

Cellobiose Dehydrogenase from *Schizophyllum commune* Bcc26414: Purification and Characterization

Vinita Yadav¹ and Sadhana Nighojkar^{2*}

¹School of Biotechnology, Devi Ahilya University Indore (M.P) India.

²Mata Gujri College of Professional Studies, Indore (M.P) India.

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CDH, an enzyme produced by wood-decomposing fungi, has diverse applications in biosensor production, bioremediation, and biomedical industries. In this study, CDH from *Schizophyllum commune* BCC26414 was purified using ammonium sulfate precipitation, DEAE-cellulose chromatography, and Sephadex G-200 chromatography. The purification fold achieved was 65.81 with a specific activity of 1612.34 U/mg. The purity and molecular weight of CDH was confirmed using native and SDS PAGE. Optimal temperature and pH were found to be 30°C and 5, respectively. The purified CDH exhibited stability over a wide pH range (3.5 to 6.5) for 24 hrs and retained complete activity at 40°C, with reduced activity at 50°C when observed for 150 min. KCl, MgSO₄, ZnSO₄, and NiCl₂ at a concentration of 5 mM enhanced CDH activity and HgCl₂ and CuSO₄ inhibited the enzyme activity. The kinetic constants, Km and Vmax of CDH for lactose were observed to be 125 mM and 13.26 U/ml, respectively. The purified CDH may be utilized commercially in various applications.

Keywords: DEAE-cellulose anion exchange chromatography; Purification fold; *Schizophyllum commune* BCC26414; Sephadex G-200 gel filtration chromatography; Specific activity.

Fungi and bacteria have evolved to secrete several extracellular enzymes to degrade the lignocellulosic biomass¹. This lignocellulosic biomass consists of cellulose, hemicellulose, and lignin. Cellulose is the main structural component of the plant cell wall, and is made up of glucose monomers connected by β -1-4 glycosidic bonds. Many hydrolytic enzymes such as Endoglucanase, Exoglucanase and β -glucosidase are involved in the degradation of cellulose². All these enzymes promote the complete degradation of cellulose into glucose, and a partial degradation of crystalline cellulose. The complete metabolism of crystalline cellulose is catalyzed by the oxidoreductase

enzyme which is also involved in the electron transfer mechanism. Cellobiose dehydrogenase (CDH) is one of these enzymes which catalyzes the degradation of crystalline cellulose by an oxidoreduction mechanism³. Due to CDH oxidoreduction mechanism, hydroxyl free radicals are produced that randomly attack on cellulose⁴.

CDH enzyme causes oxidation of disaccharides, such as cellobiose, lactose, and maltose, as well as other oligosaccharides, producing corresponding lactones. These lactones then transform into carboxylic acids, like cellobionic acid, lactobionic acid, and maltobionic acid. Two domains, a flavin containing FAD

*Corresponding author E-mail: sadhana0301@gmail.com



and heme domain containing cytochrome b type heme have been described in the CDH monomer. Both of these domains are linked by an amino acid linker rich in serine and threonine, and this linker is sensitive to protease. FAD-containing fragments within the enzyme possess catalytic and cellulose-binding properties. CDH acts on beta 1,4 glycosidic linkages, thus monosaccharides remain unaffected. Also CDH exhibits a slow interaction with molecular oxygen according to Baminger et al.⁵ and Gupta et al.⁶.

A variety of applications in the fields of biomedical, biocatalysis, bioremediation and biosensor production have been reported for CDH⁷. There is a need to purify and characterize CDH for these industrial applications. *Schizophyllum commune* BCC26414 isolated from degraded wood was used for CDH production by SSF of groundnut shell⁸. In the present study, CDH has been homogenously purified and this purified CDH was further characterized.

MATERIALS AND METHODS

Organism and culture conditions

Schizophyllum commune BCC26414 was isolated from a degraded wood sample obtained from School of Biotechnology, DAVV Indore India. The fungus was maintained on Potato dextrose agar plate at 4°C.

CDH has been produced by solid-state fermentation using *Schizophyllum commune* BCC26414. SSF was carried out by taking 100 g of groundnut shell (0.5 mm to 1 mm particle size and moisture content 50%) in a 500 ml Erlenmeyer flask and autoclaved at 121 ° C for 20 mins. 2 ml inoculum was mixed in the SSF flask and incubated for 9 days at 30°C under static conditions. For enzyme extraction, 100 mM sodium acetate buffer (pH 4.5) was thoroughly mixed with the fermentation medium in (1:15 w/v). The mixture was incubated for 1 hr at 30°C and 120 rpm. The mixture was filtered using 4 layers of muslin cloth. The supernatant obtained after centrifugation of the filtrate at 7000 rpm for 15 mins at 4 ° C was used as crude enzyme for enzyme assay⁸.

Enzyme assay and protein quantification

CDH activity assay was performed using Dichloro Phenol Indo phenol (DCPIP). The reaction mixture contains 3mM DCPIP (in 10%

ethanol), 300mM lactose, 80mM NaF and 100 mM sodium acetate buffer (pH 4.5). The absorbance at 520 nm was measured after adding enzyme to the reaction mixture. One unit of CDH activity is defined as the quantity of enzyme necessary to reduce one μmol of DCPIP per minute under standard conditions⁸.

The protein content was measured by Folin-Lowry method using bovine serum albumin as standard⁹. Each experiment was carried out three times.

ESI- LCMS

ESI mass spectrometric analysis was used to identify the reaction product generated after the oxidation of lactose by CDH¹⁰. The reaction mixture containing 300 mM lactose and 3 mM DCPIP as described in enzyme assay was incubated for 5 hrs with CDH. The reaction was stopped by placing the mixture for 10 mins in boiling water bath and subsequently centrifuged for 10 mins at 8000 rpm. All the protein was removed from the supernatant following the protocol of Sutherland and Wilkinson (1971)¹¹. The final sample was analyzed by MicroTOF-Q ESI mass Spectrophotometer.

Purification of CDH

Ammonium sulphate precipitation

The crude enzyme was used for purification. The enzyme protein present in the crude enzyme was concentrated by ammonium sulphate at 90% (603g/l) saturation and incubated overnight at 4°C. Centrifugation at 8000 rpm for 15 min was used to separate the precipitate. Pellets were collected after centrifugation and suspended in a minimum volume of 100 mM sodium acetate buffer, pH 5. The concentrated enzyme suspension was desalted using a Sephadex G-25 column which is equilibrated and eluted with sodium acetate buffer.

Anion exchange chromatography

The desalted enzyme was applied to an anion-exchanger DEAE-cellulose column, pre-equilibrated with 100 mM sodium acetate buffer (pH 5). Fractions were collected after eluting with the same buffer, and the enzyme activity and protein content were examined. A linear 0-1 M NaCl gradient prepared in the buffer was used to elute bound proteins. The fractions containing protein (absorbance 280 nm) were subjected to protein estimation and enzyme assay.

Reverse dialysis against solid sucrose was used to concentrate the fractions showing highest CDH activity.

Gel filtration chromatography

Sephadex G 200 column equilibrated using 100 mM sodium acetate buffer pH 5 was used for further purification of concentrated CDH. After loading the sample, the column was eluted with the same buffer and each fraction was analysed for enzyme activity and protein. CDH active fractions were similarly concentrated using sucrose. The purified enzyme solution was used for characterization.

Electrophoresis

The purity of the enzyme was determined by native PAGE (stacking gel 4% and resolving gel 10%) using a gel electrophoresis system¹². Coomassie brilliant blue R-250 was used to stain and visualize the protein bands.

SDS-PAGE using 4% stacking gel and 10% resolving gel was carried out to check the presence of protein as well as determine the subunit structure of enzyme after purification^{13,14}. CDH mixed with 2X sample buffer was heated for 10 mins in boiling water bath and quickly chilled in ice bath prior to loading on electrophoresis gel. Protein bands were visualized after staining with Coomassie brilliant blue R-250.

Biochemical characterization of the enzyme

Effect of temperature and thermal stability

The effect of temperature on CDH activity was investigated by incubating the enzyme-substrate reaction mixture for 10 min at different temperatures ranging from 20–90°C with an interval of 5°C, at pH 5 using lactose as a substrate.

CDH's thermal stability was assessed by incubating the enzyme alone at various temperatures between 20 to 90 °C (with a 10-degree interval). The sample was taken out after every 15 minutes intervals till 150 mins to check the relative activity under optimum conditions¹⁰.

Effect of pH and pH stability

CDH activity was evaluated at different pH levels, using various buffer systems between pH 3 to 8, with a 0.5 interval. The pH range was prepared using sodium acetate buffer (pH 3 to 7.0) and Tris-HCl buffer (pH 7.5 to 8). The pH stability of CDH was examined by incubating the enzyme alone for 24 hrs at room temperature at various pH ranges, 3.0 to 8.0 (with an interval of 0.5). The sample was taken out and enzyme activity was evaluated. The enzyme activity at zero-time incubation was used as a control (100% activity)¹⁰.

Determination of K_m and V_{max}

The K_m and V_{max} values of purified CDH for lactose were calculated from the Lineweaver-

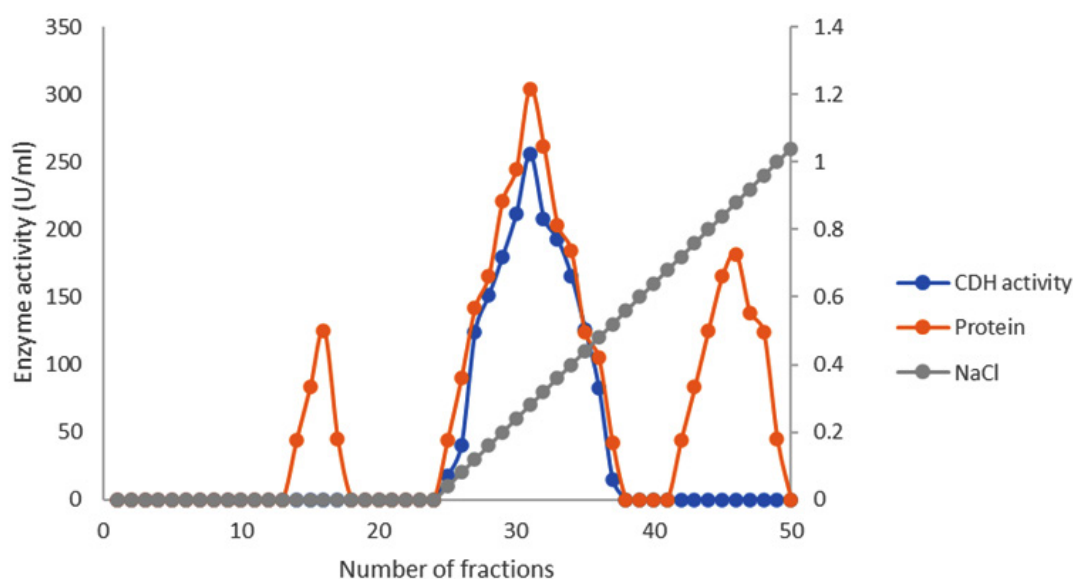


Fig.1. DEAE cellulose anion exchange column chromatography for CDH

Burk plot. CDH activity was measured with Lactose concentrations ranging from 100 to 1000 mM. Enzyme activity was determined at each concentration at 520 nm with 3 mM DCPIP as an electron acceptor. Each experiment was carried out three times.

Effect of metal ions

The effect of metal ions on CDH activity were studied using various ions mainly Ca^{2+} , K^+ , Na^+ , Mg^{2+} , Mn^{2+} , Cu^{2+} , Hg^{2+} , Zn^{2+} , and Ni^{2+} at concentrations of 2 and 5 mM. CDH activity was measured after incubation of the enzyme with the above metal ions for one hour at room temperature

under optimized experiment conditions. The enzyme assay was carried out using DCPIP, the electron acceptor and lactose as a substrate. The CDH activity was regarded as 100% when there were no metal ions present¹⁵.

Molecular weight

Molecular weight of CDH was calculated using Rf value of the standard molecular weight (MW) markers (Himedia) and comparing it with Rf of purified CDH protein.

Native and SDS PAGE has been used for molecular weight determination of purified CDH. Both the protein standard, and enzyme were

Table 1. Purification profile of CDH from *Schizophyllum commune*

Purification steps	Total enzyme activity Units ($\mu\text{mol}/\text{min}$)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	54192	2211.918	24.5	-	100
Ammonium sulphate fractionation(0-90%)	32354	619.45	52.23	2.132	59.70254
DEAE Cellulose Chromatography	17341	16.94	1023.17	41.76	31.99919
Sephadex G 200 Chromatography	6503	4.03	1612.34	65.81	12.00011

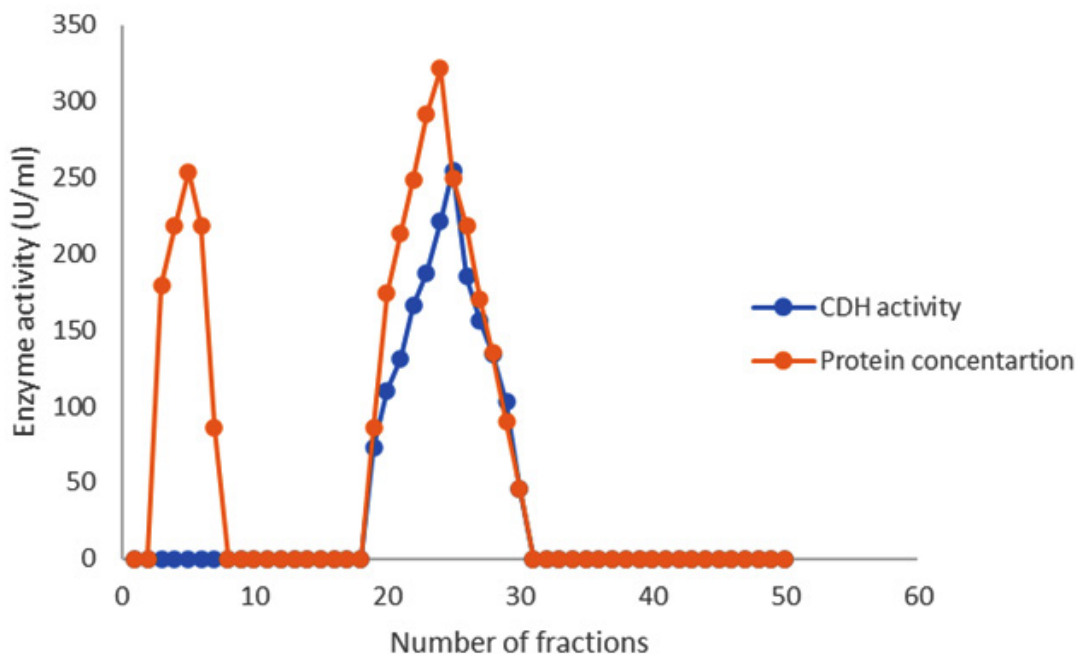


Fig.2. Elution profile of CDH on Sephadex G-200

electrophoresed on the same gel under identical separation conditions. The proteins were separated through the gel based on their molecular weight in an electric field. SDS gel was analysed to obtain

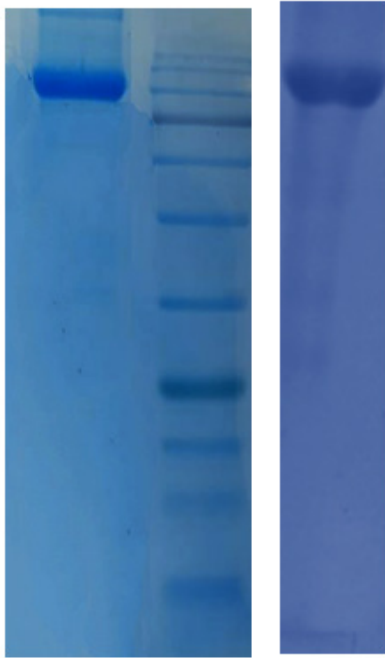


Fig. 3. Electrophoretic profile of purified CDH. Lane1- Molecular standard markers, Lane 2- Purified CDH on SDS PAGE gel, Lane 3- Purified CDH on Native PAGE gel

the Rf value for each band. The Rf is defined as $Rf = \frac{\text{Distance of migration of protein}}{\text{Distance of migration of the dye front}}$

A plot of MW versus Rf value on semi-logarithmic graph paper was generated from the bands in the gel to determine the MW of the unknown protein.

Statistical Analysis
All the experiments were performed in triplicates and mean values were calculated. The data were examined using the Analysis of Variance (ANOVA) and the P value of $d > 0.05$ was considered to be significant¹⁶.

RESULTS AND DISCUSSION

Production and Purification of CDH

CDH production by *Schizophyllum commune* was performed under SSF conditions. *Schizophyllum commune* was cultivated on groundnut shell for nine days. The crude enzyme was extracted in supernatant from the SSF after centrifugation. The crude CDH preparation was further purified and characterized.

Ammonium sulphate precipitation at 90% saturation was used to concentrate the enzyme and subjected to desalting by Sephadex G-25 Gel filtration chromatography (Table 1). Anion exchange chromatography employing DEAE cellulose was further used to purify the enzyme.

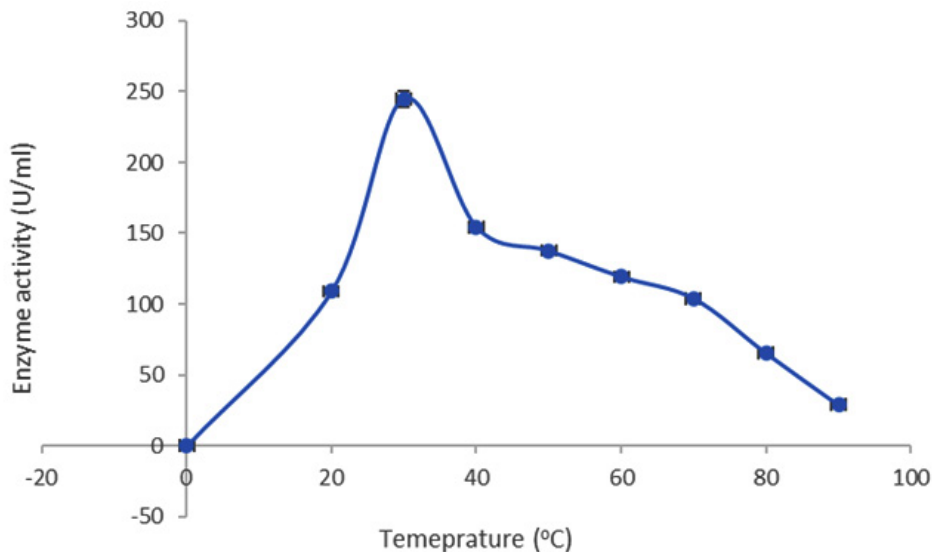


Fig. 4. Effect of temperature on CDH activity

The CDH enzyme was obtained in the form of a single peak (Fig. 1) when eluted using a linear salt gradient of NaCl ranging from 0 to 1M. After concentration, the enzyme sample was loaded onto Sephadex G-200 gel filtration chromatography and the elution profile (Fig. 2) showed the presence of a significant CDH enzyme in a single peak. The enzyme at this stage showed purification fold 65.81 with 1612.34 U/mg specific activity. 42-fold purified CDH from *Coprinellus aureogranulatus* has been reported after the final step on a HiTrap Q column¹⁶.

After purification, the specific activity of CDH was calculated to be 1612.34 U/mg, which is higher than the other reported basidiomycetes fungi for CDH^{10,17,18}. The cauCDH-specific activity was reported to be 28.9 U/mg at 37°C and pH 5.0¹⁶.

Electrophoresis

Native PAGE and SDS-PAGE analysis revealed that CDH is migrated as a single band which indicates that enzyme CDH has been purified to homogeneity (Fig. 3). It showed that CDH is a monomeric enzyme and a prominent protein in purified preparation. The molecular weight of CDH

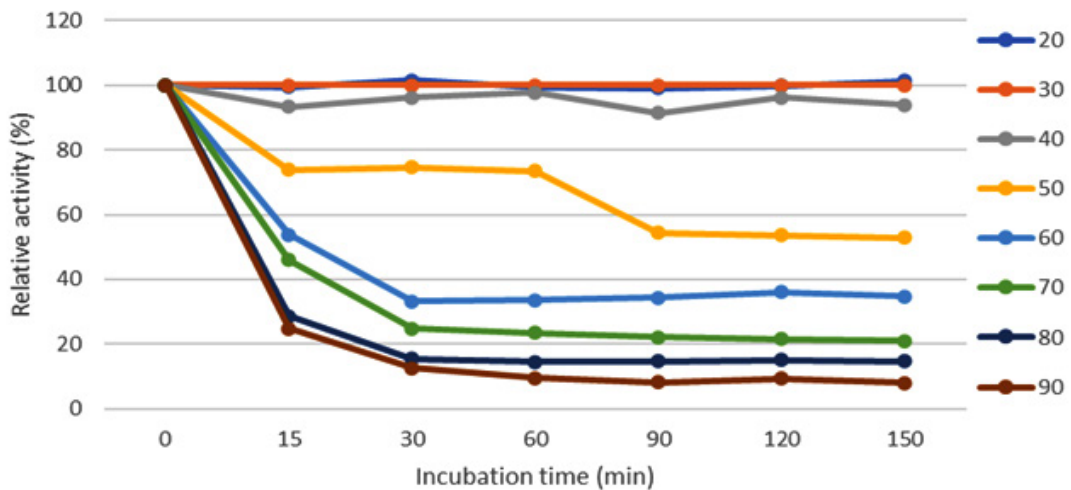


Fig. 5. Thermal stability of purified CDH at various temperatures

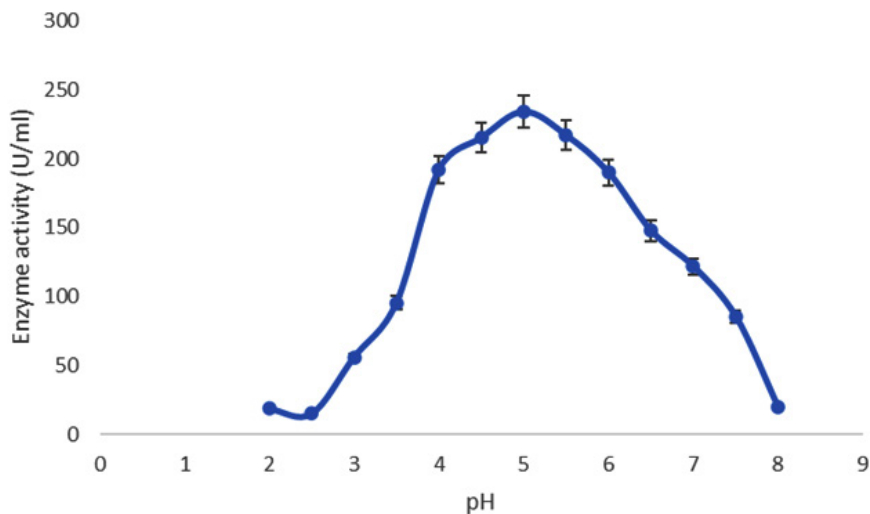


Fig. 6. Effect of pH on CDH activity

is found to be 100 kD. These results are consistent with other reports which have shown CDH to be a monomeric enzyme^{6,10,16,19}.

LCMS-ESI

Lactose is found to be a good substrate for CDH production. Lactose conversion to lactobionic acid was validated using LCMS-ESI. The lactobionic acid peak in the ESI- spectra was found at m/z 381, which was attributed to $[M^+Na]^+$. Similar results were reported by Saha et al (2008) where the spectrum only displayed one peak for lactobionic acid at m/z 381, and expected peak of lactose at m/z 383 was not seen¹⁰.

Biochemical characterization of CDH

Effect of temperature and thermal stability

CDH enzyme was analysed to determine its optimum temperature range, and it was observed that the enzyme remains active between 20°C to 70°C. (Fig 4). The optimum temperature of purified CDH from *S.commune* was found to be 30°C, and on increasing the temperature further, the enzyme activity decreases. Similar results were reported for CDH purified from *T.clypeatus*, where the optimum temperature was 30°C¹⁰.

Thermal stability of purified CDH was determined under varying temperature. The

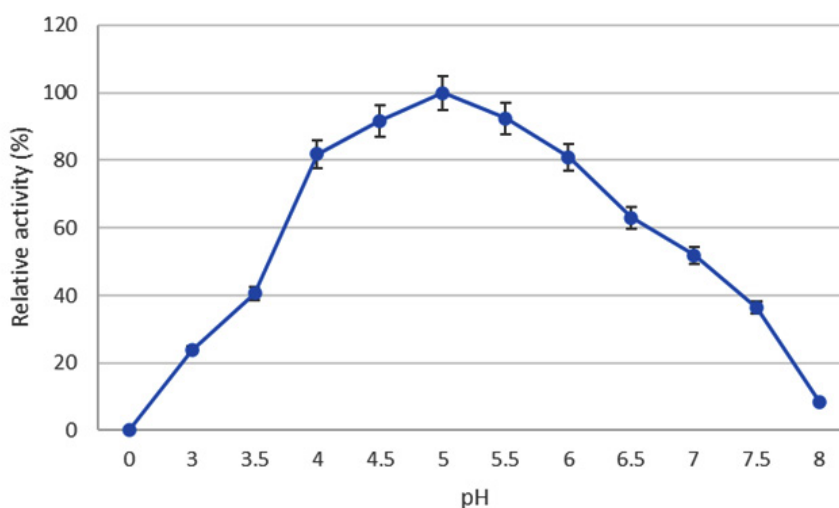


Fig. 7. pH stability of purified CDH after 24 hrs

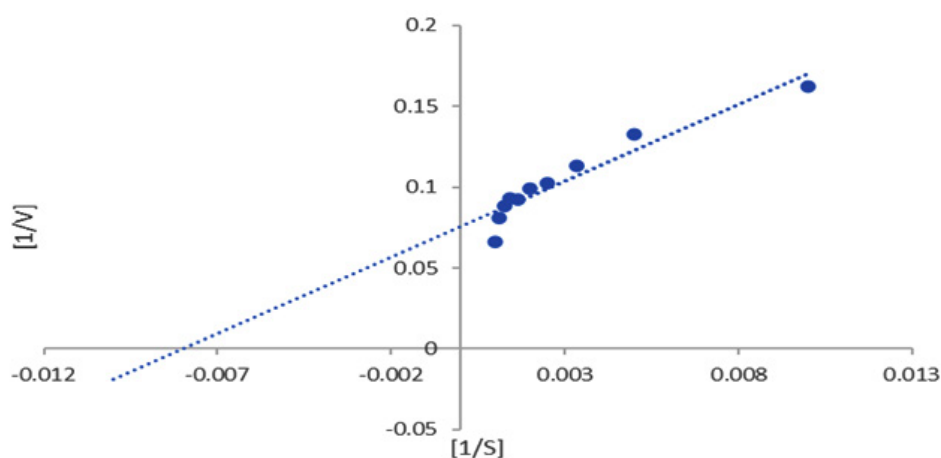


Fig. 8. Lineweaver-Burk plot for CDH at lactose concentration (100-1000 mM)

analysis of thermal stability demonstrated that the enzyme maintained its full functionality till 40°C, but a decrease in activity was observed at 50°C, after being incubated for 150 minutes (Fig. 5). The possible reason for this might be because enzymes denature at high temperature. Rai et al. (2020) investigated that CDH was stable at 50°C and enzyme activity declined after 50°C and the enzyme was unstable at 60°C²⁰. CDH obtained from

T. clypeatus was found to be sensitive to heat, as it lost 50% of its initial activity after being incubated at 50°C for a period of 30 minutes¹⁰.

Effect of pH and pH stability

The effect of pH on CDH activity was examined at different pH ranging from 3.0 to 8.0. The optimum pH was found to be pH 5 (Fig. 6). Similar results were reported for CDH from *T. clypeatus*¹⁰, *C. thermophiles*²⁰ *Trametes hirsute*¹⁷ where optimum pH was 5.0. On the contrary, optimum pH for other basidiomycetes CDH such as of *P. chrysosporium*, *Schizophyllum commune*, *T. hirsute* and *Trametes versicolor* has been reported at 5.5¹⁶.

To determine pH stability, CDH activity was evaluated at various pH ranges from 3.0 to 8.0 after a 24-hour incubation period (Fig. 7). The enzyme remained active after being subjected to incubation at a pH level ranging from 4.5 to 5.5. The enzyme exhibited considerable stability over a wide range of pH values, ranging from 4 to 6 when assayed with DCPIP. 50% CDH activity was retained at pH 7.0. The majority of CDHs experience a loss of their activity when exposed to pH below 3.0¹⁷.

Table 2. Effect of different concentrations of metal ions on CDH activity. The values represent relative activity with control being 100%

Metal ions	2mm	5mm
CaCl ₂	97.60±1.20	95.86±.99
KCl	99.42±0.72	102.90±0.25
NaCl	96.60±1.2	96.89±0.34
MgSO ₄	96.97±3.4	101.60±0.177
MnCl ₂	99.53±0.07	100.75±0.02
CuSO ₄	6.45±0.64	5.93±0.19
HgCl ₂	7.45±2.98	5.93±0.54
ZnSO ₄	97.08±1.06	102.83±0.05
NiCl ₂	100.75±0.02	103.08±0.3

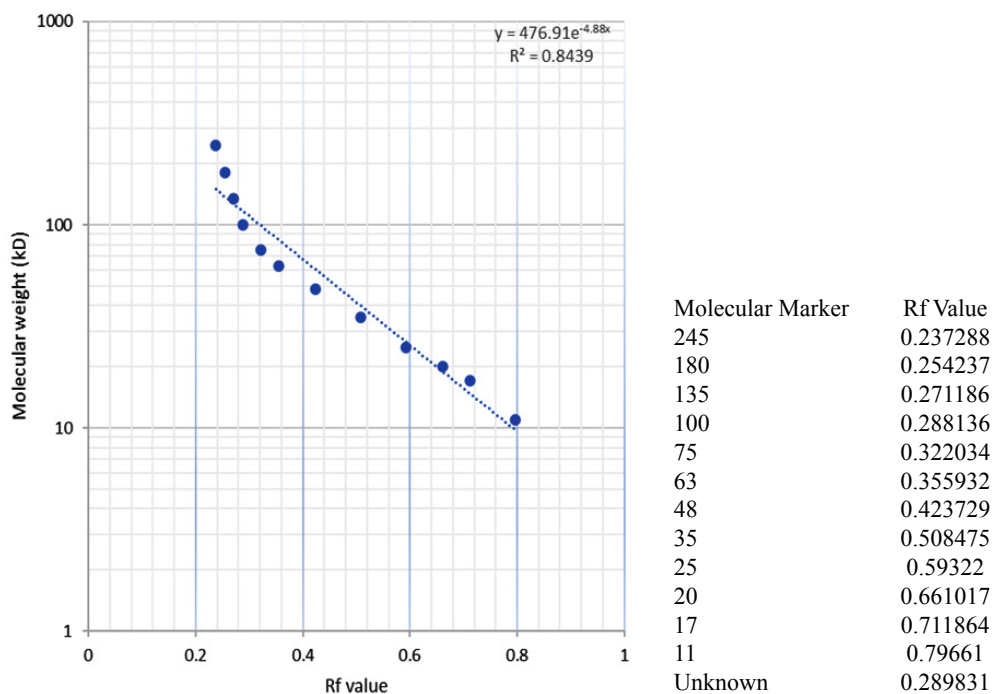


Fig. 9. Molecular weight Determination of CDH protein by SDS PAGE. Using common molecular markers, a standard curve of MW vs Rf was constructed

Effect of metal ions

Metal ions are potential effectors for the activity of CDH enzyme. The activation and inhibition effect of metal ions on CDH activity was related to their concentration. 5mM concentration of KCl, MgSO₄, ZnSO₄, NiCl₂ enhances CDH activity. HgCl₂ and CuSO₄ inhibit enzyme activity. *C. unicolor* and *Pycnoporus sanguineus* CDH are also reported to be sensitive to higher concentrations of CuCl₂^{14,21}. Enzyme activity was observed to be activated by the action of divalent cations²¹.

Determination of K_m and V_{max}

The K_m and V_{max} values of CDH for lactose were found to be 125 mM and 13.26 U/ml, respectively. The substrate affinity of CDH for lactose and cellobiose has also been observed from *Volvariella volvacea* (K_m is 1.95 U/mg), *T. clypeatus* (22.53 U/mg) and CauCDH (39.3 U/mg)^{10,23,15}.

Molecular weight

CDH is a monomeric protein with molecular weight 100 kD as estimated by native and SDS PAGE analysis using the Rf value of the prestained Protein standard (Fig. 3). A similar result was reported by Saha et al (2008), where CDH from *Termitomyces clypeatus* was reported to have 100 kD molecular weight¹⁰. The molecular weight of the present CDH is in accordance with that of other reported CDHs²⁴.

CONCLUSION

By employing the agricultural waste, ground nut shell as the solid substrate for SSF, *Schizophyllum commune* BCC26414 has been used to produce CDH. The CDH enzyme was isolated, and purified with a specific activity of 1612.34 U/mg and purification fold of 65.81. The molecular weight of monomeric CDH was found to be 100 kD, and enzyme homogeneity was revealed by Native and SDS-PAGE. The influence of metal ions on CDH activity was also investigated, and the findings showed that KCl, MgSO₄, ZnSO₄, and NiCl₂ at 5mM Concentrations increased CDH activity. CuSO₄ and HgCl₂ suppressed the CDH activity. The optimum temperature and pH of purified CDH was found to be 30°C and 5.0, respectively. Thermal stability demonstrated a decrease in activity at 50°C after 150 minutes

of incubation. The enzyme showed stability throughout a wide pH range of 4.0 to 6.0 when incubated for 24 hours. CDH was shown to have K_m and V_{max} values of 125 mM and 13.26 U/ml, respectively for substrate lactose.

The study focuses on producing CDH using *Schizophyllum commune* BCC26414 on a low-cost substrate, dried groundnut shell. The purified CDH enzyme had a high level of purity and activity, and showed stability over a wide range of pH and sensitivity to certain metal ions. The results suggest that the purified CDH has potential for commercial applications.

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Conflict of interest

The authors state that they have no competing interests, either financial or otherwise.

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