertiary alcohols, and sugars were purchased from hi-media (India). All chemicals were of analytical grade. Silica gel 60 (0.040-0.063 mm, 230-400mesh) was purchased from E. Merck Pvt. Ltd., Mumbai, India.

2. Microorganism and Lipase: - Lipase lyophilized enzyme was prepared from freeze drying of cell free supernatant of fermented broth having 85U/ml of *B. tequilensis* lipase. *Bacillus tequilensis* was originally isolated from a soil sample by selective enrichment technique at 45°C. The culture was purified by repeated sub-culturing on a Nutrient agar medium (NB) medium supplemented with 1% (v/v) tributyrin. Nutrient agar medium contained (g/ml) (peptone 0.5g, beef extract 0.3g, Nacl 0.5g , Agar 2% and tributyrin 1 ml & pH 7.0 (Lawrence *et al.* 1967a).

3. Production of Lipase by *Bacillus tequilensis*:- Seed culture of *Bacillus tequilensis* was prepared by inoculating 50 mL of broth with a loopful culture of the bacterium maintained on NA (nutrient agar) slants. The culture was allowed to grow for 24 h at 37°C under shaking condition at 120 rpm. Thereafter, 2.0% (v/v) of actively growing seed culture (OD_{600nm} = 0.8±0.1) was used to inoculate 1000 mL of the lipase production medium (50 mL each in 250 mL capacity Erlenmeyer flasks). The seeded production medium was incubated at 37°C and 120rpm for 24 h Lipase production medium contained (gm/liter) (peptone 10g , Yeast extract 5g, Sodium sulphate 2.0 g , KH_2PO_4 1.0 g , K_2HPO_4 3.0 g , $MgSO_4.7H_2O$ 0.1g , Dextrose 2.0g, Olive oil 5.0, pH 7.0)

4. Determination of Lipase Activity:- The lipase activity was determined by a colorimetric method using p-NPP (p-Nitrophenyl palmitate) assay (substitute of lipase activity) 30 mg p-NPP was dissolved in 10 ml Iso-propanol & mixed with 90 ml of Tris-Buffer (0.05 M, pH 8.5). To 2.4 ml freshly prepared p-NPP solution, 0.1 ml of culture supernatant was added. The reaction was performed by incubating the reaction mixture at 50°C for 5 min. The reaction was terminated by addition of 0.1 ml of 100mM $CaCl_2$ (fused) and keeping the tubes on ice. The reaction mixture was then centrifuged to clarify the solution. The absorbance of the yellow color was read at 410 nm.(Winkler and Stuckmann1979, Mahadik *et al.* 2002).

Enzyme unit: One unit (IU) of lipase activity is defined as micromole(s) of p-Nitrophenol released per minute by hydrolysis of p-NPP by one ml of soluble enzyme.

(a) Synthesis of Flavour and Fragrance Precursors: Synthesis of these esters was performed through lyophilized lipase catalyzed esterification of fatty acids ($C_{4:0}$-$C_{18:1}$) with alcohols (primary, secondary and tertiary alcohols).

Experimentally, 100mM fatty acid (of different chain length viz. $C_{4:}$ - $C_{18:1}$) was reacted with 100mM of primary, secondary and tertiary alcohols in 4 ml of *n*-hexane in 15 ml screw capped vials in individual sets. The reaction mixtures were equilibrated for 24 h in an evacuated desiccators containing $MgCl_2.6H_2O$ at the bottom to attain an initial water activity (a_w) of 0.33. 100 mg of lyophilized lipase (containing 10 IU/mg) was added to the reaction mixture and the vials were incubated at 50 °C and 150 rpm for 24h. The reactions were terminated by adding 5 ml of

acetone: ethanol (1:1). Qualitative analysis of the products was carried out using Thin layer chromatography (TLC) plates (silica gel 60 F_{254} plates, Merck Ltd., Germany) using a solvent system comprising of **petroleum ether: diethyl ether: acetic acid** in the ratio of **80:30:1**. Quantitative estimation of ester synthesis was carried out by titrating remaining fatty acids with 0.01N NaOH using a pH-stat.

(b) Synthesis of Biosurfactants: Synthesis of these esters was performed through lyophilized lipase catalyzed esterification between sugars/sugar alcohol and fatty acids ($C_{4:0}$ - $C_{18:1}$).

Esters of sugars/sugar alcohols are used as biosurfactants in food industry, besides which these are also used in many other areas, such as enhanced oil recovery, environmental detoxification process, detergents, cosmetic, agrochemical and pharmaceutical.

Experimental

Fatty acids (100 mM) of different chain length (C_4-C_{18}) were reacted with 100 mM of glucose, sucrose, fructose and sorbitol in the presence of 100 mg of lyophilized lipase in 4 ml of tetrahydrofuran (THF) as the solvent. The reaction was carried out at 50ºC for 24h in an incubator shaker. Qualitative analysis of the reaction mixture was done on TLC plates. The solvent system used for TLC analysis comprised of **petroleum ether: ethyl acetate** in the ratio of **19:1 (v/v)**. The extent of esterification (% conversion) was determined by titrating the leftover fatty acids using 0.01N NaOH.

Observation and Results

Synthesis of Flavour and Fragrance Precursors

The results of TLC analysis for the synthesis of flavor and fragrance esters are shown in Fig. 1(A). It is clearly visible from the TLC plates that the lipase catalyzed the synthesis of esters of fatty acids with all the four types of alcohols investigated i.e. primary, secondary, and tertiary. The observations are presented as the % conversion of the substrate to the product in the **Table 1.**

The results of quantitative analysis (% conversion) presented in above table show that this lipase catalyzed the synthesis of esters of all the fatty acids with different alcohols, irrespective of their chain length. Further, saturation or unsaturation of fatty acids as well as the position of the hydroxyl group in the alcohols (primary, secondary and tertiary) did not prevent synthesis. However higher syntheses were seen for long chain fatty acids i.e. palmitic, stearic and oleic acids, wherein respectively 68 %, 70 % and 78 % conversions were obtained with *Iso*-propanol. On the other hand, percent conversions of ester yield with butyric acid were the lowest. Short-chain fatty acid esters of alcohols are the most significant contibutors of flavors and fragrances in different natural compounds. These esters are widely used and are in great demand in the beverage, food, cosmetic, and pharmaceutical industries. Industrial production of flavor esters is done through chemical synthesis; however the preference for "natural" flavors is on rise and people are turning away from chemically synthesized flavors and fragrances (Gillies *et al.* 1987). In this context microbial lipases have been reported for the synthesis of flavor and fragrance esters, e.g., *Candida antarctica* fraction B (CAL-B) has been used for the production of flavor and fragrance esters of short-chain acids having linear (acetic and butyric acids) and branched chain (isovaleric acid) structures (Larios *et al.* 2004). Similarly, Su e*t al.*

Table 1: Important differences between lipases and esterases

Fatty acids	% Conversion			
	Methanol	***Iso*-propanol**	***Iso*-amyl alcohol**	***Tert*-Butanol**
Butryic (C4:0)	33.0	43.0	26.0	10.0
Caproic (C6:0)	35.0	46.0	22.0	19.0
Caprylic (C8:0)	34.0	31.0	34.0	18.0
Capric (C10:0)	42.0	48.0	37.0	22.0
Lauric (C12: 0)	59.0	62.0	58.0	26.0
Palmitic (C16:0)	64.0	68.0	61.0	36.0
Stearic (C18:0)	68.0	78.0	69.0	31.0
Oleic (C18:1)	62.0	70.0	64.0	28.0

Table 3: Esterification of fatty acids with different sugars and sugar alcohols in THF after 24h at 50ºC.

Fatty acids	% Conversion			
	Glucose	Fructose	Sucrose	Sorbitol
Butryic (C4:0)	27.0	24.0	27.0	33.0
Caproic (C6:0)	26.0	32.0	29.0	42.0
Caprylic (C8:0)	32.0	30.0	34.0	46.0
Capric (C10:0)	45.0	49.0	52.0	59.0
Lauric (C 12:0)	53.0	58.0	62.0	68.0
Palmitic (C16:0)	55.0	62.0	66.0	74.0
Stearic (C18:0)	59.0	67.0	71.0	78.0
Oleic (C18:1)	46.0	49.0	51.0	57.0

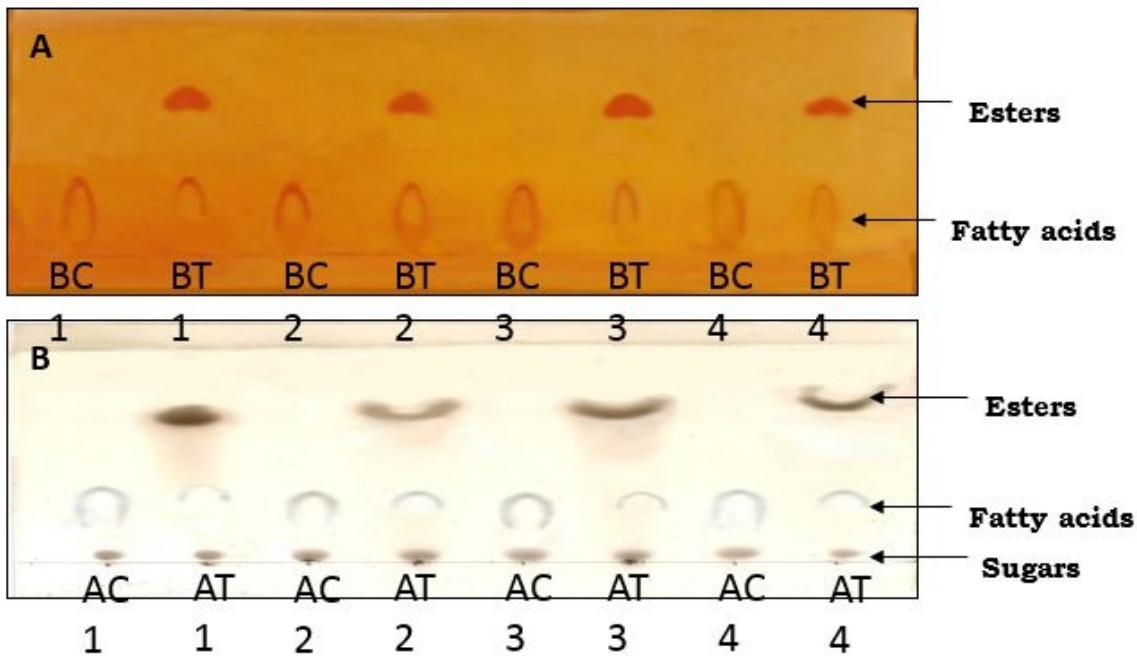

Fig 1: TLC analysis of esterification reactions catalyzed by *B. tequilensis* lipase (A) Esterification reaction between fatty acids (butyric, caprylic, lauric and stearic acid) and iso-Propanol (B) Esterification reaction between fatty acids (butyric, caprylic, lauric and stearic acid) and sugars (glucose, fructose, sucrose and sorbitol).

(2010) and Jin *et al.* (2012) have also used CAL-B lipase for the synthesis of flavour esters. Recently, de Lima *et al.* (2018) have reported the use of different immobilized lipases in the synthesis of Short- and Long-Chain Carboxylic Acid Esters.

Synthesis of Biosurfactants

The results of TLC analysis of products of biosurfactant synthesis reaction are shown in Fig. 1(B). It is evident from the figure that the lipase successfully catalyzed the esterification reaction between fatty acids and sugars. Spots of esters were visible in all the fatty acid-sugar combinations investigated. The results of quantitative determination of sugar-fatty acid esters produced (presented in the **Table 2)** clearly show that the lipase efficiently catalyzed synthesis of fatty acid esters of different sugars. The three sugars investigated underwent esterification reaction with sorbitol showing higher conversions than other sugars. It is clearly evident from the table that this lipase catalyzed higher synthesis with long chain fatty acids ($C_{10:0}$-$C_{18:1}$). Highest esterification was observed for stearic acid ($C_{18:0}$) with all the sugars (78 % conversion with sorbitol). On the other hand, lowest yields were obtained for butyric acid with only 24 % conversion with fructose.

Sugar esters of fatty acids used

commercially are presently being produced through chemical processes. However, the chemical synthetic methods are non-specific and normally result in a variety of isomers of the product exhibiting acylations at different positions and to varying degrees (Kobayashi 2011). Whereas, lipase-catalyzed synthesis of sugar esters by transesterification can be relatively easily applied for food processing in comparison to the general chemical synthetic processes which rely on using harmful reagents or solvents like N,N dimethylformamide, and pyridine (Kobayashi 2011). The lipase mediated production of sugar esters also has an advantage of being accepted by customers as a 'natural product'. In this regard Afach *et al.* (2005) have used *Candida antactica* lipase for the synthesis of D-allose fatty acid esters. In another paper *Pseudomonas* sp.- lipase has been used to catalyze the synthesis of glucose and palmitic acid esters (Tsuzuki *et al.* 1999). Sugar esters of fatty acids used commercially are presently being produced through chemical processes. However, the chemical synthetic methods are non-specific and normally result in a variety of isomers of the product exhibiting acylations at different positions and to varying degrees (Kobayashi 2011). Whereas, lipase-catalyzed synthesis of sugar esters by transesterification can be relatively easily applied for food processing in comparison to the general chemical synthetic processes which rely on using harmful reagents or solvents like N,N dimethylformamide, and pyridine (Kobayashi 2011). The lipase mediated production of sugar esters also has an advantage of being accepted by customers as a 'natural product'. In this regard Afach *et al.* (2005) have used *Candida antactica* lipase for the synthesis of D-allose fatty acid esters. In another paper *Pseudomonas* sp.- lipase has been used to catalyze the synthesis of glucose and palmitic acid esters (Tsuzuki *et al*. 1999).

Conclusion

The *B. tequilensis* lipase successfully catalyzed the synthesis of flavor and fragrance esters (alcohol esters of fatty acids) and biosurfactants (sugar esters of fatty acids) with various fatty acids and different alcohols and different sugars. This reflects the broad specificity of this enzyme. Although the lipase was able to esterify fatty acids with short to long alkyl chains, it showed a clear preference for medium to long alkyl chain fatty acids. Among alcohols it exhibited preference for the secondary alcohol i.e. –iso-propanol and among sugars it showed preference for sugar alchol-sorbitol. Overall, it can be concluded that the *B.tequilensis* lipase is a potential candidate for application and food and pharmaceutical industry and in environmental remediation .

Acknowledgement

Authors sincerely acknowledge the financial support given for this research work by Science & Engineering Research Board (SERB), New Delhi vide grant sanction NO. SB/YS/LS-146/2014

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