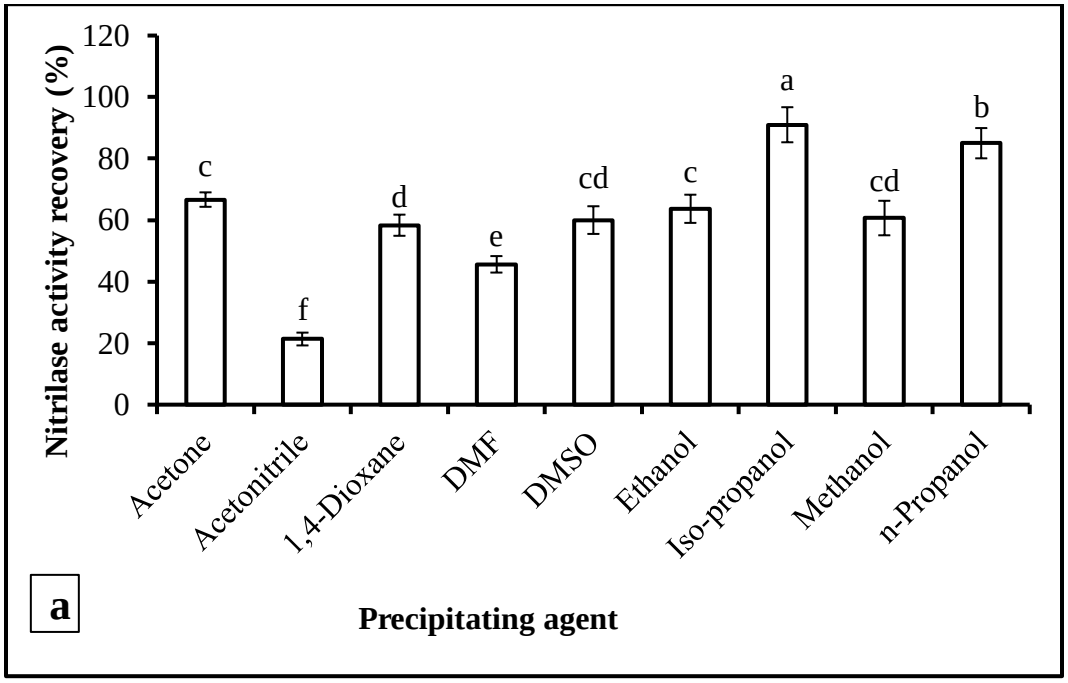
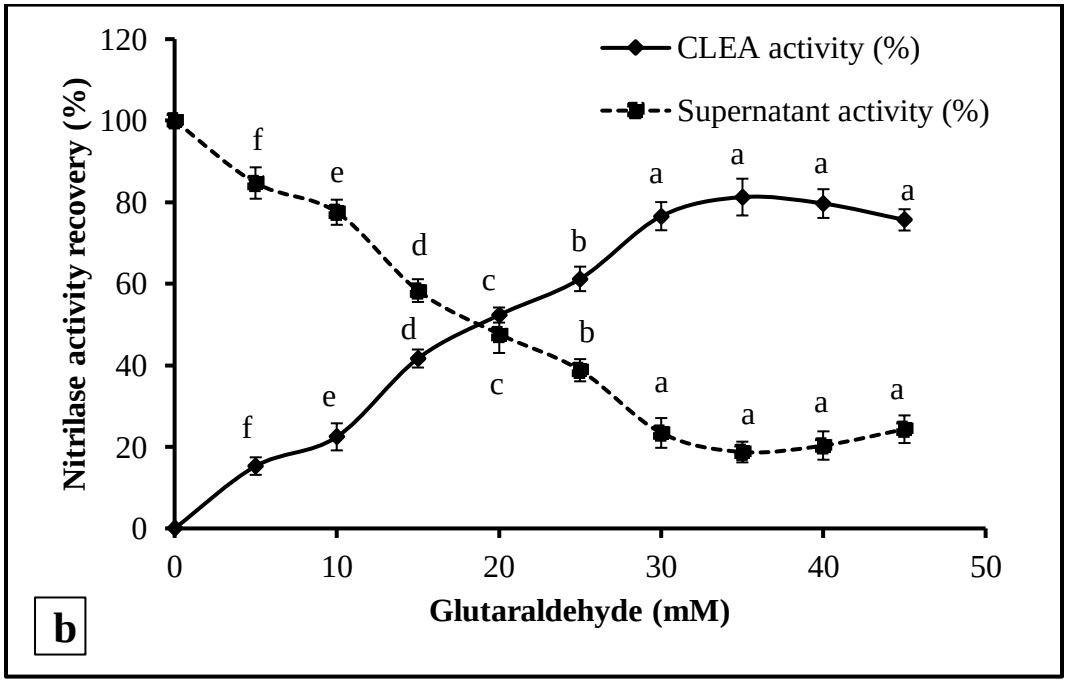
Figure legends:





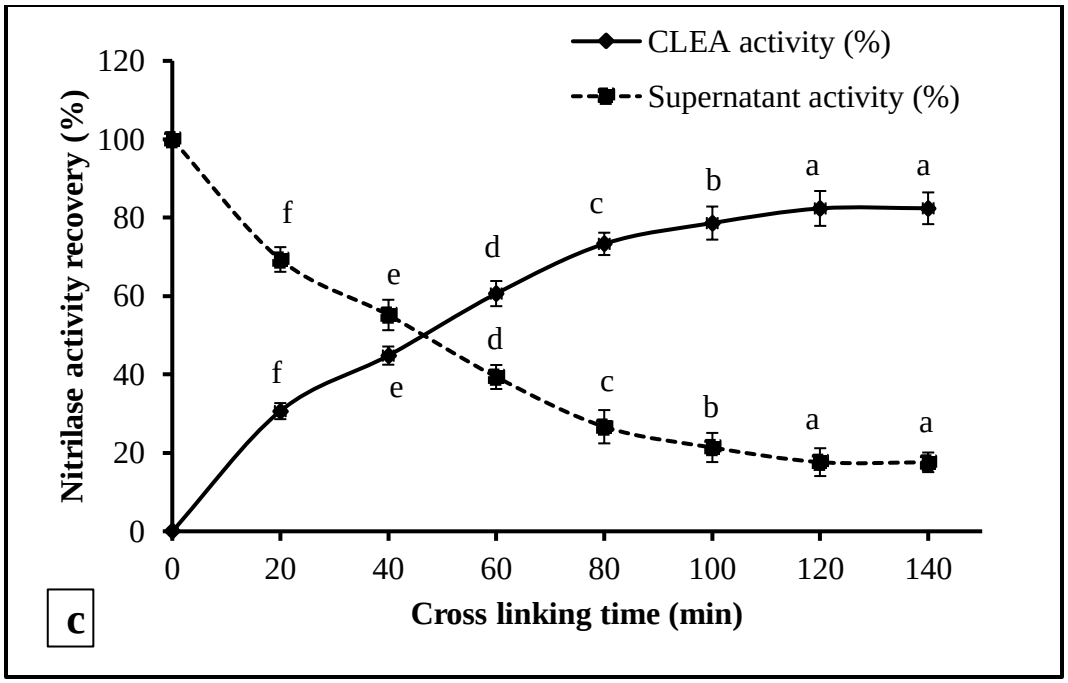
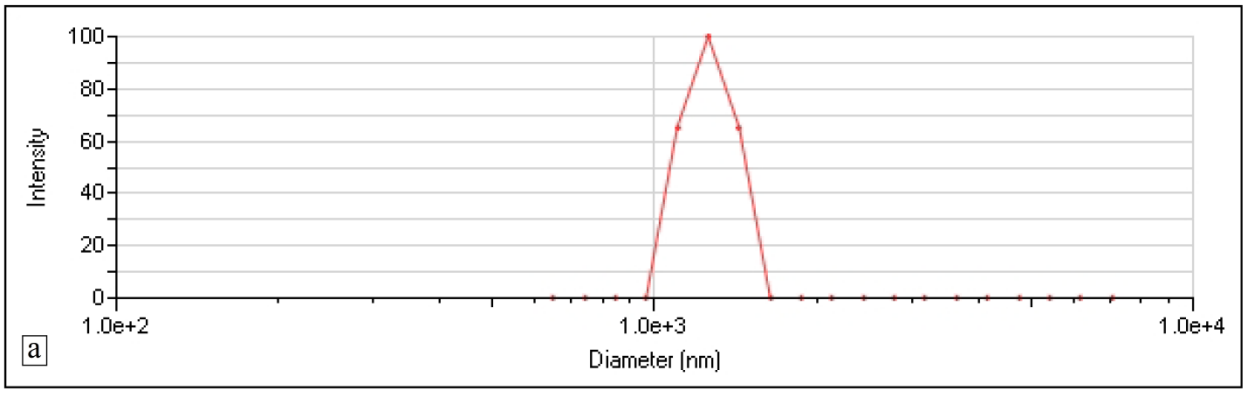
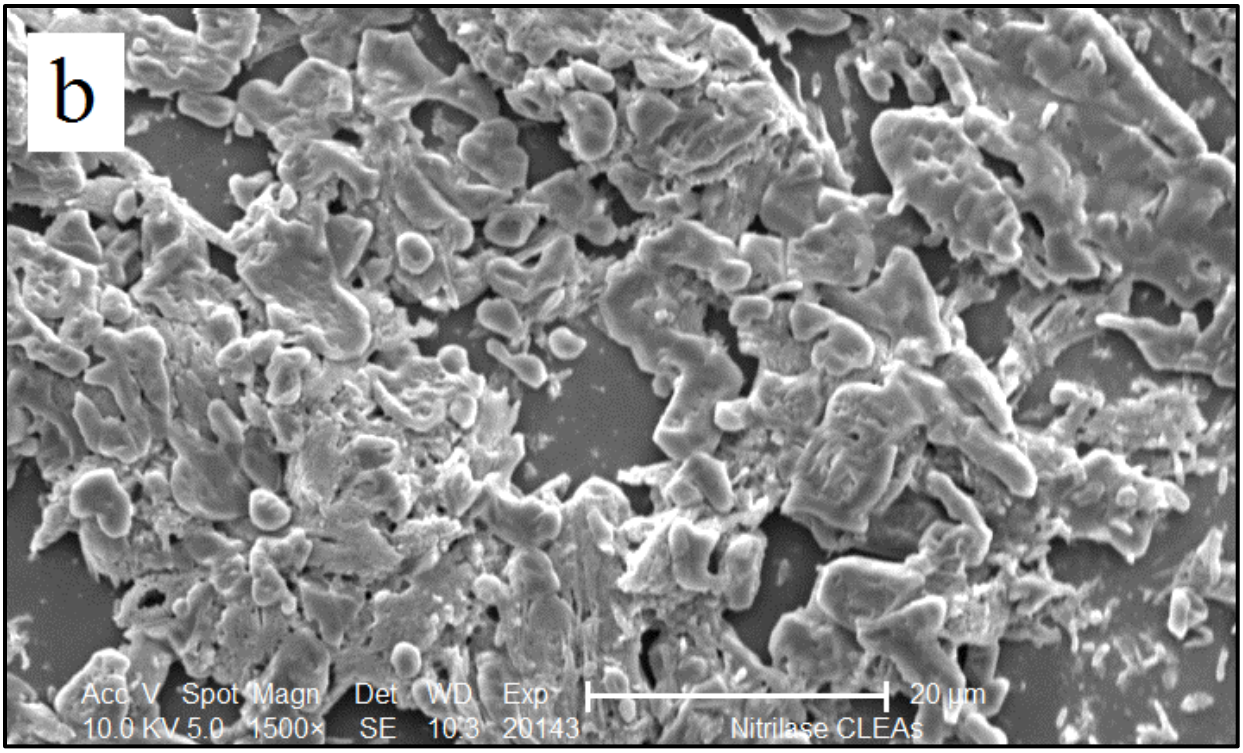


Fig.1(a) Effect of miscible organic solvents on the recovery of nitrilase activity; Effect of operational parameters (b) glutaraldehyde concentration (mM), (c) cross-linking time (min) on the preparation of nitrilase-CLEAs


100
80
60
40
20
0
Intensity
1.0e+2
1.0e+3
1.0e+4
Diameter (nm)
a


b
Acc.V Spot Magn Det WD Exp
10.0 kV 5.0 1500x SE 10.3 20143
20 µm
Nitrilase CLEAs

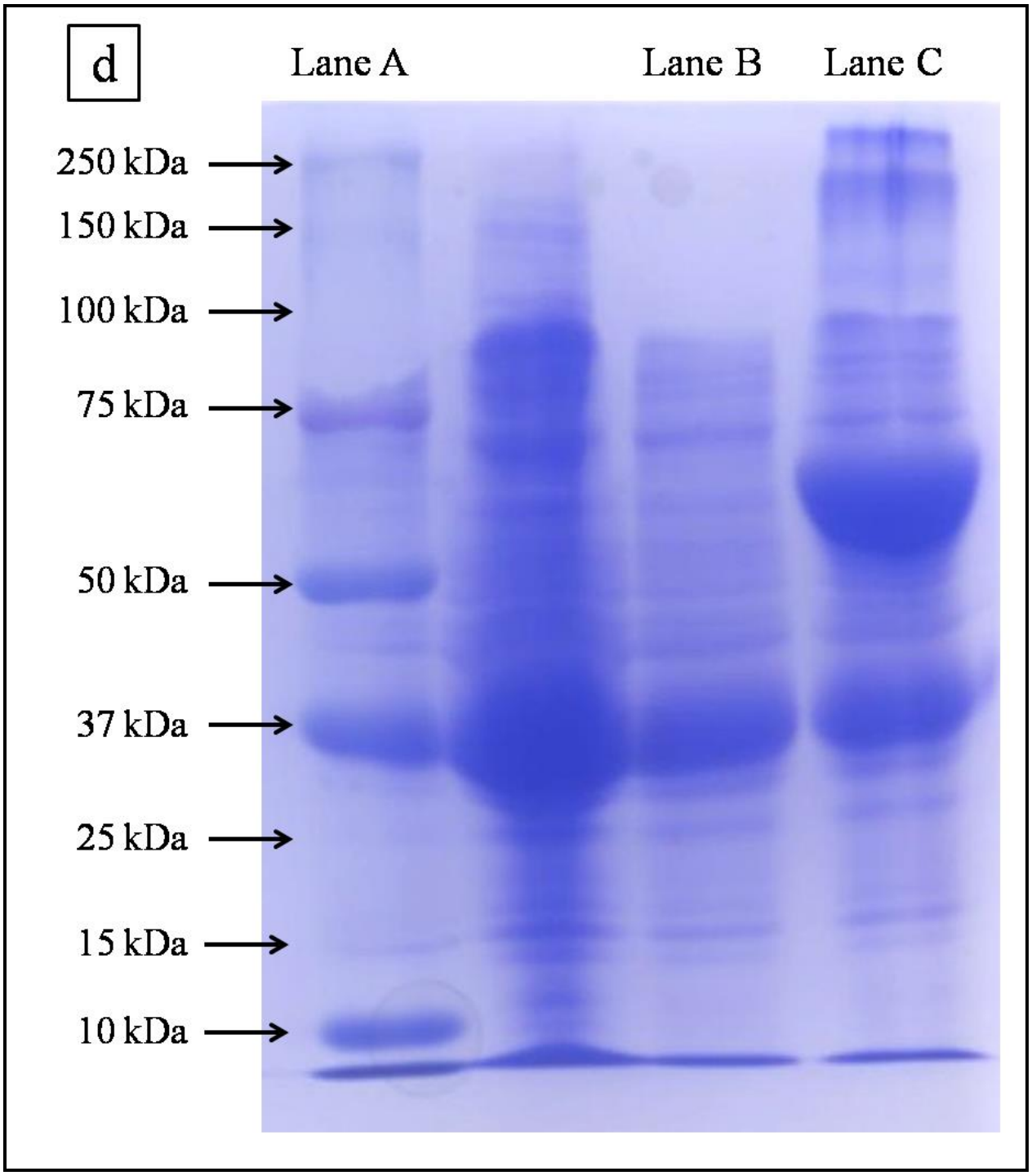


Fig.2 Instrumental characterization (a) particle size distribution of nitrilase-CLEAs; (b) Scanning electron microscopic image of nitrilase-CLEAs at 1500 × magnification; (c) FTIR spectra of free nitrilase and nitrilase-CLEAs, (d) SDS-PAGE analysis of free nitrilase and nitrilase-CLEAs. Lane A: standard molecular weight markers, Lane B: free nitrilase, and Lane C: nitrilase-CLEAs

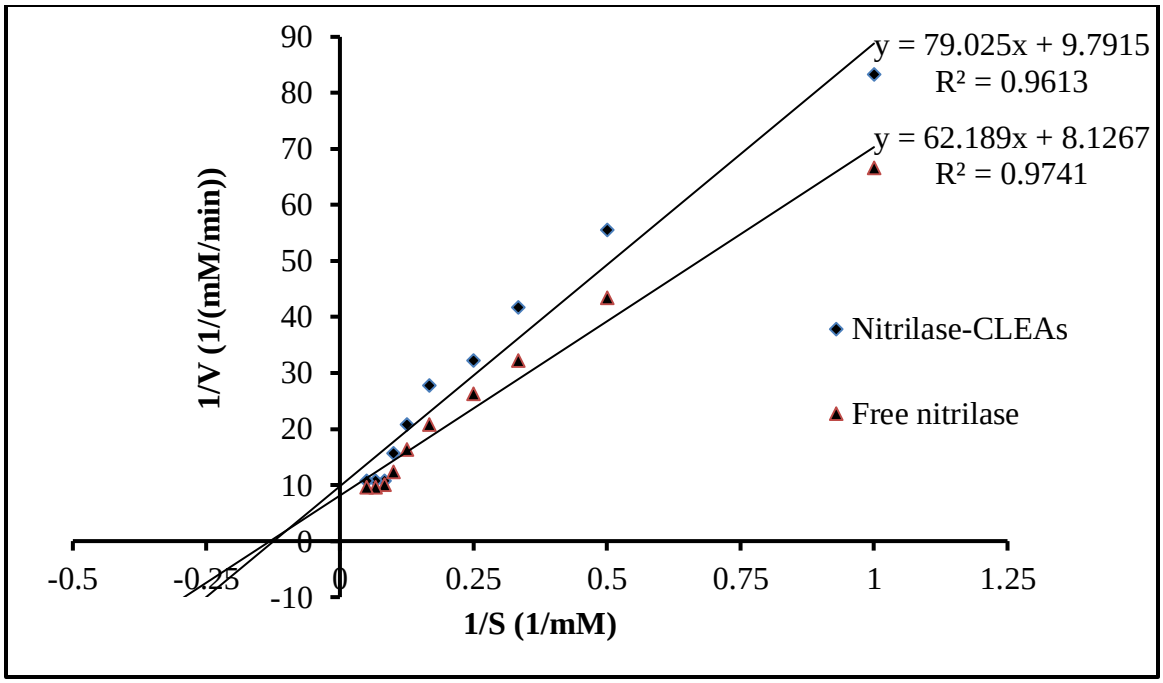


Fig.3 Lineweaver-Burk plot for determination of kinetic parameters of freenitrilase and nitrilase-CLEAs

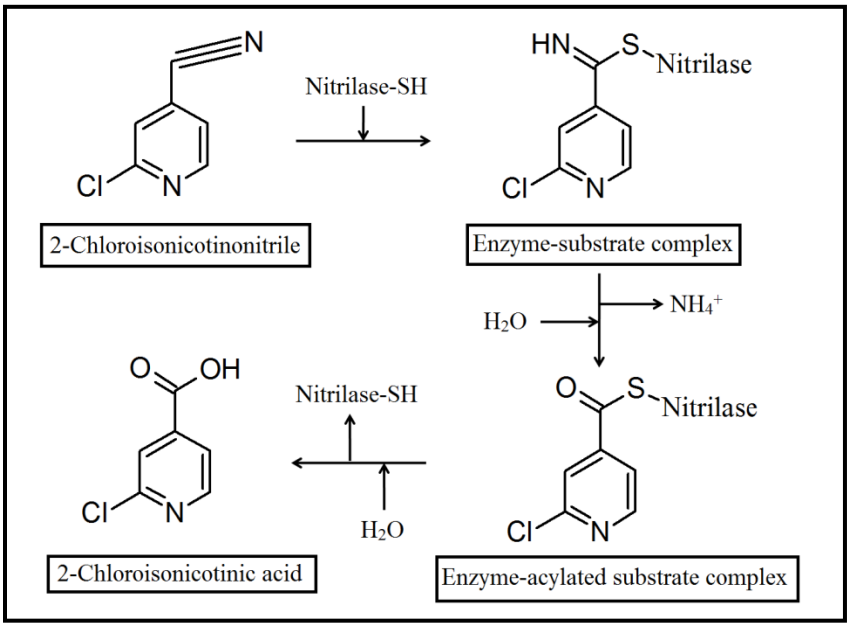


Fig. 4 Reaction pathway of hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acidwith nitrilase

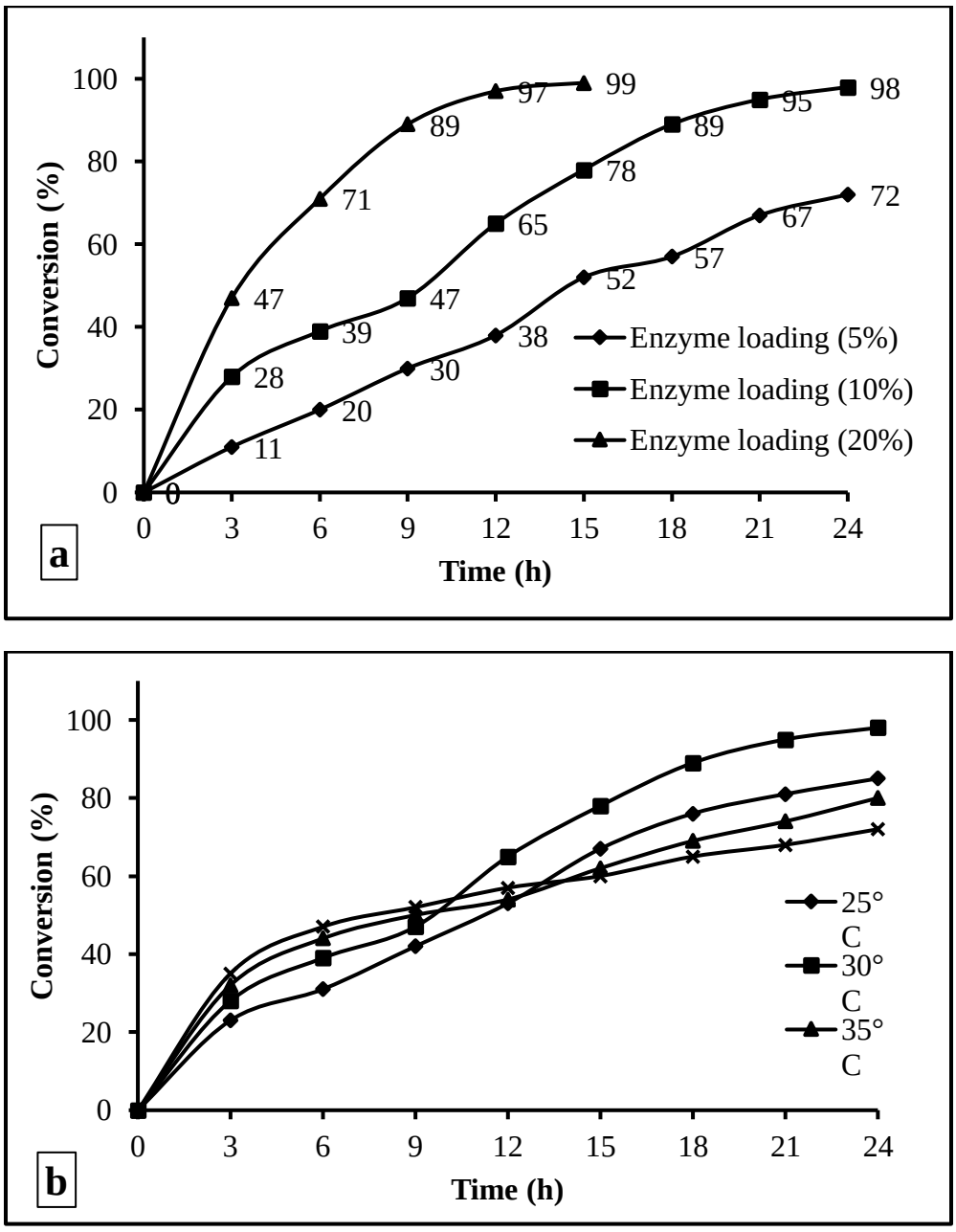


Fig. 5 Effect of (a) nitrilase-CLEAs loading, and (b) temperature with incubation time (h) on conversion of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

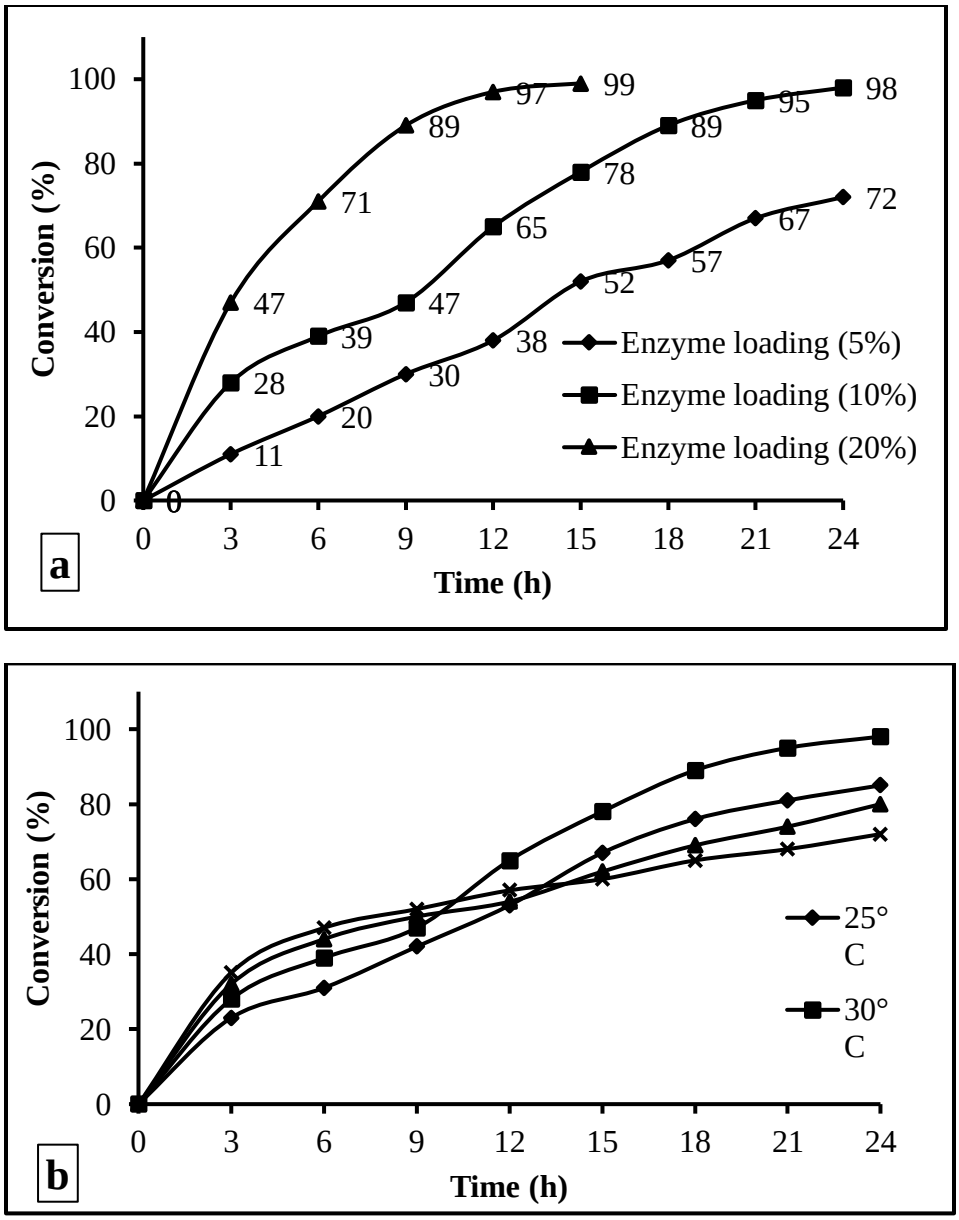


Fig. 6 Effect of (a) nitrilase-CLEAs loading, and (b) temperature with incubation time (h) on conversion of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

FORM-2

THE PATENTS ACT, 1970

(39of 1970)

PROVISIONAL/COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION

"Preparation, characterization and stability of cross linked nitrilase aggregates (nitrilase-CLEAs) for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid"

2. APPLICANT

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3. PREAMBLE TO THE DESCRITION

COMPLETE

The following specification particularly describes the invention and the manner in which it is to be performed

Preparation, characterization and stability of cross linked nitrilase aggregates (nitrilase-CLEAs) for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

FIELD OF INVENTION:

[0001] The invention involves development of novel process for the synthesis of 2-chloroisonicotinic acid from 2-chloroisonicotinonitrile using nitrilase-CLEA and recycling of its upto 4 cycles.

BACKGROUND OF INVENTION:

[0002] 2-Chloroisonicotinic acid is a vital pharmaceutical ingredient due to its unique anti inflammatory propertyand acts as a primary derivative in the synthesis of active drug intermediates such as Homocamptothecin derivative and anticancer agent, (phenylmorpholinyl)pyrimidinones (, trans-N-[1-(2-fluorophenyl)-3-pyrazolyl]-3-oxospiro-[6-azaisobenzofuran-1(3H),10-cyclohexane]-40-carboxamide, and 11-oxo-11 H-pyrido[2,1-b]quinazoline-7-carboxylic acid, as well as some monocomposite films. The 2-chloroisonicotinic acid is synthesized by using isonicotinic-N-oxide, phosphorous pentachloride and phosphorous oxychloride. This method requires harsh chemicals and conditions during operation and this leads to product with very less yield and some impurities. Further, there are risks during processing and operation, may add on to environmental burdens, and may also cause deterioration of process reactor. Nowadays, biocatalysis based alternative to chemical process and/or synthesis for hydroxylation by nitrilases are becoming highly popular, due to stereo-, chemo-, and regio- selectivity, and substrate specificity.

[0003]Nitrilases (E.C. 3.5.5.1) belong to hydrolases class of enzymes which has gained tremendous focus due to their unique capability of hydrolyzing a wide array of nitriles and yielding important carboxylic acids and amides by carbon chain extension at ambient processing conditions. The wide application of nitrilases including synthesis of carboxylic acids, drugintermediates, nicotinamides, cyanoverlamide, and valuable amides like acrylamide with specific chiral center have been explored in a review by Shen et al. (2021). Although nitrilases are green, versatile, and specific biocatalysts for numerous catalytic and transformation process and reactions, their stability during process and unit operations along with kinetic and thermal instability, and reusabilty are major bottlenecks for exploring them on commercial scale. The mentioned challenges and/or problems for application of nitrilases can be resolved by stabilization and immobilization. The various approaches previously used for immobilization of nitrilases includes entrapment in polysaccharide matrices like agar, carrageenan, and alginate, encapsulation in zeolite imidazole framework-90, covalent attachment on SiO_2 nanoparticles, cross linking using glutaraldehyde.

[0004] In current times, the cross linked aggregates of enzymes (CLEAs) have gathered invigorating attention of worldwide researchers to ameliorate as one step direct carrier independent crosslinking or immobilization process. In addition to simplicity, facile nature and

robustness, the retardation to heat, organic solvents, and proteolysis are extra added features. The preparation protocol for CLEAs is unique as it can be performed with partially purified enzyme/s. Further, the enzyme/s can be partially purified and immobilized in single unit operation of the preparation process. Previous reports have stated a superior volumetric productivity, better operational stability, ease in recovery for repeated application, and eco-friendly nature of CLEAs for desired industrial applications over the native free form. Although CLEAs have numerous benefits, the limitations such as (i) a broad distribution of of particle size, (ii) poor mechanical stability, (iii) non-uniform crystallinity cannot be ignored. These problems mainly alter the reaction kinetics, mass transfer, leaching out of enzymes, and loss of enzyme activity.

[0005] The preparation of CLEAs is carried out by firstly precipitating the enzyme in active state under non-denaturating conditions followed by covalent cross linking with bifunctional cross-linking entity such as citric acid, formaldehyde, and glutaraldehyde. Solutions or enzyme formulations with lower content of protein can be also be processed with additives like bovine serum albumin, polylysine, and other proteins. It is worth noticing that the activity of CLEAs with lower activity can also be enhanced with application of surfactants such as Triton X-100, sodium dodecyl sulphonate, and Tween 80. Further, the parameters involved in governing the characteristic features of CLEAs are amount of protein loading, nature and concentration of additives and precipitant used, concentration of cross linker, period of cross linking, pH and temperature of medium used for CLEAs preparation. Therefore the optimization of parameters used for CLEAs preparation is utmost importance, and this can be achieved by one variable at a time method or statistical method using response surface methodology as an approach. Earlier, the one variable at a time method was used for preparation of acrylamidase-CLEAs, cutinase-CLEAs, nitrilase-CLEAs, formate dehydrogenase-CLEAs, lipase-CLEAs. On the other side, statistical method using response surface methodology was previously used for lipase-CLEAs, β-mannanase-CLEAs, cellulase-CLEAs, esterase-CLEAs, hydroxy nitrile lyase-CLEAs reported in literature.

OBJECTIVE OF INVENTION

1. Synthesis of a vital pharmaceutical ingredient having its unique anti inflammatory property2-Chloroisonicotinic acid from 2-chloroisonicotinonitrile using green chemistry, a commercially viable process.
2. Preparation of nitrilase-CLEA and its recycling for providing enzyme stability towards temperature, pH and shear forces generated during the reaction.

SUMMARY OF THE INVENTION:

Screening of enzymes for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

[0006] A total of 40 nitriliases were screened for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid. For this, nitrilase (25 mg) was added slowly in 900 µL of potassium phosphate buffer (pH 7.5, 100 mM) with proper care to prevent foam formation. After this, substrate (50 mg dissolved in 100 µL dimethyl sulfoxide) was added slowly to this, and the resulting reaction mixture was incubated at 30±2°C on a thermomixer (Model Matrix Orbital Delta Plus, IKA Thermoshaker, Germany) for 24 h and 1000 rpm at 1000 rpm. The reaction was stopped by adjusting the pH to 2-2.5 with 6 M HCl solution, and the samples were extracted in dichloromethane (3 × 1 mL). The collected organic layers were combined and dried by evaporation under reduced pressure. Further, the dried samples were dissolved and diluted to a final concentration of 0.25 mg/mL with a mixture of 0.1% trifluoroacetic acid in water (55%) and acetonitrile (45%), followed by filtration through 0.45 µm filter, and analyzed on C18 column (Inert sustain AQ-C18 (5µm × 4.6 ×250mm)) HPLC system (Shimadzu LC-2030C 3D plus, Japan). The mobile phase used was 0.1% trifluoroacetic acid in water : acetonitrile :: 55:45, column temperature was 30±2°C, flow rate was 1 mL/min, and detected at 210 nm with a run time of 20 min. The enzyme yielding maximum product formation was selected for preparation of CLEAs and further scale-up studies.

Preparation of nitrilase-CLEAs

[0007]Various miscible organic solvents such as acetone, acetonitrile, 1,4-dioxane, N,N-dimethylformamide, dimethylsulfoxide, ethanol, *iso*-propanol, methanol, and *n*-propanol were screened for the precipitation of nitrilase in active form. For this, chilled organic solvents were

added drop wise from the side of tube to a chilled enzyme solution (5 mg/mL in 100 mM potassium phosphate buffer pH 7.5, 0.127 U/mg), and incubated on ice bath with intermittent shaken. These tubes were then centrifuged (Remi centrifuge, Model C-24PLUS, Remi Elektrotechnik, Ltd., Vasai, India) at 6000 × g, for 10 min at 4±2°C. The collected precipitate was assessed for nitrilase activity and the activity recovery of nitrilase after precipitation was enumerated as

$$\text{Nitrilase activity recovery (\%)} = \frac{\text{Activity of nitrilase after precipitation}}{\text{Activity of nitrilase in free form}} \times 100$$

Step 2: Cross-linking of nitrilase

[0008]The optimization of cross-linking of nitrilase with glutaraldehyde was performed with one variable at a time approach by considering two parameters namely glutaraldehyde concentration (5-45 mM) and cross-linking time (0-140 min) for the best probable recovery of nitrilase. The nitrilase-CLEAs formed after cross-linking with glutaraldehyde with optimal concentration and ambient time were recovered by centrifugation at 6000 × g, for 10 min at 4±2°C, and were assessed for its activity. The activity recovery of nitrilase after cross linking was enumerated as

$$\text{Nitrilase activity recovery (\%)} = \frac{\text{Activity of nitrilase in CLEAs}}{\text{Activity of nitrilase in free form}} \times 100$$

Determination of nitrilase activity and protein content

[0009]The standard activity of free nitrilase and nitrilase-CLEAs was calculated by using mandelonitrile (30 mM) as a substrate as explained by Zhang et al. (2014). In a reaction mixture of 2 mL, nitrilase (5 mg) was added slowly in 1.8mL of potassium phosphate buffer (pH 7.0, 100 mM) with proper care to prevent foam formation followed by addition of substrate (200 µL in DMSO). The resulting reaction mixture was incubated on a thermomixer for 30min at 30±2°C and 1000 rpm. The reaction was stopped by adding 100 µL 2 M HCl solution, and the samples were extracted in dichloromethane (3 × 1 mL). The organic layers were collected, combined and dried by evaporation under reduced pressure. Further, the dried samples were dissolved and diluted to a final concentration of 0.25 mg/mL with a mixture of 0.1% trifluoroacetic acid in water (60%) and acetonitrile (40%), followed by filtration through 0.45 µm filter, and analyzed on C18 column on a HPLC. The mobile phase used was 0.1% trifluoroacetic acid in water :

acetonitrile :: 60:40, column temperature was 30±2°C, flow rate was 1 mL/min, and detected at 210 nm with a run time of 20 min. One unit of nitrilase activity was defined as the quantity of enzyme required for production of 1 µmol of mandelic acid per min at standard defined conditions of pH, temperature, and buffer strength. The soluble protein content was quantified by Bradford method.

Instrumental characterization

[0010]The size of prepared nitrilase-CLEAs was estimated with DLS-Particle size analyzer (Dynamic light scatteringsystem, DLS Nanobrook 90 plus PALS Instrument, New York) as statistical diameter. The shape, structure and morphology of external surface of nitrilase-CLEAs was seen with SEM analysis (Scanning electron microscope, Philips XL 30 SEM). The distinctive variations in functional, characteristic and structural groups of free nitrilase and nitrilase-CLEAs were studied with ATR-FTIR spectrophotometer (Bruker Alpha II, Eco, Bruker Corporations, Germany). The comparative molecular weight of free nitrilase and nitrilase-CLEAs was determined by SDS-PAGE assembly using 12%polyacrylamide (1:29:1 :: bisacrylamide:acrylamide) in 250 mM glycine and 25 mM tris electrophoretic buffer (pH 8.2, 0.1% SDS).

Kinetic parameters of free nitrilase and nitrilase-CLEAs

[0011]The kinetic parameters of free nitrilase and nitrilase-CLEAs were enumerated by determining the activities at different concentrations of mandelonitrile in a regime of 1-20 mM. The Michaelis-Menten equation and Lineweaver-Burk double reciprocal plot were utilized for estimation of kinetic parameters.

Biochemical characterization

pH optimaand pH stability

[0012]The pH optima was determined by measuring the activity of free nitrilase and nitrilase-CLEAs at 30±2°C in 100 mM buffers systems with varying pH regime (4-10). The pH stability was quantified by incubating free nitrilase and nitrilase-CLEAs in 100 mM buffers with a varying pH domain of 4-10 for 24 h at 30±2°C.

Temperature optimaand thermalstability

[0013]The temperature optima was analyzed by measuring the activity of free nitrilase and nitrilase-CLEAs in 100 mM potassium phosphate buffer (pH 7.0) in varying temperature domain of 25-65°C. Further, the thermal stability was determined by incubating free nitrilase and nitrilase-CLEAs in 100 mM potassium phosphate buffer (pH 7.0) with a varying temperature domain of 4-10 for 24 h at 30±2°C.

Application of free-nitrilase and nitrilase-CLEAs for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

[0014] The reaction set-up with pH stat was adjusted. The substrate was prepared by adding 50 g of 2-chloroisonicotinonitrile in 50 mL dimethyl sulfoxide. In a reaction vessel, 450 mL potassium phosphate buffer (100 mM, pH 7.5) was added and stirring was started and temperature was maintained at 30±2°C. The pH stat was started and maintained at 7.5 with 10% NaOH solution, followed by addition of 5 g enzyme (10%). Further, the reaction was initiated by slow addition of substrate (within 1-2 h) with continuous stirring and maintenance of pH. The reaction was monitored for 24 h by HPLC (as mentioned above). The reaction mixture was filtered through Whatman filter paper no. 42 to isolate the nitrilase-CLEAs. This filtration step was excluded for free nitrilase. The pH of the filtrate was adjusted to 11-12 with 20% NaOH solution, followed by extraction in dichloromethane (2 × 1 volume) to remove un-reacted 2-chloroisonicotinonitrile. Further, the pH of collected aqueous layer was adjusted to 2-2.5 with 6 M HCl solution, and this was extracted in dichloromethane (3 × 1 volume). The collected organic layers were combined and distilled on rotary evaporator (model RV 8V, IKA® India Private Limited, Bangalore) at45-50°C under vacuum. The product i.e. 2-chloroisonicotinic acid was recovered, dried in vacuum tray dryer for 20-22 h at 45-50°C under vacuum, and the yield was calculated on molar basis.Finally, the effect of reaction parameters namely enzyme loading (5%, 10% and 20%) and temperature (25°C, 30°C, 35°C and40°C) linked with hydroxylation process were optimized with one factor at a time approach.

Instrumental characterization of 2-chloroisonicotinonitrile and 2-chloroisonicotinic acid

[0015]Along with HPLC, the confirmation of 2-chloroisonicotinonitrile and 2-chloroisonicotinic acid was done by FTIR, ^{1}H NMR, AND LC-MS analysis. The FITR was performed on ATR-FTIR. LC-MS was carried out by using the same mobile phase on C18 column. (LCMS 2020,

Shimadzu, Japan)). ^{1}H NMR was recorded on NMR (Varian 400 MR, NMR spectrometer, USA) by dissolving both the compounds in DMSO.

Reusability studies of nitrilase-CLEAs

[0016] The reusability potential of nitrilase-CLEAs was assessed by their subsequent use in repeated batches of hydroxylation reaction as mentioned above. The recovery of nitrilase-CLEAs was done by filtration and then washing by 100 mM potassium phosphate buffer (pH 7.5) to remove any impurities adhered on them. After each batch, the conversion (%), product (g/L) and yield (%) were calculated.

Statistical analysis

[0017] All the experimental reactions were carried outthree times and the obtainedfindings are expressed as average ± standard errors. The recorded data was examined on Microsoft Excel, 2013, and SPSS Version16 by applying one way ANOVA to assess the differences in mean and statistical significant differences amongst the attained average values established at $p \leq 5\%$ and Dunnett's post hoc test.

DETAILED DESCRIPTION OF THE INVENTION

Screening of nitrilases for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid

[0018]The hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid was carried out by enzymatic pathway using nitrilase to overcome the limitations of chemical route which include harsh treatment with corrosive acids and alkalies, high processing temperature, discharge of toxic compounds, amongst many others, leading to lower yield, and purity. The enzymatic route may impart higher specificity, metal free reactions under mild conditions, minimum requirement of energy, less by product formations, and many others. For the hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid, 40 nitriliases were screened at 50% enzyme loading and 10% dimethylsulfoxides as co-solvent in the potassium phosphate buffer. Amongst the nitrilases investigated, ES-NIT-102, ES-NIT-103, ES-NIT-129,ES-NIT-131, ES-NIT-132 and ES-NIT-138 have shown promising conversion of 90%, 85%, 80%, 71%, 29%, and 78% after 24 h of incubation, and the yield of the reaction was 86.9%, 82.1%, 77.3%, 68.6%, 28%,

and 75.3%, respectively (Table 1). The other enzymes used in screening namely ES-NIT-101, ES-NIT-104, ES-NIT-105, ES-NIT-106 ES-NIT-107, ES-NIT-108, ES-NIT-109, ES-NIT-110, ES-NIT-111, ES-NIT-112, ES-NIT-113, ES-NIT-114, ES-NIT-115, ES-NIT-116, ES-NIT-117, ES-NIT-118, ES-NIT-119, ES-NIT-120, ES-NIT-121, ES-NIT-122, ES-NIT-123, ES-NIT-124, ES-NIT-125, ES-NIT-126, ES-NIT-127, ES-NIT-128, ES-NIT-130, ES-NIT-133, ES-NIT-134, ES-NIT-135, ES-NIT-136, ES-NIT-137, ES-NIT-139, ES-NIT-140 did not show any significant conversion during this hydroxylation reaction

The higher conversion with the best enzymes could be due to the desired specificity of substrate towards its active cleft. Nitrilase may show activity towards either aliphatic nitriles, aromatic nitriles, or arylacetonitriles, and some on all. In aliphatic nitriles, the short chain aliphatic di-nitriles are preferred over the medium-chain aliphatic mononitriles and aliphatic substituted mononitriles. Whereas, in aromatic nitriles, mono cyclic aromatic nitriles are more selective than the hetero cyclic aromatic nitriles. This specificity could also be due to the action of specific enzymes on the specific structure of the substrate moieties. Further, stated that different nitrilases may act in a different way on dinitriles but they may not show similar catalytic effect on similar substrate.

From the different nitrilases studied, ES-NIT-102 was selected as an ideal candidate for carrier free immobilization because of its ability to convert maximum substrate and also yield highest product formation. In addition, the selected enzyme also showed desired results for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid (described below). The carrier free immobilization was performed by cross linking the enzyme moieties together using glutaraldehyde as a bifunctional agent. Further, mandelonitrile was used as a substrate for determination of optimization parameters during preparation of nitrilase-CLEAs, its kinetics study, and biochemical characterization.

Preparation of nitrilase-CLEAs

[0019]The main stages engaged during preparation of CLEAs of enzymes are aggregation of proteins in their active form by precipitation followed by cross-linking with each other using a desired bi-functional agent. Here, the activity of nitrilase was determined by analyzing the conversion of mandelonitrile to mandelic acid. The standard HPLC profiles of mandelonitrile

(0.25 mg/mL in 0.1% trifluoroacetic acid in water (60%) and acetonitrile (40%)), mandelic acid (0.25 mg/mL in 0.1% trifluoroacetic acid in water (60%) and acetonitrile (40%)), and enzymatic reaction with around 90% conversion are shown in Figure S1-a, S1-b, and S1-c (Supplementary file).

Precipitation of nitrilase

[0020]During precipitation *i.e.* the first stage, enzymes are converted into aggregated state (*i.e.*insoluble form) from the free state (*i.e.*soluble form). For the maintenance of active state along with the native conformation a desired precipitating agent with proper concentration is a pre-requisite. Herein, different miscible organic solvents such as acetone, acetonitile, 1,4-dioxane, N,N-dimethylformamide, dimethylsulfoxide, ethanol, *iso*-propanol, methanol, and *n*-propanol were screened for precipitation of ES-NIT-102 in active state. Highest activity recovery of 91±5.41% and 85±4.92% with an actual activity of 0.115 ± 0.007 U/mg and 0.108 ± 0.006 U/mg were seen with *iso*-propanol and *n*-propanol, while least recovery of 45.62±2.68% and 21.33±2.08% with an actual activity of 0.058 ± 0.003 U/mg and 0.027 ± 0.003 U/mg with N,N-dimethylformamide, and acetonitrile, respectively (Fig. 1a).

The miscible organic solvents are known for removal the aqueous layer present in the vicinity of enzyme forcing different attraction dipolar chargesandelectrostatic forces to cause aggregation. These solvents may also cause a change in polarity of the liquid medium and lead to dispersion of hydrophobic domains that may hamper the enzyme activity. This water layer plays a crucial part in maintenance of structural conformation, and hence the activity of enzyme. A substantial reduction in nitrilase activity may be due to loss of active 3D structural conformation caused by deep penetration of these solvents. Previously, *iso*-propanol was also found to the best precipitating agent for nitrilase and it was also suitable for other enzymes like lipase, and cellulase from fermentation broth.

Cross-linking of nitrilase

[0021]During cross linking *i.e.* the second stage, enzymes are attached with each other using a bi-functional cross linking agent such as glutaraldehyde, epichlorohydrin or glyoxyl-containing compounds, ethylene glycol-N-hydroxysuccinimide, genipin, and many others. Herein, glutaraldehyde was utilized as a cross linking agent for ES-NIT-102 because of unique features

like low cost, abundant availability, ease in handling and processing, and predominant ability to introduce covalent linkages with a wide array of enzymes.

For preparing the CLEAs of nitrilase, two parameters namely glutaraldehyde concentration and period of cross linking were optimized by one factor at a time aspect. The concentration of cross linking agent has a major impact on particle size, shape and operational stability of the prepared CLEAs. The ambient concentration of glutaraldehyde (mM) for firm cross linking of nitrilase with each other in its active state was 35 mM. The residual activity and actual activity of ES-NIT-102 after cross linking at this concentration were 81.26 ± 4.51% and 0.103 ± 0.006 U/mg, respectively (Fig. 1b). For effective activity of nitrilase, the Glu-Lys-Cys is a conformational catalytic triad, and the disturbances in this triad will hamper the active performance of the enzyme. At higher glutaraldehyde content (above 40 mM), all the nitrilase molecules could have got cross-linked with each other which might have caused excess molecular crowding due to distortion in the structural conformation. On the other hand, lower glutaraldehyde content (below 25 mM) could have not been sufficient to cross link all the nitrilases together.

Cross linking period is other pivotal factor that majorly affects the robustness, rigidity and activity recovery of the enzyme CLEAs. The ambient duration required for cross linking nitrilase with each other in its active state was 120 min. The residual activity and actual activity of nitrilase after cross linking with 35 mM glutaraldehyde for 120 min were 82.36 ± 4.45% and 0.105 ± 0.005 U/mg, respectively (Fig. 1c). A cross linking duration below or above ambient cross-linking time resulted in sparingly lower residual activity and actual activity of nitrilase. After a shorter period (below 100 min) the cross linking was incomplete, whereas prolonged cross linking period (above 120 min) may have caused excess cross-linking and impeded the active cleft of nitrilase.

Instrumental characterization

Particle size and morphology of nitrilase-CLEAs

[0022]The verification of cross linking with glutaraldehyde after aggregate formation of nitrilase with *iso*-propanol was assessed by analysis of particle size and surface morphology. Particle size analysis revealed the formation of wide range of aggregates and the average particle size was 1544.93 nm and polydispersity index was 0.271 (Fig. 2a). The scanning electron microscopy studies indicated the presence of abundant pores on the surface nitrilase CLEAs (Fig. 2b).

The porosity and size of CLEAs is not only associated with type and amount of crosslinking agent and additives used, but also the steps and process followed during preparation. Further, CLEAs are differentiated as Type-I (ball like spherical structures) and Type-II (less defined structures with more hydrophilicity) on the basis of degree of glycosylation of enzymes . The structure of prepared nitrilase-CLEAs was in a close resemblance with Type-II structure. In addition, CLEAs with less defined and large structure (Type-II) may have constrictions for transfer of substrate to active site of enzyme and vice versa. On the other hand, CLEAs with defined and small structure (Type-I) may possess challenges during recovery from the medium.

FTIR analysis

[0023]The FTIR of free nitrilase (ES-NIT-102) and nitrilase-CLEAs displayed a distinguishing spectrum of usual protein with primary functional peaks in 3400–3300 (1/cm) and 1700–1600 (1/cm) region which specified the stretching of OH and/or stretching vibration of NH, CO=N–H binding, and peptide linkages, while the minor peaks at 597(1/cm) indicated S-S linkages, respectively (Fig. 2c). Further, cross linking of nitrilase with each other by glutaraldehyde caused a reduction in intensity of functional peaks 3400–3300 (1/cm) and 1700–1600 (1/cm). A shift in amide I bond from 1630 (1/cm) to 1640 (1/cm) due to imine reaction amongst the amino group of enzyme and aldehyde group of glutaraldehyde was also noticed. This reduction in the intensities of peak proved the alterations in conformation amide domain of enzyme structure. Similar findings were also reported after preparation of β-mannanase CLEAs, and acrylamidase-CLEAs.

SDS PAGE analysis

[0024]The molecular weight of free nitrilase (ES-NIT-102) and nitrilase-CLEAs was enumerated by comparing the distance migrated against the proteins with standard molecular weight in the gel. The free nitrilase showed a single prominent band whereas nitrilase-CLEAs showed four distinguishing bands (Fig. 2d). The molecular weight of free nitrilase was found to be ~ 37 kDa. Cross linking of nitrilase with each other using glutaraldehyde forms large molecules with higher molecular weight of ~70 kDa, ~100 kDa, ~150 kDa, and ~250 kDa, indicating successful formation of nitrilase-CLEAs.

Kinetic constants

[0025]The kinetic constants of free nitrilase (ES-NIT-102) and nitrilase-CLEAs were enumerated by Michaelis-Menten equation and using Lineweaver-Burk double reciprocal plot with a varying concentration of substrate (Fig. 3). The K_m values of free nitrilase and nitrilase-CLEAs were 7.65±0.31 mM and 8.07±0.39 mM. An increase in K_m values after cross linking with glutaraldehyde indicated a reduction in affinity of substrate to enzyme, and this may lead to an increased hurdle for movement of substrate to catalytic domain of the enzyme. This can be further associated with a decline in conformational flexibility of enzyme after cross linking with each other and thus successively causing alterations in microenvironment in the nearby vicinity of the active domain of the enzyme. The V_{max} values of free nitrilase and nitrilase-CLEAs were 0.123 and 0.102 mM/min. A slight decline in V_{max} values after cross linking may lead to a decrease in conversion of initial substrate to desired product. The current results are in accordance with previous findings of nitrilase-CLEAs and immobilized nitrilase. A similar phenomenon was previously recorded for esterase-CLEAs, β-mannanase-CLEAs, formate dehydrogenase-CLEAs, acrylamidase-CLEAs, hydroxy nitrile lyase-CLEAs, and lipase-CLEAs.

Biochemical characterization

[0026]The reaction parameters usually practiced for carrying out enzyme mediated transformations are generally mild. The evaluation of biochemical parameters such as pH and temperature optima, along with pH and thermal stability are a pre-requisite for best performance of biocatalysts, and subsequently attain subsequently higher conversion and yields of desired product/s.

pH optimaand pH stability

[0027]The determination of optimum pH for free nitrilase and nitrilase-CLEAs was carried out by analyzing the activity in a wide pH regime ranging from 4.0-10at 40°C (Fig. 4a). The results revealed that the activity of both the nitrilases increased from the pH 6.0 to pH 7.5, and highest activity of both free nitrilase and nitrilase-CLEAs was seen at pH 7.0 in 100 mM potassium phosphate buffer. Nitrilase-CLEAs exhibited a better activity under both acidic and alkaline over the free nitrilase. Further, the pH stability study revealed a higher stability of nitrilase-CLEAs under acidic conditions, while free nitrilase to be stable under alkaline conditions. The free nitrilase in pH regime of 7-8 and nitrilase-CLEAs in the pH range 6-8 retained more than 50% of their initial activities (Fig. 4b). The interaction of glutaraldehyde and free amino groups of

enzyme nitrilase is by the development of Schiff bases and this may lead to transformation of its original ionization state and/or electrostatic charge/s, folding, and native conformation of enzyme.

Temperature optimaand thermalstability

[0028]The determination of optimum temperature for free nitrilase and nitrilase-CLEAs was carried out by analyzing the activity in a wide temperature regime ranging from 25-65°Cat pH 7.0 in 100 mM potassium phosphate buffer (Fig. 4c). The results revealed that free nitrilase and nitrilase-CLEAs displayed variations in sensitivities, and the optimum temperature was 40°C for and 45°C. The increase in optimum temperature by 5°C for nitrilase-CLEAs can be corroborated with the stability provided by strong covalent bond amongst glutaraldehyde and the aggregated enzymes.

A comparative investigation of thermal stability of free nitrilase and nitrilase-CLEAs was carried out in a temperature regime of 35-55°C in 100 mM potassium phosphate buffer of pH 7.0 (Fig. 4d and Fig. 4e). It can be seen that the activity of both free nitrilase and nitrilase-CLEAs reduced gradually with a rise in temperature and incubation time. The residual activity of nitrilase-CLEAs has reduced less and at a slow rate than free nitrilase, and this could be due enhanced thermal stability. The free nitrilase have retained 78.89%, 58.65%, 21.21%, 2.89%, and 1.80% activity after incubation for 24 h, 24 h, 24 h, 16 h, and 9 h at 35°C, 40°C, 45°C, 50°C, and 55°C, respectively. On the other hand, nitrilase-CLEAs were more tolerant to heat treatment as evident from the superior activities at time duration at each studied temperature. The subsequent residual activities of nitrilase-CLEAs at 35°C, 40°C, 45°C, 50°C, and 55°C after 24 h of incubation were 91.34%, 78.08%, 62.73%, 27.66%, and 0.52%, respectively.

Hydroxylation of 2-chloro-4-cyanopyridine (2-chloroisonicotinonitrile) to 2-chloropyridine-4-carboxylic acid (2-chloroisonicotinic acid)

[0029]The selection criteria of variables such as pH, temperature, enzyme loading, incubation time, amongst many others for optimization of process to obtain a desired product with required specifications are depended on characteristic features of product, substrate and reaction conditions that are possible to be followed. Herein, the hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid was carried out at different nitrilase-CLEAs loading (5%, 10, and 20%) at 30±2°C, and different temperature (25±2°C, 30±2°C, 35±2°C, and

40±2°C) at 10% enzyme loading. The progression of reaction with increasing incubation period was monitored by HPLC. The schematic presentation of nitrilase assisted reaction pathway for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid is displayed in Fig. 5. Moreover, the standard HPLC profiles of 2-chloroisonicotinonitrile (0.25 mg/mL 0.1% trifluoroacetic acid in water (55%) and acetonitrile (45%)), 2-chloroisonicotinic acid (0.25 mg/mL in 0.1% trifluoroacetic acid in water (55%) and acetonitrile (45%),), and enzymatic reaction with around 40% conversion are shown in Figure S2-a, S2-b, and S2-c (Supplementary file)

Effect of enzyme loading

[0030]The hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid was directly affected by the amount of nitrilase-CLEAs loaded. This could be attributed to the fact that product formation is linearlyassociatedwith the number of accessible active sites of enzyme to the substrate. Maximum conversion of 99% was seen at 20% loading of nitrilase-CLEAs after 15 h, while minimum conversion of 72% after 24 h incubation was seen at 5% loading (Fig. 6a). A considerably fair conversion of 98% was also seen with 10% loading after 24 h of incubation. The final product formation at 5%, 10% and 20% nitrilase-CLEAs loading was 69.59g/L, 94.72 g/L, and 95.69 g/L with a product yield of 61.20%, 83.30%, and 84.15%, respectively.

Effect of reaction temperature

[0031]The influence of incubation temperature on the progression of the hydroxylation reaction was analyzed due to its direct effect on rate of reaction, solubility of respective substrate and its corresponding product, and stability of enzyme and its activity. The hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid was carried out at 25±2°C, 30±2°C, 35±2°C, and 40±2°C at nitrilase-CLEAs loading of 10% for 24 h. A steady and consistent increase in conversion was seen with an increase in temperature in the early phase and which significantly got reduced with further progression of reaction (Fig. 6b). The conversion was 85%, 98%, 80%, and 72% at 25±2°C, 30±2°C, 35±2°C, and 40±2°C after 24 h with a product formation of 82.16 g/L, 94.72 g/L, 77.32 g/L, and 69.59 g/L with a product yield of 72.25%, 83.30%, 68%, and 61.20%, respectively. The slight reduction in conversion, product formation, and yield at higher temperature may be corroborated with the fact that enzyme progressively losses its activity at elevated temperature with prolonged incubation.

Instrumental characterization of 2-chloroisonicotinonitrile and 2-chloroisonicotinic acid

The instrumental characterization was carried out to confirm the formation of 2-chloroisonicotinic acid from 2-chloroisonicotinonitrile with ATR-FTIR, ^{1}H NMR, and LC-MS analysis.

FTIR analysis

[0032]The FTIR spectra of 2-chloroisonicotinonitrile and 2-chloroisonicotinic acid is presented in Fig. S3-a (Supplementary file). The presence of characteristic peak at 1590-1660 (1/cm) and 1500 (1/cm) indicated C-C and C-N in the pyridine rings. The presence of CN (nitrile) group in substrate was confirmed by prominent peak at 2250 (1/cm), while its conversion to –COOH (carboxylic group) was inveterated by distinct peaks at 3000 (1/cm) and 1720 (1/cm) revealing –OH stretching and C=O presence. The presence of C=C in pyridine was ascertained by peak at 1700 (1/cm). Finally the presence of C-Cl in the structure was revealed by sharp peak at 750 (1/cm).

LC-MS analysis

[0033]The liquid chromatogram profile of 2-chloroisonicotinic acid in LC-MS was same as that obtained in HPLC with a retention time of 4.475 min (Fig. S3-b (Supplementary file)). The mass fragments of the compound were determined by mass split in the instrument (Fig. 7a). The mono-isotopic mass of 2-chloroisonicotinic acid is 156.9 and this can also be confirmed by m/z of 156.87. The m/z fragments at 79.79, 114.77, 124.42, and 158.33 indicates the $(M+H)^{+}$of pyridine ring, chloro-pyridine, pyridine carboxylic acid, chloropyridine carboxylic acid, respectively.

^{1}H NMR analysis

[0034]NMR studies revealed the presence of three and four protons in 2-chloroisonicotinonitrile and 2-chloroisonicotinic acid, respectively.

In the substrate (2-chloroisonicotinonitrile), the proton at 3rd position gives a singlet peak (S) at 8.28 ppm, equivalent to single proton, and the proton present at the 5th position gives doublet of doublet (dd) at 7.94 ppm equivalent to single proton, and proton present at the 6thposition also gives doublet of doublet (dd) at 8.68 ppm and also equivalent to single proton (Fig. 7b).

^{1}H NMR (400 MHz, d_6, DMSO): δ7.94 (dd, 1H, J = 1.44 Hz, 1.44 Hz), δ8.26 (S, 1H), δ8.66 (dd, 1H, J = 5.0 Hz, J = 5.08 Hz).

In the product (2-chloroisonicotinic acid), the proton at 5th position gives the doublet at 8.61 ppm which is equivalent to single proton (S), and the proton present at the 3rd and 6th position were merged and gives multiplet at 7.82 and these were equivalent to two protons (Fig. 7c).

^{1}H NMR (400 MHz, d_6, DMSO): δ7.82 (m, 2H), δ8.61 (d, 1H, J = 4.63 Hz), δ14.00 (bS, 1H).

Reusability of nitrilase-CLEAs

[0035]The assessment of reusability of nitrilase-CLEAs was done for five consecutive cycles for a hydroxylation reaction performed in batch mode (Table 2). There was continuous reduction in conversion of substrate to product, product formation, and yield of product with each subsequent batch. Herein, the nitrilase-CLEAs were efficient in converting 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid till the third batch where the conversion was 81%, product formation was 75.53 g/L and yield was 66.42%, this was substantially less than the findings of first batch. In the fifth batch, the conversion reduced to 30% from 100%, product formation declined to 25.24 g/L from 96.65 g/L, and yield got down to 22.2% from 84.99%, when compared to the first batch. The continuous decline in the nitrilase-CLEAs after each progressive batch might be because of leach out of enzyme moieties from CLEA structure, generation of CLEA clumps, and/or reduced loading of enzymes caused by low shear resistance. In addition, the factors such as mechanical and physical forces exerted on nitrilase-CLEAs because of compression and firmness during/after centrifugation and/or filtration could have also added up in hampering of nitrilase activity.

CLAIMS:

We claim,

1) Process of preparation and useof nitrilase-CLEAs for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid.
2) The nitrilase claimed in claim-1 having average particle size was 1544.93 nm and polydispersity index was 0.271
3) The preparation of CLEAs of enzymes is carried out by precipitation followed by cross-linking with each other using a desired bi-functional agent.
4) Bi-functional cross linking agent claimed in claim-3 is glutaraldehyde, epichlorohydrin or glyoxyl-containing compounds, ethylene glycol-N-hydroxysuccinimide, genipin.
5) Bi-functional cross linking agent claimed in claim-3 is glutaraldehyde and is utilized as a cross linking agent for ES-NIT-102
6) The substrate used is 2-chloroisonicotinonitrile for conversion in to 2-chloroisonicotinic acid.
7) Temperature for reaction conversion is 25-35°C, nitrilase-CLEAs loading of 10%, pH 6-7.0 in 100 mM potassium phosphate buffer
8) The nitrilase-CLEAs used for conversion is reusable.

Abstract

Nitrilases capable of performing hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid were screened, and ES-NIT-102 was the best nitrilase for said biotransformation. Nitrilase was immobilized as cross linked enzyme aggregates (nitrilase-CLEAs) by fractional precipitation with *iso*-propanol, and cross linked with glutaraldehyde. The nitrilase-CLEAs prepared with optimized 35 mM glutaraldehyde for 120 min cross linking time had 82.36±4.45% residual activity, and displayed type-II structural CLEAs formation as confirmed by particle size, SEM, FTIR, and SDS-PAGE analysis. Nitrilase-CLEAs had superior pH and temperature stability, showed a shift in optimal temperature by 5°C, and retained nearly 1.5 to 1.7 folds activity over free nitrilase at 50°C and 55°C after more than 9 h incubation. Nitrilase-CLEAs showed reduced affinity and decreased conversion of substrate as indicated by slightly higher K_m values by 5.19% and reduced V_{max} by 17%. Further, these nitrilase-CLEAs showed 98% conversion, 94.72 g/L product formation, and 83.30% recovery after 24 h when used for hydroxylation of 2-chloroisonicotinonitrile to 2-chloroisonicotinic acid. Nitrilase-CLEAs were catalytically active for 3 cycles showcasing 81% conversion, 75.53 g/L product formation and 66.42% yield. The recovered product was confirmed by HPLC, FTIR, LC-MS, and ^{1}H NMR, and displayed >99% purity.