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Stability Enhancement of Aldehyde Dehydrogenase from *Anoxybacillus geothermalis* Strain D9 Immobilized onto Seplite LX120

Wahhida Latip [1], Nur Ezzati Rosli [1], Mohd Shukuri Mohamad Ali [1,2], Nor Hafizah Ahmad Kamarudin [1,3,*] and Raja Noor Zaliha Raja Abd Rahman [1,4,*]

1 Enzyme and Microbial Technology Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), Serdang 43400, Malaysia
2 Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), Serdang 43400, Malaysia
3 Centre of Foundation Studies for Agricultural Science, Universiti Putra Malaysia (UPM), Serdang 43400, Malaysia
4 Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), Serdang 43400, Malaysia
* Correspondence: hafizah_kamar@upm.edu.my (N.H.A.K.); rnzaliha@upm.edu.my (R.N.Z.R.A.R.); Tel.: +60-3-97691416 (R.N.Z.R.A.R.)

Abstract: Enzyme stability is regarded as an important criterion for an industrial biocatalyst. Aldehyde dehydrogenase (ALDH) from *A. geothermalis* strain D9 was previously reported to exhibit good thermostability. However, this enzyme is still not suited to use in harsh environments. In this current work, we aim to see the viability of ALDH in terms of stability when immobilized into Seplite LX120. The purified ALDH was successfully immobilized via physical adsorption at 4 h with 1.25 mg/mL enzyme loading. The immobilized ALDH exhibited improved stability compared to free ALDH as the optimum temperature increased up to 80 °C and was stable with temperatures ranging from 30 to 90 °C. It was also stable in broad pH, ranging from pH 4 to pH 12. Moreover, more than 50% of the immobilized ALDH activity was retained after being stored at 25 °C and 4 °C for 9 and 11 weeks, respectively. The reusability of immobilized ALDH is up to seven cycles. The corroboration of ALDH immobilized on the Seplite LX120 was verified via Fourier-transform infrared spectroscopy, scanning electron microscopy, and a reduction in the surface area. The improved features of immobilized ALDH, especially in enzyme stability, are important for future applications.

Keywords: immobilization; enzyme stability; aldehyde dehydrogenase; polystyrene–divinylbenzene copolymer; scanning electron microscopy; Fourier-transform infrared

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1. Introduction

Aldehyde dehydrogenase (ALDH) is an enzyme involved in the oxidation of aldehydes and mainly used in production in the food industry, such as in prawns and wine, and in the synthesis of carboxylic acid. Carboxylic acid has been reported to be an important compound for the production of polymers, biopolymers, pharmaceutical drugs, food additives and flavors in food [1,2]. Moreover, ALDH has been widely used in the environment as a biosensor and for bioremediation purposes [3]. One of the important criteria for an industrial enzyme is the ability to work in harsh conditions. For instance, exposure to high temperature, pH, and solvent may affect the activity of the enzyme and lead to enzyme denaturation. This limits the use of enzymes in many applications.

To control this problem, enzyme immobilization is a frequently used approach to improve enzyme function and extend the enzyme life cycle for industrial purposes. According to [4], numerous immobilization strategies have been used; including adsorption, covalent binding, entrapment, and affinity immobilization. Each immobilization strategy

has its advantages and/or drawbacks and might contribute to a new feature of the enzyme. The advantages of using immobilized enzymes in broad temperature, pH and solvent environments have been described by [5–7]. Although immobilization was reported to enhance enzyme features, in certain cases it can hinder the enzyme activity. For instance, the synthesis of ethyl hexanoate using immobilized AMS8 lipase onto kaolin is lower than the free enzyme [8]. Thus, the selection of support is important to build a good interaction between the enzyme–support to protect the enzyme from denaturing.

The immobilization of enzymes on solid supports has many advantages, including quick recovery, which allows for repeated use of the catalyst, facile separation of an enzyme from its product, the capacity to operate continuously and improved enzyme stability [8,9]. A larger surface area, high stiffness, appropriate shape and particle size, reusability and resistance to microbial adhesion are all desirable characteristics of the support. These characteristics will improve enzyme stability [10,11]. Previously, ALDH has been immobilized onto a few supports such as montmorillonite, metal–organic framework capsulation and nylon [12–14]. To the best of our knowledge, this is the first study of ALDH immobilized onto polystyrene–divinylbenzene copolymer.

Hydrophobic support such as polypropylene, polymethacrylate (butyl) and polystyrene–divinylbenzene copolymer is commonly used to immobilize enzymes. These supports were reported to have a high ability to absorb water and a solid organic compound including enzymes [15–17]. Polystyrene–divinylbenzene copolymer has a porous structure, is easy to manipulate for preparing different properties (surface area, pore size, size distribution, pore volume) and is a low-cost monomer [17–19]. Due to these properties, this compound can serve as a good support for biocatalysts. In a study conducted by [18], polystyrene–divinylbenzene copolymer was manipulated to become more hydrophobic and have an enlarged pore size. As a result, an improvement in the recovery activity of immobilized lipase B from *Candida antarctica* A was obtained.

Previously a new ALDH from *Anoxybacillus geothermalis* strain D9 was identified as a thermostable and organic solvent-tolerant enzyme [20]. Despite the potential of this enzyme as a biocatalyst, the stability of ALDH towards temperature, pH and solvent is considered moderate for industrial purposes, and studies on the durability of ALDHs towards extreme environments have not been widely discussed. Therefore, in this study, enzyme immobilization was performed as a strategy to enhance the stability and potential of this enzyme. ALDH was immobilized onto Seplite LX120. The efficacy of immobilization was confirmed using FTIR, SEM and BET. In addition, this paper studied and discussed the biochemical characterization of immobilized ALDH at different storage temperatures and its reusability.

2. Results and Discussion

2.1. Immobilization Process

The effect of ALDH immobilization time using Seplite LX120 at different times was studied from 1 to 6 h. From Figure 1a, the best immobilization time for the ALDH is 4 h with 81% and 1184.31 U/g of immobilization yield and enzyme activity, respectively. From the result, it showed that at 4 h of immobilization, almost all of the ALDH was completely bound to the support, and the activity of the immobilized ALDH was recorded as high. However, a prolonged immobilization time caused the immobilization yield to decrease to 20% and 40% at 5 h and 6 h, respectively, from the optimum time. Prolonged stirring during immobilization may provide a mechanical force effect on the enzyme and lead to denaturation [11]; as a result, the enzyme might leak or detach from the support [21]. Sufficient time is needed during the immobilization for ALDH equilibrium to bind with the support. Thus, the lowest yield and activity were detected at the first hour of incubation and started to increase gradually after that.

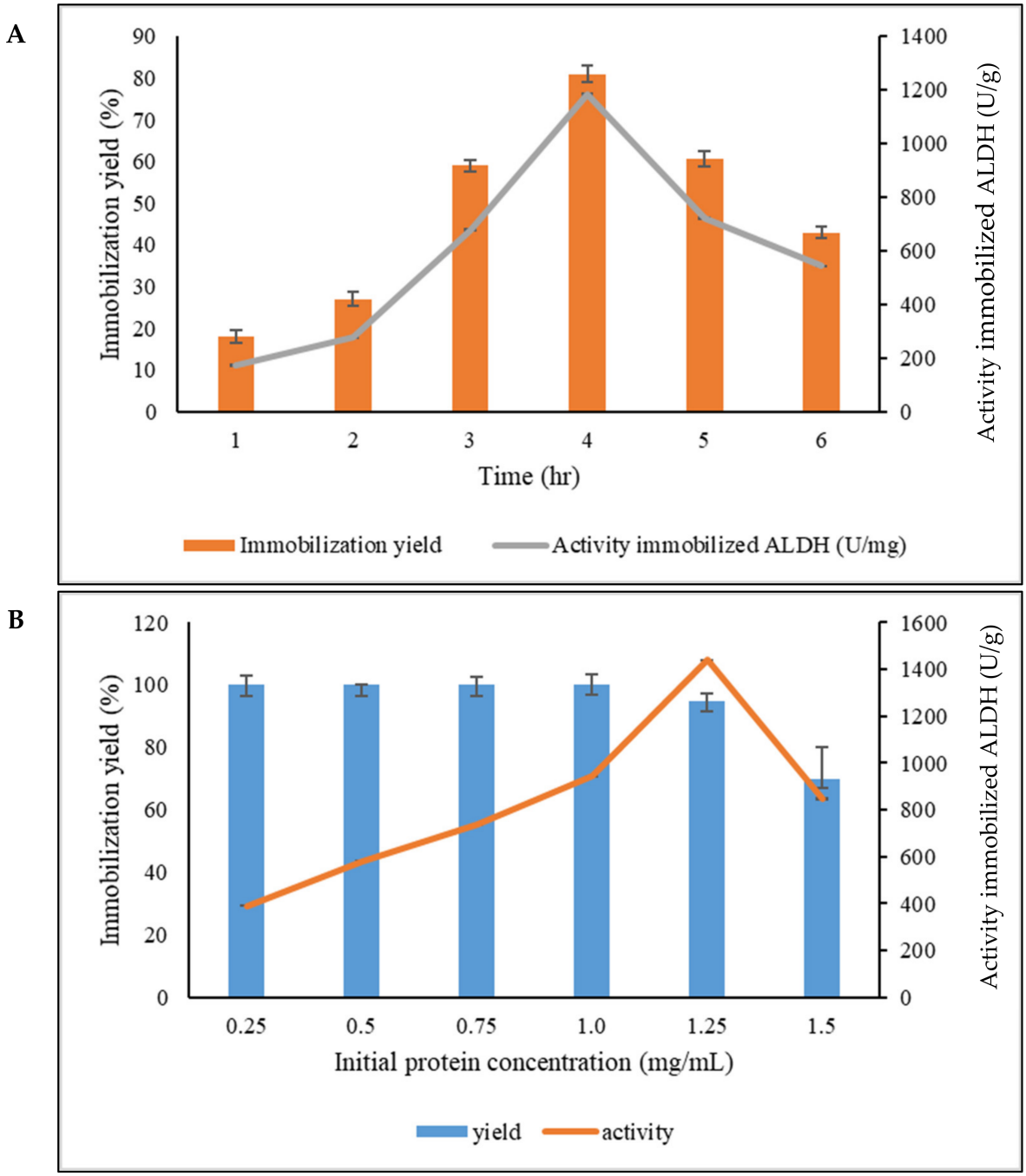


Figure 1. Optimization condition of the purified ALDH immobilized onto Seplite LX120. (**A**): The effect of immobilization time for the purified ALDH. (**B**): The effect of enzyme loading for ALDH immobilization. The bar chart shows the immobilization yield (%) for the ALDH. The line graph indicates the activity (U/mg) of the immobilized ALDH. Each point represents the mean of three experiments ± S.E.

The effect of the amount of ALDH loaded into supports, ranging from low (0.25 mg/mL) to high (1.50 mg/mL), on enzymes was studied. Figure 1b illustrates the immobilization yield and activity of the immobilized ALDH. From the results, when a low amount of ALDH was loaded, the immobilization yield achieved 100% as the space for the enzyme to bind to support was sufficient. As the protein concentration increased, the immobilization yield started to decrease. This is likely due to the maximum adsorption achieved, with fully-coated Seplite LX120 and the addition of more ALDH resulting in leakage. Moreover,

each support has a limited adsorption area depending on the availability of the surface and size of protein adsorption [22]. The immobilization yield decreased to 95% when loaded with 1.25 mg/mL of ALDH, but the activity was the highest (1439.22 U/g) among all tested concentrations.. Further treatment with higher concentration of ALDH (>1.25 mg/mL) caused the activity to drop. Excessive enzyme loading always causes protein–protein interaction and inhibits the flexible stretching of enzyme conformation, which will result in steric hindrance and thus the inactivation of an enzyme [23]. That is, for the enzyme molecule, it may be difficult to modulate its most suitable conformation for catching the substrate molecules and releasing the product molecules under molecular crowding conditions [24].

2.2. Confirmation of ALDH Immobilized onto the Seplite LX120

2.2.1. FTIR Analysis

The confirmation study of the enzyme–protein interaction, either in free or immobilized form, can be explained by the infrared (IR) technique. The FTIR spectrum in Figure 2 reveals the changes that occurred on the peak between 1700–1600 cm^{-1}, as with the C=O stretching vibration of the peptide bond [25,26]. This signal represents an amine band, which is a functional group for Seplite LX120. This signal is overlapped with the group signals that contain information on the secondary protein structure of the enzyme. The stretching vibration of C=O was also detected when ALDH was immobilized onto montmorillonite [12]. The carbonyl group at the immobilized ALDH may be detected at wavelength 1148 cm^{-1}. At wavelength 1064 cm^{-1}, an obvious difference can be seen in the immobilized and free ALDH, where the C-O bond was detected, whereas the empty Seplite LX120 has no C-O bond. It can thus be concluded that the ALDH was successfully immobilized into Seplite LX120.

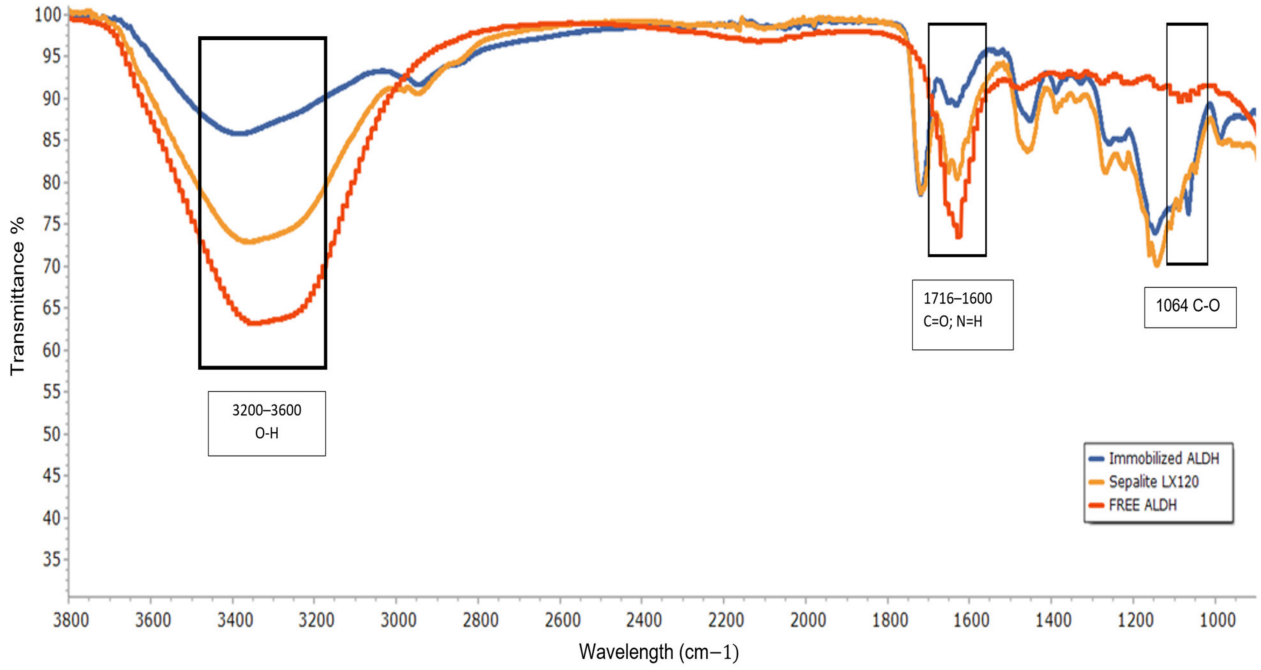


Figure 2. FTIR spectra of empty Seplite LX120, immobilized and free ALDH. Blue line: immobilized ALDH; orange line: empty Seplite LX120; red line: free ALDH.

2.2.2. Morphological Characterization of Immobilized ALDH by Scanning Electron Microscopy

The surface morphologies of the empty and immobilized ALDH were investigated by using scanning electron microscopy (SEM). Figure 3 shows the SEM for the empty Seplite LX120 and immobilized ALDH on Seplite LX120 under three magnifications, 20×, 500×

and 5000×. The difference between empty support and immobilized ALDH was seen, especially on uneven and cracked surface areas. After immobilization, the support was covered with ALDH, making the surface look more compact with fewer cracks. The morphology differences reflected a positive indication that the purified ALDH was successfully immobilized onto Seplite LX120. A similar pattern of SEM images was reported by [25] where multienzymes were immobilized on a polymeric membrane reactor (PVDF). The bare PVDF membranes were initially fairly porous, and after immobilization, a substantial change in the membrane pores was observed. The pore size shrank as the enzymes attached to the membrane [25].

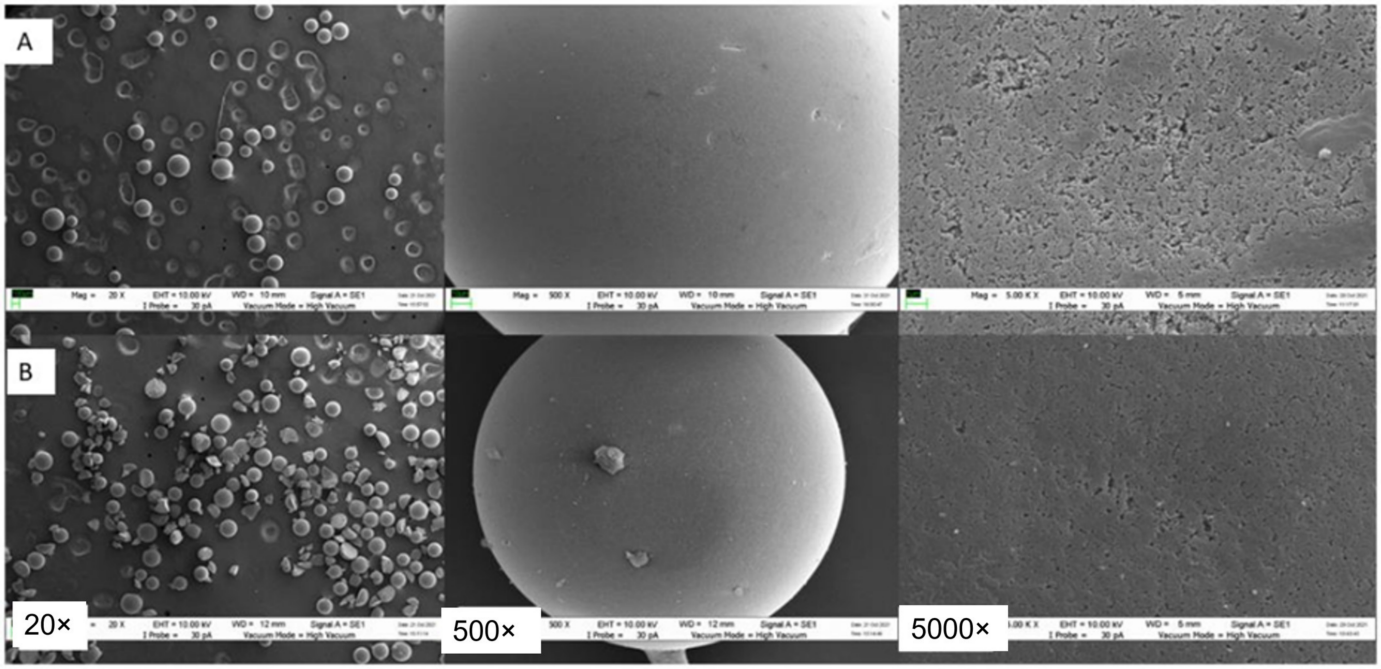


Figure 3. Scanning electron microscopy images of (**A**) empty Seplite LX120 and (**B**) immobilized ALDH. The images were captured at magnifications 20×, 500× and 5000×.

2.2.3. Surface Area and Porosity Analysis

The results of the BET surface area, pore volume and pore size of the empty support (Seplite LX120) and immobilized ALDH on Seplite LX120 are summarized in Table 1. The reduction in the surface area of the empty supports indicated that the immobilization of the purified ALDH was successful. The surface area of the empty Seplite LX120 was 122.3 m^2/g, whereas after immobilization, the area reduced to 93.6 m^2/g. Similar to N_2 adsorption–desorption isotherms, a reduction in N_2 gas uptake into the support was observed when immobilized with ALDH. The analysis provides clear evidence for protein immobilization involving pore channels. In agreement, the pore size of the support (immobilized ALDH) also showed a reduction in N_2 adsorption–desorption isotherms. These data confirmed the entrance of ALDH into the pore channel. Similar results were obtained when *Streptomyces griseus* HUT 6037 enzyme was immobilized in three different mesoporous silicas, namely, mesoporous silica film, microcellular foam and rod-like SBA-15. The surface area and pore size of the silicas decreased after the immobilization process [27].

Table 1. BET surface area, pore volume and pore size of empty Seplite LX120 and immobilized ALDH.

Parameters	Empty Seplite LX120	Immobilized ALDH
Surface area (m^2/g)	122.33	93.61
Pore volume (cm^3/g):		
Adsorption	0.02	0.01
Desorption	0.22	0.18
Pore size (nm):		
Adsorption	0.74	0.73
Desorption	7.28	7.18

2.3. Biochemical Characterization of Immobilized ALDH

2.3.1. Effect of Temperature on the Immobilized ALDH Activity and Stability

To examine the effects of immobilization on the enzyme's properties, biochemical characterization of free and immobilized ALDH was conducted. Figure 4a shows the comparative study for the effect of temperature on free and immobilized ALDH. Interestingly, the immobilized ALDH was found to be most active at 80 °C, 20 °C higher than the optimal temperature of free ALDH. The marked increase in the optimal temperature upon immobilization suggested that immobilization could be one of the strategies to improve enzyme thermostability. In general, the improvement in the activities of the immobilized ALDH was also noticeable for all tested temperatures. Immobilization is seen to be likely to impart changes in the physical and chemical properties of the enzyme, leading to less activation energy being required to catalyze the substrate [28]. While the activity of immobilized ALDH was retained at a broad temperature range and only declined at 90 °C, the free ALDH was found to be more susceptible to temperature changes. A drastic increase in enzyme activity was observed at 60 °C, while a sharp decline in activity was recorded at a temperature above 80 °C. The free ALDH almost completely lost its function at 90 °C with 21.5 U/mL.

These data showed that the immobilization of ALDH managed to prevent the denaturing of the enzyme caused by high temperature. In some cases, the immobilized enzyme showed a decline in optimum temperature but a higher thermal stability. This report was obtained when *Pseudozyma hubeiensis* (Strain HB85A) lipase was immobilized with similar support (polystyrene–divinylbenzene). The immobilized lipase showed a slight decline in the optimum temperature compared to the free enzyme (68 °C to 52 °C) but was higher in thermal stability [29].

The temperature stability for free ALDH and immobilized ALDH is depicted in Figure 4b. Both free and immobilized ALDH were pre-incubated at various temperatures for 30 min and assayed at 80 °C. The free ALDH was stable at 30 to 70 °C; however, a broader stability profile of the immobilized ALDH was observed. When the immobilized ALDH was incubated at 90 °C, more than 50% of the activity of the enzyme was retained. This proved that the immobilized ALDH was more stable compared to the free ALDH at 90 °C. The improved stability is probably due to the restraining of thermal movement at higher temperatures imposed by immobilization (Figure S1). As a result, there is a reduction in the conformational change in the enzyme due to the reduction in the flexibility of the enzymes [30]. Similarly, immobilized carboxylic acid reductase (CAR) from *Mycobacterium phlei* (MpCAR) on Seplite LX120 showed an improvement in stability by retaining 60% of the relative activity at 90 °C, while the free CAR displayed a major reduction in activity at the same temperature [31].

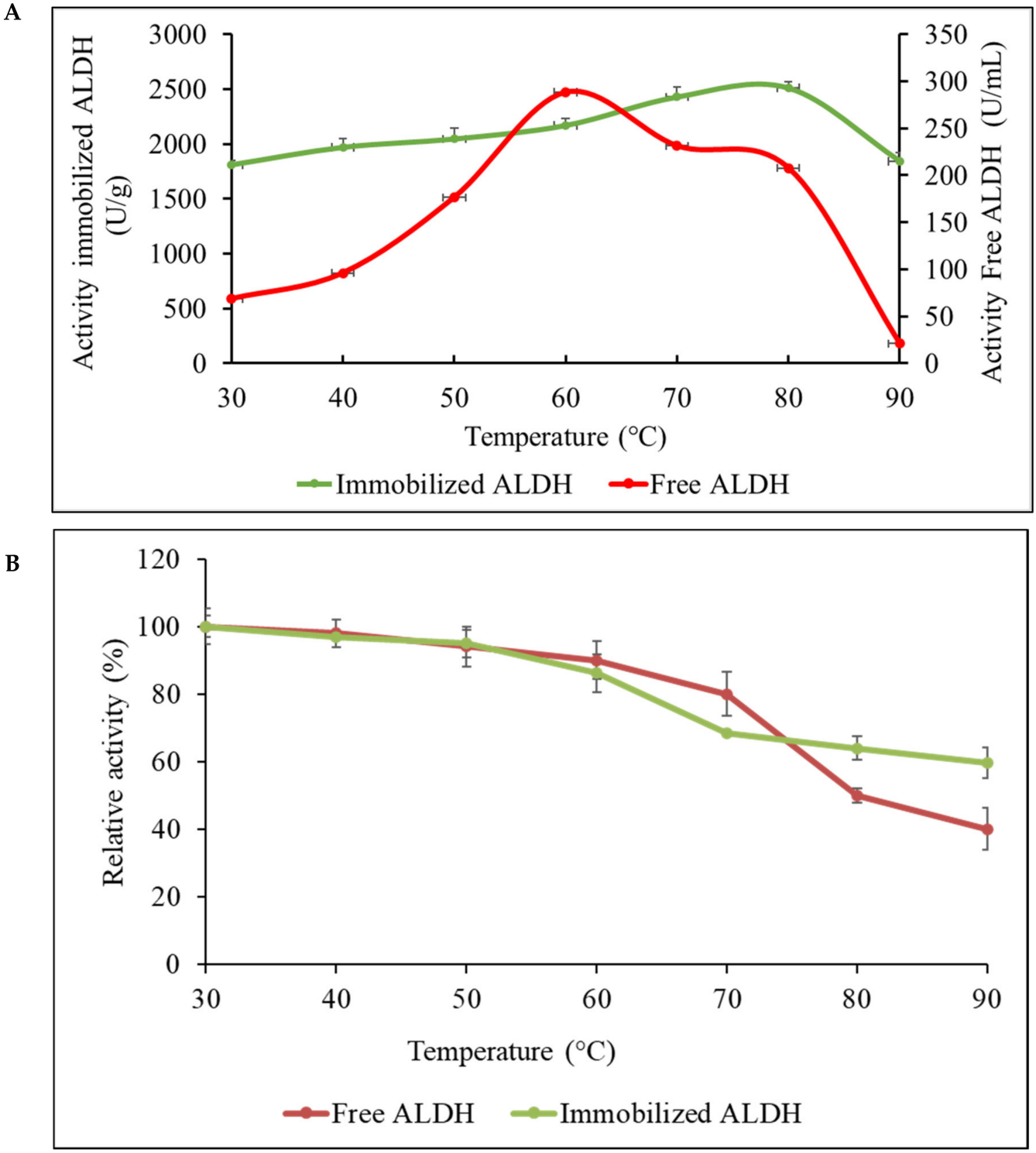


Figure 4. Effect of temperature on the activity and stability of immobilized and free ALDH. (**A**): The activity of immobilized and free ALDH. (**B**): Thermal stability of immobilized and free ALDH. Red line: free ALDH; green line: immobilized ALDH. Each point represents the mean of three experiments ± S.E.

2.3.2. Effect of pH on the Immobilized ALDH Activity and Stability

The influence of pH in the immobilization process or immobilized enzymes is crucial. Changes in pH can either activate or deactivate the enzyme. The effect of pH on the activity and stability of immobilized and free ALDH is shown in Figure 5. The optimum pH for both

immobilized and free ALDH is at pH 8. However, the overall pH profile of the immobilized ALDH was slightly altered from the profile of the free ALDH. The immobilized ALDH displayed a higher activity at pH 6 than the free enzyme. The activity of immobilized ALDH gradually increased up to pH 8 and slowly decreased above the optimal pH. Both immobilized and free ALDH are found to be deactivated in highly acidic and alkaline environments. Changes in pH value are known to affect the conformation and the degree of dissociation of a substrate, thus resulting in high or low catalysis conditions [32,33]. It is presumed that the influence of pH on immobilized ALDH may be contributed to by the enzyme–support interactions, the enzyme itself or the support.

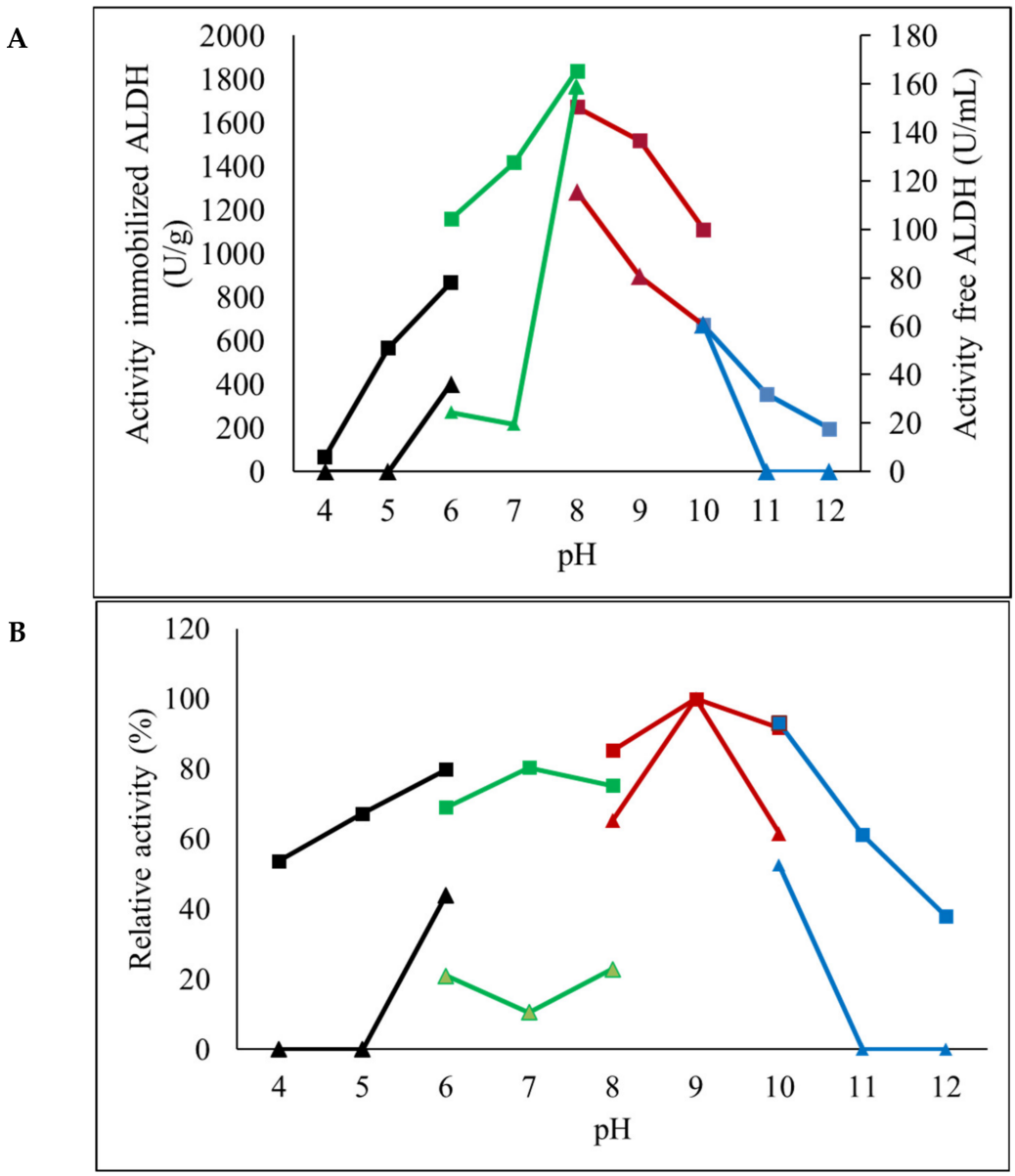


Figure 5. Effect of pH on the activity and stability of immobilized ALDH and free ALDH. The box line ■ is the immobilized ALDH, while the triangle line ▲ is the free ALDH. The pH stability for the immobilized ALDH was from pH 4 to 11, while the free ALDH was only stable at pH 8 to 10. Each point represents the mean of three experiments ± S.E. Black line represents the sodium acetate buffer, green line represents sodium phosphate buffer, red line represents Tris-HCl buffer, blue line represents glycine-NaOH buffer.

The application of ALDH as a biocatalyst in industries required the enzyme to possess high stability in a wide pH range. Figure 5b shows the effects of pH on the stability of immobilized and free ALDH. Although both immobilized and free ALDH are highly stable at pH 9, their stability trends are far too different. The immobilized ALDH is stable up to 50% relative activity at broader pH (4–11). Meanwhile, the free ALDH is only stable at moderately alkaline pH (8–10). The results suggest that changes in pH become less detrimental to the enzyme structure when the enzyme is immobilized onto support. Covalent bonding and the type of support that contains a functional group at the surface area may contribute to high tolerance to broad pH [34,35]. This finding was in agreement with [36], in which they reported that immobilization can reduce the sensitivity of the enzymes towards acidic and alkaline conditions.

2.3.3. Effect of Organic Solvents of the Immobilized ALDH Stability

Enzymes that can catalyze reactions in an organic solvent environment as well as an aqueous solution can add versatility for industrial biocatalysts. To explore the robustness of immobilized ALDH in organic solvents, the activity was tested in 25% (v/v) and 30% (v/v) of various organic solvents (Figure 6). Previously, Rosli et al. (2022) reported that free ALDH was highly tolerant to several organic solvents. In general, the immobilization technique has been reported to promote improved enzyme stability in organic solvents [16,37]; however, this was not the case for the immobilized ALDH reported in this study. Different organic solvents impart different effects to the activity of immobilized ALDH. 2-propanol, 1-propanol, benzene and octanol were found to reduce the activity of the immobilized ALDH by 90%, 80%, 110% and 60%, respectively. In contrast, the free ALDH was highly activated in the presence of the aforementioned organic solvents, 25% (v/v). This suggests that Seplite LX120 might not be able to maintain its structure under certain organic solvents and therefore disrupt the support–enzyme interaction. On the other hand, immobilized ALDH was activated when treated with 25% (v/v) of DMSO, toluene and n-heptane, by increments of more than 100% of the activity. Interestingly, these solvents are commonly used in the hydrocarbon industry, which is in line with the application of ALDH. The overall trend depicts that the support is able to protect the enzyme from total damage by securing the enzyme conformation at a higher concentration (30% (v/v)) of non-polar organic solvents with higher log p (>2.0). Non-polar organic solvents tend to alter the active site shape for better contact with the substrate [38]. In addition to that, a high concentration of non-polar solvent may damage the interaction between amino acids or the enzyme structure that promotes a hydrophobic environment [39,40].

2.3.4. Effect of Storage and Reusability of the Immobilized ALDH

The stability of the immobilized and free ALDH in retaining the activity during storage at two different temperatures (4 °C and 25 °C) is shown in Figure 7. The immobilized ALDH is able to retain the relative activity for up to 11 weeks (60%) when stored at 4 °C. The enzyme activity began to show a gradual decrease at 11 weeks of storage. In contrast, the activity of free ALDH gradually declined as early as the first week, and a total loss of activity was observed at 4 weeks of storage. At higher temperatures (25 °C), immobilized ALDH retained its activity up to 9 weeks, while the stability of free ALDH became shorter and the activity began to diminish after the first week of storage. In general, it can be concluded that immobilization brought about significant improvement in the storage stability of ALDH. This is in agreement with a study by [5], who reported better stability of immobilized oxidoreductase during prolonged storage. The short half-life of free ALDH at elevated temperatures portrayed the high sensitivity of the free enzyme. Fluctuations in the temperature in the microenvironment also affected the free enzymes of lipase, amylase and xylanase in terms of relative activity, where the activity was reduced by more than 50% [41–43]. Other factors that may cause the loss of enzyme activity include humidity, irregular environment temperatures and light. Moreover, the humidity was reported to decrease the enzyme activity of immobilized enzymes when stored at ambient

temperatures [44]. The presence of water during the storage may result in the disruption of enzyme conformation and lead to aggregation. The unfolded enzyme or aggregation will consequently affect the interaction between the enzyme and support and therefore cause leakage.

The high demand for robust biocatalysts, especially for repetitive and continuous processes, is often addressed by the use of immobilized enzymes. Immobilized enzymes are known for their effective reusability and feasibility. The reusability of immobilized ALDH was evaluated for up to 10 cycles. The immobilized ALDH retained more than 50% of its activity even after seven cycles. The results achieved the purpose of immobilization; that is, to generate biocatalysts with higher reusability rates and make them more interesting to be chosen as industrial enzymes. The decline in the activity after seven cycles may be due to changes in the microenvironment during storage and assay temperatures. This will lead to alterations in enzyme conformation [12]. The changes can also break the van der Waals bonds between the hydrophobic groups of the enzyme and support and cause the enzyme to be detached from the support [45]. This report was in line with ALDH immobilized onto montmorillonite, where the activity dropped to 24% after 5 times of usage [12].

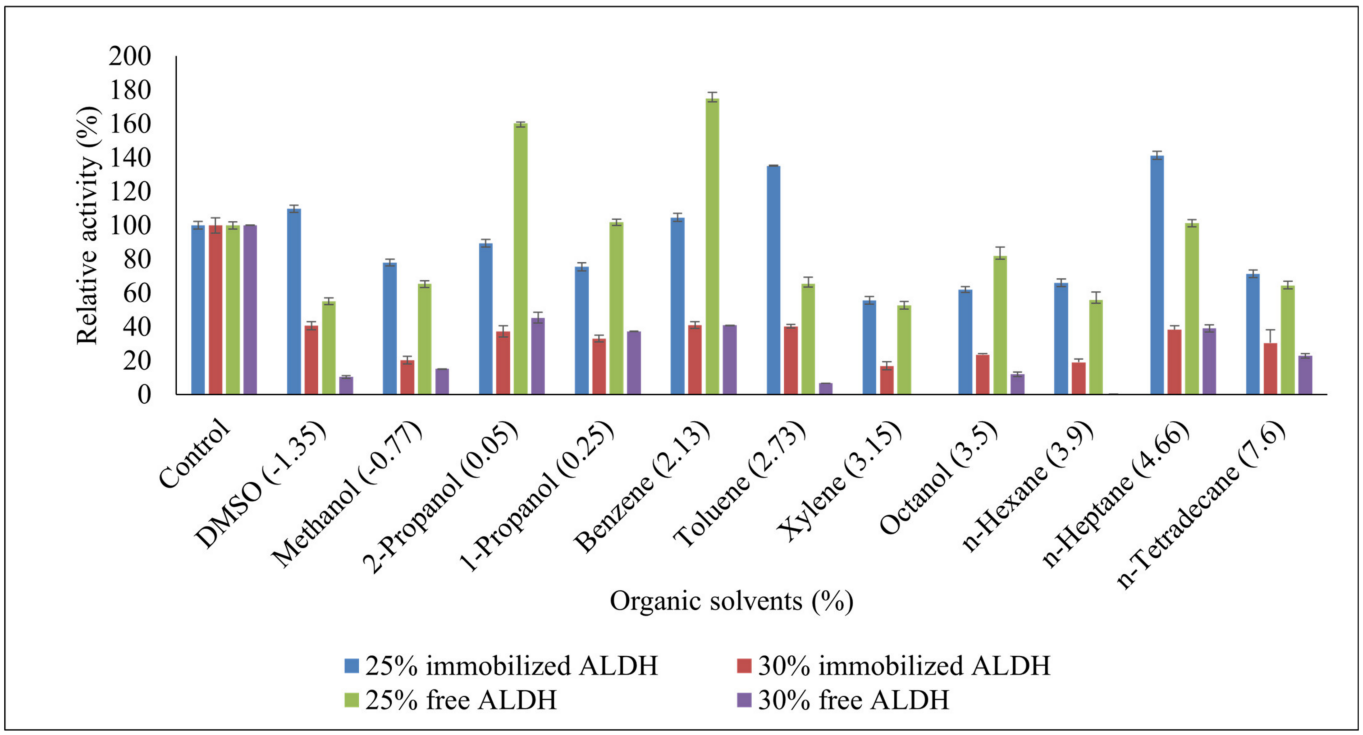
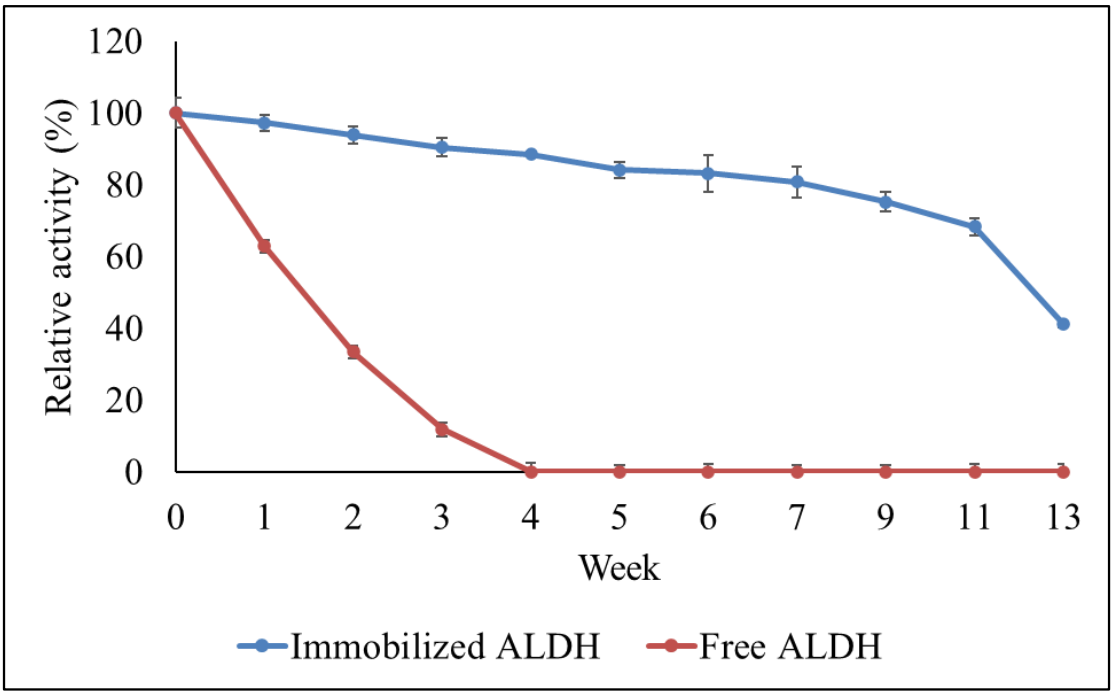


Figure 6. Comparison of effect of organic solvents on the stability of immobilized ALDH and free ALDH. Blue bar: 25% v/v immobilized ALDH; red bar: 30% v/v immobilized ALDH; green bar: 25% v/v free ALDH; purple bar: 30% v/v free ALDH. Each point represents the mean of three experiments ± S.E. The value in the bracket represents the log p-value of the organic solvents.

A



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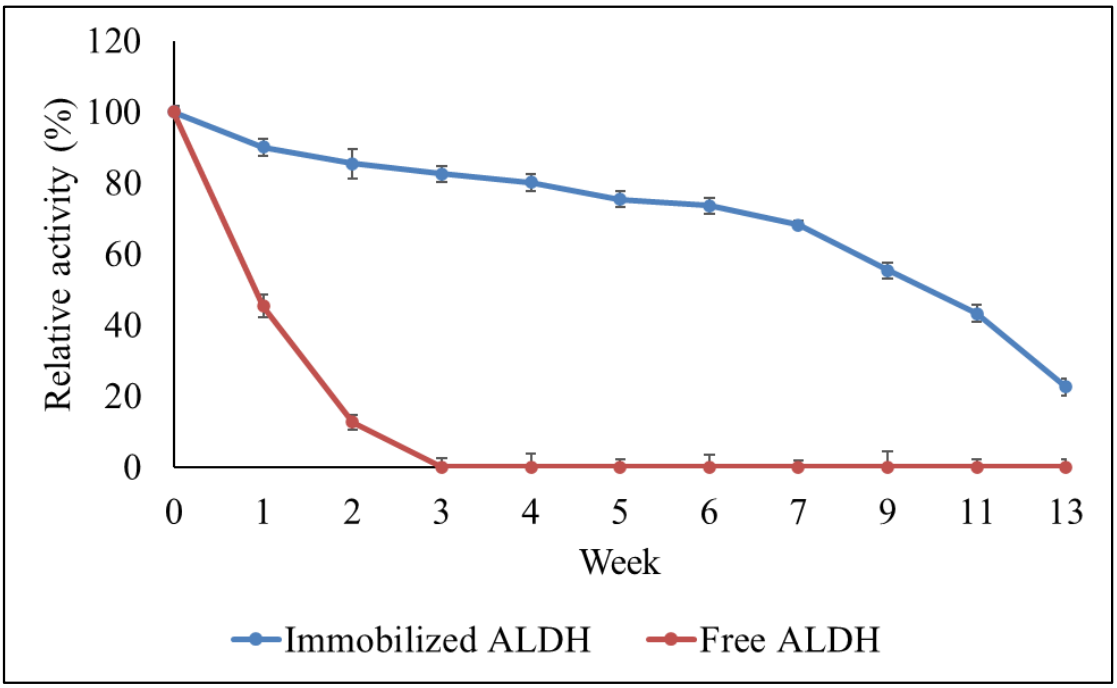

Figure 7. *Cont.*

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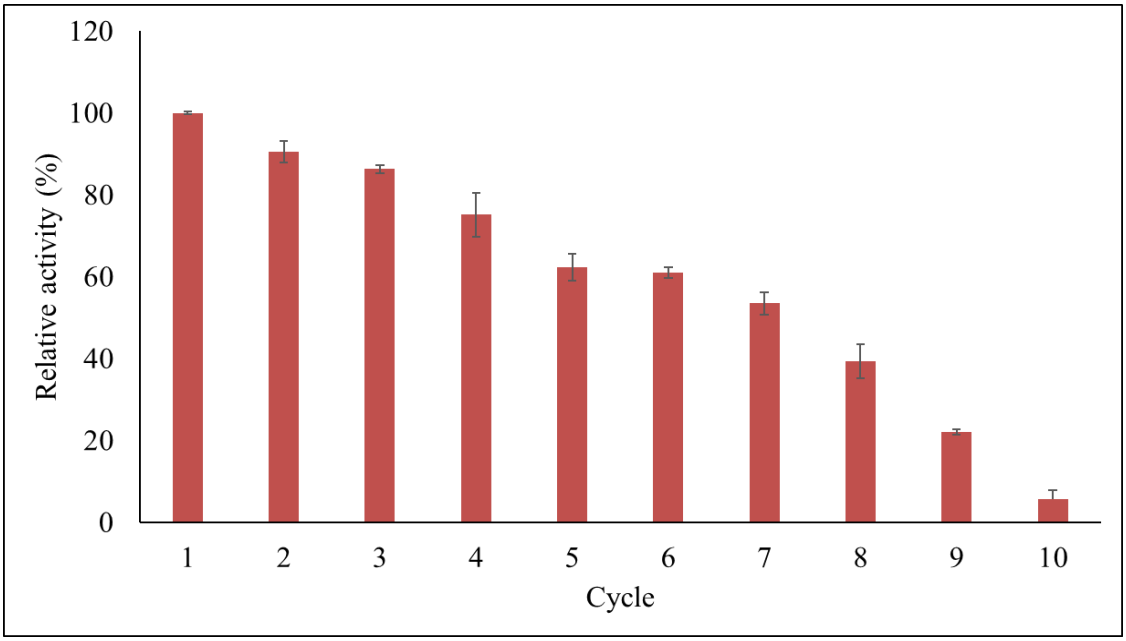

Figure 7. Effect of storage and reusability of the immobilized ALDH and free ALDH. (**A**) Storage stability at 4 °C; (**B**) storage stability at 25 °C; (**C**) reusability test. Each point represents the mean of three experiments ± S.E.

3. Materials and Methods

3.1. Chemical, Reagents and Equipment

Chemicals, reagents and equipment for this project were obtained from Microbial Technology (EMTech) Research Center, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia

3.2. Purification of Recombinant ALDH

The purification method of ALDH was carried out as described by Rosli et al. (2022). A single-step purification method, affinity chromatography, was used to purify recombinant ALDH. The crude ALDH from *E. coli* Transetta (DE3) was obtained by expressing the recombinant ALDH using 0.75 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) at 25 °C for 20 h. The culture was harvested by centrifuging the sample at 10,000 rpm for 10 min. The pellet was resuspended with 20 mL binding buffer containing 35 mM PBS buffer, 300 mM NaCl and 10 mM of imidazole (pH 7.4). The resuspended pellet was sonicated for 4 min and cleared by centrifuge at 10,000 rpm for 10 min. The ALDH was loaded into the column containing Ni-Sepharose resin. Prior to loading, the column (XK 16) was equilibrated with a binding buffer. Then, the enzyme was eluted using 20 mM of PBS buffer, 300 Mm of NaCl and 500 mM of imidazole. The activity for purified ALDH was measured and stored at 4 °C for future experiments.

3.3. ALDH Assay

The assay was conducted using a method from [46] with some modifications [20]. The reaction mixture was composed of 50 mM Tris buffer (pH 8), 20 mM NADH, 50 mM of cinnamaldehyde and 10 µL of the immobilized enzyme. The mixture containing empty support was used as a control. The assay was conducted at 60 °C for 10 min. Then the mixture was measured at 340 nm using the spectrophotometer. One unit of ALDH catalyzes the oxidation of 1.0 mmol reduced nicotinamide adenine dinucleotide, NADH, to the oxidized form, NAD per minute under assay condition.

3.4. Determination of Protein Concentration

Protein concentration was measured using the Bradford assay [47]. It was performed using the commercial reagent from Sigma, Saint Louis, MO, USA. The mixture was measured at an absorbance of 595 nm.

3.5. Immobilization Process

3.5.1. Effect of Adsorption Time

For the adsorption time optimization, 10 mL of purified ALDH containing 1.0 mg/mL protein was mixed with the 1 g of Seplite LX120 and was stirred at different hours from 1 h, 2 h, 3 h, 4 h, 5 h and 6 h at room temperature. The supernatant obtained from the sampling was assayed based on the ALDH activity assay. The immobilized ALDH was filtered and recovered using a Buchner funnel with vacuum filtration. The wet immobilized enzyme was dried using Fluid Bed Dryer. The immobilization yield and activity of the immobilized enzyme were calculated using the following formula:

$$\text{Immobilized enzyme activity (U/mL)} = \frac{(\text{Absorbance of control} - \text{Absorbance of enzymes})/\text{Gradient of NADH curve}}{\text{Incubation time} \times \text{weight of immobilized enzyme}} \quad (1)$$

$$\text{Immobilization yield (U/g)} = \frac{\text{Initial protein concentration} - \text{initial protein concentration in supernatant}}{\text{Initial protein concentration}} \times 100 \quad (2)$$

3.5.2. Effect on the Amount of Enzyme Loading

Determination of best enzyme loading onto the support was performed by varying the protein concentration of purified ALDH protein (0.25 mg/mL, 0.50 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.25 mg/mL and 1.50 mg/mL). Approximately 10 mL of purified ALDH containing different protein concentrations was mixed with 1 g of Seplite LX120 and stirred for 1 h at room temperature. The protein concentration of the supernatant was calculated using Bradford and assayed based on the ALDH activity assay. The immobilized ALDH was filtered and recovered using a Buchner funnel with vacuum filtration. The wet immobilized enzyme was dried using Fluid Bed Dryer.

3.6. Confirmation Study ALDH Immobilize onto Seplite LX120

3.6.1. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

The structural study of immobilized ALDH was performed using FTIR. The presence of a functional group from the free ALDH, immobilized ALDH and empty support (Seplite LX120) was determined. The analysis was carried out at the resolution of 4 cm^{-1} between 4000 and 500 cm^{-1} with an average of 32 scans using FTIR spectrometer Nicolet 6700, Thermo Scientific at Institute of Tropical Forestry and Forestry Products (INTROP), Universiti Putra Malaysia.

3.6.2. Scanning Electron Microscopy (SEM) Analysis

The morphology of the empty and immobilized ALDH was investigated by using SEM. The analysis was carried out using JSM-IT200 InTouchScopeTM (JEOL, Tokyo, Japan). The supports were mounted on the specimen stub and dried overnight to remove any moisture on the samples. Then, the sample was coated with gold before being scanned by SEM. The morphologies and shapes of the supports were observed at various magnifications (20×, 5000× and 5000×).

3.6.3. Surface Area and Porosity Analysis

The surface area characterization of the empty Seplite LX120 and immobilized ALDH was carried out by nitrogen gas adsorption–desorption at 150 °C, and the surface area was calculated using the Brunauer–Emmett–Teller (BET)method. The pore volume and

pore size were calculated using the Barrett–Joyner–Halenda (BJH) method included in the MicroActive TriStar II Plus 2.03 surface area analyzer.

3.7. Biochemical Characterization of Immobilization ALDH

3.7.1. Effect of Temperature on the Immobilized ALDH Activity

The effect of temperature on immobilized ALDH was determined at 30 to 90 °C with 10 °C intervals for 10 min. The immobilized ALDH activity was determined based on the method mentioned in Section 3.5.1. The activity of immobilized ALDH was calculated and compared with the activity of free ALDH.

3.7.2. Effect of Temperature on the Immobilized ALDH Stability

For the temperature stability of immobilized ALDH, the immobilized ALDH was pre-incubated at different temperatures ranging from 30 to 90 °C with an interval of 10 °C for 30 min. Then, the treated ALDH was assayed for 10 min at 80 °C (optimum temperature) using cinnamaldehyde as a substrate. The activity of immobilized ALDH was calculated and compared with the activity of free ALDH.

3.7.3. Effect of pH on the Immobilized ALDH Activity

The effect of pH on immobilized ALDH activity was performed by using different buffers replacing the 1M Tris-HCl (pH 8). The buffer systems involved were sodium acetate (pH 4.0–6.0), phosphate buffer (pH 7–9), Tris-HCl (pH 8.0–9.0), glycine-NaOH (pH 9.0–11.0) and sodium phosphate (pH 11.0–12.0). The assay was conducted at 80 °C for 10 min. The activity of immobilized ALDH was calculated and compared with the activity of free ALDH.

3.7.4. Effect of pH on the Immobilized ALDH Stability

The pH stability test was performed by pre-incubating the immobilized ALDH with various pHs for 30 min at 70 °C. The buffer systems involved were sodium acetate (pH 4.0–6.0), phosphate buffer (pH 7–9), Tris-HCl (pH 8.0–9.0), glycine-NaOH (pH 9.0–11.0) and sodium phosphate (pH 11.0–12.0). The assay was conducted at 80 °C for 10 min. The activity of immobilized ALDH was calculated and compared with the activity of free ALDH.

3.7.5. Effect of Organic Solvents on the Immobilized ALDH Activity

A stability study of the immobilized ALDH towards different organic solvents was conducted. The immobilized ALDH was pre-incubated with the mixture of pH 8 buffer and 25% v/v and 30% v/v of organic solvent for 30 min at 70 °C. The pre-incubated enzymes were then assayed for enzyme activity. The same procedure was applied to the free enzyme. The untreated enzyme was assigned a value of 100% activity. The assay was conducted at 80 °C for 10 min. The activity of immobilized ALDH was calculated and compared with the activity of free ALDH.

3.7.6. Effect of Storage and Reusability of Immobilized ALDH

Effect of Storage Temperature on the Immobilized ALDH Stability

The storage stability of immobilized ALDH was studied under the following storage conditions; 4 °C and ± 25 °C (room temperature). The activity of immobilized ALDH was measured every week until the remaining ALDH activity was at 50% of the initial activity.

Reusability Test of the Immobilized ALDH

The reusability test of the immobilized ALDH was determined by assaying the enzyme sample for up to 10 cycles. The enzyme activity assay was conducted in the above section and incubated at the optimum temperature. After a cycle of the activity assay, the reaction medium and immobilized ALDH were separated by centrifugation. The immobilized ALDH was washed and resuspended with 50 mm Tris-HCl buffer to remove any residual

cinnamaldehyde substrate. The immobilized enzyme was dried using a fluid bed dryer and kept at 4 °C. The process was repeated 10 times. Enzyme activity of the first cycle was considered to be 100% and served as a control.

3.8. Statistical Analysis

All experiments were performed in triplicate, and the results reported as mean of the value and the standard deviation was calculated using Excel spreadsheets available in Microsoft Excel.

4. Conclusions

As a conclusion, ALDH from *Anoxybacillus geothermalis* strain D9 was successfully immobilized on the Seplite LX120 via physical adsorption. The immobilization of the ALDH requires 4 h with 1.25 mg/mL of enzyme to reach maximum loading. The confirmation of ALDH immobilized onto Seplite LX120 was performed using FTIR, SEM and the reduction in pores and surface area. The immobilized ALDH showed an improvement in thermal stability towards low and high temperatures. The ability of immobilized ALDH to withstand a broad buffer system from pH 5 to 11 is an interesting feature that can be exploited further. Moreover, the immobilized ALDH is able to retain more than 50% of the enzyme activity after being stored at 4 °C for 11 weeks and can be reused for up to seven cycles. The immobilized ALDH was proven to be more stable compared to the free ALDH and has the potential for further improvement in industrial applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal13020368/s1, Figure S1: Schematic diagram: How immobilization is able to enhance the thermostability of the ALDH toward temperature.

Author Contributions: R.N.Z.R.A.R. and N.H.A.K. designed the study. N.E.R. performed the experiment; R.N.Z.R.A.R., N.H.A.K., M.S.M.A. and W.L. provided critical revision and data analysis. W.L. prepared the manuscript. R.N.Z.R.A.R., N.H.A.K. and W.L. provided final approval of the manuscript. All authors have read and agreed to the published version of the manuscript.

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