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JATROPHA CURCAS SEED OIL AS NEW SUBSTRATE FOR ENZYMATIC METHYLHYDROXYLAMINOLYSIS

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Synthesis of methyl fatty hydroxamic acids (MFHAs) was carried out using lipozyme RM IM based on *Jatropha Curcas* seed oil. Optimized conditions were obtained at a mole ratio of 6/1 of methyl hydroxylamine/oil, temperature at 41˚C, enzyme 30mg/mmol and 72 h of reaction time. At this optimal condition, the yield% of methyl fatty hydroxamic based on *Jatropha Curcas* seed oil was 93.92%. The product was characterized by Fourier transform infrared (FTIR), and proton and carbon nuclear magnetic resonance (^{1}H-NMR and ^{13}C NMR) spectra.

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Keywords: Methyl Fatty hydroxamic acid; lipase; Jatropha Curcas seed oil; enzymatic reaction

1. Introduction

The biosynthesis of hydroxamic acid compounds has been extensively considered, due to their wide range of various applications and biological properties. Hydroxamic acids (R-CO-N-OH) and their derivatives, which are weak organic acids with low toxicities, have a very large range of applications, such as tumour inhibitors [1] ; enzyme inhibitors, such as ureases [2] ; collectors for scarce earth mineral metal ions extraction from aqueous media; food additives; growth factors; antibiotics; antifungal agents and cell division factors [3]. Their pharmacological, toxicological, and pathological properties have been studied, for instance, they have been found to be a good inhibitor of matrix metalloprotease, which concerns tissue remodeling [4,5]. In fact these enzymes are ubiquitous in human diseases, such as arthritis, for example, osteoarthritis and rheumatoid arthritis [6]. Besides their biological activity their uses in analytical chemistry as reagents for gravimetric and spectrometric metal determination have also been considered [7]. Furthermore several methods for waste water treatment by using hydroxamic acids homopolymers or copolymers have been reported. Their property in heavy metal conveyors in liquid-liquid extraction in the nuclear industry have been studied [8]. In addition, long chain hydroxamic acids have been studied as efficient surfactants in the detergent industry [9]. Recently, the anti cancer activity in organic compounds containing the hydroxamic acid functional group have been reported [10,11].

In view of the above, synthesis of methyl fatty hydroxamic acid based on *jatropha curcas* oil has been carried out. It is pointed out that *jatropha curcas* is an attractive and multipurpose plant, with plentiful potential. The seed kernel oil contains 40-60% (w/w) oil [12]. The dominant fatty acids of the oil are oleic and linoleic acid by 43.709% and 34.934%, respectively, followed by palmitic acid (13.418%), stearic acid (7.345%), and palmitoleic acid (0.595%). The seed oil has

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been discovered to be useful and valuable in medicinal and veterinary purposes, as well as an insecticide [13]. The oil was traditionally utilized as a medicine to heal diseases like dysentery, hemorrhoids, gonorrhea, coated tongue, infertility, smallpox, and skin infections. The noticeable properties and reasonable potential of hydroxamic acids along with jatropha seed oil, especially its medicinal effects, led to this investigation. The present work involves the synthesis based on *jatropha curcas* seed oil, characterization, and optimization condition of synthesis of methyl fatty hydroxamic acid. This synthesis of MFHAS based on jatropha oil catalyzed by lipozyme RM IM is presented for the first time. The process variables for optimization were the ratio of methyl hydroxylamine/oil (mol/mol), reaction temperature (C°), reaction time and nature of the solvent. The oil is easily available, often at low cost, and the reaction was catalyzed by an immobilized lipase catalyst, thus, it contains overall advantages of enzymatic reactions, such as high selectivity, mild reaction conditions and is environmental friendly.

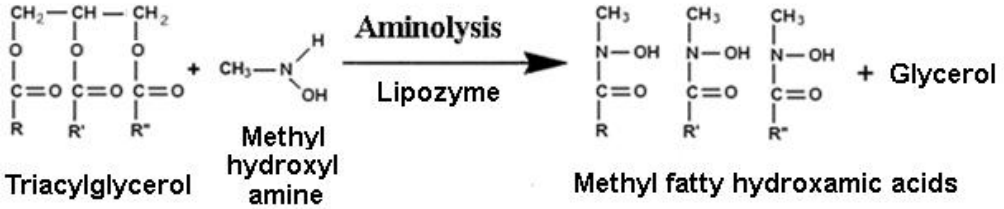
2. Materials and methods

2.1 Material and apparatus

Malaysian *Jatropha curcas* seeds were obtained from the UKM experimental plot and it's oil was gained by the soxhlet extraction method, methyl hydroxylamine hydrochloride, was purchased from Merck and the immobilized lipase used was the product of Novo Nordisk (Denmark), Methanol and hexane were from T.J. Beaker (USA). Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer GX FTIR Spectrophotometer (USA). Characterization test was carried out by using carbon and proton nuclear magnetic resonance (^{1}H-NMR and ^{13}C NMR) (Bruker AV-III-600 FT-NMR 600 MHZ spectrometer with a cry probe).

2.2 Experimental procedure

In a typical enzymatic methylhydroxylaminolysis of jatropha oil, the reaction medium consists of 850 mg N-methyl hydroxylamine dissolved in 20 ml distilled water and 2.6 g jatropha oil dissolved in 30 ml hexane in the presence of the enzyme in a 250 ml flask sealed. The mixture was incubated in a water shaker bath at 150 rpm and specific temperature. After the required time the enzyme was removed by filtration and then the filtrate was transferred using a separation funnel. The aqueous layer was removed and 20 ml distilled water was added to the organic phase to remove the glycerol and then 30 ml HCl (2M) was added to the organic phase to remove the unreacted MFHAs. Hexane was then evaporated from the organic layer. Absolute methanol (50ml) was added to extract the product from the unreacted oil, methanol was evaporated to gain the purified product. The yield% was measured, the coloured metal complexes test was done, and the FTIR and ^{1}H, and ^{13}C NMR were analyzed. Scheme 1 shows the possible reaction involved in the bioconversion.



Scheme. 1. Possible reactions for the biosynthesis of methyl hydroxamic acids.

3. Results and discussion

This study demonstrates the optimization of the methyhydroxylaminolysis catalytic process based on *Jatropha Curcas* seed oil. In this synthesis, methylhydroxylaminolysis of *Jatropha Curcas* seed oil was carried out in biphasic organic/aqueous medium using lipozyme as a catalyst. Hence, the various reaction parameters that have been studied include enzyme

concentrations, nature of the solvent molar ratio of methyl hydroxylamine to oil, temperature and reaction time.

3.1 Effect of organic solvent

In order to study the effect of various solvents on the synthesis of methyl fatty hydroxamic acids, several solvents were screened. The enzyme showed some activity in all the solvents studied but a proper solvent in biosynthesis should have the ability to dissolve substrate and the products and portioning them into different phases [14]. In addition, hydrophobic solvents, such as hexane and heptane preserve the catalytic activity without disturbing the micro aqueous layer of the enzyme contrary to hydrophilic solvents that strip the essential water from the enzyme and thus distort the catalytic conformation leading to enzyme inactivation. As seen in Table 1, heptane and hexane are the most suitable solvents that were used. However, for subsequent experiments hexane was used because it was cheaper and more available than heptane. In addition, the difference between the obtained amount of yield by hexane and heptane was not significant.

Table 1. The effect of solvent on the enzymatic synthesis of MFHAS: Reaction time = 48 hours, Temperature = 36°C, H_2O = 20ml, pH = 7, Jatropha Curcas seed oil = 2.62 g, Shaking rate = 150 rpm, MHA.HCl = 12 mmol, Lipozyme = 50 mg, Organic solvent = 30 ml, NaOH (6 M) ≈ 2 ml

Solvent	Yield (%)
Heptane	77. 1
Hexane	74.0
Petroleum ether	68.5
Xylene	52.2
Chloroform	25.6

3.2 Effect of reactants molar ratio

In view of the fact that the mole ratio of reactants is one of the most important parameters for obtaining a higher yield, the influences of their amounts on yield% were studied. This was determined using varying amounts of methyl hydroxylamine (1-7mmol) in return for 1mmol of oil at a fixed enzyme concentration. As shown in Figure 1., increasing the amount of methyl hydroxylamine led to an increase of the product, hence, the best mole ratio for maximum yield is 6mmol MHA/1mmol Jatropha Curcas seed oil.

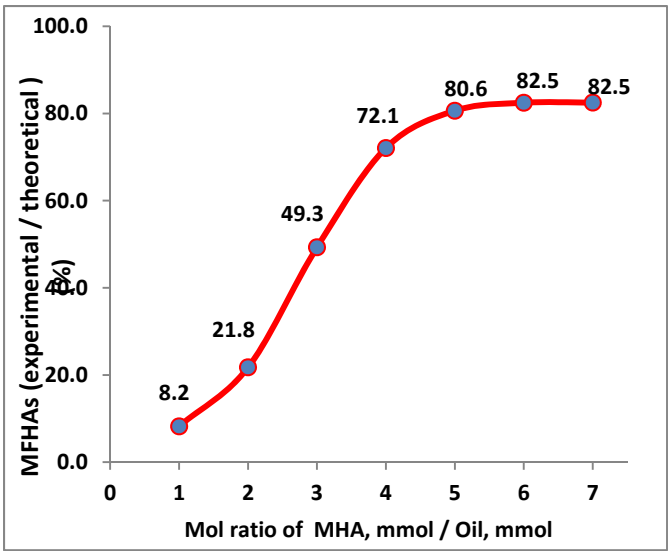


Fig. 1. Effect of mol ratio on synthsis of MFHAs : Jatropha Curcas seed oil = 2.62 g, Temperature = 37°C, Reaction time = 48 h, Shaking rate = 150 rpm, Lipozyme = 50 mg, Hexane = 30ml, H_2O = 20ml, NaOH (6 M) ≈ Variable, pH = 7

3.3 Effect of Amount of enzyme

The amount of enzyme used is crucial from the viewpoints of both conversion and economics. Therefore, the reactions were carried out using different amounts of enzyme levels, as shown in the Figure 2. The best yield of methylhydroxylaminolysis reaction was obtained when the amount of enzyme was 30mg per mmol of oil. Figure 2. shows that increasing the amount of enzyme led to an increase in the reaction yield, however, additional lipase does not affect the yield percentage. The reason for this behaviour has been reported before [15]. The extra enzyme remained in bulk and the active sites could not be exposed to the substrates. It is important to note that different enzymes can be utilized as acyltransferses for the synthesis of various hydroxamic acids. This depends on the carbon chain length of the substrate as an acyl donor [16].

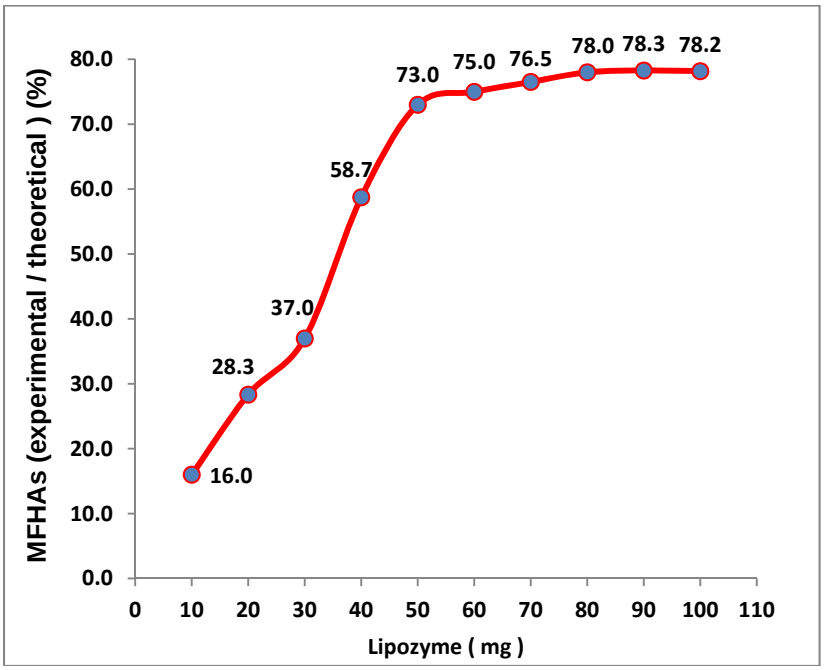


Fig. 2. Enzyme load effect on synthsis of MFHAs : MHA.HCl = 12 mmol, Reaction time = 48, Temperature = 37°C, Jatropha Curcas seed oil = 2.62 g, Shaking rate =150 rpm, Hexane= 30ml, H_2O = 20ml, NaOH (6 M) ≈ 2ml, pH = 7

3.4 Influence of temperature

To study the influence of temperature on methylhydroxylaminolysis different temperatures between 31-45˚C were studied. In biocatalyzed reactions, enzymatic activity may also be lost due to temperature effects. Lower temperatures result in poor conversion levels because of the relatively low enzyme activity. As expected, conversion was found to increase with increasing temperature. According to Figure 3. the bioconversion could be improved by raising the temperature, however, a greater increase resulted in lower product content in the reaction mixture, which is related to the inactivation of the enzyme due to its denaturation by high temperature. At higher temperatures the enzyme obtains a more open configuration, which may increase the accessibility to sensitive amino acids in the inner regions of the enzyme, and thus decrease its catalytic activity [17]. Hence, the best temperature was found to be 41˚C

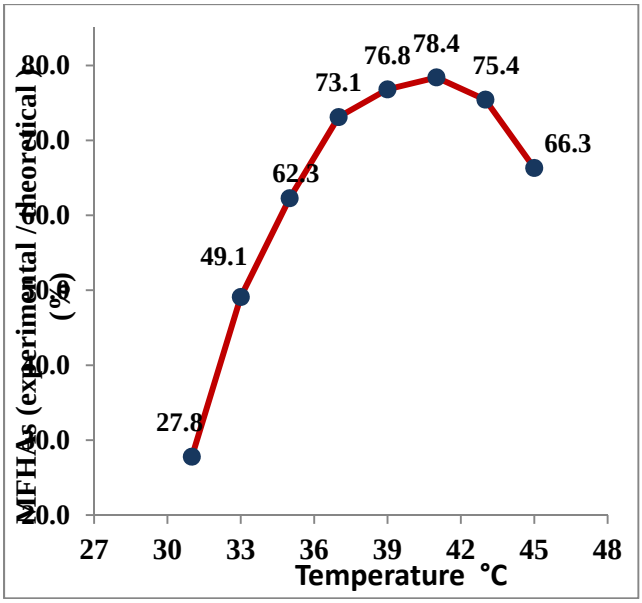


Fig. 3. Effect of temperature on synthsis of MFHAs : Reaction time = 48 hours, Shaking rate = 150 rpm, MHA.HCl = 12 mmol, Lipozyme = 50 mg, Hexane= 30ml, H_2O = 20ml, NaOH (6 M) ≈ 2ml, pH = 7, Jatropha Curcas seed oil = 2.62 g.

3.5 Effect of reaction time

As can be seen in Figure 4., the reaction proceeded rapidly for about 24h. The rate of methylhydroxylaminolysis decreased after 26h. This may be due to some mass-transfer limitations and reaching the reaction to equilibrium condition. The best yield was achieved after 72h.

A series of bioconversion was carried out six times in the optimal condition and the achieved yields of methylhydroxylaminolysis are shown in Table 2. The average of the yield % was 93.92 where the optimal conditions were: reaction time, 72 h; organic solvent, hexane; temperature, 41°C; mol ratio of metylhydroxylamin/oil, 6/1; amount of lipozyme, 30mg lipozyme per one mmol oil, also other conditions were Shaking rate, 150 rpm; hexane, 30 ml; H_2O, 20ml; NaOH (6 M) ≈ 2ml, pH = 7.

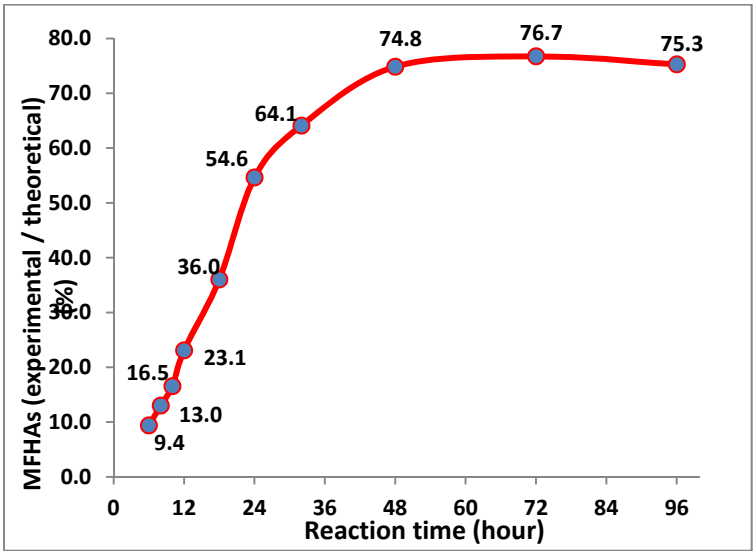


Fig. 4. Effect of reaction time synthsis of MFHAs : Temperature = 38°C, Shaking rate = 150 rpm, MHA.HCl = 12 mmol, Lipozyme = 50 mg, Hexane = 30 ml, H_2O = 20ml, NaOH (6 M) ≈ 2ml, pH = 7, Jatropha Curcas seed oil = 2.62 g.

Table 2. Product yield in optimum condition: reaction time, 72 h; temperature, 41°C; metylhydroxylamin, 18 mmol; lipozyme, 90 mmol; Jatropha Curcas seed oil, 2.62 g (3 mmol); shaking rate, 150 rpm; hexane, 30 ml; H_2O, 20ml; NaOH (6 M) ≈ 2ml, pH = 7 and MFHAs (theo.) = 2.763 g

Test No.	MFHAs (exp. / theo.) (%)	MFHAs (exp.), g
1	93.0	2.569
2	93.7	2.589
3	93.9	2.594
4	94.5	2.610
5	94.3	2.605
6	94.1	2.599

Note: exp = experimental, theo.= theoretical, g = gram

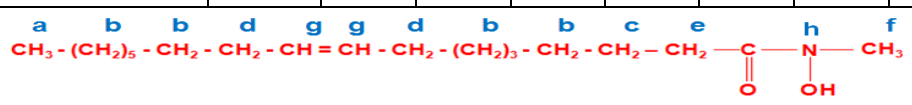
3.6 Characterization of product

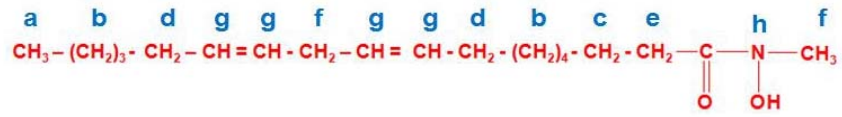
The most important property of hydroxamic acids are their ability to form coloured and very stable chelates with a number of metals. This property has been used for qualitative identification of the product. For this purpose a solution of MFHAs in hexane was mixed with 0.01 M of copper (II), iron (III) and vanadium (V) aqueous solutions and agitated for about 5 minutes. The colour complexes of the product were green, brownish red and purple, respectively [18]. Figure 5. shows the FTIR spectrum of substrates and product. The main peaks and the functional groups of the product show characteristic strong absorption bands at 2855, 2921 cm^{-1} for the C-H stretching long alkyl chain that were confirmed by peak 1456 cm^{-1}, which corresponds to C-H bending. The peaks at 3200 cm^{-1} and 3007cm^{-1} correspond to the OH group and C-H alkenes (C=C-H), respectively, and 1540 cm^{-1} corresponds to C=C. Additional characteristic absorption bands for the product appear at 1709 cm^{-1} for the C=O functional group and the disappearance of the absorption bands at 1740 cm^{-1} is related to the C=O stretching group of substrate (*Jatropha Curcas* seed oil). In addition, the disappearance of the absorption band at 3401 cm^{-1} corresponds to the N-H group of substrate (methyl hydroxylamine).

Signals of the ^1H NMR and ^{13}C NMR spectra confirmed the biosynthesizing of the product. The sample was dissolved in deuterochloroform and analysis was performed using a frequency of 600 MHZ. The important point is the ^1H NMR spectrum appearance signal at 9.5-9.6 ppm, which is assigned to the –OH proton, which is attributed to the trans geometric form [19] ^{13}C NMR: δ 179.98 (C=O), δ 130.11, 129.94, 129.89, 129.62, 128.05, 127.89 (CH=CH), δ 34.26 (C-C=O), δ 31.92, 29.60, 29.68, 29.45, 29.15, 29.10, 27.20, 27.18, 24.82, 22.66, (CH-CH), δ 14.04 (terminal CH3). The signals of ^1H NMR are summarized in Table 3.

Table 3. The main Signals present in ^1H-NMR spectra of methyl oleyl hydroxamic acid (A) and methyl linoleyl hydroxamic acid (B)

Proton family	a	b	c	d	e	f	g	h
(A)	0.87	1.27	1.59	1.98	2.33	2.78	5.33	9.5
(B)	0.87	1.32	1.62	2.04	2.33	2.76	5.35	9.5





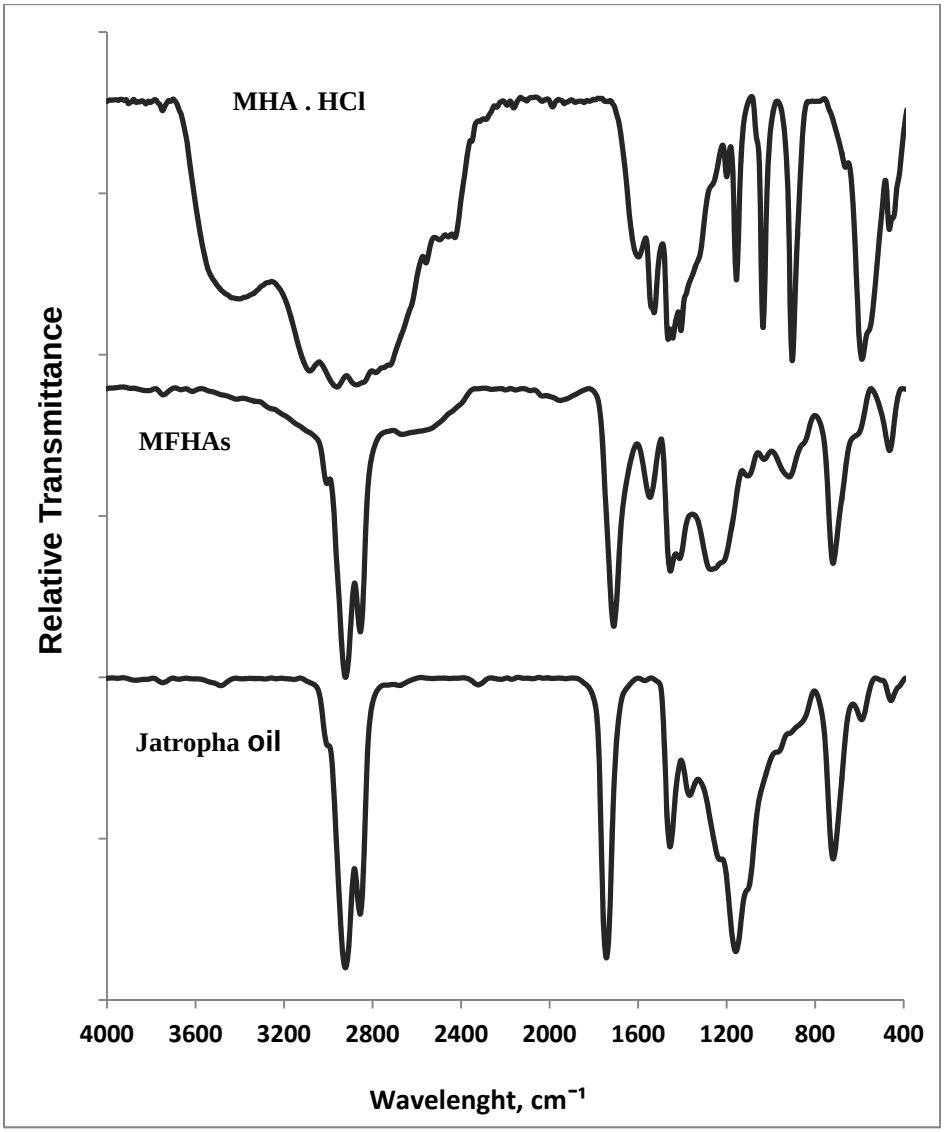


Fig. 5. FTIR Spectrum of MHA.HCL, Jatropha Curcas seed oil as substrate and the product MFHAs.

4. Conclusion

The study describes the optimization of the methylhydroxylaminolysis based on *Jatropha Curcas* seed oil. The reaction was facilitated in biphasic media, by using lipase as acyltransferase. Large amounts of product can be achieved with a few tens of milligrams of enzyme. The reaction described in this paper is an effective method for preparing methyl fatty hydroxamic acids from *Jatropha* seed oil and methyl hydroxylamine. Also it could be applicable for production on a large scale. The procedure employed offers operation simplicity and easy removing of the enzyme from products. Moreover, it allows being worked up under mild reaction conditions, which improves the yield and reduces unwanted compounds.

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