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Research Article

LIPASE CATALYZED ESTERIFICATION AND TRANSESTERIFICATION TO SYNTHESIZE BIOFLAVORS AND FRAGRANCES

Amita Madhukar Kocharekar and Meenal Shriniwas Dukhande

Department of Microbiology, Guru Nanak Khalsa College, Matunga, Mumbai-400 019, Maharashtra, India

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ABSTRACT

Flavors and fragrances of long/short chain esters have representative fruity and pleasing aroma. They have marketable significance in cosmetic, fragrance, food and pharmaceutical commerce. This has led to the development of identical artificial flavor esters. In this study, the biosynthesis of esters from different acids and alcohols and esters/transesters of coconut oil and palm oil were carried. The esterification and transesterification reaction was catalyzed using lipase from *Staphylococcus gallinarum.* The synthesized product was examined by using hydroxylamine test for esters. Based on the results obtained from this test, the products were confirmed using gas chromatography associated with high resolution mass spectrometry. Lipase was able to catalyze the synthesis of esters such as butyl acetate, isobuty acetate, isoamyl acetate and transesters of palm oil such as isobutyl stearate and butyl palmitate and coconut oil as butyl palmitate. Occurrences of all these esters are perceived in fruits like apple, pineapple, banana, cherry, berry, strawberry, etc. Transesters of fatty acids have application in cosmetic products. The process can be suitably scaled up for commercial production of bio flavors and fragrances.

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INTRODUCTION

Flavors and fragrances are largely used compounds in the food, cosmetic, detergent, chemical and pharmaceutical industries. Flavor esters of long/short-chain or medium-chain fatty acids and alcohols are important aroma active compounds used as flavor enhancers in the food industry and fragrances in the cosmetic industry. Chemically, aroma-active substances are organic volatile compounds like alcohols, aldehydes, ketones, acids, esters, phenolics, terpenes, lactones and pyrazines with characteristic smell. (Hassan *et al*., 2013). They contribute desirable flavors and fragrances to a variety of food products such as cheese, wine, soft-drinks, beer, candies, etc. and cosmetics product such as lipstick, skin lotion, perfumes, etc. Traditionally flavoring compounds have been produced by chemical synthesis or extracted from plant materials. However, high costs plus low yields of natural compounds made this technique inadequate to be used on a large scale. Also esterification requires a catalyst to increase the rate of reaction. Concentrated sulfuric acid is used in the manufacturing of chemical flavor esters but its use is dangerous and can cause chemical burns when it comes in human contact(Contarini and Povolo, 2013).

Raising awareness among consumers about the safety of the products they use, especially food and beverages they choose, preference for natural additives like flavor esters has increased. Products derived from bioprocesses starting with natural substrates can be considered as 'natural' if they have already been identified to be present in natural sources. However, natural flavors are still limited because they are expensive compared to chemically synthesized flavors. Thus there is a high demand for natural flavors. This has led to extensive biotechnological research to develop alternative production methods. Interest in lipases has increased markedly in the last two decades owing to their applications in detergent industry, leather industry, environmental management, cosmetics and perfume industry, biomedical devices and biosensors. Lipases being a versatile enzyme are exploited in various industries especially in the food processing industries.

Lipases are produced by microorganisms, plants and animals. Lipase enzymes as biocatalyst offer a wide variety in synthesis of flavor active and volatile compounds due to their specificity and stereoselectivity. Triacylglycerol acyl hydrolases, commonly called as lipase promotes esterification and transesterification reaction. Esterification is formation of ester from an acid and alcohol. While in transesterification reaction

Corresponding author:* **Amita Madhukar Kocharekar
Department of Microbiology, Guru Nanak Khalsa College, Matunga, Mumbai-400 019, Maharashtra, India

exchange of ester group take place with alcohol. Left over cooking oil such as palm oil, sunflower oil, etc and less use edible oil such as coconut oil can be used for transesterification reaction(Kadivar *et al*., 2013). Generally, coconut oil and palm oil contains long chain fatty acids and triglycerides which can be used to produce esters and transesters of long chain fatty acids. Long and short chain fatty acid ester can be used as bioflavors and fragrance imparting agents having application in food and cosmetic industry. Lipase catalyzed esterification is a simple process where ester linkage is formed between acid and alcohol. Whereas, the first stage in transesterification of oil is esterification of fatty acid with the alcohol. Excess of alcohol is generally used so that esterify fatty acids can be transesterify to give esters of biofragrances and bioflavor importance(Sun *et al*., 2012; Tan *et al*., 2011). Lipases of microbial origin have gained considerable attention in the field of biotechnology and a large number of microbial strains have been used for the enzyme production. Microbial commercial lipases are mainly produced from *Pseudomonas* species, *Mucor* species, *Rhizopus* species, *Penicillin chrysogenum*, *Bacillus subtilis, Aspergillusoryzae*, *Candida rugosa,* etc.Garlapati and *et al* in India in 2013 have studied the esterification of lactic acid and alcohols using a lipase of *C. antarctica* in hexane. This lipase was used to produce different flavors like geranyl acetate (rose) and isoamyl acetate (banana)(Garlapati and Banerjee, 2013).Porcine pancreatic lipase was immobilized into calcium alginate gel beads and was used to produce industrially important flavor esters, namely isoamyl acetate (banana flavor), ethyl valerate (green apple flavor) and butyl acetate (pineapple flavor) by Ozyilmaz and *et al* in Turkey in 2009 (Ozyilmaz, 2015). Liu and *et al* in 2013 in China investigated the bio production of isoamyl esters in coconut cream by lipases (Liu *et al*., 2010). Although the natural flavor and fragrances may contain many different compounds, but certain single ester resembles the natural odor. In the present study, lipase catalyzed esterification of acid and alcohol and transesterification reaction of coconut oil/palm oil and alcohol to form bioflavors and fragrances is been investigated. Bacterial culture was isolated from spoilt butter sample on Goradkowa's tributyrin agar medium. 16s rRNA sequencing revealed that the isolate is *Staphylococcus gallinarum*. Lipase produced by *Staphylococcus gallinarum* was used to catalyze the process(Kocharekar, 2017).Different acids and alcohols were used as substrate for the esterification reaction. And coconut oil and palm oil with different alcohols were used for the transesterification reaction. Final analysis of flavoring esters was done using GC-HRMS. Synthesized natural flavors and fragrances can be used in future in bakeries, food industry and cosmetic industry.

MATERIALS AND METHODOLOGY

Culture maintenance

Staphylococcus gallinarum was maintained on sterile nutrient agar slant. It was preserved at 4° C and was periodically subcultured until used. Lipase enzyme of 8329.61 U/mg specific activity was purified from *S. gallinarum* by ammonium sulphate precipitation method and was used to catalyzed esters and transesters.

Material

All chemicals used in this study were of analytical grades. All experiments were performed in triplicates.

Methods

Esterification reaction

To synthesize fruity esters, different combination of acids and alcohols as mentioned in table no 1 were tried. The reaction mixture consists of 10 ml of 1M acid and 1 M alcohol in 1:1 ratio followed by adding 1 ml of lipase. Incubation was carried at 35°C for 48 hours at 155 rpm under shake flask condition. Appropriate control was also maintained containing acid and alcohol with no lipase. After the time elapse, the reaction cocktail was filtered through whatman filter paper no.1 to remove the enzyme. The filtrate was used as samples for confirming the products(Raghavendra *et al*., 2014).

Table no 1 Different combination of acids and alcohol and their respective esters with its occurrence in natural food.

Acid	Alcohol	Ester	Type of flavor
Acetic acid	Ethyl alcohol	Ethyl acetate	Grapes, cherry
Acetic acid	Methyl alcohol	Methyl acetate	Rum, wine, whiskey
Acetic acid	Propyl alcohol	Propyl acetate	Pear, banana, honey
Acetic acid	Butyl alcohol	Butyl acetate	Ripe banana
Acetic acid	Iso butyl alcohol	Isobutyl acetate	Banana
Acetic acid	Iso amyl alcohol	Isoamyl acetate	Banana
Acetic acid	Octanol	Octyl acetate	Orange, mushroom, waxy, apple
Formic acid	Ethyl alcohol	Ethyl formate	Rum, raspberries, wine
Formic acid	Methyl alcohol	Methyl formate	Plum
Formic acid	Propyl alcohol	Propyl formate	Rum, berry
Formic acid	Butyl alcohol	Butyl formate	Plum, rum, brandy
Formic acid	Iso butyl alcohol	Isobutyl formate	Rum
Formic acid	Iso amyl alcohol	Isoamylformate	Apple, wine, fatty
Formic acid	Octanol	Octylformate	Oily, orange, waxy, citrus
Butyric acid	Ethyl alcohol	Ethyl butyrate	Pineapple, apple
Butyric acid	Methyl alcohol	Methyl butyrate	Apple, banana, dairy, acidic
Butyric acid	Propyl alcohol	Propyl butyrate	Apricot, rancid, pineapple
Butyric acid	Butyl alcohol	Butyl butyrate	Pineapple, rip fruits, banana
Butyric acid	Iso butyl alcohol	Isobutyl butyrate	Pineapple, berry, cherry, apple, rum
Butyric acid	Iso amyl alcohol	Isoamyl butyrate	Apricot, pear, banana, melon, apple, berry, waxy
Butyric acid	Octanol	Octyl butyrate	Oily, creamy

Esterification and transesterification of coconut and palm oil

The reaction was carried in 250 ml sterile flask containing 10 ml of coconut oil/palm oil and 30 ml of alcohol (methyl alcohol/ethyl alcohol/propyl alcohol/ isobutyl alcohol/butyl alcohol). 10ml of lipase enzyme was added to the reaction mixture. Incubation was carried at 35°C for 48 hours at 155 rpm under shake flask condition. Appropriate control was also maintained containing oil and alcohol with no lipase. After the reaction time, the mixture was filtered through whatman filter paper no.1. The mixture was placed in a separating funnel, where the upper phase containing esters and transesters of fatty acids was separated from lower phase containing unreacted oil, other products (mono and diglycerdies) and other impurities.

Qualitative detection of esters by Hydroxyl amine test

Samples were checked for ester formation by using Hydroxylamine test of Holman (1961) with some

modifications. 1 ml of standardester (isoamyl acetate) was used as positive control. Hexane was used as negative control and samples from above esterification reaction were used as test samples. 1 ml of sample was mixed with 1 ml of hydroxylamine hydrochloric reagent. All the tubes were incubated at 50°C for 10minutes followed by addition of 1 ml of ferric chloride reagent. Red brown or orange coloration indicated positive results whereas yellow coloration indicated negative result.

GC-HRMS analysis to confirm the esters and transesters:

Samples testing positive for hydroxylamine tests, where further confirmed for presence of the esters and transesters using gas chromatography associated with high resolution mass spectrometry.

RESULTS AND DISCUSSION

Hydroxylamine test for detection of esters and transesters

The basis of the detection in this test is the reaction of the ester with hydroxylamine hydrochloride in alkaline pyridine solution to form hydroxamic acid of ester. This further reacts with ferric chloride to form orange/red ferric hydroxamate complex. Ester/transester production was checked for using Hydroxylamine test. Lipase could catalyze esterification of butyl alcohol and acetic acid to form butyl acetate; isobutyl alcohol and acetic acid to form isobutyl acetate; isoamyl alcohol and acetic acid to form isoamyl acetate. It was unable to catalyze esterification with other combination of acid and alcohol. Coconut oil contains fatty acid such as caprylic acid, decanoic acid, lauric acid, myristic acid, palmitic acid and oleic acid. Lipase was able to catalyze esterification reaction between palmitic acid and butyl alcohol to form butyl palmitate. No transesters of fatty acid were formed.

Whereas palm oil contains fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid. Lipase was able to catalyze esterification reaction between stearic acid and isobutyl alcohol to form isobutyl stearate. However, presence of butyl palmitate was also observed along with isobutyl stearate as detected by GC-HRMS. Transesters are generally formed by interchanging the groups between reacting molecules. Lipase was able to catalyze transesterification reaction between stearic acid and isobutyl alcohol to form butyl palmitate.

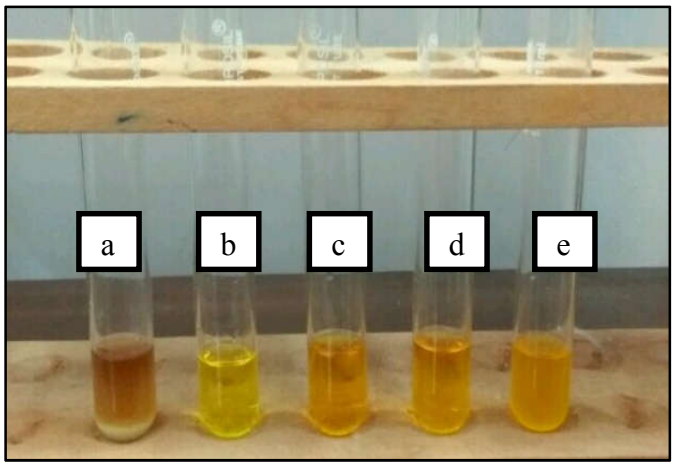

Figure no 1 Figure showing results of esters as detected by hydroxylamine test. a) positive control b)negative control and test samples-c) butyl acetate d) methyl acetate e)isoamyl acetate

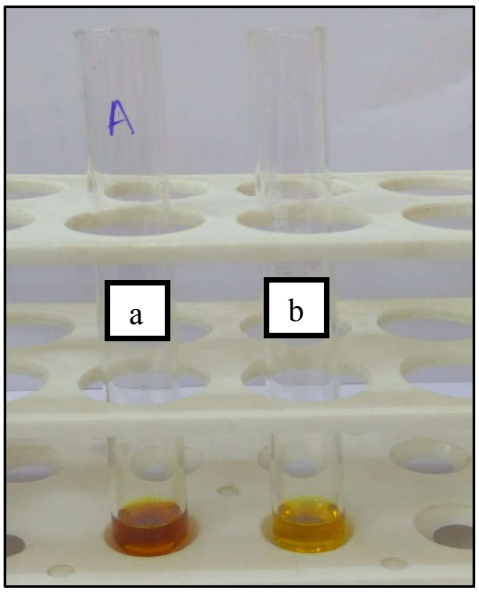

Figure no 2 Figure showing results of transester of fatty acid from coconut oil as detected by hydroxylamine test. a)positive test sample b)negative control

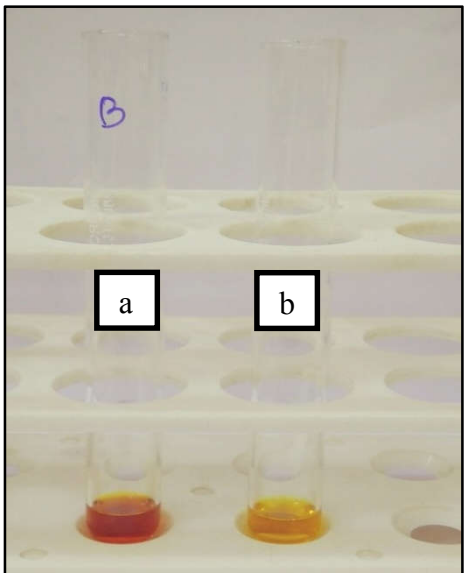

Figure no 3 Figure showingresults of transester of fatty acid from palm oil as detected by hydroxylamine test. a)positive test sample b)negative control

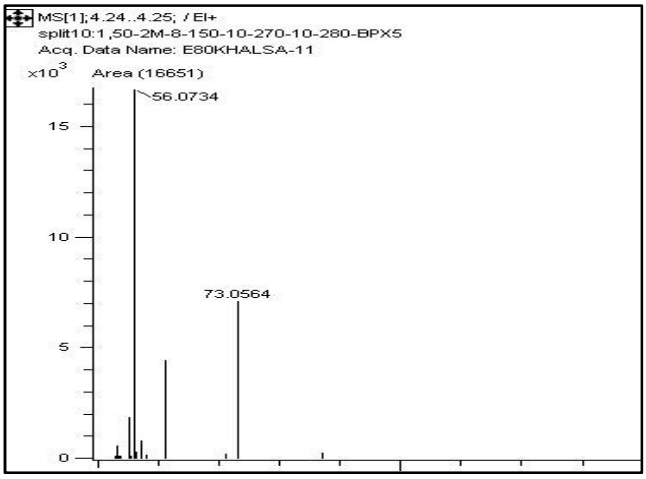

Figure no 4 Electron ionization mass spectrum of butyl acetate

Samples testing positive for this test were confirmed using Agilent gas chromatography (fused silica capillary column BPX5 Neat,30m x 250µ) equipped with a mass spectrometer

detector. The column temperature was programmed to increase from 50° C to 250° C at 8° C/min rise. Helium was used as carrier gas. The sample injection volume was 1.0 µl with split ratio of 10:1. Figure no 4-9 depicts GC-HRMS result of positive ester samples as detected by Hydroxylamine test.

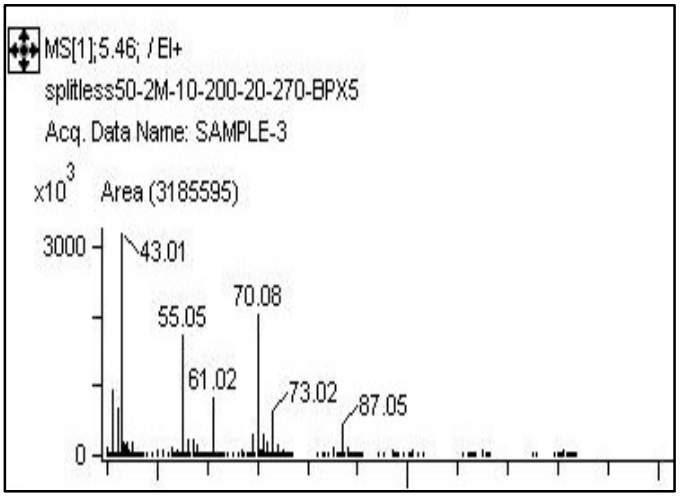

Figure no 5 Electron ionization mass spectrum of isoamyl acetate

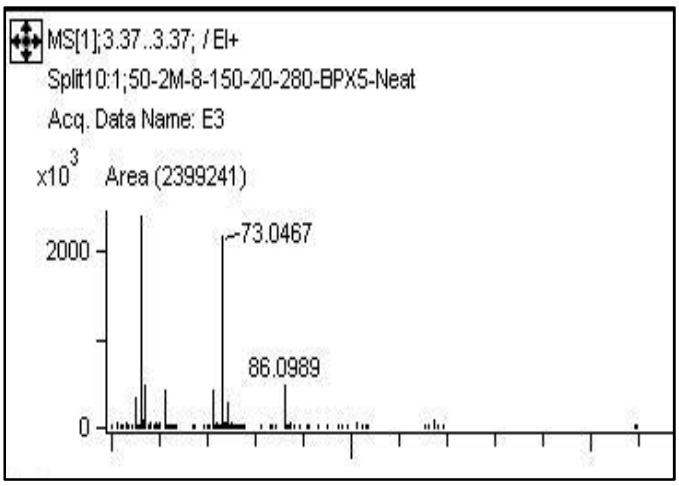

Figure no 6 Electron ionization mass spectrum of isobutyl acetate

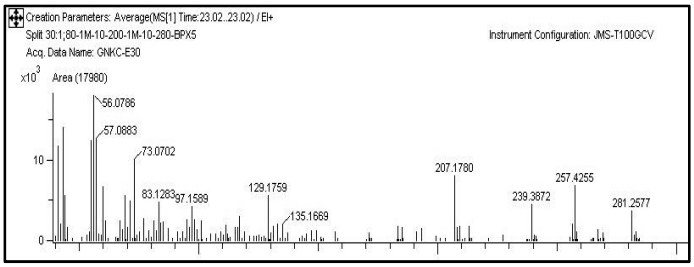

Figure no 7 Electron ionization mass spectrum of butyl palmitate (transesterfrom palm oil)

It is observed that lipase from *Staphylococcus gallinarum* was able to form esters such as butyl acetate, isobutyl acetate, isoamyl acetate and transesters of palm oil such as isobutyl stearate and butyl palmitate; and coconut oil as butyl palmitate. Microbial lipases are regiospecific. They act on the substrate in specific or non-specific manner.

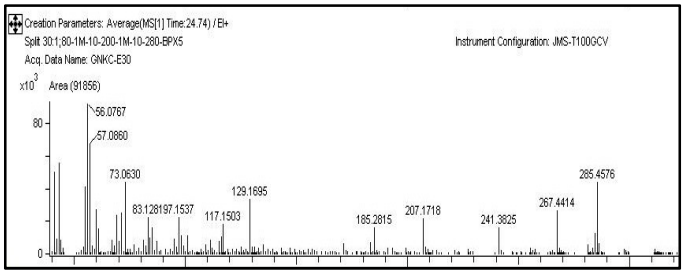

Figure no 8 Electron ionization mass spectrum of isobutyl stearate (ester from palm oil)

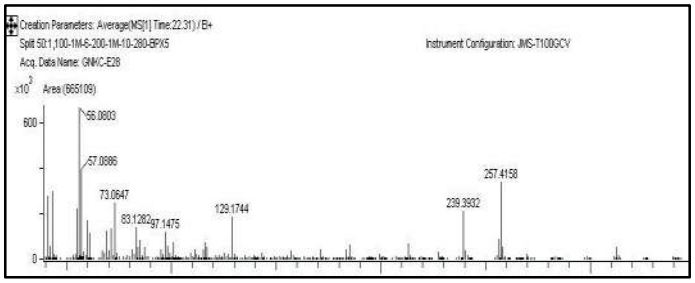

Figure no 9 Electron ionization mass spectrum of butyl palmitate (ester from coconut oi)

In the absence of water, besides being lipolytic, lipases are able to catalyze esterification and interesterification reaction. The capacity to catalyzed esterification reaction is influenced by number of variables such as molarity of alcohol and acid, reaction time, water, temperature, agitation and amount of enzyme. Performance of esterification also depends on alcohol structure(primary, secondary and tertiary alcohol)and length of fatty acid chain. In our study five primary alcohols (ethyl, methyl, propyl, butyl and octyl alcohol) and two secondary alcohols (isobutyl and isoamyl alcohol) were used. Whereas acid chain varies from one carbon(formic acid), two carbon(acetic acid) and four carbon (butyric acid). Fatty acids from coconut oil such as caprylic acid, decanoic acid, lauric acid, myristic acid, palmitic acid and oleic acid contain eight, ten, twelve, fourteen, sixteen and eighteen carbon respectively. Whereas palm oil contains fatty acids such as myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid having fourteen, sixteen, eighteen, eighteen (monounsaturated) and eighteen (polyunsaturated) carbon respectively. Lipase was specific to acetic acid , stearic acid (palm oil), palmitic acid (coconut oil) and butyl alcohol, isobutyl alcohol and isoamylacohol to esterify /tranesterify them.

CONCLUSION

Lipase catalyzed esters/transester can be used as bioflavors in food industries and as fragrances in cosmetic products. In the present study, lipase was able to catalyzed synthesis of butyl acetate, isobutyl acetate, isoamyl acetate and transesters of palm oil such as isobutyl stearate and butyl palmitate and coconut oil as butyl palmitate. Butyl acetate, isobutyl acetate and isoamyl acetate has common occurrence in bananas and pineapple. Thus, these can be used as alternative to artificial chemical flavors in food and bakery articles. Isobutyl stearate and butyl palmitate can be used in cosmetics and personal care products. They are frequently used in formulation of eye makeup, skin makeup, lipstick and skin care products. It is oily liquid and act as lubricants on skin surface which gives the skin

a soft and smooth appearance. The process can be scaled up for commercial production. Future prospective of the present study lies in down streaming and purification of esters.

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