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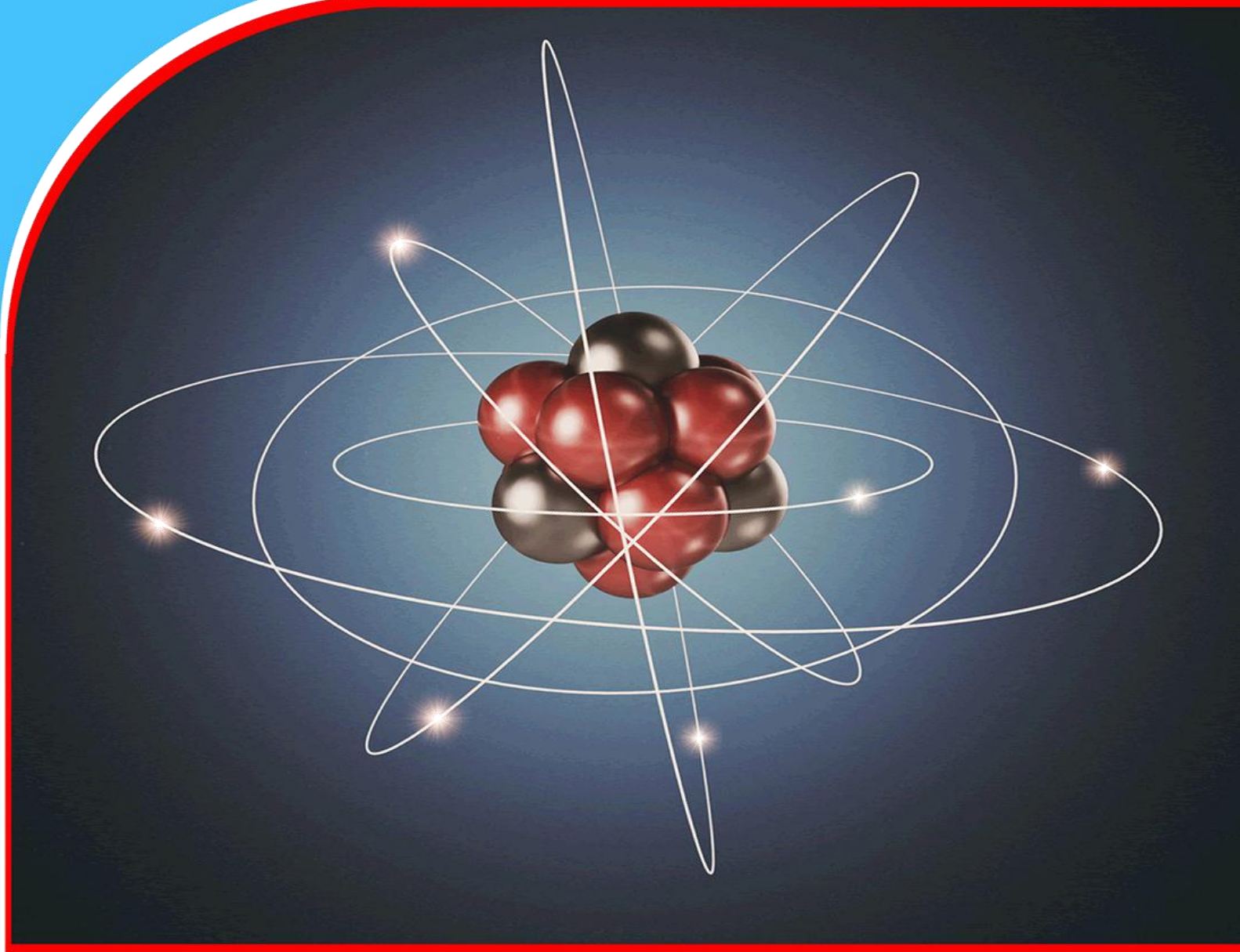

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ABSTRACT

Purpose: The purpose of the study was to establish the optimal conditions required for the attachment of Candida rugosa lipase (CRL) onto silica extracted from ash of acid treated oil palm leaves, for maximum catalytic efficiency.

Methodology: Six different concentrations of CRL solution ranging from 1 mg/mL to 6 mg/mL, immobilization time of 4, 8, 12, 16, 20 and 24 h as well as immobilization temperature of 4, 25, 30, 35 40 and 45 ℃ were independently investigated. In this study, the parameter to be investigated was varied while others were fixed. The effectiveness of the immobilization protocol were assessed using four catalytic parameters – protein loading, immobilization yield, specific activity and ester yield. Statistical analysis was performed using one way ANOVA (IBM SPSS -20.0) software while significant differences within ranges in a parameter, if any was given as $p < 0.05$.

Findings: The study revealed that the optimal values of concentration of CRL solution, immobilization time and immobilization temperature required to immobilize CRL onto SiO_2-MNPs derived from oil palm leave were 5. 0 mg/mL, 16 h and 25 ℃ respectively. At this optimal conditions, protein loading (33.3, 38.1, 20.5 mg/g), immobilization yield (57.8, 70.0, 59.0 %), specific activity (74.6, 63.5, 72.2 U/g) and ester yield (85.0, 74.1, 85.5 %) respectively were achieved.

Recommendation: Optimization of the immobilization protocol for immobilizing CRL onto silica support extracted from the highly abundant oil palm leave – an agricultural biomass, will not just produce a biocatalyst of with high catalytic efficiency but would circumvent the environmental pollution arising from dumping of large quantities of the biomass into the ecosystem. It is recommended from the findings of this study that 5.0 mg/mL CRL solution be immobilized onto glutaraldehyde activated SiO_2-MNPs support matrix derived from oil palm leave for 16 h at 25℃.

Keywords: *Immobilization, Oil palm leave, Optimal, Silica*

1.0 INTRODUCTION

CRL is a versatile biocatalyst used widely for biotransformations in industries, particularly for the production of biodiesel (Adachi *et al.*, 2013). Its vast industrial usage is associated with several unique features of the lipase, such as, stereospecificity, stability in organic solvents, thermal stability, high enantioselectivity and its ability to catalyse a wide range of reactions (McCabe *et al*., 2005). Immobilization of CRL onto various support matrixes and its utilization for catalyzing several reactions such as hydrolysis, esterification, interesterification and transesterification had been well documented (Elias *et al.*, 2017). Catalytic efficacy of CRL would be greatly enhanced by its immobilization, as this helps to overcome the severe drawbacks associated with the use of CRL in its free form. Findings of earlier studies have indicated that CRL in its free form suffers rapid deactivation under extreme industrial settings such as high temperatures and extreme pH (Manan *et al.*, 2018; Sheldon and van Pelt, 2013).

1.1 Statement of the Problem

Hitherto, literatures on the assessment of immobilization parameters of *Candida rugosa* lipase (CRL) onto silica coated magnetite of oil palm leave origin that will give optimal catalytic activity is yet to be seen. Oil palm leaves – an agricultural biomass produced in large quantities in countries like Malaysia, Indonesia and Nigeria have been indiscriminately disposed, resulting in environment hazard. Earlier researchers (Onoja *et al.*, 2018) have published their findings on the utilization of silica extracted from acid treated ash of oil palm leaves for CRL immobilization. However, a detailed assessment of immobilization parameters such as immobilization time, temperature as well as concentration of CRL solution required to give the most effective CRL/SiO_2-MNPs biocatalyst has not been documented. The present study was prompted by the quest to establish optimum conditions for immobilization parameters.

1.2 Objective of the Study

The objective of the present study is to assess the optimal conditions, using three parameters – immobilization time, immobilization temperature and concentration of CRL solution needed for attaching CRL onto silica-magnetite support derived from oil palm leave acid treated ash.

2.0 LITERATURE REVIEW

2.1 Theoretical Framework

It is known that only an insignificant percentage of oil palm biomass has been converted into useful bio-products for various industrial applications. These include; nanocellulose (Elias *et al.*, 2017), biogas (Chaikitkaew *et al.*, 2015), cellulose nanocrystal (Chieng *et al.*, 2017), bio-composite (Abdulrazik *et al.*, 2017) and biofuel (Kurnia *et al.*, 2016). In view of the circumstances, it is evident that natural polymers present in large quantities in oil palm leave (OPL) are highly underutilized. According to literature, OPL is a rich source of cellulose, hemicellulose and lignin with substantial quantity of ash that is rich in silica (SiO_2) (Samiran *et al.*, 2015). Likewise, recent studies have reported on the extraction of nanocellulose as well as SiO_2 from OPL (Elias *et al.*, 2017; Onoja *et al.*, 2017). From the industrial and biotechnological perspectives, OPL ash appears to be a promising renewable source of silica. It is a versatile material that can be converted into secondary raw materials or composites useful for manufacturing purposes. In view of this, scientific researches that explore the inexpensive and renewable SiO_2 present in OPL as intermediates for preparing value-added products, appears relevant.

2.2 Empirical Review

Silica (SiO_2) has been rated high amongst the available inorganic support materials used for enzyme immobilization. The compound has found technological importance for a myriad of applications due to its high thermal and mechanical stability and rigidity (Hartmann and Kostrov, 2013; Onoja *et al.*, 2018). This has somewhat to do with the abundance of surface polar groups on SiO_2 i.e. silanols (Si–OH) and siloxanes (Si–O–Si). These functional groups are easily converted into functional biomaterials for lipase immobilization, hence one of the few reasons for SiO_2 being a popular choice of support (Hung *et al.*, 2015).

So far, studies on SiO_2 have largely resorted to using SiO_2 sourced from tetraethyl orthosilica (TEOS) as adsorbent for biomaterials, filler in polymer industry and support for the immobilization of enzymes (Hung *et al.*, 2015). Nonetheless, concerted efforts focusing on acquiring greener and sustainable source of SiO_2 from bio-based materials i.e. agricultural biomass have significantly gained momentum over the past decade. This development has focused on the use of agricultural wastes as renewable sources of SiO_2. This is a conceivable feat as a myriad of agricultural biomass sources are available all year round and, contribute to low carbon dioxide release (Ghani *et al.*, 2010).

Lipase is one of the several technologically relevant enzymes owing to its broad specificity and high activity. Specifically, this study used *Candida rugosa* lipase (CRL), a versatile enzyme known for its general ability to catalyze a number of important reactions. Among the reactions that CRL catalyzes are hydrolysis, transesterification, esterification and interesterification (Elias *et al.*, 2017). Considering the wide commercial utilization of CRL, its physical modification may prove useful, as the free form of CRL is rapidly deactivated under extreme industrial settings (Sheldon & van Pelt, 2013).

Interestingly, the focus on investigating the optimal conditions necessary for immobilizing CRL on a SiO_2-based support extracted from OPL ash, followed a well-reported compatibility for supporting proteins or enzymes (Onoja and Wahab, 2020). Past studies have mostly resorted to using mesoporous SiO_2-based matrices for supporting enzymes, prepared from the hydrolysis of tetraethyl orthosilicate (TEOS) (Meléndez-Ortiz *et al.*, 2013). In this milieu, for this study to feasibly consider the use of renewable SiO_2 sourced from agricultural biomass as CRL support, innovative techniques of extracting SiO_2 from OPL at quantities exceeding and effective attachment of CRL onto this support must, therefore, be established.

Additionally, a well-executed immobilization protocol would heighten the structural stability of CRL and enhance its lipase activity, while prolonging the half-life of CRL. The technique would be greener, too, as it allows the repeated use of the biocatalysts, hence an avenue for possible cost reduction. Reduced enzyme inhibition and the ability for high repeated use of CRL for successive esterification or hydrolytic reactions would be highly advantageous. In fact, these are among the key considerations in devising appropriate enzyme immobilization protocol (Rodrigues *et al.*, 2013). Meeting these requirements can favorably result in higher interests of manufacturers into adopting newer and greener technology for large-scale manufacturing activities.

3.0 RESEARCH METHODOLOGY

3.1 Preparation of Oil Palm Leave SiO_2-MNPs Support

The method of Onoja and Wahab, (2019) was adopted with slight modifications, for the preparation of oil palm leave SiO_2-MNPs support. In this study, freshly prepared magnetite nanoparticles (MNPs) (1.0 g) were dispersed in ultrapure water (5.0 mL), acidified with 3.0 M HCl (1 mL) and sonicated for 15 min. In a separate plastic vial, 3.0 M HCl solution (1.0 mL) was added to sodium silicate solution (25.0 mL) previously extracted from oil palm leave acid treated ash, to lower the pH of solution to pH 12.0. The acidified sodium silicate solution was transferred to the MNPs solution and further sonicated for 15 min before transferring into a two-necked round bottom flask equipped with magnetic stirrer (200 rpm) and heated at 30℃. Hexadecyl trimethyl ammonium bromide (Sigma-Adrich, USA) (1.0 g), dissolved in ultrapure water (40.0 mL) was transferred into the flask with the temperature raised to 85 ℃ at rising temperature of 1 ℃/min. Ethyl acetate (QReC, New Zealand) (3.5 mL) was rapidly added to the mixture and stirred at 500 rpm for 15 min. the mixture was cooled at room temperature. The resultant SiO_2-MNPs was then functionalized and activated with 3-aminopropytriethoxysilane (APTES) and glutaraldehyde. The method described by Onoja *et al.,* (2018) was adopted for both product estimation and statistical method.

3.2 Optimization of Immobilization Protocol

To investigate the efficiency of CRL immobilization onto SiO_2-MNPs (CRL/SiO_2-MNP), three parameters which include i) concentration of initial CRL solution, ii) immobilization time and iii) immobilization temperature were investigated. Efficacy of the immobilization protocol was assessed based on the yield of butyl butyrate produced in the CRL/SiO_2-MNP-catalyzed enzymatic synthesis.

3.3 Effect of CRL Concentration on Immobilization

The concentration of bound CRL onto glutaraldehyde activated SiO_2-MNPs support after immobilization was investigated using CRL solutions of various initial concentrations ranging from 1.0 mg/mL to 6.0 mg/ml. Immobilization was carried out on the 4 % (v/v) SiO_2-MNPs, (0.5 g) using supernatant from 1 mg/mL CRL solution, while variables *viz.* temperature, stirring rate and time of immobilization were kept constant. The same procedure was repeated for the 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, 5.0 mg/mL and 6.0 mg/mL CRL solutions, respectively.

3.4 Effect of Immobilization Time

Effect of time was investigated by monitoring the progress of the enzymatic esterification for up to 4 h of immobilization using an optimised enzyme concentration. The procedure was repeated for incubation durations, 8−24 h, with every 4 h increments, while other factors were maintained constant.

3.5 Effect of Immobilization Temperature

The effect of immobilization temperature was investigated for the following temperatures: 4, 25, 35, 40 and 45℃, using an optimized lipase concentration while other factors were held constant.

4.0 PRESENTATION OF FINDINGS, ANALYSIS AND INTERPRETATION

4.1 Effect of CRL Concentration

Assessing the optimum enzyme concentration for any immobilization studies is imperative as the cost of enzymes can contribute substantially to the high cost of immobilized enzyme preparation (Kuperkar *et al.*, 2014). The effect of CRL concentration on CRL immobilization onto Gl-A-SiO_2-MNPs was investigated and the data are tabulated in table 1.

Table 1: Assessments on the effects of CRL concentration on protein loading and immobilization yield.

	Lipase concentration (mg/mL)	Protein loading (mg/g)	Immobilization yield (%)	Specific activity (U/g)	Ester yield (%)
1	1.0	15.0	54.1	33.3	41.1
2	2.0	18.5	53.6	42.9	52.9
3	3.0	20.8	54.3	61.9	76.5
4	4.0	21.3	55.2	65.1	80.6
5	5.0	33.3	57.8	74.6	85.5
6	6.0	24.9	57.1	71.4	81.8

Immobilization conditions: 25^{o}C, 16 h, 180 rpm. Efficacy of CRL/SiO_2-MNPs in catalysing the enzymatic esterification was assessed for parameters specific activity and yield of butyl butyrate.

Results revealed that elevating CRL concentrations exerted a positive effect on the amount of protein bound to the surface of glutaraldehyde activated oil palm leave derived SiO_2-MNPs support. There was significant difference ($p < 0.05$) in the immobilization efficiency as the concentration of CRL solution changes from 1 mg/mL to 5 mg/mL. Further increasing the lipase concentration was counterproductive as demonstrated by a corresponding decrease in the values of all the parameters assessed. The optimal concentration of CRL (5 mg/mL) gave the highest enzyme loading at 33.3 mg/g, followed by 6, 4, 3, 2 and 1 mg/mL that corresponded to decreasing protein loadings of 24.9, 21.3, 20.8, 18.5 and 15 mg/g respectively.

Most importantly, the results conveyed the direct correlation on the use of high concentrations of CRL to produce high protein loadings on SiO_2-MNPs derived from oil palm leaves. Moreover, a large number of free CRL protein in solution would also increase the likelihood of effective collisions between the $-NH_2$ groups on CRL with the C=O groups on SiO_2-MNPs. This outcome seen here is concomitant with the ascending trend of immobilization yield and high specific activity of the resultant CRL/SiO_2-MNPs.

Since more CRL proteins are rendered available to bind to the support, maximum immobilization and ester yield as well as specific activity were attained at 57.8 %, 85.5 % and 74.6 U/g, monitored over the course of 3 h (Table 1, entry 5). In contrast, using the least concentration of CRL of 1 mg/mL (Table 1 entries 1, column 3) was consistent with the observably lowest protein loading, 15 mg/g and immobilization yield, 54.1 % (table 1; entries 1, column 4). The data seen here for

CRL/SiO_2-MNPs therefore, support previous study (Xie & Ma, 2010) which describes that using low concentration of enzyme during immobilization process led to production of immobilised biocatalysts showing low activity. Results of the enzymatic esterification using a constant enzyme loading of 3.5 mg/g for all reaction mixtures, to produce butyl butyrate are also shown in table 1.

The results clearly implied a direct correlation between the initial concentration of CRL with specific activity of the resultant CRL/SiO_2-MNPs and the percent yield of butyl butyrate (Table 1). It was evident that the developed biocatalyst exhibited varying specific activities and yields of butyl butyrate that ranged between 33.3−74.6 U/g and 41.1−85.5 %, respectively. The best specific activity of 74.6 U/g (Table 1 entries 5, column 5) and percent butyl butyrate of 85.5 % (Table 1 entries 5, column 6) were attained when 5 mg/mL of CRL was used. The high degree of conversion of the ester hence, supports the literature describing the use of suitably high concentrations of enzymes which can improve yield of the desired product (Xie and Ma, 2010). The findings in this study agreed with results of similar work by Fonseca *et al.*, (1993). Based on the data, it was affirmed that an optimal protein loading is 5 mg/mL afforded the best result, hence the protocol was applied in the subsequent work.

4.2 Effect of Immobilization Time

In general, the use of CRL immobilization time between 12−20 h largely gave rise to CRL/SiO_2-MNPs exhibiting higher protein loading and immobilization yield (table 2). Protein loadings and their corresponding immobilization yields for the assessed immobilization times 4−24 h was found to range between 17.7−28.1 mg/g and 46.0−70.0 %, respectively. The highest protein loading (28.1 mg/g) and immobilization yield (70.0 %) were attained following a 16 h immobilization (Table 2 entries 4, columns 3 and 4). The data imply that a relatively long immobilization time is required to allow sufficient contact time for covalent bonds to form between CRL and the glutaraldehyde activated oil palm leave SiO_2-MNPs supports. This observation concurred with a recent finding (Manan *et al.*, 2018). However, ANOVA result shows that there was no significant difference ($p > 0.05$) between the different immobilization times for all the parameters investigated, except for the immobilization time of 24 h.

Correspondingly, assessment of CRL/SiO_2-MNPs catalysed esterification revealed that the biocatalyst catalysed with specific activities and yields of butyl butyrate that generally ranged between 58.7−65.1 U/g and 66.1−74.1 %, respectively. Maximum yield of butyl butyrate was attained for a 12 h immobilization time whereas the highest specific activity of 65.1 U/g (Table 2 entries 5, column 5) was obtained following a 20 h immobilization duration. Remarkably, the use of a 24 h CRL immobilization duration (Table 2 entry 6) gave the worst results in all assessed factors. The study believed this can be attributed to denaturation of CRL protein, resulting from prolonged exposure to extended mechanical stress of stirring.

Table 2: Assessments on the effects of immobilization time on protein loading and immobilization yield.

	Immobilization time (h)	Protein loading (mg/g)	Immobilization yield (%)	Specific activity (U/g)	Ester yield (%)
1	4	26.5	68.9	60.3	73.1
2	8	25.5	66.2	60.3	67.9
3	12	27.9	69.3	63.5	74.1
4	16	28.1	70.0	63.5	74.1
5	20	26.0	67.6	65.1	73.2
6	24	17.7	46.0	58.7	66.1

Immobilization conditions: 25°C, 5 mg/mL, 180 rpm. Efficacy of CRL/SiO_2-MNPs in catalysing the enzymatic esterification was assessed for parameters specific activity and yield of butyl butyrate.

In terms of leaching, the study found that the use of an 8 h immobilization time was ideal to reduce the leaching of CRL/SiO_2-MNPs from the support. Leaching constant was seen consistent at 7.3 % for immobilization durations of 8−24 h, as compared to 12.5 % to a 4 h lipase immobilization time. The low leaching constant observed for immobilization times of 8–24 h is attributed to firm attachment of CRL onto glutaraldehyde activated oil palm leave SiO_2-MNPs by multipoint covalent bonds. Although an extended immobilization duration is said to allow formation of covalent bonds between lipase and the support, the results seen in this study, have shown otherwise.

Contrastingly, it was revealed that a short contact time of 4 h was insufficient to allow the adequate formation of covalent bonds between the CRL and SiO_2-MNPs. Presumably, a short incubation time would likely result in lipases being largely adsorbed to the surface of SiO_2-MNPs by weak non-specific interactions, instead of forming covalent bonds, hence are easier dislodge. The results obtained in this study closely agreed with findings of similar studies by Mendes *at al.*, (2011). Following this observation, the study chose an immobilization time of 12 h as the optimum duration for immobilising CRL onto SiO_2-MNPs. While it was shown that a 16 h incubation time yielded marginally higher protein loading, the similar lipase activity (63.5 %) and yield of butyl butyrate (74.1 %) seen for the 12 h incubation, appears economically more feasible.

4.3 Effect of Immobilization Temperature

Stability and activity of an enzyme are imperative parameters to be considered when dealing with enzymatic reactions. Results showing the effect of various temperatures on protein loading and immobilization yield in preparing highly functional CRL/SiO_2-MNPs are presented in Table 3. It is a known fact that for most chemical reactions, increasing the temperature of reaction would increase the rate of reaction (Arcus *et al.*, 2016). However, lipases are prone to destabilisation when subjected to high temperatures by thermally induced enzyme inactivation. Hence, determination of a suitable immobilization temperature is essential to achieve a high enzyme

activity while achieving a high enzyme loading. As such, the study must determine the optimum temperature for preparation of CRL/SiO_2-MNPs to maintain the high CRL activity for an extended duration. The immobilization temperatures assessed in this study afforded protein loadings that ranged between 13.2−20.5 mg/g, and immobilization yields between 41.6−59 percent.

Table 3: Assessment of the effect of immobilization temperature on protein loading and immobilization yield.

	Immobilization temperature (℃)	Protein loading (mg/g)	Immobilization yield (%)	Specific activity (U/g)	Ester yield (%)
1	4	15.3	44.1	54.0	61.8
2	25	20.5	59.0	72.2	85.5
3	35	19.2	55.2	69.5	85.5
4	40	13.7	42.0	50.8	83.6
5	45	13.2	41.6	50.8	83.8

Immobilization conditions: 6 mg/mL, 12 h, 180 rpm. Efficacy of CRL/SiO_2-MNPs in catalysing the enzymatic esterification was assessed for parameters specific activity and yield of butyl butyrate.

It was clear that immobilization protocol carried out at 25 ℃ afforded the best protein loading and immobilization yield that corresponded to 20.5 mg/g (Table 3 entries 2, column 3) and 59.0 % (Table 3 entries 2, column 4), as compared to other tested immobilization temperatures. In contrary, the highest immobilization temperature (45 ℃) gave the lowest protein loading at 13.2 mg/g that corresponded to 41.6 % immobilization yield. A significant difference ($p < 0.05$) existed between the parameters assessed at the various temperatures investigated for the immobilization process, as indicated by ANOVA result. This means the parameter of immobilization temperature does have a significant impact influencing a high immobilization yield. Considering the economics of process, CRL immobilization carried out at ambient temperature, is more desirable because no additional cost on energy is required to sustain such condition within the immobilization system.

In terms of reaction rate of the produced immobilised CRL, reaction rate for CRL/SiO_2-MNPs prepared at 25 ℃ was evidently the highest. A high specific activity of 72.2 U/g (Table 3 entries 2, column 5) and 85.5 % (Table 3 entries 2, column 6) of butyl butyrate was attained, thus implying good correlation between the use of ambient immobilization temperature to result in high lipase activity. As can be seen, employment of immobilization temperatures beyond 25 ℃ resulted in the corresponding decrease in values of all parameters investigated. This is because the stability of CRL during immobilization tend to decline with every increase in immobilization temperature. Such an outcome is likely due to denaturation of CRL protein structures at higher temperatures, which explains the observed decline in specific activities and percent yield of butyl butyrate.

Moreover, it has been indicated in literature that hydrophobic interactions between the nucleophilic -NH_2 group of the protein and the electrophilic groups on the surface of supports i.e. SiO_2-MNPs are increased at relatively high temperatures (Arica *et al.*, 2001). Kinetic energy of the reacting

molecules would increase with increase in temperature of the reaction system, with consequent effect of attachment of more lipases onto the support. Consequently, the high temperatures tend to cause more buried hydrophobic amino acid residues within CRL to become exposed to the hydrophobic groups on SiO_2-MNPs. Such structural changes can further elevate hydrophobic interactions between CRL and SiO_2-MNPs (Arica *et al.*, 2001). Essentially, these interactions are likely the reason behind the low values for all the tested parameters (Table 1 entries 1, columns 3, 4, 5 and 6) observed when the immobilization was carried out at 4℃. Hence, it was demonstrated that 25 ℃ is the optimal temperature to immobilise CRL onto glutaraldehyde activated oil palm leave SiO_2-MNPs.

5.0 SUMMARY, CONCLUSIONS AND RECOMMENDATION

5.1 Summary of Findings

This study demonstrated that the optimized immobilization conditions required to effectively attach CRL onto oil palm leave SiO_2-MNPs that achieved approximately 80 % immobilization yield of CRL onto SiO_2-MNPs were 5.0 mg/mL CRL solution stirred for 12 h at 25 ℃. The activated.

5.2 Conclusion

This study showed that at immobilizing CRL onto glutaraldehyde activated SiO_2-MNPs derived from oil palm leave at the optimal conditions of 5.0 mg/mL CRL solution stirred for 12 h at 25 ℃. The activated.

5.3 Recommendation

Based on the findings of this research work, it is recommended that CRL be immobilized onto silica-base support matrix at the stated optimized values for optimum performance of the biocatalyst.

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