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Research Article

Synthesis of Oleoyl-diethanolamide by Using Purified Lipase from Thermotolerant *Bacillus subtilis* TTP-06

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Abstract

Present study aimed to synthesize oleoyl-diethanolamide in a greener way by using lipase as a biocatalyst. The lipase enzyme used in present work was purified from a thermotolerant *Bacillus subtilis* TTP-06. The synthesis of oleoyl-diethanolamide was determined by checking the % conversion of oleic acid (OA). Therafter, oleoyl-diethanolamide synthesized in the laboratory was characterized by Fourier Transform Infra-Red (FTIR) spectroscopy. Maximum 92.64 ± 0.62% conversion of OA was achieved after the optimization of certain parameters *viz.* concentration of substrates, reaction time, reaction temperature and amount of biocatalyst.

Keywords: Fourier Transform Infrared Spectroscopy; Oleoyl-Diethanolamide; Synthesis, Oleic Acid; Diethanolamine

Abbreviations

FTIR: Fourier Transform Infrared; OA: Oleic Acid; DEA: Diethanolamine; W/O: Water/Oil; mM: Millimolar; %: Percent; wt/wt: Weight by Weight; v/v: Volume by Volume

Introduction

Many medicinal and cosmetic products use fatty diethanolamides (FDAs) as surfactants. Being a nonionic surfactant with a high dispersion and a favourable impact on water/oil (W/O) foam stability, fatty alkanolamide offers a wide range of possible uses. Fatty alkanolamides are commercially produced by condensing fatty acids and amines. These surfactants are widely employed in many formulations, including detergents, shampoos, antibacterial agents, antifoaming agents, cosmetics, medicinal chemistry and lubricants. The fatty amide surfactants can be employed as solublizing, foaming, wetting and emulsion agents [1,2].

Typically, fatty alkanolamide is produced at a temperature of 180ºC while being chemically catalyzed [3]. When alkanolamide is made from fatty acids using chemical catalysts, impurities such as monoesteramines, diesteramines, monoesteramides and diesteramides typically result. Also, using high temperatures causes odor and makes the color of the product less appealing [4,5].

The use of enzymes in the manufacture of amides has significantly improved. Enzymatic synthesis provides a number of benefits, including easy product isolation, gentle reaction conditions, low contaminants, low energy requirements and eco-friendly biocatalyst [6]. Other researchers have reported the use of commercial enzymes in the manufacture of fatty alkanolamides [7]. Yet, there is no report on the enzymatic amidification of oleic acid (OA) and diethanolamine (DEA) by using the enzyme which has been purified in the laboratory only. Also, on the optimization of conditions for the synthesis of oleyl-diethanolamide, there are surprisingly few reports.

Fatty diethanolamide can be manufactured commercially by combining chemical catalysts with an oil substrate and an alkanolamine. Nevertheless, only extremely high pressures and temperatures make this synthesis feasible [8,9]. Therefore, by employing lipase as a biocatalyst, an enzymatic method has been devised for the synthesis oleyl-diethanolamide from fatty acids (OA) and diethanolamine (DEA). In a brief period of time, at low temperatures, and under low pressure, lipase can catalyze the process. The optimization reactions for the synthesis of oleyl-diethanolamide from fatty acid (OA) were carried out in present study with the following variables: reaction time, temperature, substrate concentration and enzyme amount. Enzyme purified from *Bacillus subtilis* TTP-06 is the one employed as a catalyst.

Materials and Methods

Lipase enzyme was purified from thermotolerant Bacillus subtilis TTP-06. The purification was done in a step-wise manner by using ammonium sulphate precipitation, Anion-exchange (DEAE-Sepharose) and Gel-permeation (Sephadex G-100) chromatography. Lipase producing bacterium (Accession No.: MW828331.1) was previously isolated from Tattapani hot spring (31.2487°N, 77.0878°E) situated in Himachal Pradesh, India at an altitude of 2,182 feet above the sea level. DEA and OA were purchased from CDH Pvt. Ltd. in *n*-Hexane, Tris-HCl were obtained from Sigma-Aldrich U.S.A. All chemicals used were of analytical grade.

Amidification reaction for the synthesis of oleoyl-diethanolamide by purified lipase

In graduated tubes with a working volume of 15 ml, amide synthesis was carried out by reacting the suitable concentrations of the reactants (OA and DEA) *n*-hexane [10]. The addition of 20 µg (0.318 U) of lipase purified from *Bacillus subtilis* TTP-06 started the reaction. The reaction mixture was then incubated for 12 hours at 55ºC with shaking. The control reaction mixture was devoid of lipase.

Purification and characterization of oleoyl-diethanolamide

By evaporating the crude amide product at 70ºC, the solvent (*n*-hexane) was removed. The product was then rinsed with acetone to remove any remaining OE and excess DEA. The top product was the remaining OA with acetone and the bottom layer contained amide [11]. The following equation was used to compute the conversion of OA:

$$\% \text{ conversion} = \frac{Acid\ value(initial) - Acid\ value(final)}{Acid\ value(initial)}$$

At Panjab University, Chandigarh, Fourier Transform-Infra Red (FTIR) instrument series 1100 from Perkin Elmer was used to record the FTIR spectra in order to characterize the final product.

Optimization of reaction parameters for synthesis of oleoyl-diethanolamide using purified lipase from *Bacillus subtilis* TTP-06

Effect of molarity of reactants

Concentration of OA and DEA was varied to check the synthesis of oleoyl-diethanolamide. Firstly, DEA concentration was kept constant at 6 mM and OA concentration was varied from 0.2-2.0 mM. While, in the second set, OA concentration was kept constant as optimized in the previous set and the concentration of DEA was varied from 2-20 mM. *n*-Hexane was added to make up the reaction volume (5 ml). The reaction was started by the addition of 20 µg (0.318 U) purified lipase and the reaction mixture was incubated at 55ºC for 12 hrs.

Effect of reaction time

Reaction mixture was incubated for different time intervals *viz.* 6, 12, 18, 24, 30 and 36 hrs at 55ºC.

Effect of reaction temperature

Reaction mixture was incubated at different temperatures (35, 40, 45, 50, 55, 60 and 65ºC).

Effect of enzyme amount

Optimized reaction mixture was incubated with varying amount (10-60 µg) of purified lipase (0.318 Units).

Results and Discussion

Identification and characterization of oleoyl-diethanolamide

The FTIR spectrum of oleoyl-diethanolamide is shown in Figure 1, where absorption peak in the wavelength region of 3434-3451 cm^{-1} indicated the vibration of –OH group stretching. In the structure of an amide, there is a N-H bond; no peaks were found at wavenumber region of 3118-3191 cm^{-1}, which is attributed to the fact that diethanolamide is a tertiary amine. In the region of 2922.98

cm^{-1}, the absorption of –CH alkanes is visible. The absorption band at 865.05 cm^{-1} wavenumber typically represented the vibration of $-(CH_2)_n$ or long chain alkyl hydrocarbons. The successful synthesis of oleyl-diethanolamide is indicated by the appearance of absorption peak occurring in the wavenumber region of 1645.23-1651 cm^{-1} which is an area for –C=O stretching of carbonyl of the amide. FTIR results are in concordance with those obtained during the synthesis of fatty diethanolamide from Calophyllum inophyllum L. Kernel oil by using immobilized lipase of *Thermomyces lanuginosus* [7]. In the present study, the percent conversion of OA was calculated to be 74. Previously, OA conversion of 78.01%, has been obtained by using lipase from *Candida antarctica* under optimized conditions [11]. However, in another study % conversion of about 44% fatty diethanolamide was obtained from nyamplung oil under the optimum reaction conditions by using *Thermomyces lanuginosus* lipase as biocatalyst [7].

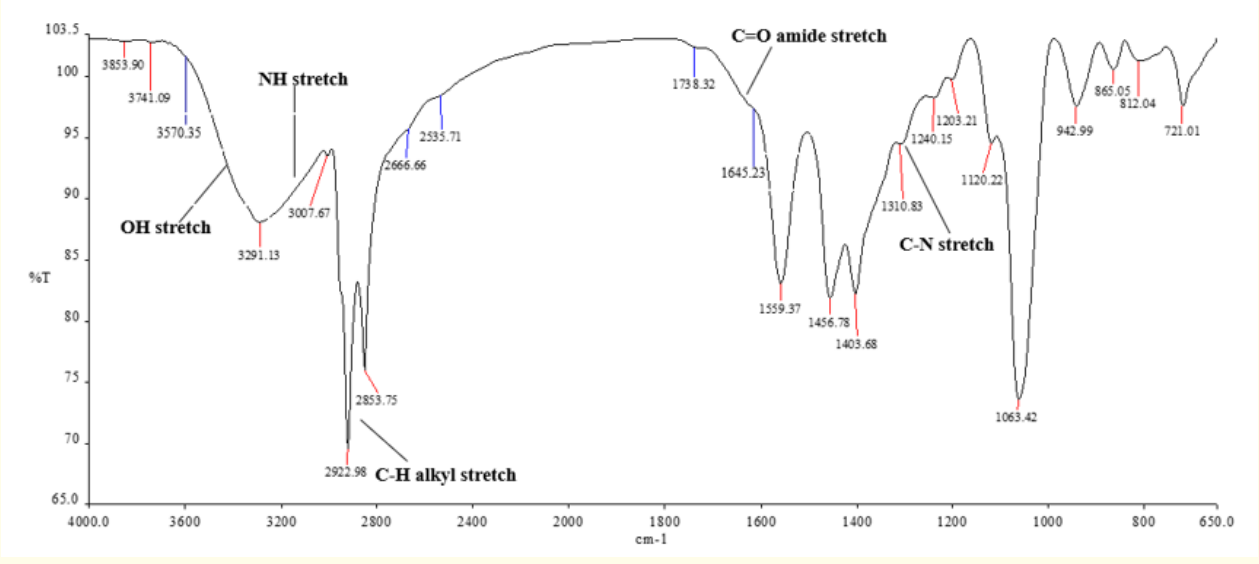

Figure 1: FTIR spectrum of oleoyl-diethanolamide synthesized by using lipase purified from *Bacillus subtilis* TTP-06

Effect of molarity of reactants

Maximum OA conversion of 78.76 ± 1.7% was achieved by using 1 mM OA and 10 mM DEA (Table 1). No increase in the OA conversion was seen on further increase in the molar concentrations of substrates. This could be attributed to the reason that when molar ratio of substrate exceeds the optimum then the active space of the reaction is lowered which in turn leads to a reduction in active collision of particles. In a previous study, 50 mM of each of OA and DEA were found optimum to give oleylethanolamide yield of 73.5% [10]. 61.35% OA conversion was noted down in another experiment by using DEA/OA molar ratio of 7 in the presence of immobilized lipase from Candida antarctica [1]. Another study reported 78.01% conversion of OA by using diethanolamine to OA molar ratio in the range of 1:1 to 3:1 in the presence of lipase from *Candida antarctica* [11].

Substrate	Concentration (mM)	Oleic acid conversion (%)
Oleic acid[1]	0.2	68.75 ± 1.9
	0.4	69.48 ± 2.3
	0.6	71.91 ± 1.8
	0.8	72.31 ± 0.9
	1.0	74.5 ± 2.1
	1.2	74.39 ± 1.3
	1.4	74.02 ± 1.7
	1.6	74.01 ± 1.2
	1.8	74.05 ± 2.4
	2.0	74.1 ± 1.2
Diethanolamine[2]	2	67.83 ± 2.2

	4	69.74 ± 1.6
	6	74.38 ± 0.8
	8	76.92 ± 1.3
	10	78.76 ± 1.7
	12	78.72 ± 2.3
	14	78.02 ± 1.5
	16	77.39 ± 0.7
	18	77.23 ± 1.5
	20	77.12 ± 1.9

Table 1: Effect of substrate concentration on synthesis of oleyl-diethanolamide.

[1]The concentration of OA was varied from 0.2 to 2.0 mM, while the concentration of DEA was 6.0 mM. [2]The concentration of DEA was varied from 2.0 to 20 mM, while the concentration of OA was 1.0 mM. Values are mean ± SD of three observations.

Effect of reaction time

Maximum OA conversion of 84.87 ± 1.5% was observed by incubating the reaction mixture for 18 hrs under the previously optimized conditions. However no further increase in the OA conversion was seen with increase in incubation time (Table 2). The decrease in fatty acid conversion rates along the time could be explained by the formation of by-products. In a similar study, 61.35% OA conversion has been achieved by incubating the reaction mixture at the optimum conditions for 24 hrs [1]. However in another study, approximately 100% OA was converted in 2-3 hrs to yield maximum content of oleoylethanolamide [10].

Reaction time (hrs)	Oleic acid conversion (%)
6	73.34 ± 1.7
12	78.97 ± 2.3
18	84.87 ± 1.5
24	81.63 ± 2.1
30	78.23 ± 1.9
36	76.78 ± 0.8

Table 2: Effect of reaction time on the conversion of oleic acid to product.

Effect of reaction temperature

Maximum % conversion of OA (84.87 ± 1.6) to the product was achieved at 55ºC. Enhanced accessibility of substrate to the catalytic site can be achieved at a particular temperature. However further increase in reaction temperature decreased the conversion of OA (Figure 2). This could be attributed to the reason that high temperatures change the conformation of enzymes that alter the free energy of the system, potentially affecting substrate binding capacity and reducing the yield of the reaction. In a previous study, 70°C temperature has been found to be optimum to yield maximum oleoyl-diethanolamide in a solvent-free system [1]. In another study, 100% OA was converted to the product (96.8% yield) at 60°C by using commercial lipase Novozym 435 from *Candida antarctica* [10].

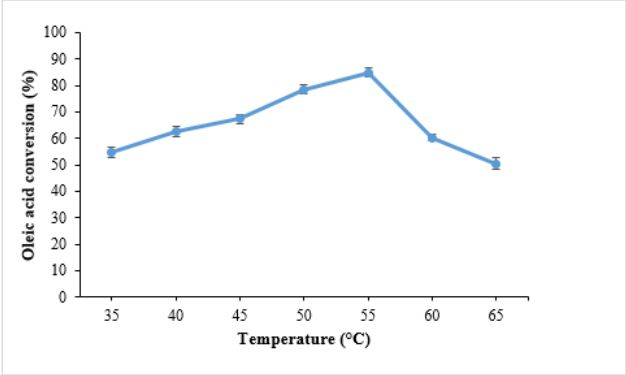

Figure 2: Effect of temperature on the conversion of oleic acid to oleoyl-diethanolamide by using purified lipase.

Effect of amount of enzyme

30 µg (0.478 Units) of purified lipase was found to convert 92.64 ± 0.62% OA to the product. Further increase in the enzyme amount did not enhance the conversion of substrate to the product. The presence of optimum amount of enzyme provides more active sites for the acyl-enzyme complex formation and also increases the probability of enzyme substrate collision and subsequent reactions. However further increase in enzyme concentration did not affect the conversion much (Figure 3). In a previous study, maximum OA conversion of 78.01% was achieved by using 5-9% (wt/wt OA) commercial lipase from *Candida antarctica* [11].

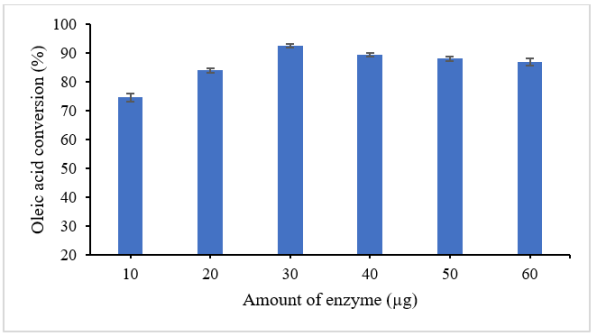

Figure 3: Effect of amount of enzyme on conversion of oleic acid to oleoyl-diethanolamide.

Conclusion

This is the first investigation which reports the synthesis of oleoyl-diethanolamide by using an enzyme purified from thermotolerant *Bacillus subtilis.* An economical, effective and scalable reaction system was developed during the present study. Efficient synthesis of oleoyl-diethanolamide was achieved by using enzymatic biocatalyst rather than chemical methods which utilize high temperature and pressure conditions. Maximum conversion of OA (92.64 ± 0.62%) was achieved when reaction mixture containing 1 mM OA, 10 mM DEA, *n*-hexane (to make final volume 5 ml) was incubated with 30 µg purified lipase (0.478 Units) at 55ºC for 18 hrs. Oleoyl-diethanolamide can further be used as a surfactant in various industries. Also, its antimicrobial and anti-cancerous profiles can be studied in future.

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Conflict of Interest

Author(s) do not have any conflict(s) of interest(s).

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