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Efficient microwave-assisted hydrolysis of triolein and synthesis of bioester, bio-surfactant and glycerides using *Aspergillus carneus* lipase

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Microwave irradiations are known to alter the rate of chemical reactions. Often this results in enhanced reaction rates; higher yields, purity of products; more efficient and homogeneous distribution of energy and heating effects, and easier water elimination. The enzyme lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is a special class of esterase enzyme that acts on fats and oils and hydrolyses them in steps into the substituted glycerides and fatty acids, and finally on complete hydrolysis into glycerol and fatty acid. Hydrolysis of triolein using a fungus, *Aspergillus carneus* lipase has been carried out both under normal conditions and microwave irradiations of LOW 10 (175 W, 38–40°C) and HIGH 100 (800 W, 90°C). Analysis of the hydrolytic products on TLC plates showed the presence of triolein, diolein, monolein and oleic acid in 24 h under normal conditions, compared to microwave irradiations where it took 45–90 s under LOW power level and 15–30 s under HIGH power level for the production of monolein and oleic acid. However, complete hydrolysis of triolein took place in 160 s at LOW power and 75 s at HIGH power level. The synthesis of bioester, biosurfactant and glycerides could successfully be carried out rapidly within 30 s under both solvent-containing and solvent-free condition under HIGH power microwave irradiation.

Keywords: *Aspergillus carneus*, hydrolytic and synthesis reactions, lipase, microwaves.

MICROWAVES (0.3–300 GHz) fall between the infrared and radio frequency region of the electromagnetic spectrum[1]. In recent years, besides other uses, microwaves have gained considerable attention for their utilization as domestic ovens and other scientific applications[2]. Microwave irradiation is becoming an increasingly popular method of heating, replacing the classical one, because it proves to be a clean, cheap and convenient method[3]. Often, it affords higher yield and results in shorter reaction times. These reactions are especially appealing as they can be carried out in open vessels, thus avoiding the risk of development of high pressures. Gedye *et al.*[4] were the first to use the microwave oven in organic synthesis. Since then, this technique has evolved into a useful tool[5–9]. Currently, besides other uses in biological reactions, microwaves are being utilized to assist hydro-

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lytic and synthetic reactions using enzymes such as lipases, proteases, etc.[10–12]. Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3) are hydrolytic enzymes[13,14]. They are a special class of esterase enzymes that act on fats and oil and hydrolyse them in steps into the substituted glycerides and fatty acids, and finally on complete hydrolysis into glycerol and fatty acid. This enzyme is used as versatile biocatalyst in modern organic chemistry, especially for modifications of fats and other lipids via hydrolysis, esterification and interesterification reactions[15–18]. On the other hand, the enzyme efficiently carries out the reverse reaction of synthesis under water-limiting conditions[2].

In the present study, the rate of hydrolysis and synthetic reactions catalysed by the lipase enzyme produced by the fungus *Aspergillus carneus* were studied under normal and microwave irradiation conditions. Two different power levels, viz. LOW 10 (175 W, 38–40°C) and HIGH 100 (800 W, 90°C) of microwave irradiation were employed for the study.

A natural isolate of *A. carneus* produced 12,000 IU/l lipase on the modified medium[18] with composition (in %): sunflower oil (1.5), peptone (0.5), glucose (1), KH_2PO_4 (0.25), KCl (0.05) and $MgSO_4.7H_2O$ (0.05); pH 8.0. The crude enzyme was concentrated and partially purified using 30 kDa membrane and was freeze-dried at –70°C in a freeze-drier. The freeze-dried enzyme was used to carry out hydrolytic and synthetic reactions using conventional method and microwave irradiations. Product analysis was carried out using TLC and per cent yield was determined by titration of initial and residual fatty acids contents according to the procedure[19].

One International Unit (IU) of lipase activity is defined as the amount of enzyme required to release 1 µmol of fatty acid per ml per minute under the standard assay conditions.

Hydrolytic reaction was carried out in the aqueous system and was monitored both under normal condition and microwave irradiations (i) LOW 10 (175 W, 38–40°C) and (ii) HIGH 100 (800 W, 90°C). Next, 1 ml triolein and 5 ml of the enzyme sample (culture filtrate of the production medium) were incubated at 37°C for 24 h with shaking at 100 rpm for reaction under normal conditions. Aliquots of 200 µl were withdrawn at regular intervals of time up to 24 h and the reactions were terminated using 5 ml diethyl ether each time. The extracts were frozen and later analysed by thin layer chromatography (TLC; silica gel 60 F_{254} Merk, Germany). The hydrolysis of triolein was studied under low (10) and high (100) power levels for 5–90 s using a microwave (model no. BMC 900T; BPL Sanyo, India) oven. The products formed were analysed on TLC plates.

For the synthesis of bioester, 2 ml of 200 mM oleic acid (fatty acid) was reacted with 2 ml propan-2-ol (alcohol) in the presence of 10 mg of lyophilized *A. carneus* lipase. For synthesis of biosurfactant (sugar alcohol ester), 200 mM oleic acid was reacted with 100 mM sorbitol (sugar/sugar alcohol). However, for synthesis of glycerides, 1 ml of glycerol was reacted with 200 mM oleic acid. Reactions under normal conditions were carried out at 37°C, 200 rpm for 24 h under both solvent (*n*-hexane) and solvent-free conditions. Synthetic reactions were also carried out under microwave conditions at HIGH power level for 30 s in two different ways: using *n*-hexane as solvent and under solvent-free conditions. Products were analysed using TLC. Percentage yield was also estimated and expressed as the percentage molar conversion of acid to ester after titrating the residual fatty acid against 0.01 N KOH using phenolphthalein as indicator.

The reaction and the product were monitored on TLC plates. The solvent system consisted of petroleum ether : diethyl ether : acetic acid in ratio 80 : 30 : 1. Spots were visualized in a saturated iodine chamber.

Hydrolysis of triolein using *A. carneus* lipase was studied in relation to time both under normal and microwave conditions. Analysis of the hydrolytic products on TLC plates showed the presence of triolein, diolein, monolein and oleic acid (Figure 1 *a*). Results under normal condition showed that maximum accumulation of diolein and

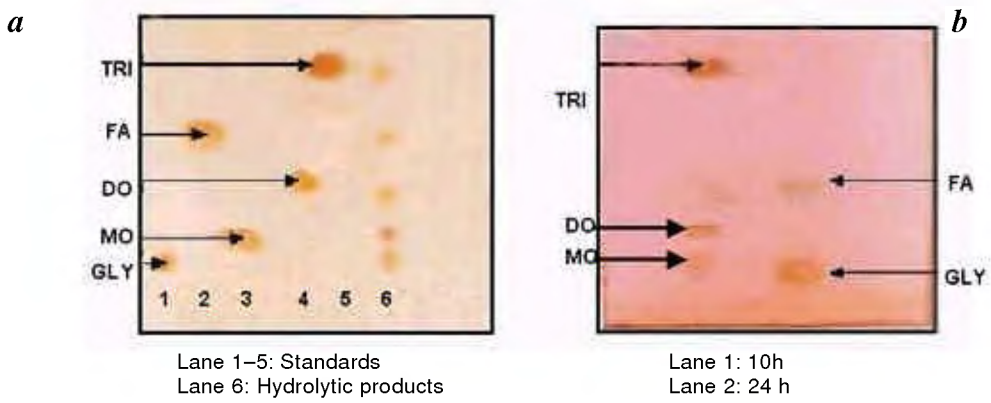

Figure 1. TLC analysis of hydrolysis of triolein using *Aspergillus carneus* lipase at 37°C, pH 9 at different time intervals. ***a***, Standards. Lane 1; Glycerol (GLY); Lane 2, Monolein (MO); Lane 3, Diolein (DO); Lane 4, Fatty acid (FA); Lane 5, Triolein (TRI) and Lane 6, Mixture of triolein, diolein, monolein, fatty acid and glycerol. ***b***, Hydrolysis of triolein under normal conditions. Lane 1, Hydrolysis after 10 h showing triolein, diolein and monolein; lane 2, Hydrolysis after 24 h showing fatty acid and glycerol.

Table 1. Microwave-promoted *A. carneus* lipase-catalysed hydrolysis of triolein at LOW 10 and HIGH 100 power levels at different time intervals

	Power level			
	LOW 10 (175 W)		HIGH 100 (800 W)	
Time (s)	Compounds (seen on TLC)	Per cent yield	Compound (seen on TLC)	Per cent yield
5	TO, DO	–	TO, DO, MO	–
10	TO, DO	–	TO, DO, MO	–
15	TO, DO, MO	–	DO, MO, FA	18
20	TO, DO, MO	–	MO, FA	38
25	TO, DO, MO	–	MO, FA	52
30	TO, DO, MO	–	MO, FA, GLY	76
45	DO, MO, FA	11	MO, FA, GLY	84
60	DO, MO, FA	33	MO, FA, GLY	90
75	MO, FA	48	FA, GLY	97
90	MO, FA	60	FA, GLY	98
120	MO, FA, GLY	77	FA, GLY	98
145	MO, FA, GLY	83	FA, GLY	98
160	FA, GLY	94	FA, GLY	98
180	FA, GLY	96	FA, GLY	98

TO, Triolein; DO, Diolein; MO, Monolein; FA, Fatty acid; GLY, Glycerol.

Table 2. Synthesis of bioester, biosurfactant and glycerides under normal and microwave conditions and their per cent yield in relation to time period

	Normal conditions (24 h) per cent yield		Microwave conditions (25 s) per cent yield	
Product	Solvent condition (*n*-hexane) condition	Solvent-free condition	Solvent condition (*n*-hexane) condition	Solvent-free condition
Propyl oleate (bioester)	25	35	75	86
Sorbitol oleate (biosurfactant)	60	68	87	90
Triglyceride	35	40	42	50

monolein was observed at 10 h after which the concentration of oleic acid increased in the reaction system. However, complete hydrolysis of triolein into oleic acid occurred at 24 h (Figure 1 *b*). On the other hand, under microwave irradiations, extremely rapid hydrolysis of triolein was achieved within 45–90 s under LOW power level and 15–30 s under HIGH power level (Figure 2 *a*, *b*), with the production of monolein and oleic acid (Table 1). Still higher accumulation of monolein and oleic acid with traces of triolein and diolein was observed in 120 s and complete hydrolysis took place in 160 s under LOW power level. However, under HIGH power level when the reaction was monitored up to 90 s, complete hydrolysis of triolein was over in 75 s (Figure 2 *c* and Table 1). This showed that *A. carneus* lipase could efficiently catalyse the hydrolysis of triolein under microwave irradiation conditions as compared to normal conditions, where it took 24 h for the complete hydrolysis. Bradoo *et al.*[7] have also suggested microwave-assisted rapids characterization of lipase selectivities. Kidwai *et al.*[20] have reported that the reaction time for the stereoselective synthesis and antibacterial activity of new fluoroquinolinyl-β lactam derivatives can be brought down from hours to minutes with improved yields using microwave irradiation. Further, Lin and Lin[21] have attributed high reactivity under microwave heating to directed absorption of energy by functional groups, and the release of this energy into the surrounding solution.

Synthesis reactions under normal and microwave irradiations were carried out for the production of bioester (2-propyl oleate), biosurfactant (sorbitol oleate) and glycerides (mono, di and triolein) in two different ways: (i) using *n*-hexane as the solvent, (ii) under solvent-free conditions. *A. carneus* lipase could successfully mediate the esterification reactions for the synthesis of bioester, biosurfactant and glycerides under both conditions. Microwave irradiations enhanced rate of reaction. Higher per cent yield was obtained within 30 s when reaction was carried out in a microwave oven, which otherwise takes at least a 48 to 96 h (Table 2).

The higher rates observed in the microwave oven both for hydrolytic and synthetic reactions could be because of the high temperatures achieved in a short time during microwave heat transfer, which depends on the dielectric losses

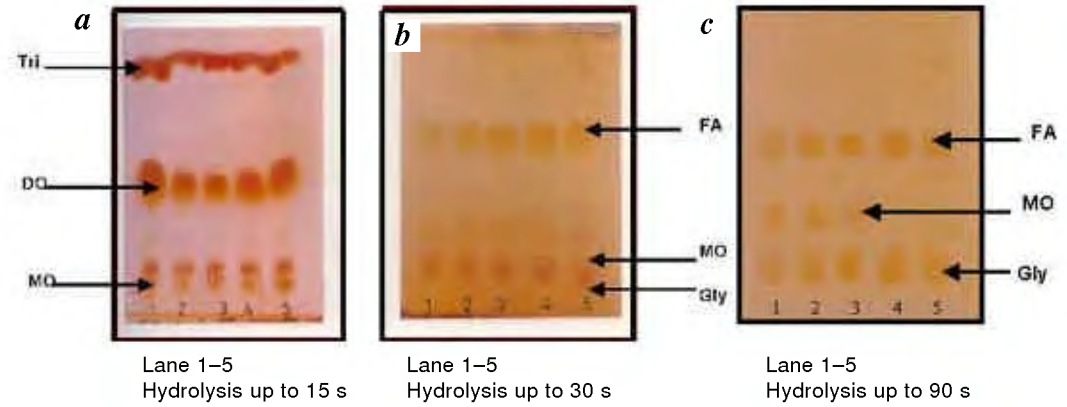

Figure 2. TLC analysis of hydrolysis of triolein at different time intervals using *A. carneus* lipase under HIGH power level 100 (800 W, 90°C) of microwave. Hydrolysis of triolein after ***a***, Lane 1, 5 s, triolein, diolein, monolein; lane 2, 8 s: triolein, diolein, monolein; lane 3, 10 s: triolein, diolein, monolein; lane 4, 12 s: triolein, diolein, monolein and lane 5, 15 s: triolein, diolein, monolein; ***b***, Lane 1, 18 s: monolein, fatty acid, glycerol; lane 2, 20 s: monolein, fatty acid, glycerol; lane 3, 25 s: monolein, fatty acid, glycerol; lane 4, 27 s: monolein, fatty acid, glycerol and lane 5, 30 s: monolein, fatty acid, glycerol. ***c***, Lane 1, 40 s: fatty acid, glycerol; lane 2, 50 s: monolein, fatty acid, glycerol; lane 3, 60 s: monolein, fatty acid, glycerol; lane 4, 75 s: fatty acid, glycerol (complete hydrolysis) and lane 5, 90 s: fatty acid, glycerol (complete hydrolysis).

of solvents; hence water having the maximum dielectric loss gets heated fast. Therefore, as soon as the reactants for (i) hydrolytic reactions, viz. liquid enzyme in the medium and triolein, and (ii) synthetic reactions, viz. 10 mg lyophilized *A. carneus* and fatty acid + propan-2-ol (for bioester synthesis); sorbitol (for biosurfactant) and glycerol for (glycerides) under solvent-free and *n*-hexane are kept in a microwave oven, the environment gets heated up immediately attaining around 90°C within 30 s. The reaction at 90°C was possible as lipase is stable for 5 min at this temperature[18]. Moreover, the whole system is heated uniformly unlike the convection currents, where the temperature gradient exists. Therefore, the activation energy of the enzyme when exposed to microwave heating is lowered at a much faster rate and hence a boost in the catalytic rate is observed when reactions are carried out with microwave irradiations.

From the above experiments, it has been concluded that many industrially important hydrolytic and synthetic reactions can efficiently be carried out using microwave irradiation. This method will not only be economical as it saves a lot of time and energy, but also offers the following advantages: (i) microwaves speed up the rate of enzyme catalysed reactions; (ii) these do not harm the enzyme properties such as its stability and substrate specificity and (iii) during microwave heating, the whole system is heated uniformly.

This will prove to be a potential tool for accelerating biocatalysis and thus a boon for enzyme-based reactions.

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