Abstract

The present study is mainly focused on the screening of seven microorganisms for the production of most efficient lipase enzyme for the formation of glycerides (mainly monoglycerides and diglycerides). It was found that *Mucor racimosus* lipase enzyme was superior to all the other tested microbial lipases for the production of partial glycerides and triglycerides. The experiments were designed on the esterification reaction between mixed free fatty acids and glycerol as well as that of between the fatty acids oil and glycerol. The latter deals with the preparation of partial glycerides namely monoglycerides and diglycerides as well as triglycerides. These compounds can be used as emulsifiers for different industries. Enzymatic esterification parameters were carried out at a various incubation temperature, incubation period, enzyme concentration and substrate concentration to reveal the optimum conditions of esterification reaction. The highest esterification percent (89%) was achieved at the temperature of 30°C and incubation period of 24 hours using the enzyme concentration of 2.5 mg/ml with the substrate concentration of 0.002M oleic acid and 0.12M glycerol.

**Key words:** Lipase, *Mucor racimosus*, esterification, monoglycerides, diglycerides, triglycerides.

Introduction

Lipolytic enzymes are one of the most important groups of biocatalysts for biotechnological application (1, 2). It is known that lipases (EC 3.1.1.3) hydrolyze triacylglycerols to fatty acids, diacylglycerols, monoacyl glycerols and glycerol whatever under certain conditions, catalyze reverse reactions such as esterification and trans-esterification (3, 4). Moreover, lipases are glycerol ester hydrolases (EC: 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface (5, 6). Much interest in microbial lipase production has been increased due to its characteristics features of broad substrate specificity, versatile molecular structure, and stability in organic solvents (7, 8). Secreted lipases from various fungi and bacteria recently have attracted considerable attention due to its vast biotechnological potential (9, 10). It has a wide range of enzymatic properties and substrate specificities, making them very useful for industrial applications, such as the processing of fats and oils, additives, detergents, cosmetics, paper manufacturing, and pharmaceuticals (11-13). Moreover, it has been also used medical applications as in digestive aids, malignant tumors, gastrointestinal disturbances and dyspepsia therapy (12, 13).

Monoacylglycerol (MAG) constitutes the major type of food emulsifiers in many food

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**Biosynthesis of Partial Glycerides via Enzymatic Esterification of *Mucor racimosus* Lipase for Edible and Industrial Purposes**

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systems like bakery products, margarine, and dairy products and used as a basic starting material to prepare several other derivatives with modified functional properties (14, 15, 25). MAGs have been used as surface-active agents in many consumer and industrial cleaning products such as detergents, shampoos, lotions, and toothpaste or as raw materials for the synthesis of chemical compounds such as alkyl resins (15, 16, 18, 26). Moreover, it is also used as texturizing agents and hair care additive. MAG is also used as binders in tablets and as emollients for transdermal, slow-release drugs in the pharmaceutical industry (25). Owing to the fact that the edible oil contained at least 80% of 1, 3-DAG, the later has been used as cooking oil with health benefits (27). DAG is also used as precursors for the organic synthesis of prodrugs for the treatment of lymphoma and Parkinson’s disease in medicine (28, 29).

MAG can be prepared by either direct esterification of glycerol with fatty acids or glycerolysis of glycerol with oils or fats (indirect esterification). Recently many approaches have been investigated in the enzymatic synthesis of MAG (monoacylglycerol) and DAG (diacylglycerol). These are selective hydrolysis, esterification of fatty acids or transesterification of fatty esters with glycerol, and glycerolysis of fats or oils (19-24). Lipases can be used as biocatalysts for the glycerolysis process which have many advantages over the chemical process such as mild reaction condition, high catalytic efficiency and stereo- and positional specificities (15, 17, 18).

The aim of this study to screen seven different microbial lipases from different microorganisms for the most efficient enzyme in the esterification reaction. The most efficient microbial lipase was selected and the results were implemented on glycerol/ olive oil mixed fatty acids as a general trend for the utilization of vegetable oil fatty acids in the production of glycerides namely monoglycerides, diglycerides and triglycerides. Moreover, preparative thin layer chromatography and gas liquid chromatography are also applied for the evaluation of esterification and hydrolytic products.

Materials and Methods

Microorganisms and culture conditions: All the microbial cultures (Mucor racemosus, Asp niger NRRL3, Asp. niger 7H, Asp niger (oil), Asp. niger 16H, Asp. terreus 2H and Asp. terreus 1H) were obtained from National Research Centre (NRC), Cairo, Egypt and Northern Regional Research Laboratory (NRRL). The microbial cultures were grown on potato dextrose agar (PDA) medium. The culture was inoculated 100 ml of the fermentation medium in 250 ml Erlenmeyer flasks and incubated at 30 °C in shaking incubator 200 rpm.

Production, evaluation and partial purification of lipases: Productions of lipases from different microorganisms were carried out according to Akhtar et al. (30), while the determination of the lipase activity was evaluated as per Parry et al. (31). Partial purification of lipase from different microorganisms was done as the method described by Abbas et al. (32).

Screening of different partially purified lipases on the esterification reaction: The seven different active hydrolyzing lipases belonging to the previously mentioned microorganisms are tested for the esterification reaction. The model esterification medium consists of; 0.2 ml of oleic acid (95 %) and 1.8 ml of glycerol. Each fungal enzyme which contains the equal amount of protein was dissolved in 1 ml of tris buffer (pH 7.5, 0.2 M) and then added to the reaction mixture followed by incubation at 30°C for 24 hours in rotating shaker (New Brunswick Scientific Edison, N.J., U.S.A.) at 200 rpm. The esterification processes were carried out under various following conditions.

Effect of enzyme concentration: The reaction mixtures were contained the oleic acid (0.5ml) and 95% glycerol (9ml) with different

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concentrations (0.5 – 20mg) of enzyme solution diluted in 1ml of tris buffer (pH7.5 & 0.2M). The reaction mixtures were incubated at 30°C for 24 hours using a rotating shaker at 200 r.p.m. The reaction was stopped by adding 10ml of chloroform and the sample was taken for investigating the tri oleoyl glycerol, (T.O.G), partial glycerides (monoalkylglycerol) (M.O.G.), dioleoyl glycerol (D.O.G.) and non-reacted oleic acid. The chloroform was evaporated by rotary evaporator under reduced pressure.

**Effect of substrate concentrations (Glycerol and oleic acid):** The influence of varying concentrations of glycerol (0.1, 0.12, 0.14, 0.16 M) with a constant concentration of oleic acid (0.002 M) was carried out. Another varying concentration of oleic acid (0.001, 0.002, 0.003 and 0.0045 M) with a constant concentration of glycerol (0.12 M) was also studied under the previously mentioned esterification conditions using 2.5 mg of partially purified lyophilized lipase diluted in 1 ml of tris- buffer (0.2 M, pH 7.5) and incubated at 30° C for 24 hours at 200 rpm. The estimation was calculated on the basis of 100 mg of the products.

**Effect of incubation time and temperatures:** The effect of various incubation periods (12.0, 24.0, 36.0, 48.0 and 72.0 hours) and incubation temperatures (25–45°C) was also studied. The reaction mixture was studied using 0.12M glycerol and 0.002 M oleic acid under the previously mentioned esterification conditions. The esterification products were evaluated and estimated as previously described as above.

**Toxicity test of Mucor racemosus strain:** Extractions of mycotoxins (AFs) were carried out according to Munimbazi et al. (33) while determination and derivatization of aflatoxins (B1, B2, G1, and G2) were carried out according to the method described by Deabes et al. (34, 35).

**Evaluation of chemical esterification reaction:** The esterification products from oleic acid/ glycerol medium were evaluated using preparative T.L.C as previously described and the estimation was calculated on the basis of 100 mg of the products, as usual. The optimal esterification optimal conditions on mixed olive oil fatty acid / glycerol were carried out using saponification method (36).

**Evaluation of enzymatic esterification reactions products:** After the preparation of fatty acids from olive oil, certain amount of mixed fatty acid (equivalent to 0.002 M (0.5/g) of oleic acid) was mixed with glycerol (0.12 M) (1.0 /g) at optimum concentration of partially purified Mucor racemosus lipase under the previously mentioned optimum conditions to obtain the (monoacylglycerol, diacyl-glycerol, non-reacted fatty acids and triacylglycerol). 100 mg of esterified products were fractionated by preparative thin layer chromatography (TLC) (coated with silica gel E-Merck, 200U). The resulting products were applied as bands (alongside the standard compounds) on each preparative silica gel plate (300 microns) and the plate was developed with the solvent system (diethyl ether : hexane : glacial acetic acid) (70 : 30 : 1 v/v/v). The fractions corresponding to the mono oleoyl-glycerol, dioleoyl glycerol, unesterified fatty acid and trioleoy glycerol were visualized with iodine vapor and then scrapped off the plate. The scrapped fractions were extracted two times with moistened diethyl ether. The filtrate was taken by Pasteur pipette and evaporated gently to obtain a residue and weight of each fraction was determined (37).

**Results and Discussion**

From the results of screening analysis of seven active lyophilized lipase enzymes, it seems that the seven lipases perform esterification reaction at different rates between oleic acid and glycerol. Monoolein, diolein and triolein, were the main product of the reaction which was isolated by preparative T.L.C. on the basis of 100 mg of the products. The lipase purified from Mucor racemosus was superior to all other microorganisms and has shown maximum esterification percentage of 59.14 %. The two isolates of Aspergillus terreus (1H, 2H) have
shown high yield production of monoolein of 21.78 % and 21.8 %, respectively. On the other hand, higher yields of diolein (24.11, 21.03, 26.16, 28.99 and 24.30%) were obtained by Aspergillus niger 7H, Aspergillus niger 16H, Mucor racemosus, Aspergillus niger NRRL3 and Aspergillus niger (Oi1), respectively (Fig.1). Moreover, Mucor racemosus was found to be superior to all other microorganisms in performing the esterification reaction.

The detailed studies on various factors (enzyme concentration, substrate concentration, incubation period and incubation temperature) affecting the rate of esterification were also conducted. The optimal concentration of the partially purified from Mucor racemosus was determined as 2.5 mg/ml for the esterification reaction. Generally, it was observed that the enzyme concentration of Mucor racemosus lipase was proportional to the esterification percent until the concentration of 5.0 mg/ml after which the esterification percentage was slightly decreased (Fig.2). All these results were in agreements with previously published reports (38, 39, 40).

![Fig. 1. Screening of active partially purified lipases from different fungi for the esterification reaction between glycerol and oleic acid.](image1)

![Fig. 2. Effect of the concentration of partially purified Mucor racemosus lipase on the esterification reaction. The estimation of products(%) monoolein, (E%) diolein, (T) Triolein, (%) esterified oleic acid and (%) non-esterified oleic acid were evaluated on the basis of 100 mg of the esterification products.](image2)
The optimal substrate concentration was estimated by taking various molar ratio molar ratio of glycerol to oleic acid. With respect to the (A), 1: 0.008, 1:0.16, 1: 0.25 and 1:0.375 in which glycerol is in a constant concentration and oleic acid is in a variable concentration. The other molar ratio (B) of oleic acid: glycerol : 1:50, 1:60, 1:70 & 1:80 in which the oleic acid is constant while the glycerol is at variable concentrations (B). The optimum esterification percent was amounted to 89.50 % with a high level of diolein (48.30 %) and moderate amounts of monoolein and triolein (21.20 % and 20.0%, respectively) (Fig.3). This may be attributed to the possibility of the enzyme to enhance the formation of diolein. It is noteworthy to mention that some authors used high concentrations of enzyme 15 % (w/w) at a molar ratio of 1:2 for 24 hours and reached > 80 % of esterification level [41]. The presented data were confirmed with earlier reports (40) who observed that large amounts of glycerol increased the velocity of esterification and attained a higher degree of esterification. On the other hand, they reported that large amounts of glycerol repressed synthesis of diacylglycerol and increased the content of monoacylglycerol and the degree of esterification reached to 89.9 % after 48 hours.

**Fig. 3a.** Effect of oleic acid concentrations (at fixed glycerol concentration) on the esterification reaction using *Mucor racemosus* lipase. The estimation of products, (l%) monoolein, (E%) diolein, (*) triolein, (%) esterified oleic acid and (a%) non-esterified oleic acid, were evaluated on the basis of 100 mg of the esterification products.

**Fig. 3b.** Effect of substrate concentrations (glycerol) at fixed oleic acid concentrations on the esterification reaction using *Mucor racemosus* lipase. The estimation of products, (l%) monoolein, (E%) diolein, (*) triolein, (%) esterified oleic acid and (a%) non-esterified oleic acid, were evaluated on the basis of 100 mg of the esterification products.
The effect of different incubation periods starting from 12 to 72 hours was also studied. The optimum values of esterification percent (89.5 and 88.7 %) were obtained after 24 and 36 hours, respectively (Fig. 4). However, the esterification percent after 24 hours was preferable to that after 36 hours which was attributed to the possible short time of 24 hours that led to similar esterification capacity. Thus, much time was saved in the esterification reaction. Accordingly, 24 hours incubation period is selected as the optimum incubation period. These results were supported by finding of earlier reports (40) who reported that the optimum incubation period 24 hours for the production of monoacylglycerol and diacylglycerol by *Penicillium camembertii* lipase with the esterification to 86.1%.

Referring to the effect of temperature on the esterification of oleic acid with glycerol, the esterification capacity amounted to (89.50%) at 30°C (Fig. 5). There was a drastic decrease in the esterification value reaching to 66.69 % at 25°C with lowering the temperature while the differences in the esterification capacities
As a trial to apply the results of glycerol / oleic acid esterification, it was planned to use olive oil mixed fatty acid of olive oil as a representative of sources of a fatty acid of natural origin. Mixed free fatty acids from olive oil have the following composition as determined by G.L.C. (palmitic acid, 12%; stearic acid, traces; oleic acid, 81.1%; linoleic acid, 5.34% and linolenic acid traces) (Table-2a). It was noted that, esterified fatty acids (oleic acid, 71.1% palmitic acid, 5.0%, linoleic acid, 3.0 % and unknown acid, 0.33 %) amounted to 79.4% while the non-esterified fatty acid (palmitic acid, 7.0%, oleic acid, 10.0%; linoleic acid, 2.0% unknown acid, 1.17% and stearic acid, traces) amounted to 20.17% (Table-2b). Thus, lipase enzyme probably selects the unsaturated fatty acids (oleic acid and linoleic acid) more than saturated fatty acid (palmitic acid) to form partial glycerides and triglycerides. Thus, the affinity of the reaction was arranged in descending order. Oleic acid > linoleic acid > palmitic acid. Generally, *Mucor racemosus* lipase enhanced the formation of monoglyceride (24.20 %), diglyceride (41.8 %) and low amounts of residual triglyceride (14.0 %).

**Fig. 6.** Quantitative fractionation via preparative thin layer chromatography (TLC) of the enzymatically esterified products of *Mucor racemosus* (monolein, diolein, residual (non-esterified) oleic acid and triolein) to-Scaled Chromatogram of sample Auto-Scaled Chromatogram of standard Auto-Scaled Chromatogram of sample between 30°C and 40°C was very less. These results are approximately supported by earlier studies (40) who found that decrease in the temperatures hindered the synthesis of diacylglycerol and monoacylglycerol and almost the same amounts of monoacylglycerol and diacylglycerol were formed by *Penicillium camemberti* lipase after 24 hours in the reaction at 30°C and 40°C reaching degree of esterification of 86.1%.

**Fig. 7.** HPLC auto-scaled chromatogram to show the detection of aflatoxins in *Mucor racemosus*

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Table 3. Detection of mycotoxins (Aflatoxins) in *Mucor racemosus* by Toxicity test

<table>
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<th>Sample</th>
<th>Aflatoxin</th>
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<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>ND</td>
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ND = Non detected

from the esterification reaction between the prepared mixed fatty acids of olive oil with glycerol (Fig. 6; Table-2). Although *Aspergillus niger* (oil) exhibited maximum lipolytic activities, *Mucor racemosus* has been selected for the studies due to its non-pathogenicity as confirmed by the toxicity test as shown in Fig. 7; Table-3 (34)

Conclusion

It can be concluded that the value added products namely, monoacylglycerol and diacylglycerol, produced from esterification reactions, are useful in the field of biotechnology which is mainly used as emulsifiers in different industries, particularly in the food industry. In other words, *Mucor racemosus* lipase has the ability to produce appreciable amounts of unsaturated monoglycerides, diglycerides of oleic acid and linoleic acid.

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