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Copper dependence, Active-Site residues, and immobilization of LYSYL oxidase from penicillium chrysogenum

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Abstract

Lysyl oxidase (LOX, EC: 1.4.3.13) is a copper-inclined enzyme consisting of wider biotechnological and biomedical essence. In this paper, LOX from Penicillium chrysogenum was cleaned to contain homogeneous element to attain a special activity of 314 Umg-1 of protein and a 20-fold means of purification, to demonstrate the efficacy of the utilized isolation plan. SDS-PAGE examination to reveal a molecular weight of nearly 45KDa. The Metal-ion examination indicated that Cu^{2+} with strong activation of LOX at both IMM and 5 MM and 5MM, to confirm the essence of the function of a cofactor, while activated LOX at both 1mM and 5 mM, Co^{2+} and Zn^{2+} evidently repressed the enzyme function. Ca^{2+} and Mn^{2+} applied inhibitory impacts at 5 mM, while Mg^{2+} indicated a slim effect. Chemical changes experimentation showed that showed histidyl and tyrosyl deposits are important for LOX catalysis. The kinetic examination showed that about two molecules of Diethylpyrocarbonate (DEPC) as well as one of the molecules of trinitromethane (TNM) may react with each of the enzyme molecule. To support the highest immobilization efficacy at (95%), to be followed by chitosan (87%) and chitin (69%). The restrained enzyme as shown the enhanced operative steasdiness, to retain 70% of its early function after te eight reuse cycles. In general, these results may contribute to the treasured insights into the biochemical elements, and catalytic demands, and application possibilities of fungal LOX. Carregenan was much better than the chitosan and chitin for enzyme halt. The optimum pH rate for the free and immobilized LOX contains values to immobilized LOX and 7 and 8 correspondingly. However, the optimum temperatures were 40°C and 50°C.

Keywords: Copper dependence, Active-Site residues, Immobilization of LYSYL oxidase, Penicillium chrysogenum

Introduction

Many biochemical reactions in living system have been catalysed by specific enzymes. Enzymes are biological catalysts with specific reaction rate and specificity for adaptation of specific substratum into projected artefact molecules. At present state, the enzymes contain notable demand in various industrial sections owing to their qualities, their catalytic efficacy and sustainable nature (El-Shora *et al*., 2025a).

Enzymes obtained through microbial source can be preferred to plant and animal ones, because of their high reliability, yields, steadiness and feasibility with regards to their economic value, and high catalytic function and swift development of microbes on cheap media. Most of the enzymes applied in cosmetics, pharmaceutical and food factories are from microbial origin from microbial (Fusco & Aulitto, 2025). Some are said to be bound from cell membrance and are grouped as intracellular, while others are extracellular. So, the extracellular ones are sent across membrane and moved to the outer fermented broth (Yadav *et al*., 2023).

Conversely, these enzymes are said to have faced some obstacles which include low catalytic efficiency with no physiological reactions, instability, high cost and complexity in their usage, the use of enzymes (Sharma & Kumar, 2021). Enzyme control on solid elements can attend to the existed shortcomings. In the perspective of biotechnology, the element can be immobilised by which biocatalyst can be attached to the insoluble procedure and become insoluble resources. In addition, the justification for the immobilization is not only for the purpose of reuse but due to the fact that it can include stability, turnover number and its activity (Sharma *et al*., 2022).

LOX is a copper-dependent amine oxidase belongs to the oxido-reductase family of enzymes and is secreted in the extracellular space. LOX catalyzes the

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oxidative deaminization of the L-amino acids through alpha amino acids that get hydrolysed acids in consonance with the use of ammonia through nonenzymatic corrosion (Izidoro *et al*. 2014; Ullah 2020 & Costa *et al*., 2024). The enzyme initiates the crosslinking of collagen and elastin by oxidatively deaminating specific peptidyl lysine and hydroxylysine residues to form highly reactive aldehydes known as allysine (Trackman, 2016). LOX enzyme is usually involved in defense mechanisms against parasites and other threats (Abed & Hussein, 2025). LOX is in high demand in most industrial sectora owing to its catalystic function for oxidation deamination of the projected L-amino acids containing dimeric system (Ullah *et al*. 2014, Costal-Oliveira *et al*. 2019 & Ullah, 2020).

LOX is widely distributed in fungi including *Trichoderma harzianum* (Smirnova *et al*., 2017), *Trichoderma cf. aureoviride* (Arinbasarova *et al*., 2024). Also, it is present in bacteria such as *Pseudomonas aeruginosa* (Abed & Hussein, 2025). The use of these microbes for LOX production is advantageous because they contain very simple development requirements, and relative low production expenses and simple act of processing. The additional essence of the L-amino acid can be oxidised through to the therapeutic values to exhibit strong antibacterial antiprotozoal antiviral, anti-HIV, antifungal elements have (Yadav *et al*., 2023).

Cytotoxic studies of LOX showed it to be a strong mechanism in the arena of oncology biology. The cytotoxic element is as a result of the direct impact of hydrogen peroxide which is founded during the oxidation of the L-amino acid, the foundation of the reacting oxygen items to lead cell apoptosis owing to the activation of caspases 3 of which the release of mitochondrial cytochrome C (Guo *et al*. 2012; Ullah *et al*., 2014 & Lukasheva *et al*., 2021).

The present work aimed to isolate, purify and immobilize LOX from *P. chrysogenum.* In addition, the work aimed to compare the characteristics of the free and immobilized enzyme.

Materials and Methods

Experimental microorganism

Penicillium chrysogenum Thom AUMC 14100 gb (Accession No. MN219732) was attained from Assiut University Mubasher Mycological Center (AUMMC), Assiut-Egypt-71516.

Culture media

1. Modified Dox medium according to El-Shora *et al*. (2025b)

The liquid medium contained L-lysine 0.5%, glucose 0.3%, KH_2PO4 0.1%, $MgSO4.7H_2O$ 0.05% and KCl 0.05%. The pH of the medium was adjusted to 7.0 using 0.1N HCl and 0.1NNaOH before sterilizing in an autoclave at 121^{o}C for 15 min.

2. Potato-dextrose agar medium (PDA) (Da Silva *et al*. 2018)

This medium used for 1) preservation of fungal strain at 6-7^{o}C and 2) the inoculum preparation at a final pH 5.6. It consists of the following (gL^{-1}): Extract of 200 g potato scrubbed and dried, dextrose 20 g and agar 15 g. These ingredients were boiled to dissolve the medium completely then sterilized by autoclaving at 15 lbs. pressure (121^{o}C) for 15 min mixed and poured into sterile slants.

Preparation of inoculum

The straining of *P. chrysogenum applied in this study was attained at* 4^{o}C on Potato Dextrose Agar (PDA) slants. Additional cultures were added by the sub-culturing fresh sterile PDA incubating and plates at approximate at 30^{o}C for 72 h (Nwodo-Chinedu et al., 2005).

Growth of experimental organism

Fresh PDA cultures of the of the bacterium were arranged and hatched at around 30^{o}C for 72 – 144 h to obtain sufficient spores. By a means of transformation of the procedure as designated by Vitale *et al*. (2002). Each of the plates was addressed with 10 ml of 0.85% to sterile with saline solution that contains 0.1% Tween-80 and the spores were cultivated into a designated sterile cotton swab. The other spore was observed in 0.1% Tween-80 and the spores were dressed into a sterile tube with the use with the use of haemocytometer raised on lighter microscope. Two ml of the designated spore suspension is (10^7 spore's ml^{-1}) with the inoculation of 100 ml of sterile liquid media in 250 ml Erlenmeyer flask. The proposed culture was placed for incubation at 30% for consecutive 6 days and continued with the use of Griffin flask shaker. The cell-free filtrate was also applied through the enzyme materials.

Purification of LOX

1. Ammonium sulphate precipitation

The initial step in the process of purification of LOX comprised of ammonium sulfate sleet. The crude enzyme extract went through a continuous ammonium sulphate level of saturation at 20% and 80% respectively. The process of saturation was meticulous for 3 hours at approximate 4stage in the purification 4 °C. The projected proteins cautiously taken off at a volume for resuspension of 100mM phosphate buffer (pH 7.0). after each of the precipitation stage, the activity of the enzyme was observed and assessed for the enrichment content of LOX.

2. DEAE-cellulose chromatography

DEAE-cellulose column was arranged and 100 nm potassium phosphate buffer (pH 7) was applied to wash up the concentration of the enzyme and later be applied to the resin. The fractions were eluted with the use of cumulative NaCl concentrations (0.1 to 1 M), and the constant flow rate retained at 1 ml 10 min^{-1} flow degree. The transmission of each number was assessed at 280 nm through a UV-VIS spectrophotometer. The LOX function of the individual fraction was established to exhibit the required activity and later be stored at 4°C for additional experimentations.

3. Gel filtration chromatography

A Sephadex G-150 column (1.5 × 40 cm) was again arranged and later get equilibrated with 100 mM potassium phosphate shield (pH 7). The active set of fractions of LOX were realised at the column and the flow was observed at 1 ml 10 min^{-1} flow rate. The elution profile was later observed through the measurement of the absorbance of the fraction at each containing 280 nm, and progressively the activity of the enzyme was evaluated.

Molecular weight analysis by SDS-PAGE

Homogeneity of purified LOX was checked by denaturing polyacrylamide gel electrophoresis according to Laemmli (1970). The markers consist of phosphorylase (97 KDa, serum albumin (66 KDa), pyruvate kinase (59 KDa), Fumarase (49 KDa), ovalbumin (45 KDa), lactate DH (35 KDa) and carbonic anhydrase (29 KDa).

Determination of LOX activity

The function of LOX was resolute as deaminase (Saurina *et al*., 1998). An aliquot of 0.5 ml of enzyme in 100 mM potassium phosphate shield and 0.5 ml of 20 mM L-lysine was again incubated for about 60 minutes at 30°C. Then, the function of the enzyme halted through the addition of 100 µl of 1% TCA, the response was centrifugated for about 5 minutes at 4000 rpm, where 0.5 ml Nessler's reagent was supplemented. The projected color was assessed at 490 nm. One of the units of LOX was articulated which later the release of one µmole of ammonium per minute under controlled standard situations. And this activity was observed by U (unit) g^{-1} protein.

Determination of protein content

The solvable protein element was established in accordance with Bradford (1976). One ml of the potential result was blended through the use 5ml dilute Coomassie Brilliant blue (CBB) G-250 then the blend was kept in a dark condition for about 1 min and then the process of absorption was evaluated at spectrophotometrically at 595nm. The proposed concentration was contained in a standard curve with the use of bovine serum albumin.

Effect of different cations on LOX activity

various metal cations were tried with regards to their impact on LOX function. These assumed cations were Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} and verified at 1 mM and 5 mM in the response medium as chloride salt after the measurement of the enzyme action.

Modification of LOX

1. Modification of LOX by diethyl pyrocarbonate (DEPC)

Chemical modification of LOX with Diethyl Pyrocarbonate (DEPC) was carried out to assess the involvement of histidyl residues in the catalytic mechanism. The solution of DEPC was freshly prepared. The purified enzyme (10 ml) was combined with 2, 4, 6, 8 and 10 mM DEPC at 25°C in the assay buffer (pH 7.0) for 100 min under controlled temperature conditions, allowing the carbethoxylation of nucleophilic histidine residues to occur. At predetermined time intervals (20, 40, 60, 80 and 100 min), aliquots were withdrawn and the reaction was quenched by adding an excess of imidazole or hydroxylamine, which reverses DEPC modification and prevents further reaction. Residual enzymatic activity was then measured relative to an untreated control, and the pseudo–first-order inactivation kinetics were analyzed by plotting ln (remaining activity) versus time to determine the number of DEPC molecules reacting per enzyme molecule (Zhang *et al*., 1992; El-Shora *et al*., 2009).

2. Modification LOX by tetranitromethane (TNM)

The involvement of tyrosyl residues in LOX catalysis was investigated using tetranitromethane (TNM) which is a tyrosine-specific chemical modification. LOX was incubated with TNM at various concentrations (2, 4, 6, 8 and 10 mM) in the assay buffer under controlled conditions (pH 7.0; 37°C) to modify phenolic hydroxyl groups of tyrosine residues. At specific intervals of (20, 40, 60, 80 and 100 min), aliquots of the response blend and it was later removed to an untreated level to have control and access for the impact of the changes. The extent of tyrosine changes was usually measured through the monitoring of the absorbance changes connected with nitrotyrosine development. Pseudo–first-order deactivation kinetics were examined through the usual plot of TNM molecules to react per enzymes element (Rao *et al*., 1999; El-Shora *et al*., 2009).

Immobilization of purified LOX

2. Immobilization on chitosan

Chitosan halt was conducted by the equilibration of chitosan bead in 100 mM phosphate buffer (pH 7.0) and the incubation with the LOX to prepare and enhance the protonated amino groups on chitosan, where the interface with the negative charged regions highlights the composition of the enzymes. Before the addition of LOX, it will allow the formation of the base between aldehyde category and the lysine residues of the enzymes and consequently reduce the leakage. After the coupling, the beads were washed thoroughly to ger rid of the unound enzyme and later be stored at a buffer of around 4°C (Datta *et al*., 2013; Jesionowski *et al*., 2014).

2. Immobilization on chitin

Immobilization of LOX on the chitin was performed through the suspension of the pre-washed chitin concentrated powder in acetate buffer (pH 5.0–6.0) and later it was mixed gently to allow excellent abdsorption of the electrostatic and hydrophobic exchanges. Following the incubation comprising of the usage of low temperature of (4–25 °C) for 1–4 h, the chitin–enzyme complex was later detached and washed to prevent the unbound protein and the efficacy was determined (Jesionowski *et al*., 2014; Zdarta *et al*., 2018).

3. Immobilization on carrageenan

For immobilization through carrageenan, the enzyme was kept in a κ-carrageenan gel by blending LOX arrangement with the warm carrageenan (4% w/v) and then dipping the blend into cold $CaCl_2$ solution to encourage immediate gelation and drip creation. The drips wer then allowed to be washed and hardened with 100 mM phosphate buffer (pH 7.0) to eliminate surface adsorbed enzyme and later stored again at 4°C. Carrageenan entrapment to offer a slight method of denaturing while the gel can be restricted with the enzymes and improves the operative steadiness (Datta *et al*., 2013; Hassan *et al*., 2019).

Determinations of immobilization efficiency for LOX

The following assienged formula was applied to calculate the immobilization efficacy of LOX: Immobilization efficacy (%) = (Activity of immobilized enzyme/Activity of free enzyme used) ×

100.

Reusability of immobilized LOX

The reusability of the immobilized LOX was assessed by repeatedly employing the same immobilized LOX preparation in successive 8 reaction cycles under the identical assay conditions. After each cycle, the immobilized beads were separated from the reaction mixture by centrifugation, gently washed with 100 mM phosphate buffer (pH 8.0) to remove any residual substrate or product, and then transferred into fresh reaction medium to initiate a new cycle. The residual activity after each run was measured relative to the initial activity in the first cycle, which was considered 100%, and the operational stability profile was constructed by plotting the remaining enzyme activity against the number of reuse cycles (Mahmod, *et al.*, 2016; El-Shora *et al.,* 2022).

Effect of pH and temperature on the activity of free and immobilized LOX

The pH effect on free and immobilized LOX activity was estimated in the presence of buffer solutions: 100 mM sodium acetate (pH 2.0 to 6.0), and 100 mM sodium phosphate (pH. 7.0 to 9.0) and LOX activity was measured as described above. The optimal temperature of LOX was evaluated at, 20°C, 25°C, 30°C, 35°C, 40 °C, 45°C, 50°C, 55 and 60°C. Enzymatic activities was assessed as described above.

Statistical analysis

The mean ± Standard Deviation (SD) of three consecutive experimentations was to express the data. The numerical paets across groups are indicated by various letters with the use of post hoc Duncan's test ($p<0.05$) as well as one-way ANOVA.

Results and Discussion

The extracellular part of the enzymes is applied for industrial justifications. These enzymes are important in biotechnology. To the present time, enzymologists have focussed mainly on microorganisms as another source of enzymes as against the microbial enzymes which have less and cheaper time consumption that the animal sources (El-Shora *et al*., 2024). The submission of purified enzyme as biocatalyst in industry may need a relatively swift purification criteria and the practical approach (El-Shora *et al*., 2025c).

Purification of LOX

The purification process involved three major steps: ammonium sulphate precipitation, DEAE-cellulose ion exchange chromatography and Sephacryl S-200 HR gel filtration. The LOX purified in the present study exhibited a specific activity of 314 Umg^{-1} proteins with an overall 20-fold purification (Table 1), indicating an efficient isolation protocol that substantially enriched the enzyme from the crude extract. Comparable purification outcomes have been reported for fungal LOXs, although specific activity values vary widely depending on the source organism and purification strategy. For example, *Aspergillus niger* LOX purified by ammonium sulfate precipitation followed by ion- exchange chromatography showed a specific activity of 280–300 Umg^{-1} protein with approximately 18-fold purification (Kim *et al*., 2018), values closely aligned with the present results. Similarly, *Neurospora crassa* LOX was purified to a specific activity of 250 U/mg^{-1} proteins with 15-fold increase (Sullivan & Jahnke, 2017), indicating that fungal LOX generally exhibits moderate purification folds due to its secretion-associated glycosylation and matrix-binding nature. LOX from *Trichoderma. harzianum* had been purified to a specific activity of 26.11 Umg^{-1} in earlier work (Costa *et al*., 2024). The LOX from *T. harzianum* achieved a specific activity of 292 U/mg^{-1} proteins with 79 % yield (Smirnova *et al*., 2025).

Table 1: Purification profile of LOX produced by P. chrysogenum

Purification step	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1} protein)	Fold of purification	Yield (%)
Crude extract	76.4	1200	15.7	1	100
Ammonium sulphate	11.4	520	45.6	2.9	43.3

-80%					
DEAE-cellulose	2.3	311	135	8.6	25.9
Sephacryl S 200 HR	0.7	220	314	20	18.3

In contrast, bacterial LOX displays broader variation. Purified LOX from *Marinomonas mediterranea* yielded a specific activity of approximately 180 U mg^{-1} protein with 12-fold purification (García-Boronat *et al*., 2017), whereas LOX-like amine oxidase from *Bacillus pumilus* reached higher specific activity (>400 U/mg) and achieved only 10–12-fold purification (Li *et al*., 2020). Also, LOX purified from *Pseudomonas aeruginosa* with specific activity of 80 Umg^{-1} proteins with 10-fold purification (Abed *et al*., 2025).

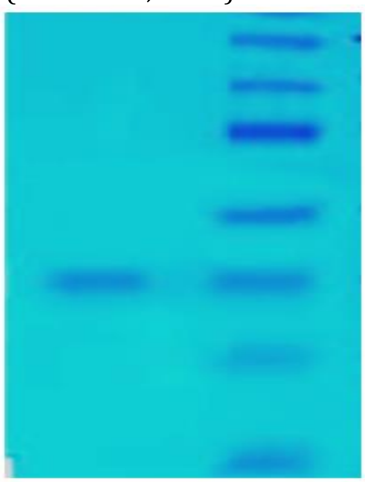

Fig. 1: SDS-PAGE of purified LOX

SDS-PAGE analysis in the present study revealed that the purified fungal LOX has an approximate molecular weight of 45 KDa (Fig. 1). This result is in good agreement with earlier studies reporting fungal LOX enzymes between 30 and 45 KDa. For instance, *Aspergillus niger* LOX was reported to have a molecular mass of 32–34 KDa (Kim *et al*., 2018). Similarly, LOX from *Neurospora crassa* exhibited a molecular weight of 36 KDa, reflecting the smaller size typically observed in fungal LOX compared to mammalian enzymes (Sullivan & Jahnke, 2017). A LOX-like amine oxidase isolated from *Aspergillus flavus* was found to be approximately 40 KDa, consistent with the value obtained in the current study (Rathod et al., 2014). The little differences found among the sources of fungal can be attributed to the variations in variations glycosylation, or excretion paths. Therfore, the projected 40 KDa molecular mass can support the conclusion of the purified enzymes found and the fungal LOX as characterised by the fungal LOX proteins. But the DS-PAGE estimation of the weight of LOX from *Pseudomonas aeruginosa* can be 54 kDa (Abed *et al*., 2025). Thus, the present purified profile can be ranked at cation profile at microbial LOX studies, which reflects both fine and substantial enrichment of the proposed enzyme.

The potent activated part of the LOX by Cu^{2+} can be observed in this study in (Fig. 2) at both 1 mM and 5 mM which is reliable with the well-founded function of copper as an important cofactor for LOX catalysis. LOX is a Cu-dependent amine oxidase, and the copper center participates directly in substrate oxidation and formation of the lysine tyrosylquinone (LTQ) cofactor. Similar enhancement of activity by Cu^{2+} has been documented for fungal LOX from *Aspergillus niger* (Kim *et al*., 2018) and mammalian LOX where exogenous copper supplementation increased the enzyme activity by up to 40% (Kagan & Li, 2003). In contrast, the strong inhibitory effects of Co^{2+} and Zn^{2+} observed in this study align with earlier reports showing that these metal ions interfere with the catalytic copper site or compete with Cu^{2+} for binding, resulting in loss of activity. Zn^{2+} has been reported to inhibit LOX from *Marinomonas mediterranea* and mammalian sources by destabilizing LTQ formation (Trackman, 2016; García-Boronat *et al*., 2017), while Co^{2+} similarly disrupts copper-dependent redox cycling essential for amine oxidation (Li *et al*., 2020).

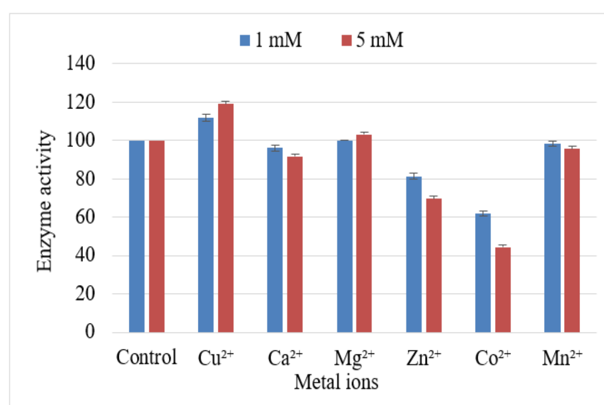

Fig. 2: Effect of metal ions on the LOX activity

The observed inhibition by Ca^{2+} and Mn^{2+} at the higher concentration (5 mM) agrees with findings that alkaline-earth metals do not play a structural role in LOX and may exert nonspecific destabilizing effects at higher ionic strengths. Mn^{2+} has been previously shown to reduce LOX activity in *Neurospora crassa* and bovine LOX, likely by altering enzyme conformation or interacting with histidyl residues involved in copper coordination (Kagan & Trackman, 1991; Sullivan & Jahnke, 2017). The negligible effect of Mg^{2+} is also consistent with prior studies that classify Mg^{2+} as a neutral ion with low affinity toward LOX active or regulatory sites (Wang *et al*., 2019). Generally, this study supports the essential reliance of LOX on Cu^{2+} and supports the inhibitory effect of transition metals that can compete with the catalytic copper centre.

Enzyme modification

Chemical adjustment of the fungal LOX with the use of diethylpyrocarbonate (DEPC) (Figs. 3a & 3b) and trinitromethane (TNM) (Figs. 4a and 4b) displayed that the histidyl and tyrosyl residues are important for catalytic functions to indicate their essential roles in the substrate and it is strongly reinforced by the earlier studies which indicate the active and functional site of the LOX for copper coordination and quinone cofactor creation (Kagan & Li, 2003; Trackman, 2016). DEPC sensitivity has also been shown for the fungal and bacterial LOX-like enzymes to reinforce the straight role of histidyl clusters in maintaining the pure catalytic structure (Payne *et al*., 2005).

The essentialness of tyrosyl deposits can be align with the well-founded requirement of the lysine and tyrosine, for substrate oxidation (Wang *et al*., 1996). The inactivation of LOX by TNM, a precise modifier of tyrosyl remains offers that tyrosyl side chains within the catalytic pocket have been found ofr other amine oxidases, where the tyrosyl remains can conrtribute to the appropriate alignment of the key amines and stabilization of transition levels (Parsons *et al*., 2014). In total, this study is in consonance with the prior description of structural and mechanistic elements of LOX and other related oxidation enzymes, to confirm that histidyl remains are critical for copper binding and tyrosyl remains for LTQ component. From Figs. 3b and 4b two molecules of DEPC and one molecule from TNM react with each enzyme molecule.

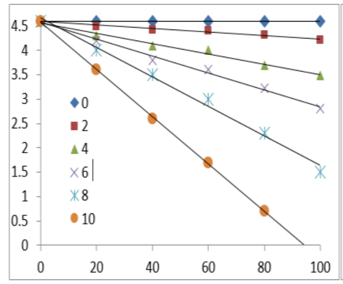

Fig. 3a: Pseudo-first- order kinetics: ln (activity) vs Time in presence of DEPC.

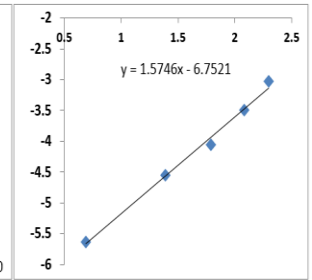

Fig.3b: Stoichiometry determination: ln (K_{obs}) vs ln (DEPC).

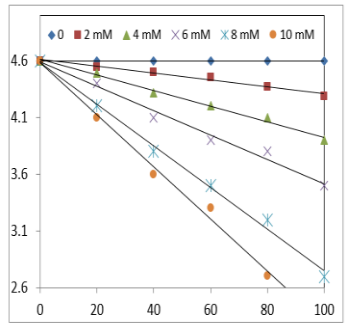

Fig. 4a: Pseudo-first- order kinetics: ln (activity) vs Time.

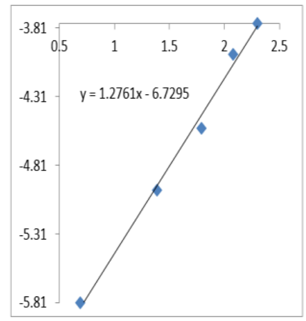

Fig. 4b: Stoichiometry determination: ln (K_{obs}) vs ln (TNM)

Immobilization of LOX

Immobilization of LOX

The halt of fungal LOX on chosan, chitin, and carrageenan in (Fig. 5) showed that there is clear variation in the matrix performance, with revealed carrageenan showing the maximum immobilization efficacy of (95%), where chitosan id (87%) and chitin (69%). These findings show important characteristics and the availability of a functional categories to determine the enzymes for retention and binding. The efficiency is also noticed and it agrees with the carrageenan previous studies to show that sulpha ted polysaccharides offer potent electrostatic and hydrogen-bonded interface that can improve the enzyme halt and reduce the leaching strong electrostatic (Nadar & Rathod, 2019). Carrageenan has as well been reported to form a stable enzyme conformation that can facilitate the loading capacities as indicated for the oxidases and hydrolases in also been κ-carrageenan matrices (Bayramoglu & Arica, 2015).

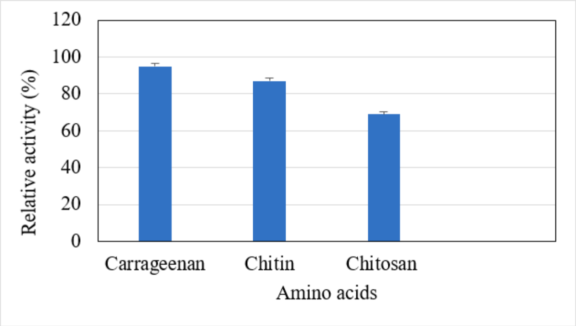

Fig. 5: Immobilization efficiency of LOX from P. chrysogenum

In contrast, the modest halt efficacy obtained with chitosan is in consonance with its known elements: similarly, the presence of amino groups can facilitate the covalent or ionic attachment and it can limit the enzyme accessibility (Sheldon & van Pelt, 2013). Chitin, as being more of crystalline and less of chemically reactive agent indicates lower halt as observed in other studies on amine oxidases as well as other copper enzymes of which chitin in supporting the produced binding and reduced catalytic maintenance in comparison with chitosan derivatives (Abdella *et al*., 2018). The outline observed in this study aligns with the wider literature, to confirm that more dynamic and functionalised polysaccharides mainly carrageenan provide more favourable microenvironments for LOX binding and stabilization. These findings show that the choice of a crucial function in the optimization of LOX halt for industrial biomedical and demonstrate biocatalytic applications.

Reusability of LOX

The reusage of profile (Fig. 6) of the immobilized (LOX) showed promising operative retention of 760% of its early activity after a progressive catalytic cycle. These functions reflect the effective immobilization matrix, which may safeguard the conformational loss and leaching during the replication usage. Comparable results also established that immobilised L-amino acid can retain an approximation of 60-70% of activity after 6-10 cycles depending on the resource supports (Musial *et al*., 2020). Likewise, immobilized amine oxidises the entrapped alginate to function silica retaining about 55-80% of activity after multiple cycles (Carta *et al*., 2018).

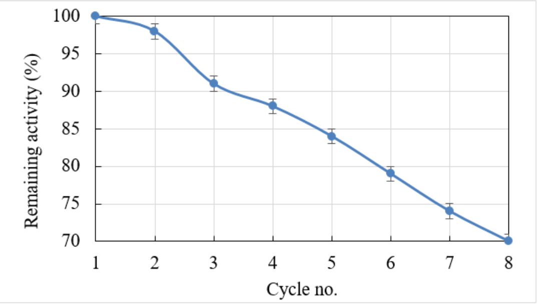

Fig. 6: Reusability of LOX from P. chrrysogenum

Although precise reports on immobilised LOX are not available, several studies on this topic including fungal laccase and phenol oxidase reliably indicate improved operative steadiness after the immobilization at 50–70% of early activity after 8–10 cycles (Min *et al*., 2015; Zdarta *et al*., 2018;). The contrastive high residual support in our LOX arrangement recommends that effective enzyme support interface and reduce active-site obstruction. These findings place immobilized LOX as a promising candidate for repeated usage like biomaterial processing, crosslinking reactions, and potential biomedical or biotechnological processes where operational stability is crucial.

The pH profile

The pH profile as in (Table 2) of fungal LOX showed a pure shift upon immobilization to reflect the changes in the microenvironment that surrounds the enzyme. The enzyme showed maximal catalytic function pH7 as reported in the microbial LOX where the optimal PH leis closer to the requirement for proper ionization and it involved in the active-site remains in substrate oxidation (Kagan & Li, 2003; Trackman, 2016).

Table 2: Effect of pH on the activity of free and immobilized

pH	LOX activity (Umg^{-1} protein)	
	Free	Immobilized
3	7.0±0.2	6.4±0.2
4	8.5±0.3	7.0±0.2
5	10.0±0.3	9.3±0.3
6	12.2±0.4	10.0±0.4
7	14.0±0.3	11.4±0.4
8	12.2±0.2	13.6±0.5
9	10.0±0.3	12.0±0.4

In contrast, the halted enzyme indicated a clear shit to optimal towards pH 8, as widely seen in immobilized oxidases and other copper-reliant enzymes. This factor can be attributed to some factors and the influence of the immobilization matrix. Which can possibly change the local proton concentrations and substrate diffusion (Sheldon & van Pelt, 2013). Relative pH shifts have been seen for the immobilized amine oxidases and fungal L-amino acid oxidases, where the support matrix can stabilize the projected enzymes for a better performance under the dedicative alkaline conditions (Carta *et al.*, 2018; Musial *et al.*, 2020). The recorded shift from pH 7 to pH 8 in this study shows that the immobilization got enhanced in industrial and biomedical domains where pH fluctuations are normal. So, the general findings reinforce that immobilization not only enhances enzyme stability but can also modulate catalytic behavior in ways that extend practical usability.

Temperature

The temperature action profile (Fig. 8) of the fungal LOX in the present study displayed that the free enzyme showed maximum action at 40°C, whereas immobilization exhibited a shift to 45°C. with this an increase in thermal optimum it can be reported that copper-inclined oxidases id broadly attributed to the improved structural rigidity as noted by the support resources, which usually reduce thermal unfolding and safeguard the key catalytic remains from the heat-induced denaturation an increase in thermal (Sheldon & van Pelt, 2013). A similar trend was observed for immobilized amine oxidases and L-amino acid oxidases, where the supplemented optimum temperature is at 5–10 °C in comparison with free form (Carta *et al.*, 2018; Musial *et al.*, 2020).

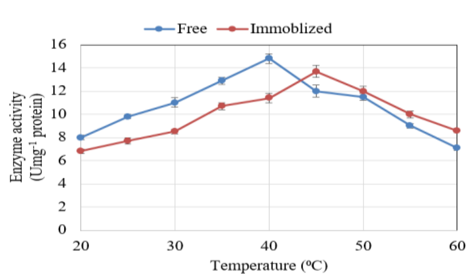

Fig. 7: Effect of temperature on the activity of free and immobilized LOX

Even though reports on immobilized LOX are insufficient, previous studies have shown that fungal LOX can display optimum action between 35–40 °C in the freely (Kagan & Li, 2003; Trackman, 2016), in consonance with this study's findings. The upward movement 45 °C in the immobilized arrangement recommends that enzyme flexibility may offer a protective barricade as against the current trepidations. This sort of tendency is in agreement with other studies on other copper oxidases, which include laccase and phenol oxidase, where immobilization on polysaccharide or nanoparticle enhances thermosteadiness and reach out to catalytic efficacy at an elevated state (Min *et al.*, 2015; Zdarta *et al.*, 2018). In general, the recorder temperature may shift to show the steady effect of immobilization and enhances the possibility of its application to LOX in processes that require increased thermal resistance.

Conclusion

The present study offers a detailed characterization of fungal LOX, to highlight its potent catalytic capacity, versatility and stability. The process of purification produced a maximum enzyme with a molecular mass of 45 kDa. The enzymes stern dependence on Cu^{2+} for action and its stoppage was inferred by Co^{2+}, Zn^{2+}, Ca^{2+}, and Mn^{2+} to reflect the sensitivity of the LOX catalytic interference. The essential histidyl identification and the remains of tyrosyl can later elucidate the mechanism and the structural requirements of the said enzyme. The immobilization can be marked by improved reusage of the special element when carrageenan was applied as a support. So, the results of the fungal LOX with regards to industries and environmental components will provide a robust pedigree for future research which basically focus on enzyme engineering, clinical evaluation. immobilization optimization.

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