



Immobilisation of lipase enzyme onto bacterial magnetosomes for stain removal

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ABSTRACT

Lipase was immobilized onto bacterial magnetosomes using glutaraldehyde cross-linking and confirmed by Fourier transform infrared spectrometry (FT-IR) and Scanning electron microscopy (SEM). Enzyme activity of immobilised lipase as well as free lipase was estimated by the release of *p*-nitro phenol due to the hydrolysis of *p*-nitro phenyl acetate (pNPA). The immobilisation yield of lipase onto magnetosome was found to be 88%. The optimal pH (7) and temperature (40 °C) for activity was standardised and found to be similar to free lipase. The stored immobilized lipase maintained higher activity even after 30 days at a temperature of 4 °C whereas compared to free lipase. Immobilized lipase found to have removed vegetable oil stain and showed higher cleaning efficiency when compared to free lipase. The results suggest that bacterial magnetosome displays great potential as a support material for the immobilization of industrial enzymes such as lipase.

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1. Introduction

Lipases are enzyme that catalyse the breakdown of triacylglycerol to glycerol and fatty acids. Industrial enzymes are often immobilised on a support material to increase the catalytic activity, reduce the operational expenses, improves enzyme stability (by multipoint covalent attachment), activity, selectivity, priority and resistance to inhibitors [1–5]. In general lipase enzyme can react with broad range of substrates with high stability at pH range (2.2–6.8) and temperature. Hence lipase has been considered as one of the most important industrial biocatalyst for biotechnological application [6,7]. However, lipase when exposed to hydrophilic surface are weak and less active attaining a closed form whereas coupling on a hydrophobic support material changes the molecular structure of enzyme to a more active form [8–10]. This occurs through interfacial activation mechanism. Lipase when exposed to aqueous solution forms a closed form which consists of a - lid that is made up of hydrophobic catalytic center blocked with polypeptide chain, a hydrophilic external face and a hydrophobic internal face. Lipase when exposed to oil or similar hydrophobic surface, the closed form changes to open form replacing the lid

with hydrophobic pockets [11,12]. To enable the use of enzymes in industrial processes, appropriate support material should be developed for enhanced activity and easier product separation and recycling [13,14].

Nanoparticle based enzyme immobilisation have been emerged as promising alternative that improved operational stability and reusability [15,16]. Magnetic nanoparticles (MNPs) act as support for enzyme immobilization and can be recovered easily with the help of external magnetic field [17–19]. Moreover, immobilization of enzyme on nanoparticles is easy, uniform coating on the surface is achieved while no diffusion problem [20]. But potential toxicity, direct exposure of enzyme to environmental factor and inconsistent magnetism of MNPs based support material hindered its industrial application potential [20–22]. Bacterial magnetosome are nanometer - sized, membrane bound organelle biomineralized by Magnetotactic bacteria (MTB) [23]. Magnetosomes are synthesised under strict genetic control in uniform size with the mean particle diameter ranges from 35 to 120 nm. The properties of magnetosomes such as narrow grain size distribution, high chemical purity, stable single domain magnetisation (SD) and optimised magnetic dipole moment are of great interest for application in biotechnology [24].

Magnetosome membrane contains primary amino groups which can be linked with lipase enzyme by means of crosslinking agent [25,26]. Crosslinking reagents have been widely used in previous studies stability and reusability of the enzymes [27]. One

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Nomenclature

Mathematical notations

| | |
|------------|---|
| Δ | Delta |
| ΔA | Difference between Final and initial absorbance |
| ΔT | Difference between Final and initial time |
| | Molar extinction coefficient |

such crosslinking reagent is glutaraldehyde which improves the multipoint covalent bonds [27,28]. Immobilisation of enzymes onto magnetosome was first reported by Matsunaga and Kamiya [29]. The immobilisation of bioactive molecules onto magnetosomes can be done either directly or indirectly with linkers. This study deals with development of an effective method for the coupling of lipase onto magnetosome via glutaraldehyde cross-linking. The enzyme activity and stability of the immobilised lipase was analysed followed by demonstration of the influence of pH and temperature on enzyme activity. The immobilized lipase was used to remove the stains from cotton cloth.

2. Materials and methods

2.1. Microorganism and culturing

Magnetospirillum gryphiswaldense strain MSR-1 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. The culture was preserved in the standard condition as per the instruction manual [30]. *Magnetospirillum* growth medium (MSGM) was used for the routine cultivation of bacteria [31]. The obligate microaerophilic bacteria were cultivated according to Hungate anaerobic technique [32]. Briefly culture medium was dispensed in anaerobic serum bottles (500 ml) leaving some air space. Dissolved oxygen was removed from the culture medium by flushing sterile nitrogen. Further the culture flasks were closed with butyl rubber stoppers and sealed with crimped metal seal. The sterile bottles were inoculated with 10 % (v/v) cells of 24 h grown culture (exponential phase). Growth in flasks was monitored at 28 °C in incubator shaker for 3 days. The magnetosome formation was checked routinely using a magnetic separator (Single tube magnet, Qiagen, Hilden, Germany).

2.2. Extraction and characterization of magnetosome

Magnetosome extraction was carried out as per modified method proposed by Alphantery [33]. The cells containing magnetosome were separated out from the culture medium through centrifugation (8000 xg, 10 min). The pellet was then resuspended in Tris-HCl and sonicated (Sonics Vibracell VCX 130, USA) at 30 W for 2 h. The extracted magnetosomes were washed until the cell debris was completely removed. Magnetosomes hence obtained were magnetically separated and lyophilized (*Lark, Penguin Classic Plus*, India). The morphology and crystal structure of magnetosome was studied using high resolution transmission electron microscopy (HRTEM, JEOL JEM2100, Japan) operating at 200 kV. Samples were mounted on a carbon coated copper grid and micrographs were captured. The extracted magnetosomes were characterized in X-ray powder diffraction analysis (XRD, BRUKER, Germany).

2.3. Lipase immobilization

The magnetosome aggregates were dispersed in PBS (pH: 7) using ultrasonicator for 30 min at 30 W. The obtained magnetosome

dispersion was filtered through 0.22 μm syringe filter (Whatman Maidstone, Kent, UK) and dried in room temperature. For activation 30 mg of purified magnetosome were mixed with 20 μl of 25 % glutaraldehyde (protein cross linking agent) dissolved in 30 μl of potassium phosphate buffer (0.2 M, pH = 7) [33]. Lipase solution (1 mg ml⁻¹) was mixed with glutaraldehyde activated magnetosomes (1 mg) and further incubated for 12 h in shaking condition [34]. Followed this, magnetosome loaded lipase were separated by centrifugation (8000 xg, 10 min) and were washed with sterile Phosphate buffer saline (PBS).

2.4. Characterization of immobilized lipase and immobilisation efficiency

FT-IR spectroscopy can be used to confirm the crosslinking of magnetosome with glutaraldehyde and coupling of glutaraldehyde activated magnetosome with lipase. Absorption peaks of pure lipase, bacterial magnetosome and immobilized enzyme were documented between 400 cm⁻¹ and 4000 cm⁻¹ (Schimadzu, Japan). The morphological changes of magnetosome before and after the immobilization of enzyme were observed using Scanning electron microscopy (SEM, Zeiss EV018, Germany) analysis. Immobilisation efficiency was determined based on the protein (enzyme) content present in the supernatant (wash) after immobilisation. Protein concentration was quantified by Bradford method with bovine serum albumin (BSA) used to plot the standard curve [35].

2.5. Enzyme activity assay

The hydrolytic activities of free lipase as well as immobilized lipase were investigated by assaying p-nitrophenyl acetate as substrate under standard conditions (pH 8) [36]. One unit of is defined as the amount of enzyme that liberates 1 μg of substrate (pNPA) min⁻¹. In general the activity of lipase from any source was calculated by evaluating the hydrolysis of p-nitrophenyl esters at 410 nm [36,37]. Enzyme activity has been determined according to the formula

$$\text{Activity} = \frac{\Delta A}{\Delta T} \times \frac{1}{\epsilon} \times \frac{V_t}{V_c} \times 100$$

ΔA = Difference between Final and initial absorbance

ΔT = Difference between Final and initial time

ϵ = Molar extinction coefficient

V_t = Total volume

pH, thermal and storage stability study of immobilized lipase

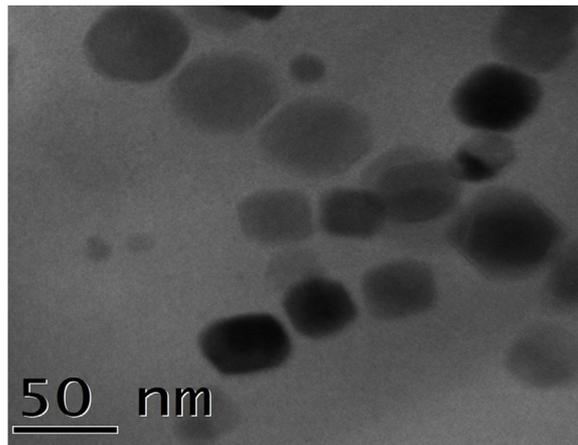


Fig. 1. Transmission electron microscopy image of bacterial magnetosomes extracted from *Magnetospirillum gryphiswaldense* cells separated using magnetic field arranged in chains (Scale bar 50 nm).

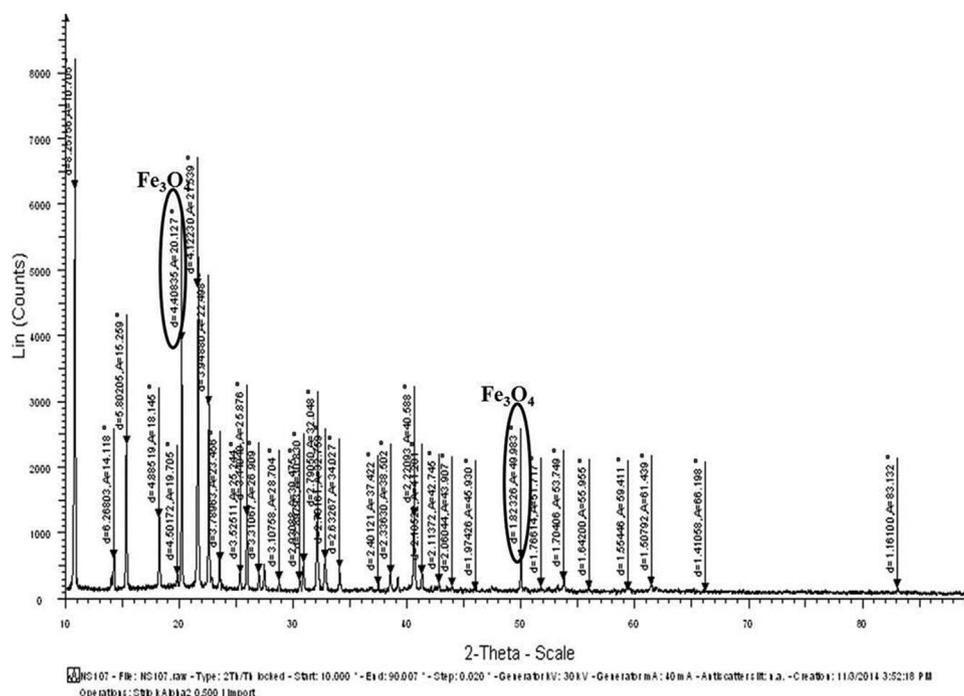


Fig. 2. XRD diffraction patterns of magnetosomes extracted from *Magnetospirillum gryphiswaldense*. The major peaks matches with that of standard Fe_3O_4 .

To investigate the effect of pH on the activity, free and immobilized enzyme was incubated at different pH. Different pH values were accomplished using citrate buffer pH 4–6, phosphate buffer pH 7–8 and glycine buffer pH 9–10 at optimum temperature (40 °C) for 4 h. The effect of temperature on the stability of free and

immobilized lipase was measured by exposure at varying temperature (10–60 °C) at the optimum pH for 4 h. The ability of free and immobilized enzyme to retain activity upon storage was analysed at regular intervals for a period of 30 days. To investigate the stability of the activity change of free and immobilized enzyme,

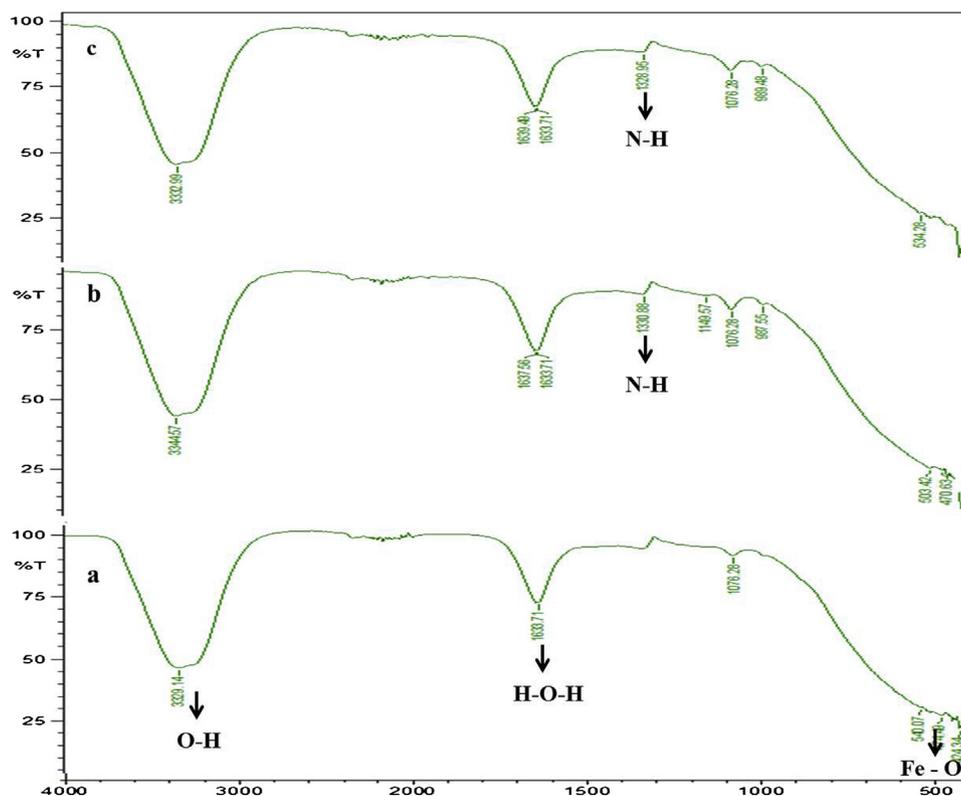


Fig. 3. FTIR spectra of (a) magnetosome shows characteristic peak of Fe-O bonding at 526 cm^{-1} (b) Immobilized lipase showed absorption peak for Fe-O bonding at 503 cm^{-1} and N–H stretch vibrations (1330 cm^{-1}) of lipase enzyme (c) pure lipase showed an absorption peak near 1328 cm^{-1} that represents the N–H stretch vibrations assigned for lipase enzyme. Absorption peak near 1330 cm^{-1} in the immobilized lipase confirms the successful immobilisation of lipase to magnetosome.

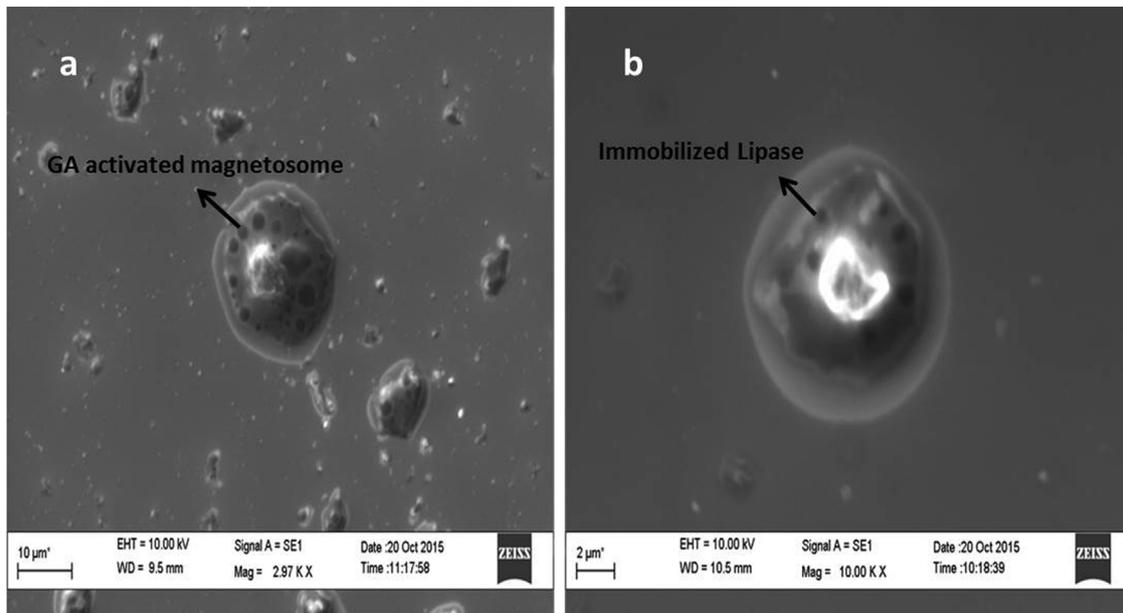


Fig. 4. SEM micrographs of (a) Glutaraldehyde activated magnetosome shows a layer around the magnetosome chain. The layer around magnetosome confirms the activation process (Scale bar 10 μm) (b) Immobilized lipase showed the presence of a second thin layer around the activated magnetosome. The second layer confirms the lipase immobilization (Scale bar 2 μm).

Table 1

Efficiency of Immobilisation. The immobilization efficiency was calculated based on the residual lipase present in the magnetosome. (All values are mean ± SD range for three observations each).

| Supernatant | Unbound Lipase (mg) | Residual Lipase (mg) |
|----------------------|---------------------|----------------------|
| Initial | 0.08 ± 0.11 | 0.92 |
| 1 st wash | 0.032 ± 0.17 | 0.89 |
| 2 nd wash | 0.01 ± 0.06 | 0.88 |

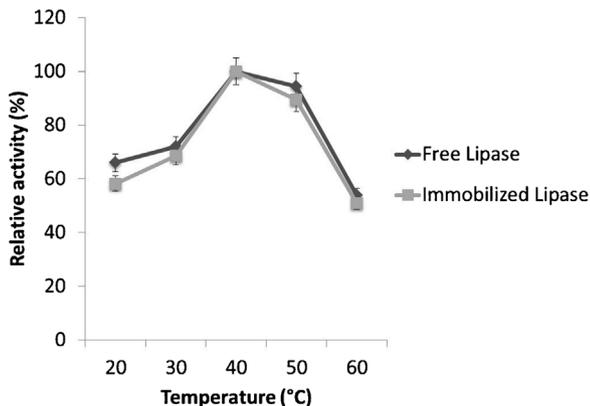


Fig. 5. Influence of temperature on the activity of immobilized and free lipase. Enzymes were assayed with p-nitrophenyl acetate as substrate at pH 7. Maximum relative activity was obtained at a temperature of 40 °C.

1 mg/ml was dispersed in PBS (pH 7, 0.1 M) stored at 4 °C. The activity was analysed every 5 days using p-nitrophenyl acetate as substrate.

2.6. Strain removal from fabric strips

Fabric strips were prepared and the clean strips were soiled with spent cooking vegetable oil. Oil stained fabric strips were dried and excess oil was removed using a paper towel. The soiled cotton fabric strips were washed with 1 mg free and magnetosome

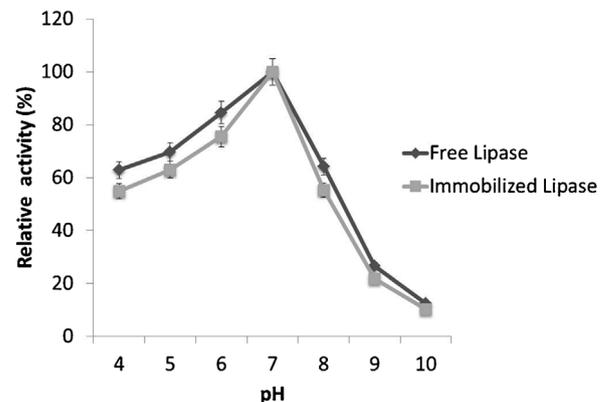


Fig. 6. Influence of pH on the activity of immobilized and free lipase. Enzymes were assayed with p-nitrophenyl acetate as substrate at temperature 40 °C. Maximum relative activity was obtained at a pH of 7.

immobilized lipase ml⁻¹ at 100 xg for 30 min while the PBS washing was considered as control [38]. The weight of soiled fabric strips was measured before and after washing process to determine the cleaning efficiency [39].

Cleaning efficiency = $\frac{\text{Soiled cloth weight} - \text{Washed cloth weight}}{\text{Initial cloth weight}}$

Each value is mean of 3 replicates. The washed cloth pieces were rinsed two times with sterile PBS and analysed visually.

3. Results and discussion

3.1. Microorganism, magnetosome and characterisation

The yield of magnetosome from MSR-1 culture was found to be 10 ± 1.4 mg l⁻¹. The magnetosomes production was significantly lesser than the previous reports of 88 mg l⁻¹ [40]. The batch culturing by hungate standard technique followed in this study is less consistent in terms of dissolved oxygen when compared to chemostat culturing method. The ratio of N₂/O₂ present in the

media while culturing has clear correlation with the magnetosome formation in MSR-1 [41].

TEM image shows the chain like arrangement of magnetosome (Fig. 1). From the TEM micrographs it is evident that magnetosome biomineralized by strain MSR-1 possess cubo – octahedral morphology.

X-ray diffraction studies validated that a number of strong reflections showed the closest similarity with the reflection of standard Fe_3O_4 (Fig. 2). This result indicated that the magnetosome consists of magnetite.

3.2. Lipase immobilization

Magnetosome was coupled with lipase using glutaraldehyde as a cross-linker. The results of the immobilization process were confirmed using FT-IR analysis by examining the characteristic chemical structures of bacterial magnetosome (Fig. 3a), Immobilised enzyme (Fig. 3b) and free lipase (Fig. 3c). The spectrum of the pure magnetosome showed six characteristic absorption bands: 3344 cm^{-1} , 1633 cm^{-1} , 1330 cm^{-1} , 526 cm^{-1} , 503 cm^{-1} , 457 cm^{-1} and pure lipase showed five bands 3332 cm^{-1} , 1633 cm^{-1} , 1328 cm^{-1} , 1076 cm^{-1} , and 534 cm^{-1} . The characteristic absorption peaks at 3344 cm^{-1} and 1633 cm^{-1} represent the O—H and H—O—H bonds while the peaks at 528 cm^{-1} assigned to stretching vibration for Fe—O bond [42]. The FTIR spectra of free lipase indicated absorption bands near 1328 cm^{-1} that represents

the N—H stretch vibrations assigned for lipase enzyme. A characteristic absorption peak near 1330 cm^{-1} in the immobilized lipase confirms the chemical cross-linking between glutaraldehyde activated magnetosome and lipase. Fig. 4 shows the SEM images of glutaraldehyde activated magnetosome (4a) and immobilized enzyme (4b). When compared with pure magnetosome a thin layer is observed around the crystal planes of magnetosomes which confirms the successful immobilisation of lipase to magnetosome [34].

The amount of lipase immobilised onto magnetosome was determined using the Bradford method [35]. Protein present in the supernatant after all the three washing step was estimated to determine the lipase immobilized on magnetosome (Table 1). All the three washes had a total of 0.122 mg of unbound lipase which indicates a total of 0.880 mg of lipase was immobilized to magnetosome. An immobilization yield of 88 % was achieved using glutaraldehyde activated magnetosome (Table 1). The immobilization yield was much higher than previous reports [43].

3.3. Enzyme activity assay

To estimate the activity of bound lipase a standard curve consist of activity of different concentrations of free lipase (0.1, 0.25, 0.5, 1 mg ml^{-1}) was plotted. By comparison to the standard curve the enzyme activity of immobilized lipase is found to be 23.43 U/ml.

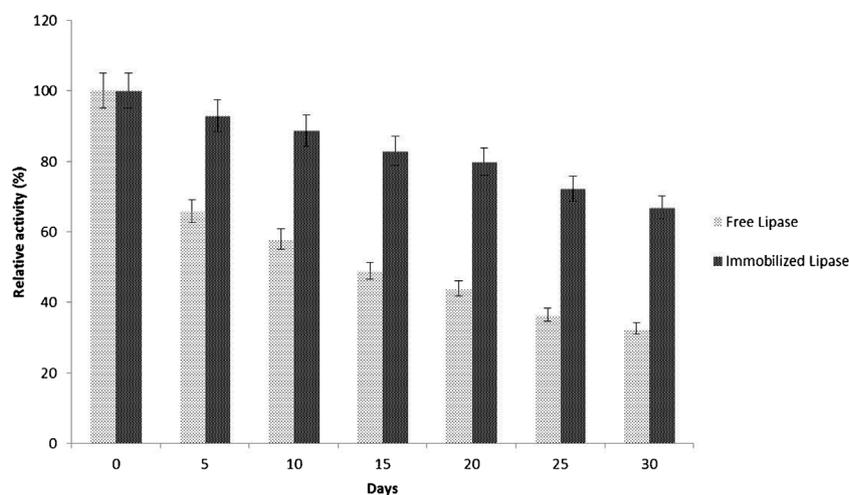


Fig. 7. Storage stability at 4°C of free and immobilized lipase. Enzymes were assayed with p-nitrophenyl acetate as substrate at temperature 40°C and pH of 7. The immobilized lipase maintained relative activity at 67 % after 30 days at 4°C whereas free lipase showed only 32 % activity over the same time.

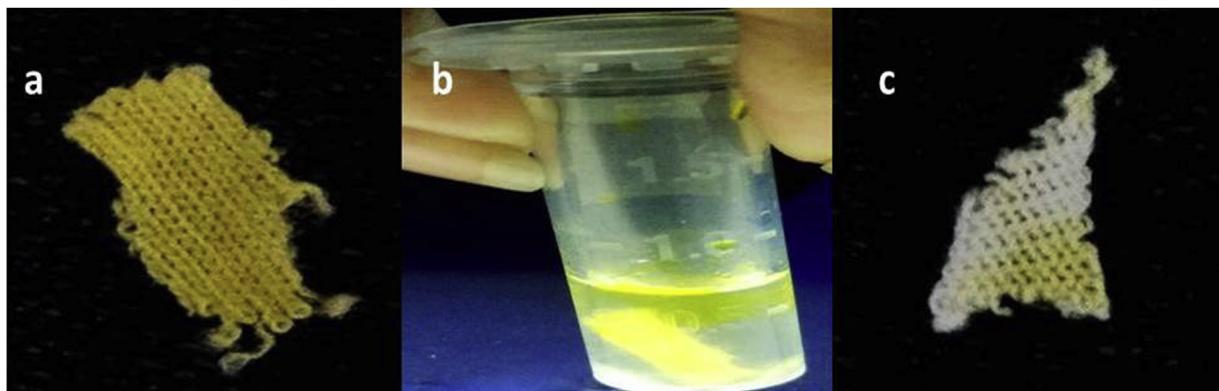


Fig. 8. Removal of oil stain from cotton fabric strips (a) oil stained cotton fabric strips (before wash) (b) Washing of cotton fabric strips with immobilized lipase (c) cotton fabric strips after washing with immobilized lipase (after wash). The cotton cloth found to be relatively clean after washing with immobilized lipase.

The value indicates 0.87 mg of lipase bound to magnetosomes based on the standard curve. Hence the immobilization efficiency based on enzyme activity assay is 87 % (Table 1) and the value is close to the measurement based on Bradford method [35]

3.4. Optimal temperature, pH and stability of free and immobilized lipase

The relationship between lipase activity and temperature is illustrated in Fig. 5. The enzyme activity is highest at 40 °C and beyond this temperature the activity is significantly reduced. Hence immobilisation or presence of magnetosome has no influence in lipid hydrolysis of the enzyme. So to achieve maximum activity the optimal temperature of 40 °C should be applied throughout the experiments.

The optimal pH of both free enzyme and immobilized enzyme showed similar activity profiles. Fig. 6 shows similar optimal pH for both free lipase and immobilized lipase. The enzyme activity is highest at a pH of 7 and further then the activity is significantly decreased. We assume that the presence of magnetosome did not influence practically the activating energy of lipase hydrolysis. Hence the optimal pH of 7 can be used for further experiments.

3.5. Storage stability of immobilized lipase

The storage stability of immobilized lipase was tested and illustrated in Fig. 7. Results of storage stability of the immobilized lipase at optimal temperature (40 °C) and pH (7) was compared with free lipase at same optimal conditions for 30 days. Fig. 7 shows the stability profiles of free and immobilized enzyme. The immobilized lipase maintained activity at 67 % after 30 days at 4 °C whereas free lipase showed only 32 % activity over the same time. The results confirm that bacterial magnetosome are suitable support material to immobilize lipase enzyme due to their ability to increase its storage stability.

3.6. Stain removal from fabric strips

The washing of cotton fabric strips using immobilized lipase gave better results when compared to free lipase (Fig. 8). Moreover free lipase is not reusable and not able to separate after enzyme reaction. The immobilized lipase was separated by the magnetic property of magnetosome and can be reused without any considerable loss of enzyme activity [38,44]. The cleaning efficiency was further calculated based on the weight difference in fabric strip before and after cleaning. Cleaning efficiency of immobilized lipase was found to be 79 ± 03 % whereas free lipase could remove only 47 ± 02 % of stain from the fabric.

4. Conclusions

Magnetosome extracted from *Magnetospirillum gryphiswaldense* strain MSR-1 were successfully used for lipases immobilization using glutaraldehyde as coupling agent. The immobilization yield was found to be satisfactory (88 %) and immobilized lipase retained 87 % activity when compared with free enzyme. The optimal pH and temperature profiles of both immobilized and free lipase showed similar activity. However, the immobilized lipase showed better storage stability compared to free lipase. Magnetosome immobilized lipase found to be promising in stain removal from cotton fabric strips. Hence lipase immobilized on GA activated magnetosome shall have a great prospective for industrial use due to simple immobilization process and easy magnetic separation.

Contributors

Jobin John Jacob has performed all the experiments and prepared the manuscript. Dr. K. Suthindhiran has verified the results and approved the final manuscript.

CRedit authorship contribution statement

Jobin John Jacob: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Suthindhiran K:** Conceptualization, Validation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

All authors declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00422>.

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