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RESEARCH PAPER

Immobilization of *Candida rugosa* Lipase on Magnetic Biosilica Particles: Hydrolysis and Transesterification Studies

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Abstract Biodiesel is a renewable fuel used mainly in diesel engines. At the present time, biodiesel is largely produced by acid or alkali transesterification reactions. A hot spring water algae isolate "*Kamptonema formosum*" was cultivated at three different temperatures, and the algae oil was extracted using chloroform and methanol (v/v, 1/1 ratio) as the solvent. The maximum amount of algal biomass (1.86 g/L) was obtained at 25°C, and the extracted oil was found to be 48.7% of the total dry biomass. Diatomic earth particles (Biosilica) were magnetized via thermal co-precipitation reaction, and then it was grafted with polydopamine (MBioSi@PDA). The lipase was covalently immobilized on the surface of the MBioSi@PDA via Schiff's base reaction. The immobilization conditions were optimized and 3.0 mg/mL as the initial lipase concentration in the immobilization medium was found to be the most favorable. At this lipase concentration, the amount of the immobilized lipase on the MBioSi@PDA particles and immobilization yield were found to be 81.9 mg/g and 67.9%, respectively. The MBioSi@PDA@lipase particles were used for conversion of *K. formosum* oil into biodiesel, and the conversion yield was found as 91.2% under optimum conditions. The fatty acid methyl ester (FAME) compositions of the alga oil were identified using a gas chromatography-mass spectrometry (GC-MS). *K. formosum* oil mainly composed of the required fatty acids (*i.e.*, 16 and 18 carbon long-saturated and unsaturated fatty acids) for biodiesel synthesis, and these were advantageous for synthesis of biodiesel from the algal oil.

Keywords: diatomic earth, *Kamptonema formosum*, algal oil, immobilized lipase, transesterification, biodiesel

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1. Introduction

Algae are aquatic photosynthetic plants including a large group of various organisms that are extensively spread in the sea, freshwater, swamp, *etc*. Algae can be used as an industrial and chemical raw material, and also a food and energy source. Compared to terrestrial plants, algae have a rapid growth rate, high efficiency CO_2 retention and, can grow in harsh environmental conditions [1-6]. The exponential increase in global atmospheric temperature is due to human activities such as burning fossil fuels and deforestation [7,8]. Biodiesel can be obtained from renewable non-toxic and biodegradable sources, and many algae species can provide these requirements [9,10]. Thus, different algal biomasses can be used as an alternative source for biodiesel production instead of fossil fuels [11,12]. Currently, large amounts of biodiesel are produced using oilseed plants such as soybean, sunflower, and palm [13,14]. In recent studies, biodiesel and synthetic gas production from different algal biomasses have been reported [10]. Selection of a proper alga strain for biodiesel manufacture is important

due to its lipid content and composition. Additionally, the biomass production potential of a particular strain should be considered together under certain growth conditions [15,16]. The biomass and oil productivity properties of algae strain should be studied under certain growth conditions in batch culture before large scale production [17,18]. From the cultivated algal biomass, biodiesel can be produced from algal oil by various transesterification reaction and, in these reactions, the oil is reacted with methanol or ethanol to produce fatty acid alkyl esters and as byproduct glycerol is obtained in the presence of catalysts [19-25]. These transesterification reactions can be realized in the presence of acid, base catalysts and enzymes [5,14].

Immobilized enzymes have a number of advantages compared to native enzymes, such as improved operational stability, increased enantioselectivity, reusability of the enzymes, and easy adaptation to various reactor configurations, reduce the process cost and easy product separation [26-28]. In general, multipoint covalent attachment on the solid support could improve the operational stability of the enzymes to various denaturation agents. Additionally, immobilization could prevent aggregation, autolysis and proteolysis by various proteases [29-32].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from different sources have been used in many biotechnological to yield many fine chemicals [22-24]. Moreover, lipases from *Candida rugosa*, *Candida antarctica*, and *Rhizopus oryzae* were frequently utilized for enzymatic synthesis of biodiesel from various oil sources [5,9,23,25]. Lipases are flexible biocatalysts that can catalyze different types of reactions, such as hydrolysis, esterification, and transesterification and inter-esterification under moderate experimental conditions, therefore, lipases were mostly used in food, pharmaceutical, and chemical industries [33-35]. Therefore, lipases have been immobilized on different support for various biotechnological applications [36,37]. Recently, silica-based materials have been used in many studies as solid support for the immobilization of enzymes [23,33]. Amongst, biosilica from diatoms is a low-cost material that has many interesting properties for a wide range of industrial applications [33,38-42]. The biosilicates from diatoms are extensive unicellular photosynthetic algae that produce distinctive highly ordered siliceous cell walls, called a frustule. These biosilicates have from micro to nanoporous structures with a high surface area that can be easily modified, high mechanical resistance, high chemical, and biological stability with good biocompatibility property, make diatom biosilica suitable raw material for use in the biotechnological area. Diatomic biosilica particles have many advantages such as natural source, non-toxic, low cost, and high surface area compared to other biosilicates. They can be readily used in the preparation of biosensors, adsorbents, support materials for immobilization, and drug delivery systems [33,42]. Additionally, it also permits coating of straightforward to imitate of mussel adhesive proteins refereed as polydopamine that can be grafted under gentle alkaline conditions [42,43].

The coated poly(dopamine) layer can be used for Michael addition or Schiff's base reactions platform. Thus, the PDA layer on the biosilica gives permission for the immobilization of the enzyme using its amine groups [44].

The novelty of this study is the preparation of a magnetically recyclable immobilized lipase on diatomic biosilica. It is a stable non-toxic particle of high quality, and has high mechanical and chemical strength. The magnetic biosilica based particles do not agglomerate in an aqueous medium solution due to the presence of paramagnetic nanoparticles within the pores of the biosilica. These properties make it a suitable lipase-carrier for enzymatic reactions and provide the application in the transesterification reaction for biodiesel synthesis. For large scale applications, the magnetic property provides the immobilized lipase to be recovered easily from the reaction medium by using an external magnet, making it economically preferable for biodiesel production. Additionally, the algae "*Kamptonema formosum*" was isolated from hot spring water and cultivated at three different temperatures. The effect of temperature on the biodiesel properties was evaluated for the first time.

To the best of our knowledge, this is the first report, for the biomass production from *K. formosum* at different temperatures, in which oil productivity and FAME profiles of the algae were evaluated. *C. rugosa* lipase was immobilized on the polydopamine coated magnetic biosilica particles (MBioSi@PDA@lipase) and used for enzymatic biodiesel synthesis from the extracted algal oil. Moreover, the presented magnetic immobilization system allows easy removal of the immobilized lipase from the reaction medium and its reuse. The produced biodiesel composition was analyzed using GC-MS.

2. Materials and Methods

2.1. Materials

Lipase from *C. rugosa* (Type VII, > 700 U/mg solid; EC 3.1.1.3), p-nitrophenyl palmitate (p-NPP), sodium cholate, Tris-HCl, dopamine hydrochloride (purity > 98%), chloroform, n-hexane, and methanol were obtained by the Sigma-Aldrich Chemical Co. Diatomic earth was gift from Karaman Mining Company, Kazan-Ankara and utilized as BioSi source after treatment with strong acid.

2.2. Isolation and cultivation of *K. formosum*

Hot spring water samples were collected from SeyHammam (Turkish bath) in Kızılcahamam / Ankara - Turkey (40°36'00.4"N 32°38'39.1"E) in September. The temperature of the hot spring water was approximately 44°C, and pH value was determined as 6.5. The algal strains were isolated using micromanipulation technique [17,18,44-46]. The light microscopy of the isolated *K. formosum* is presented in Fig. S1. The BG11 culture media was used for the growth of *K. formosum* [25,47]. Alga culture was achieved in minimal base medium (BG11 medium) containing the following ingredients per 1.0 L of distilled water: Citric acid (0.06 g), $NaNO_3$ (1.5 g, as a sole source of nitrogen), $CaCl_2$ (0.6 g), KH_2PO_4 (0.4 g), $MgSO_4$ (0.75 g), $FeSO_4$ (0.05 g), and 5.0 mL trace metal stock solution. It was comprised of EDTA·2Na (0.75 g), $ZnSO_4 \cdot 7H_2O$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.01 g), MoO_3 (0.01 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), and $Co(NO_3)_2 \cdot 6H_2O$ (0.005 g), and this medium was amended with 1.0 mL of trace solution. Trace metal ions solution composition is (g/L): H_3BO_3 (2.86 g), $MnCl_2 \cdot 4H_2O$ (1.81 g), $ZnSO_4 \cdot 7H_2O$ (0.222 g), $Na_2MoO_4 \cdot 2H_2O$ (0.39 g), $CuSO_4 \cdot 5H_2O$ (0.079 g), and $CoNO_3 \cdot 6H_2O$ (0.049 g). The initial pH of the medium was adjusted to pH 7.1, maintained at 25 ± 1°C with 16:8 h of light-dark cycle using cool-white fluorescent. The intensity of light during the light period was 6,000 mol cm^{-2} s^{-1}. The isolate was maintained either on MG-11 agar plates or liquid medium with continuous shaking. The culture medium was exposed at a photon flux density of 50 μmol photons m^{-2} s^{-1} with a photo-period of light for 16 h and darkness for 8 h. The subculture of strain was grown in BG-11 medium into Erlenmeyer flasks (50 mL) and subjected to consecutive rounds of serial dilution and streaked across BG-11 agar plates. The isolate was identified from solid culture as *K. formosum* (Bory ex Gomont) as reported previously [12,15,17]. Growth curve and rate, the amount of biomass and total lipid were analyzed at three different temperatures of 25, 35, and 45°C with a photon flux density of 50 μmol photons m^{-2} s^{-1} with a photoperiod of light for 16 h and darkness for 8 h. *K. formosum* cells were inoculated in a 300 mL growth medium in 500 mL flask and grown for 20 days under continuous shaking at a given temperature and at pH 7.1. After this period, the algal biomasses were collected by centrifugation at 4,000 g and at room temperature for 4.0 min. The algal biomass was dried under reduced pressure at 50°C for 24 h in a vacuum oven. All the experiments were studied with three replicates. The specific growth rate of *K. formosum* was calculated using the following equation:

$$\text{The specific growth rate} = \ln (X_1 / X_0) / (t_1 - t_0) \quad (1)$$

where X_1 and X_0 are the densities of algae at initial and at time t (g/L), respectively, and t_1 and t_0 are the culture time (day).

2.3. Extraction of oils from algal biomasses

The dried algae samples were powdered using a mortar. One part of powdered algal biomass (g) was mixed with five parts of chloroform/methanol mixture (1/1 ratio, v/v) to extract oil from algal biomass in an Erlenmeyer as described previously [25]. The mixture was first sonicated for 15 min then stirred magnetically for 24 h in the Erlenmeyer. After this period, the mixture was centrifuged at 6,000 rpm for 15 min. The phase containing the algal lipid was separated using a filter paper. The supernatant was contacted with pure Na_2SO_4. Then, the chloroform/methanol was removed by using an evaporator and the extracted oil was weighted using a balance and stored at 4°C until use. The oil yield (wt. %) was calculated using the following equations:

$$\text{The oil yield (wt. \%)} = [\text{Amount of oil extracted (g)}] / [\text{total mass of dry algae (g)}] \times 100 \quad (2)$$

2.4. Preparation of lipase immobilized magnetic diatomic biosilica

The diatomic biosilica samples (40 g) were washed in purified water (500 mL) while magnetically stirring at 25°C for 5.0 h. They were collected by filtration with a Buchner funnel and dried at 100°C under reduced pressure for 18 h. Then, a strong sulfuric acid solution (H_2SO_4, 30% v/v, 100 mL) was added to the diatomic biosilica samples. The mixture was stirred magnetically at 100 rpm and 100°C for 18 h. After cooling to room temperature, the acid treated BioSi was washed with distilled water until the solution pH was neutral.

The magnetization of the acid-treated BioSi was performed by thermal co-precipitation reaction. Briefly, acid treated BioSi (10 g) was transferred into a round bottomed flask containing $FeCl_3$ (7.5 g) and the solution stirred magnetically at room temperature for 6.0 h. Then, the $FeCl_3$ saturated BioSi particles were collected with filtration. The BioSi particles were transferred into NH_4OH (100 mL, 25% w/v) and combined with $FeCl_2$ solution (5.0%, 100 mL in water). During the thermal co-precipitation, the magnetic nanocrystals of Fe_3O_4 were formed within the pores of the BioSi particles. The surface of magnetic biosilica (MBioSi) was grafted with poly(dopamine) using the method described by Lee *et al*. [44]. To achieve this, magnetic biosilica particles (approximately 10.0 g) were transferred into Tris-HCl buffer solution (20 mmol/L, pH 8.5, 100 mL) and ethanol (50 mL) containing dopamine (0.5 g). The mixture was placed on a rotary incubator and rotated at 50°C for 24 h. The MBioSi@PDA particles were

collected magnetically and washed with ethanol (100 mL) and distilled water, respectively. Then they were dried under reduced pressure at 50°C for 24 h.

In order to examine the effect of the initial concentration of lipase on the amount of enzyme immobilization, enzyme solutions with different concentrations were studied. Briefly, the MBioSi@PDA particles (1.0 g) were transferred into the Tris-HCl buffer (pH 8.5, 20 mmol/L, lipase enzyme solution (50 mL) containing various amounts of lipase (0.5 to 4.0 mg/mL). Then, the immobilization mixture containing a different amount of lipase was located on an orbital shaker and rotated at 100 rpm and at 25°C for 6 h. After this period, the lipase immobilized MBioSi@PDA particles were collected magnetically using an external magnet and washed with phosphate buffer (pH 7.0, 50 mmol/L, 20 mL). Then, the MBioSi@PDA@lipase particles were stored at 4°C until use. The amounts of protein in the samples and washing solutions were determined from the alteration of the initial and final concentration of lipase in the reaction medium using the Bradford method [48].

2.5. Determination of hydrolytic activity of the lipase preparations

The activities of the native and MBioSi@PDA@lipase were determined as reported earlier [43]. The artificial substrate solution was prepared by mixing phosphate buffer (50 mL, pH 6.0, 50 mM) with ethanol containing (50 mL, 15 mM p-NPP). The hydrolysis reaction was initiated by adding the native enzyme solution (0.1 mL) or immobilized enzyme (20 mg) into 5.0 mL substrate solution. The artificial substrate p-NPP hydrolysis was carried out at 25°C and 100 rpm for 10.0 min in a rotated incubator. After 10.0 min, the reaction was finalized by adding sodium carbonate (0.25 mol/L, 2.0 mL). The MBioSi@PDA@lipase was magnetically removed, and 0.5 mL of the reaction medium was diluted by addition of 4.5 mL purified water. The optical density was recorded at 410 nm using a UV-vis spectrophotometer (PG Instrument Ltd., Model T80; PRC). One lipase unit was defined as the amount of lipase produced 1.0 μmol of p-NPP per min at 25°C. The effect of temperature on the native and immobilized lipase activities was studied between 25 and 65°C, and at in the pH range of 4.0 and 9.0.

Immobilization efficacy was designed using the following equation:

$$\text{Immobilization efficacy (\%)} = [(I_0 - I_1) / I_0] \times 100 \quad (3)$$

I_0 and I_1 are the total amounts of protein in the immobilization medium before and after immobilization. The retained enzyme activity was calculated by means of the following equation:

$$\text{Retained enzyme activity (\%)} = (\text{hydrolysis activity of immobilized lipase}) / (\text{hydrolysis activity of the native lipase}) \times 100 \quad (4)$$

2.6. Determination kinetic parameters of the native and immobilized lipase

The kinetic constants of the native and immobilized lipase preparations were studied using p-nitrophenyl palmitate (p-NPP) as an artificial substrate in the concentration range 0.1-5 mmol/L as described previously [44]. The K_m and V_{max} values for the lipase preparation were calculated from Lineweaver-Burk plots by using the initial rate of the enzymatic reaction.

2.7. Stability of lipase preparations

The thermal stability of the native and immobilized lipase was studied by incubation in phosphate buffer solution (pH 7.0, 50 mmol/L) at three different temperatures (45, 55, and 65°C) for 2.0 h in the absence of substrate. Every 15 min, a sample was taken and the activity of the enzyme was measured as described above. The storage stability of the lipase preparations was studied after storage in phosphate buffer (pH 7.0, 50 mmol/L) at 4°C during six weeks period. The activity of enzyme preparations was determined as described above.

2.8. Enzymatic biodiesel synthesis

Lipase catalyzed biodiesel synthesis was realized in a reactor. In a typical reaction medium, a mixture of algal oil (1.2 g), n-hexane (2.0 mL), methanol (0.3 mL), and the immobilized lipase (40 mg, approximately 130 U) was added to a flask. The reaction was realized at 35°C for 24 h. After this period, the MBioSi@PDA@lipase particles were collected magnetically and cleaned twice with n-hexane, and the immobilized enzyme was added into a fresh reaction medium for the subsequent biodiesel synthesis reaction. The operational stability of the MBioSi@PDA@lipase particles for biodiesel synthesis was also studied by using the same lipase immobilized particles. For every run, the immobilized lipase was operated in a fresh medium and the same MBioSi@PDA@lipase particles were operated in batch mode for 5 consequence cycles. After each run, the residual methanol and n-hexane were separated from the product at 60°C under reduced pressure using a rotary evaporator. The lipase immobilized particles separated using an external magnet and cleaned as described above.

The conversion percentage of *K. formosum* oil to biodiesel was calculated according to the following equation:

$$\text{Biodiesel conversion (\%)} = (\text{Biodiesel weight (g)} / \text{Used algal Oil (g)}) \times 100 \quad (5)$$

2.9. Gas chromatography-Mass Spectrometry analysis of FAME's

The compositional analysis of the enzymatically produced biodiesel from *K. formosum* oil was analyzed by gas chromatography-mass spectrometry (Agilent Technologies, 6890N Network GC system - 5975C VL MSD with Triple Axis Detector) as reported earlier [25]. Species separation was studied with helium as the carrier gas in a 25 m × 0.25 μm × 1.0 μm J&W CP-FFAP CB GC column. Each run the column temperature was started at 150°C for 3 min, then raised to 250°C at the rate of 10°C/min. GC conditions: column oven temperature is 150°C, injector temperature is 250°C, the column flow rate is 3.0 mL/min, and injection volume is 1 μL.

2.10. Statistical analysis

All independent experiments were repeated at least three times under the same conditions. The statistical test was performed using one-way analysis of variance (ANOVA) on OriginPro 9 software. The effects of lipase concentration, lipid hydrolysis, thermal stability, transesterification, storage stability and reusability were evaluated. The level of statistical significance was set at $p < 0.05$, and Tukey's method was used for the tests. All the results obtained in the present work were not significantly different from the test mean.

3. Results and Discussion

3.1. Properties of magnetic diatomic BioSi particles

The BioSi, MBioSi, and MBioSi@PDA particles were characterized by BET, FTIR, VSM, XRD, and SEM studies. BioSi particles were sieved and the particles sizes in the range of 25-50 μm were used for the preparation of magnetic BioSi particles. The graft polymerization of polydopamine (PDA) on the MBioSi particles was performed in Tris-HCl buffer containing dopamine at 60°C for 24 h. The grafting of PDA layers on MBioSi surface could be due to the mixed mode interactions of poly-dopamine with MBioSi surface such as hydrophobic, hydrogen bonding, and electrostatic interaction [42]. The PDA grafting gives permission for covalent attachment of many biological molecules having amine and thiol groups via Schiff's base or Michael addition reactions. The surface area and pore size of the BioSi, MBioSi, and MBioSi@PDA particles were determined by the BET method and presented in Table S1. The bare BioSi particles showed greater surface area and pore volume than those of the MBioSi and MBSi@PDA particles. The pore sizes of the BioSi based preparations were different in the range 2.74 and 1.95 nm and, thus, all the BioSi based preparations have mesoporous properties. The surface area and average pore size of the MBioSi and MBioSi@PDA grafted particles were decreased compared to the bare BioSi. This can be due to the filling of pores with Fe_3O_4 nano crystal particles and PDA polymer, thus, they can lead to the overcrowding of the vicinity of the pores of the MBioSi and MBioSi@PDA particles.

The ATR-FT-IR spectra of the BioSi based particles were obtained using ATR mode in a Spectrum 100 FTIR spectrometer (Perkin Elmer Inc., Norwalk, CT, USA). The expanded peak centered at between 3,300-3,500 cm^{-1} can be due to the hydroxyl groups of the BioSi particles (Fig. S2A). For SiO_2, the symmetric stretching of Si-O, and asymmetric stretching vibration of Si-O-Si peaks are detected at 799, and 1056, respectively (Fig. S2A). As observed in Fig. S2B, the peak at 584 cm^{-1} is due to the vibration of the Fe-O bond of Fe_3O_4 [30]. Absorption peaks for PDA can be assigned at around 3,500 cm^{-1} due to the phenolic -OH and -NH stretching vibration. Thus, the occurrence of the -NH vibration of amide groups can confirm the grafting of MBioSi with PDA (Fig. S2C).

The magnetic properties of the bare magnetic nanoparticles (Fe_3O_4, MP), MBioSi and MBioSi@PDA particles were analyzed by vibrating sample magnetometer (VSM; Model 155, Digital Measurement System Inc., Westwood, MA, USA). The magnetization curves were obtained by plotting the magnetization of the samples with the applied magnetic field (Fig. 1A). The saturation magnetization values of the Fe_3O_4, MBioSi, and MBioSi@PDA particles were found to be approximately 67.1, 42.8, and 37.6 emu/g^{-1}, respectively, at 25°C. The decrease of the saturation magnetization values of MBioSi particles, and MBioSi@PDA particles compared to bare Fe_3O_4 particles can be due to the existence of the biosilica mass and grafted PDA layers on the surface of the MBioSi particles. All of these preparations exhibited good magnetic response and were readily collected with an external magnet.

The crystalline structures of the MBioSi and MBioSi@PDA particles were studied using X-ray Powder Diffraction (XRD; Bruker D8, Advance XRD). The XRD patterns of the MBioSi and MBSi@PDA particles presented the general diffraction peaks of the pure Fe_3O_4 nano-particles. These results exhibited that the Fe_3O_4 nano-particles were formed in the BioSi and also preserved in the MBioSi@PDA during grafting reaction. The reflection peaks agree well with the X-ray diffraction data cards (ICDD number 88-0315) with eight diffraction peaks [*i.e.*, (220), (311), (400), (422), (511), (440), (620), and (533)]. As can be seen in Fig. 1B, MBioSi and BSi@MP@PDA particles participated with the same diffraction peaks harmonized with the same spinel assemblies, representing safety of the magnetic property. Thus, the magnetic properties of the samples were not deformed and changed

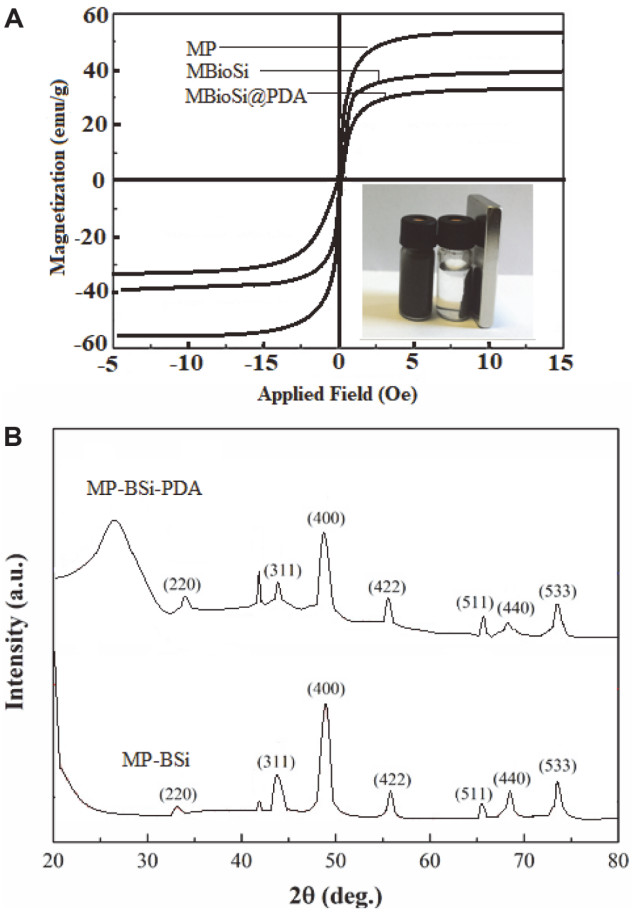

Fig. 1. (A) VSM analysis of the MP, MBioSi, and MBioSi@PDA particles at room temperature. Vibrating sample magnetometer VSM data for the Fe_3O_4 magnetic powders, and the PDA grafted magnetic biosilica particles. (B) The XRD patterns of the MBioSi and MBioSi@PDA. The XRD patterns were recorded at room temperature by an X-ray Powder Diffraction (XRD; Bruker D8, Advance XRD) using CuKα radiation (λ = 1.54Å). The scans were recorded in the 2θ range between 10 and 90° using a step size of 0.04°. The XRD patterns for the magnetic particles (Fe_3O_4) (A), and MBSi@PDA particles (B).

during the PDA grafting reaction.

SEM images of the BioSi and MBioSi@PDA-lipase were obtained using JEOL JSM 5600, scanning electron microscope, Germany. The SEM micrographs of the BioSi and MBioSi@PDA-lipase particles displayed differences between their surface morphology and pores (Fig. S3A and Fig. S3B). As observed in Fig. S3A, the particulate diatomic BioSi surface displays irregularly ordered porous surface. Whereas some alterations were observed after grafting with PDA and lipase immobilization of the on the surface of MBioSi@PDA-lipase particles compared to biosilica. The surface of MBioSi@PDA-lipase particles became slightly smooth and the pores were seemed to be filled by the grafted polymer and ligand. The porous surface and small sized particles could provide a large surface area for enzymatic hydrolysis and transesterification reaction.

3.2. The effect of enzyme loading on the retained activity of the immobilized lipase

The optimum amount of loading of lipase on the MBioSi@PDA particles was evaluated by studying immobilization experiments at different initial concentrations of lipase solutions. The chemistry of the immobilization method schematically is presented in Fig. 2. As can be seen from this Figure, the reactive functional groups of the grafted PDA layers can react with more than one amine or thiol groups of the lipase molecules via Schiff's base reaction. Thus, multipoint covalent immobilization of lipase is realized on the support. The amount of immobilized lipase on the support with varying the initial concentration of lipase in the immobilization medium is presented in Fig. 3 and Table S2. As observed from this Figure and table, the amount of immobilized lipase was steeply increased with an increase in the initial concentration of lipase from 0.5 to 3.0 mg/mL, and this relation made leveled off at around 3.0 mg/mL initial lipase concentration. Further increase in the enzyme amount (to 4.0 mg/mL) did not show a considerable change in the amount of immobilized lipase on the support. This reduction in the immobilization efficiency of the support could be due to the diminishing of the binding sites on the support by the increment of the amount of immobilized enzyme. As shown in this Figure, the highest percent of retained immobilized lipase activity (84.7%) was obtained with the lowest amount of lipase loading on the support (28.6 mg/g). In this case, the initial concentration of lipase in the solution was 0.5 mg/mL (Fig. 3). It should be noted that a high amount of enzyme loading on the support could mostly lead to a low retained activity. This can be brought by oversaturation of the support surface with the immobilized enzyme molecules, as a result of the active site of the immobilized enzyme cannot easily interact with its substrate. This could cause a decrease in the specific activity of the immobilized enzyme [25]. At 3.0 mg/mL initial lipase concentration, the MBioSi@PDA particles attained about 81.9 mg/g lipase loading, and the recovered activity of the immobilized lipase was found to be 67.9% compared to the same quantity of the native enzyme. However, the retained activity of the immobilized lipase was decreased with a further increase in the amount of lipase loading. This could be due to overcrowding of the immobilized lipase on the support, and generated diffusion restriction layers for the large substrate to reach the immobilized enzyme [24]. Therefore, a reduction of the recovered activity of the immobilized lipase was observed. Finally, for 3.0 mg/mL initial lipase concentration, the amounts of loaded enzyme

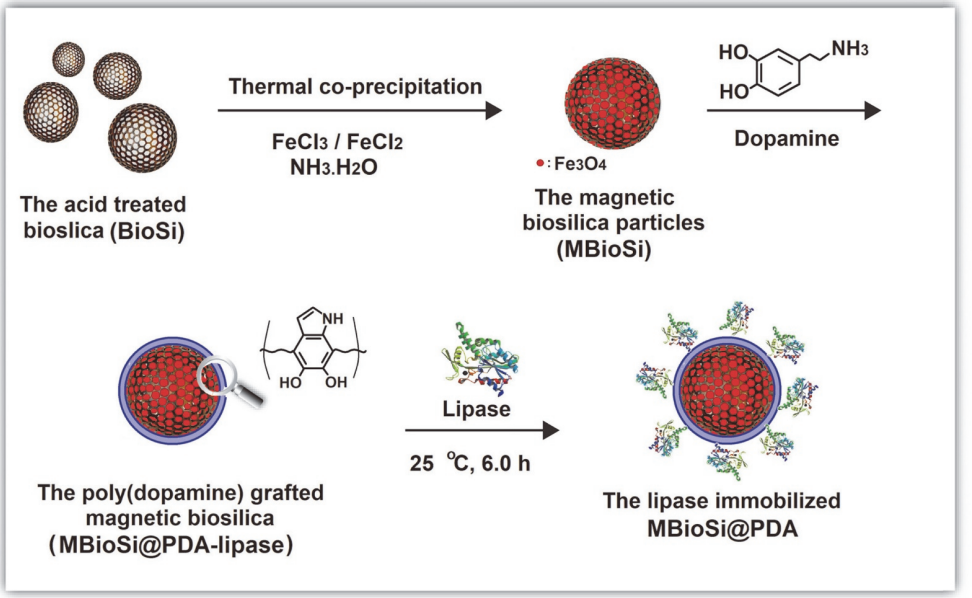

Fig. 2. Chemistry of the preparation of the MBioSi particles and the immobilization of lipase on the MBSi@PDA particles.

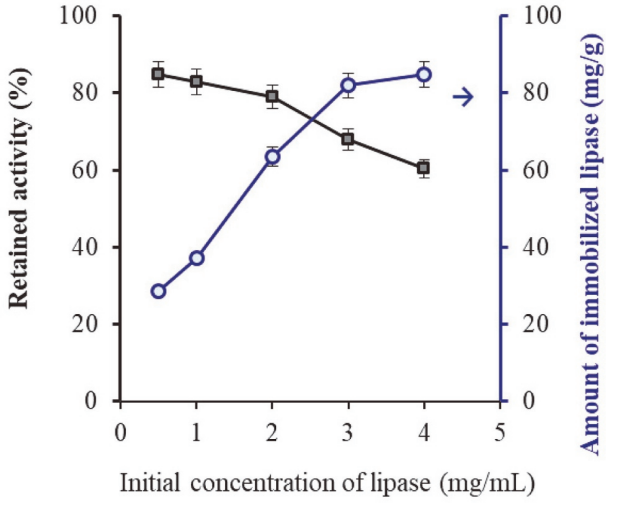

Fig. 3. The effect of initial lipase concentration on the enzyme loading and the retained activity (%). Conditions: Amount of the MBSi@PDA particles, 1.0 g 50 mL^{-1}; Rotation speed, 100 rpm; Initial concentration of lipase, 0.5-4.0 mg/mL; Temperature, 25°C; Contact time, 6.0 h; Medium Tris-HCl buffer, pH 8.5, 20 mmol/L.

were found to be 81.9 mg/g the MBioSi@PDA particles. The expected activity was 57.330 units from the commercial solid lipase preparation, on the other hand, 39.927 units of enzyme activity were obtained and the retained activity was reported as 67.9%. In the reaming experiments, the initial concentration of lipase was used as 3.0 mg/mL.

The effect of contact time on the lipase immobilization efficiency was studied by incubating enzyme solutions up to 6.0 h. The maximum amount of enzyme loading was obtained after 4.0 h reaction time, and a further increase in incubation time did not yield any enhancement in immobilized enzyme amount (data are not shown).

3.3. Effects of pH and temperature on the activities of lipase preparations

The native and immobilized lipase were operated at different pH values. The effect of pH value on the activity of the native and MBioSi@PDA@lipase is show in Fig. 4A. As observed from this Figure, the relative lipase activity for both native and MBioSi@PDA@lipase is powerfully reliant on the medium pH value, due to the effecting of the ionization state of the amino acid residue of the lipase molecule at different pH value. Maximum lipase activity was obtained for the MBioSi@PDA@lipase at pH 6.0, on the other hand, the native lipase displayed its highest activity at pH 5.0. This result showed that both native and MBioSi@PDA-lipase could be maintained three-dimensional structure in the most stable form at a definite pH condition to produce maximum catalytic activity. The pH profile of the MBioSi@PDA@lipase was found to be fairly extended compared to native lipase at the tested most pH values, this result showed that the immobilized lipase able to preserve the structural flexibility at different pH value at active forms. It means that the used immobilization method preserved the lipase activity at both acidic and basic pH regions. The alkaline nature of the polydopamine coated surface of the magnetic biosilica could interact same amount of hydrogen ions between the PDA coated surface

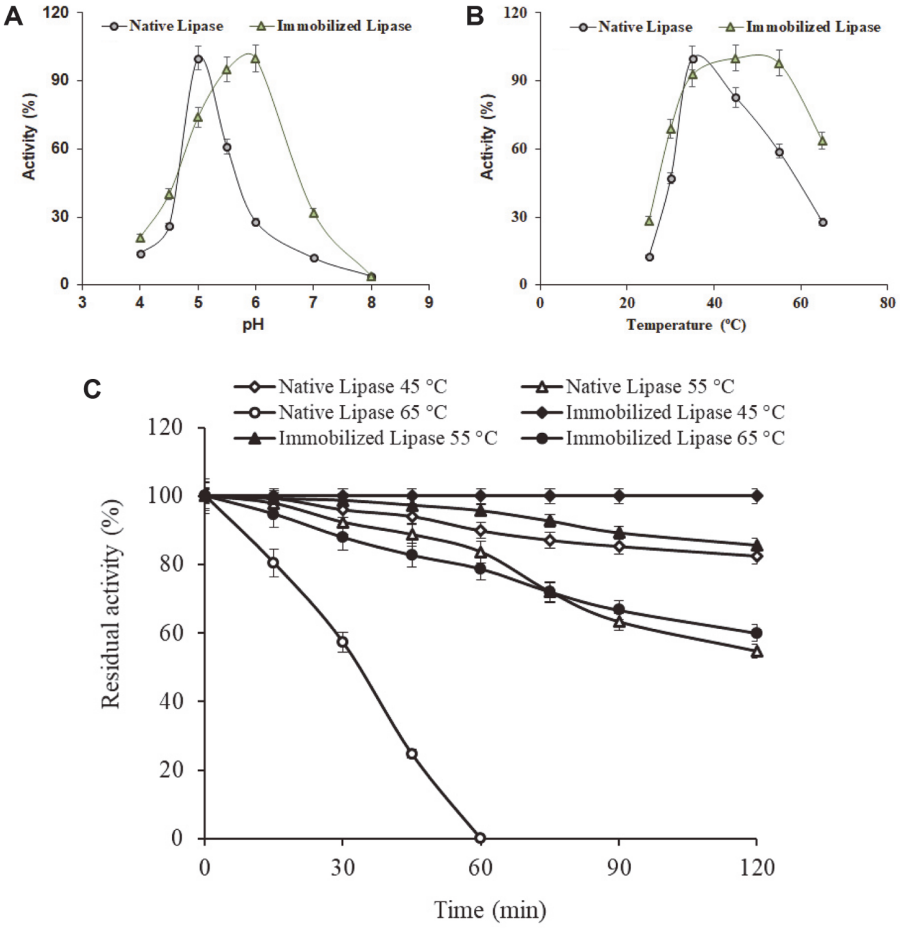

Fig. 4. Activity and stability of the native and immobilized lipase preparations. (A) Effect of pH, Conditions: Amount of the MBSi@PDA-lipase, 20 mg 5.0 mL^{-1}; Temperature, 25°C; Contact time, 10.0 min; Medium pH, 4.0-8.0; (B) Effect of temperature, Conditions: Amount of the MBSi@PDA-lipase, 20 mg 5.0 mL^{-1}; Temperature, 25 -65°C; Contact time, 10.0 min. (C) Thermal stability of the native and immobilized lipase at three different temperatures. Conditions: The free and immobilized lipase preparation incubated at three different temperature (*i.e.*, 45, 55, and 65°C) and pH 7.0 in the absence of substrate.

and the reaction medium. In general, the change in the optimum pH of the immobilized enzyme could be based on the used method, support structure and conformation change of enzyme after immobilized. Additionally, the pH of microenvironment of the immobilized enzyme is not identical with the bulk solution. It could be due to electrostatic interactions of enzyme with the matrix, and partitioning of H^+ and OH^- ions concentrations. These frequently lead to shifts in the pH activity profiles [12,33].

Fig. 4B shows the effect of temperature on the hydrolysis of p-nitrophenyl palmitate (p-NP) by the native and MBioSi@PDA@lipase. The native lipase showed optimum hydrolysis of p-NP at 35°C whereas it was at 40°C for the MBioSi@PDA@lipase. Both lipase preparations had a high variation in the temperature profiles and showed that the solution temperature had a vigorous role in the influence of the hydrolytic activity of lipases. As can be seen from Fig. 4B, the native lipase disclosed a narrow tapered temperature profile with a high enzyme activity. On the other hand, there was a substantial broadened in the temperature profile of the MBioSi@PDA@lipase at various temperatures, which was ascribed to the enhancement of structural stability of lipase molecules by multi point fixation of the enzyme on the support. Similar observations have been reported in the literature [24,33].

3.4. Determination of kinetic parameters of native and immobilized lipases

The apparent K_m and V_{max} values were determined using pNP-palmitate as an artificial substrate and calculated from the Lineweaver-Burk plots. The K_m value was increased from 0.39 to 0.89 mmol/L, whereas V_{max} value was decreased from 712 to 469 U/min upon covalent immobilization of lipase on the MBioSi@PDA particles. The K_m value of the immobilized lipase was 2.28 times higher compared to the native enzyme. These data showed that a higher concentration

of pNP-palmitate was necessary for immobilized lipase to achieve maximum reaction rate with respect to the native counterpart. Whereas the V_{max} value of the immobilized enzyme reduced approximately 1.5-fold compared to the native lipase. The reduction in V_{max} value of the immobilized lipase may be resulted from the multi-point covalent attachment of the enzyme molecules on the MBioSi@PDA particles. The multi-point fixation can be changed the binding properties to its large substrate. It should be noted that immobilization of enzyme frequently results in increment K_m and decreased V_{max} values due to improper bonding of the enzyme molecules to matrix or lower activity [33].

3.5. Thermal and low temperature storage stabilities of lipase preparations

Thermal stability is important for industrial applications of the immobilized enzymes. In the presented study, immobilized lipase presented an important enhance in thermal stability with respect to native lipase (Fig. 4C). The immobilized lipase was found to be stable and retained all its initial activity, on the other hand, the native lipase was lost approximately 16% of its initial activity at 45°C for 120 min. At 55°C, the activity of the native and immobilized enzymes was decreased gradually, whereas the immobilized lipase activity was reduced at a slower rate than that of the native lipase, and their retained initial activity approximately 54 and 87%, respectively. At 65°C, the immobilized enzyme retained approximately 59% of its initial activity whereas the native lipase was lost all its activity after 60 min incubation period. As observed from Fig. 4C the immobilized lipase was more stable at tested three temperatures compared to the native lipase. This could be due to increases in the rigidity of the immobilized lipase upon multipoint covalent fixation on the support. Thus, its tertiary structure could be preserved at elevated temperatures. Additionally, the amplified heat stability of the lipase immobilized onto MBioSi@PDA particles compared to native lipase was attended owing to the synergistic effect of the magnetic biosilica components.

The low-temperature storage stabilities of the native and immobilized lipase were studied in a batch system. The native and immobilized enzymes were incubated at 4ºC for six weeks. The native enzyme retained 16% of its initial activity. The immobilized enzyme conserved 81% of its initial activity after six weeks of storage (Fig. S4). The immobilized enzyme presented enhanced stability compared to the native enzyme. This result showed that MBSi@PDA@lipase had good long term stability compared to native lipase for storage at low temperatures. The higher storage stability of the immobilized lipase could be resulted from multi-point covalent bonding between enzyme and support. Thus, the generated self-multi-point attachment could prevent the denaturation of lipase over a long term storage. The improvement of stability of the immobilized lipase could be due to the presence of a comfortable microenvironment like in cells. The MBSi@PDA could provide a green biocompatible environment for the lipase due to the immobilization on the natural polydopamine layer and keeps from the loss of its activity. Thus, the presented immobilization method could provide a useful property to the immobilized lipase for many industrial applications.

3.6. Algal oil production using *K. formosum* at three different temperatures

In the present study, *K. formosum* was cultivated in BG11 medium at three different temperatures. FT-IR analyses were performed using the algal biomasses grown at different three temperatures (*i.e.*, 25, 35, and 45ºC) as the beginning step to detect the presence of any change in the FTIR spectra that correspond to the variation of algal biomasses (Fig. 5, partly A, B, and C, respectively). This could be a

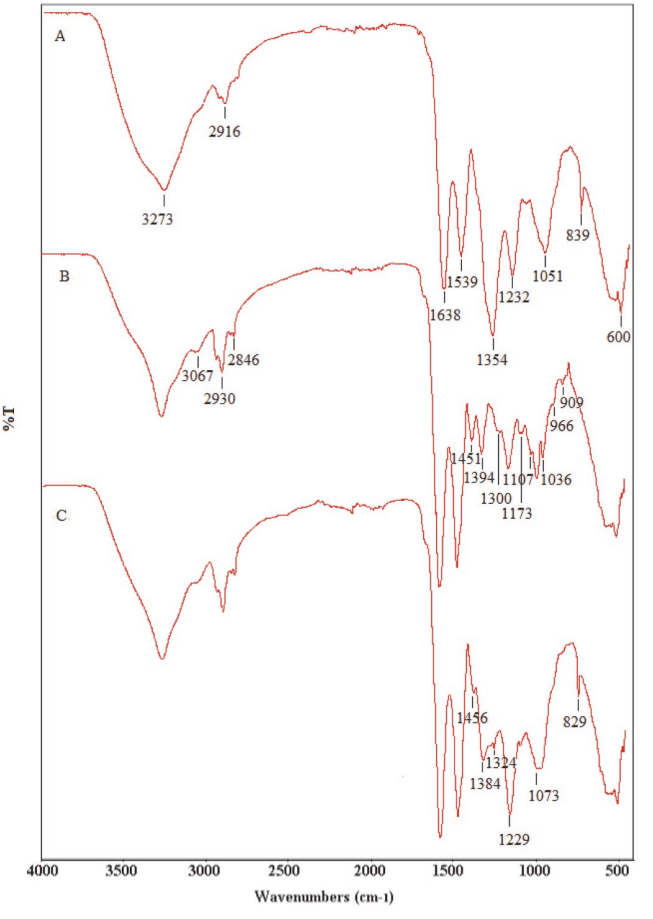

Fig. 5. FTIR spectra of the algal biomasses grown at three different temperatures. Conditions: *Kamptonema formosum* cultivated in BG11 medium at three different temperatures (*i.e.*, 25, 35, and 45ºC) under above given conditions.

useful method to detect changes in the biomass properties and lipid content of the algal biomasses grown at different temperatures. The algal biomass obtained at different temperatures showed variation in their FTIR spectrum with temperature (Fig. 5). The characteristic bands were observed for lipid fractions at 3,000-2,800 cm^{-1} for acyl chains (C-H stretch) and around 1,638 cm^{-1} for ester (C=O stretch) [1]. The algal biomasses obtained at different temperatures, a strong peak was observed at 1,549 cm^{-1} corresponding to amide groups of various proteins. Different peaks in the FTIR spectrum were observed for *K. formosum* biomasses grown at different temperatures. These results showed that the cultivation temperatures were effective on the biomass properties of *K. formosum* was yielded the highest biodiesel productivity approximately 91.2% grown at 25°C. On the other hand, alga grown at elevated temperatures (35 and 45°C) the biodiesel productivities decreased to 81.7 and 61.3%, respectively. At these studied temperatures, not only the biodiesel productivity decreased but also the oil constitutes of *K. formosum* also changed. The alga grown at 25°C was yielded of oleic acid methyl ester (23.7%, 18:1), palmitic acid methyl ester (19.3% 16:0), linolenic acid methyl ester (17.9% 18:3), linoleic acid methyl ester (15.1%, 18:2) and palmitoleic acid methyl ester (6.7%, 16:1). On the other hand, *K. Formosum* was grown at 35 and 45°C mainly constitute of similar fatty acid methyl esters but their amounts were varied (Table 1). At the end of the 20 days culture period, the total algal lipids were found to be maximum, and the lipid content of *K. formosum* was found to be 38.7, 23.6, and 13.2% at 25, 35, and 45°C, respectively. The maximum biomass productivities at three different temperatures were found to be 1.86, 1.24, and 0.94 mg L^{-1} day^{-1}, respectively. The *K. formosum* was grown in BG11 medium under the given above cultivation conditions. At 25°C, *K. Formosum* contained 38.7% lipid, whereas, the lipid content of *K. Formosum* decreased about 2.93 folds at 45°C (13.2%). Thus, the lipid percentage of *K. Formosum* decreased with increasing the growth temperature from 45 to 25°C. In an earlier study, a heat-resistant *Chlorella sorokiniana* was cultivated at 30°C and a high amount of fatty acid was obtained compared to other tested temperature (*i.e*., 37 and 40°C). For *K. Formosum* alga an increase in the growth temperature from 25 to 45°C caused an approximately 2.93 folds decreased in the lipid content. In previous studies, researchers have been reported that [8,49] temperature has a profound effect on the fatty acid composition and biomass productivity of algae, and in general, the amount of saturated fatty acids increases with increasing temperature. This could be mainly attributed to the change in membrane fluidity as an adaptation strategy to a cold environment [8]. In another study, a hot spring algal isolate from the Roman Baths was cultivated at different temperatures (*i.e*. 20, 30, and 40°C). It has been reported that the alga grew better at 20°C and did not grow at 40°C [50]. It should be noted that *K. Formosum* was grown successfully at 45°C, and also it grew at 25°C well with respect to at 35 and 45°C. This could be due to the adaptability of the cellular membrane to the temperature change as well as the thylakoid membrane as a safety measure against photo-inhibition [51].

3.7. Transesterification studies of algal oil

The lipase catalyzed transesterification could take place at different temperatures depending on the utilized oil and temperature is one of the important parameter as it effects the reaction rate and yield of the methyl esters. The effect of temperature on the transesterification efficiency of the MBioSi@PDA@lipase was examined at four different temperatures in the range of 25-55°C (Fig. 6). The transesterification reaction of algal oil was carried out with methanol. As can be seen from the Figure, as the reaction temperature increases from 25°C to 45°C, the conversion of algal oil into methyl ester was also increased. The maximum of amount ester synthesis yield was obtained at 45°C but a further increase in the temperature (*i.e*., 55°C) caused a decrease in the transesterification reaction rate. This could be due to the denaturation of the immobilized enzyme at this high temperature. Thus, the maximum methyl ester synthesis was obtained as 91.2% at 45°C for 24 h transesterification reaction.

The synthesized FAME esters oil content of *K. formosum* was measured using gas chromatography. The results were expressed as a relative percentage of the total peak area as described previously [25]. A GS-MS chromatogram was

Table 1. The fatty acid methyl esters components (FAME's) of the *Kamptonema Formosum* were determined for algal biomass grown at three different temperatures (*i.e*., 25, 35, and 45°C)

Temperatures (°C)	Fatty acid methyl esters (FAME's)					
	16:0 (%)	16:1 (%)	18:1 (%)	18:2 (%)	18:3 (%)	20:0 (%)
25	19.3 ± 0.7	6.7 ± 0.4	23.7 ± 1.1	15.1 ± 1.3	17.9 ± 0.9	5.1 ± 0.6
35	14.7 ± 0.8	11.3 ± 0.7	24.8 ± 0.3	18.2 ± 0.6	13.2 ± 0.4	6.7 ± 0.1
45	9.8 ± 0.3	10.8 ± 0.2	30.9 ± 1.3	19.7 ± 1.1	11.6 ± 0.6	8.9 ± 0.2

The fatty acid methyl esters component was synthesized via transesterification using the lipase immobilized on the MBioSi@PDA.

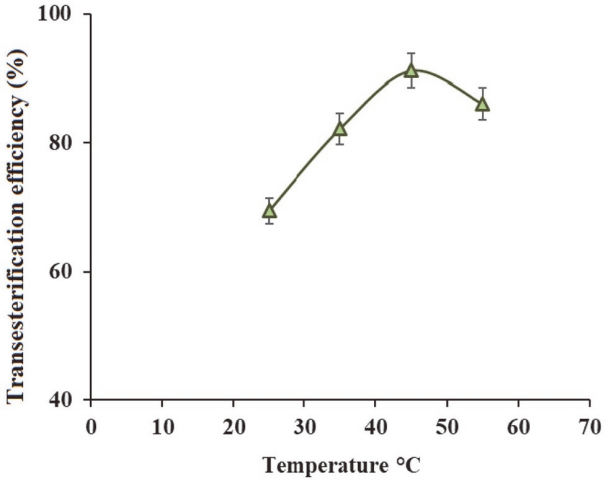

Fig. 6. Effect of temperature on the ester synthesis efficacy of MBSi@PDA-lipase particles. Condition: Temperature was varied between 25 and 55°C.

exemplified for FAME's obtained from algal biomass growing at 25°C (Fig. S5). The fatty acids constituents of the algal oil are varied with respect to chain length, degree of saturation, the configuration of double bonds, or other chemical groups (*i.e*., hydroxy, epoxy, cyclo, and keto). The FAMEs obtained from the oil of alga were found to be mostly composed of C16 and C18. The major FAMEs constitute of the synthesized biodiesel were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). The other FAMEs such as C14 and C20 are relatively in low concentrations similar to those obtained from other algal oils [2,49]. These results show that the temperature tolerant *K. formosum* is a suitable alga strain for the manufacture of biodiesel using immobilized lipase. In the earlier studies, the conversion rates of various algal oils to biodiesel using immobilized lipase have been achieved up to 96%. Our result reported in this work is compatible with the earlier reports. The use of immobilized lipase in the production of biodiesel reduces byproduct formation, purification steps, and energy requirement, and thus, reduces the production costs of biodiesel.

3.8. Operational stability of MBioSi@PDA@lipase

Compared to the native lipase, the MBioSi@PDA@lipase can be easily magnetically separated from the reaction mixture for the next run. To investigate the operational stability of the immobilized lipase for biodiesel production, the same immobilized enzyme preparation was repeatedly used in the transesterification reactions at 35°C as described above. As seen in Fig. S6, the MBioSi@PDA@lipase progressively lost its catalytic activity with increasing run number, and however, about 70.6% of original enzyme activity was preserved after 5 runs. This result indicated that the MBioSi@PDA@lipase has good activity and operational stability during the transesterification reactions.

3.9. Comparison of the obtained biodiesel production result with previous reports

It is necessary to evaluate the results of the biodiesel production in relation to previous reports. The biodiesel production performance of the earlier reported studies is presented in Table 2. As seen from the table, the biodiesel production performance of the presented system was observed to be comparable with the reported studies in the literature [33,52-59]. This finding can be recognized to the performance of the immobilization system on the magnetic biosilica. The biocompatible lipase immobilization system was showed to be favorable for transesterification of algal oil with methanol into biodiesel in hexane medium. The high biodiesel production capacity of the MBioSi@PDA@lipase can be related to the high retained activity of the immobilized lipase and increased stability to pH, temperature.

Table 2. Comparisons around the results of biodiesel production in relation to previous reports

Origin of oil	Lipase source	Support for immobilization	Biodiesel yield (%)	References
Scenedesmus quadricauda oil	*Candida rugosa*	Fibrous polymer grafted biosilica	96.4	[33]
Corn oil	*Candida antarctica*	Porous acrylic support	81.1	[52]
Scenedesmus obliquus oil	*Aspergilous niger*	Polystyrene material	53.8	[53]
Waste sardine oil	*Aspergillus niger*	Activated carbon	94.5	[54]
Jatropha oil	*Burkholderia cepacia*	Silica monolith	90.0	[55]
Coconut oil	*Pseudomonas fluorescens*	Chitosan	12.0	[56]
Nannochloropsis oceanica IMET1 microalga oil	*Candida antarctica*	Acrylic polymer	99.1	[57]
Soybean oil	*Pseudomonas cepacia*	Magnetic silica	55	[58]
Pistacia chinensis bge seed oil	*Rhizopus oryzae*	Macroporous resin	92	[59]
Kamptonema formosum oil	*Candida rugosa*	Magnetic biosilica	81.9	[In this work]

4. Conclusion

K. formosum used in this study was isolated from hot spring water and can tolerate a wide temperature range of 25-45°C. *K. formosum* was effectively cultured in BG11 medium for three weeks. Applied different temperature regimes significantly changed the biomass production efficiency, oil composition, and FAME content of the biomass of *K. formosum*. The maximum algal oil productivity of *K. formosum* was obtained at 25°C. Algal oil was obtained by extracting from algae biomass grown at three different temperatures using chloroform/methanol at a ratio of 1:1. The MBSi@PDA@lipase was used for the transesterification of algal oil obtained from the algal biomass grown at three different temperatures. The results obtained from transesterification of crude algal oil with methanol indicated that biodiesel synthesis reached 91.2% at 35°C for 24 h. In the presented lipase immobilization method, the support material was prepared using biosilica and polydopamine. These materials are fully environmentally friendly materials that could be used for many biotechnological applications and disposed of safely after any applications.

Authors Contributions

Ilkay Acıkgoz-Erkaya: Investigation and Methodology. Gulay Bayramoglu: Conceptualization, Formal analysis, Investigation, Writing - original draft. Aydın Akbulut: Investigation and Methodology. M. Yakup Arica: Conceptualization, Project administration, Writing - review & editing.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-020-0387-9) contains supplementary material, which is available to authorized users.

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