Effect of Nickel–Cobaltite Nanoparticles on Production and Thermostability of Cellulases from Newly Isolated Thermotolerant *Aspergillus fumigatus* NS (Class: Eurotiomycetes)

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Abstract In the present study, effect of nickel–cobaltite ($NiCo_2O_4$) nanoparticles (NPs) was investigated on production and thermostability of the cellulase enzyme system using newly isolated thermotolerant *Aspergillus fumigatus* NS belonging to the class Euratiomycetes. The $NiCo_2O_4$ NPs were synthesized via hydrothermal method assisted by post-annealing treatment and characterized through X-ray diffraction (XRD) and transmission electron microscopy (TEM) techniques. In the absence of NPs in the growth medium, filter paper cellulase (FP) activity of 18 IU/gds was achieved after 96 h, whereas 40 % higher FP activity in 72 h was observed with the addition of 1 mM concentration of NPs in the growth medium. Maximum production of endoglucanase (211 IU/gds), β-glucosidase (301 IU/gds), and xylanase (803 IU/gds) was achieved after 72 h without NPs (control), while in the presence of 1 mM concentration of NPs, endoglucanase, β-glucosidase, and xylanase activity increased by about 49, 53, and 19.8 %, respectively, after 48 h of incubation, against control, indicating a substantial increase in cellulase productivity with the addition of $NiCo_2O_4$ NPs in the growth medium. Crude enzyme was thermally stable for 7 h at 80 °C in presence of NPs, as against 4 h at the same temperature for control samples. Significant increase in the activity and improved thermal stability of cellulases in the presence of the $NiCo_2O_4$ NPs holds potential for use of $NiCo_2O_4$ NPs during enzyme production as well as hydrolysis. From the standpoint of biofuel production, these results hold enormous significance.

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Introduction

Cellulases find wide application in various fields, such as biofuel, detergent, and paper industries. Cellulases that bring about hydrolysis of cellulose comprise of three enzymes, namely endo-1,4-β-glucanase (also referred to as carboxymethylcellulase or CMCase; EC 3.2.1.4), exo-1,4-β-glucanase or cellobiohydrolases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) that synergistically convert cellulose into soluble sugars and glucose [1]. Thermostable cellulases can be used at high temperatures leading to increased substrate solubility and higher reaction rates resulting in high conversion of lignocellulosic biomass into fermentable sugars, essentially required for production of biofuels. Thermostable enzymes are stable at temperatures considerably higher than their optimum temperatures [2–4]. An enzyme or protein may be considered as thermostable if it maintains its half life at relatively higher temperatures for a longer time [5].

Fungi and bacteria are the most extensively studied microorganisms for the production of cellulolytic enzymes and degradation of cellulose. However, fungi are preferred over bacteria because of their high production potential and good penetrating ability [6]. Fungi belonging to the class Eurotiomycetes are well known for the production of useful secondary metabolites and enzymes [7]. Eurotiomycetes include morphologically and ecologically diverse group of fungi, and because of their broad importance in basic research, industry, and public health, several studies have started focusing on this fungal species [8]. However, to the best of our knowledge, fungi belonging to the class Eurotiomycetes have not yet been studied or exploited for the production of cellulolytic enzymes.

Presently, cellulases contribute to about 20 % of the world's enzymes market [9] and account for about 40 % of the total cost during ethanol production from cellulosic biomass [10]. High production cost and low production yields are the major bottlenecks for industrial applications of these enzymes [11]. Protein adsorption on nanomaterials can lead to enhanced activity and significant improvements in thermal stabilities of the enzymes [12, 13]. Nanoparticles have many properties which include large surface-to-volume ratio, high surface reaction activity, and high catalytic efficiency as well as strong adsorption capacity [14, 15]. Enzyme stability can be improved with support of nanoscaled and feasible modulation of the catalytic specificity [16]. Nickel–cobaltite ($NiCo_2O_4$) nanoparticles (NPs) are compounds which can be prepared in a nano-form that is apparently nontoxic. Among the various available metal oxides, $NiCo_2O_4$ is known as a low-cost environment-friendly transition metal oxide [17]. The electronic conductivity of $NiCo_2O_4$ is comparatively higher than those of nickel oxide and cobalt oxide. Nickel and cobalt ions are the important components of microbial media and play a crucial role in growth and production of microbial enzymes [18]. Therefore, the current study was focused on the synthesis of nickel–cobaltite ($NiCo_2O_4$) NPs, their characterization, and evaluating the effect of these NPs on cellulase production and thermostability of cellulase enzyme for its potential applications in different industries.

Materials and Methods

Materials

All the analytical chemicals, media components, and reagents used in the present study were purchased from Fisher Scientific (Mumbai, India) and HiMedia Laboratories Pvt Ltd (Mumbai, India). Aqueous ammonia solutions, cobalt nitrate (II) hex-hydrate [$Co(NO_3)_26H_2O$] and nickel nitrate (III) hexahydrate [$Ni (NO_3)_2·6H_2O$], were procured from Sigma-Aldrich (St. Louis, MO, USA.). Rice straw of variety PR-127 was procured from the research fields of Punjab Agricultural University (PAU), Ludhiana, Punjab, India. Rice straw was dried, cut into small pieces with chaff cutter, and ground into smaller particles of about 2 mm each and used as substrate for solid-state fermentation. Wheat bran was procured from the local flour mill.

Synthesis of Nickel–Cobaltite Nanoparticles

Nickel–cobaltite ($NiCo_2O_4$) NPs were synthesized by hydrothermal-assisted post-calcination process. The synthesis procedure involved the following steps: 1 mmol of $Ni (NO_3)_2·6H_2O$ and 2 mmol of $Co (NO_3)_2·6H_2O$ were prepared by mixing solution of distilled water (H_2O) and ethanol [H_2O: ethanol (1:1)]. The solutions of metal precursors were mixed together and stirred vigorously. Subsequently, 25 % aqueous solution of ammonia was gradually mixed to maintain the pH ~10 of the solution. The prepared solution was transferred to a 50-ml Teflon vessel for hydrothermal treatment. The reaction was carried out at 160 °C for 30 h. After the completion of reaction, the hydrothermal reactor was allowed to cool down to room temperature. The product was filtered and washed several times with deionized (DI) water and ethanol was dried at 60 °C under vacuum. The product thus obtained was further calcined at 400 °C for 2 h to obtain $NiCo_2O_4$ NPs.

Characterizations of the $NiCo_2O_4$ Nanoparticles

Phase formation and crystalline feature of the synthesized sample was investigated by powder X-ray diffraction (XRD) using a Philips X'Pert Pro-diffractometer, consisting of CuKa radiation ($\lambda = 1.54060$ Å). The surface morphology of the sample was probed through the field emission scanning electron microscope (FE-SEM) SIRIDN 2000, whereas the size and shape of the synthesized nanoparticles were studied by high-resolution transmission electron microscope (HR-TEM) Tecnai F30.

Isolation and Screening of the Fungal Isolates

Compost sample was collected from PAU, Ludhiana, India. The sample was aseptically transferred to the 250-ml Erlenmeyer flask containing 50 ml sterile distilled water. The flask was vortexed vigorously for 15 min on a vortex shaker. Fungi were isolated by serial dilution method using sterilized Rose-Bengal Chloramphenicol (RBC) agar medium plates. As the major objective of this study was to isolate thermotolerant/thermophilic fungal strains, the plates were incubated at 45 °C in an environment chamber having controls for temperature and relative humidity (RH) for 4 to 6 days and were observed regularly for colony development. Different colonies based on their morphological features, color of spores, and colony characteristics were picked, and pure fungal isolates were obtained by successive subculturing of

these colonies on the fresh potato dextrose agar (PDA) medium plates. Stock cultures were preserved at 4 °C on PDA slants in a refrigerator for further identification and characterization. Further, inoculum from the culture tubes of each of the isolate was transferred to carboxymethycellulose (CMC) agar medium. The plates were incubated at 45 °C for 48 h and were flooded with Gram's iodine for 5 min. Production of extracellular cellulase by the organisms was indicated by the zone of clearance around the colony [19]. Cellulolytic index (CI) was determined and expressed as the ratio between the diameter of the degradation halo and the diameter of the colony.

Effect of NiCo$_2$O$_4$ Nanoparticles on Cellulase Production

Solid-state fermentation was carried out in 250-ml Erlenmeyer flasks using rice straw and wheat bran (4:1) as substrate. In order to determine the effect of NPs on cellulase production, the medium was supplemented with different concentrations (0.5–3.0 mM) of NiCo$_2$O$_4$ NPs. The medium without NPs was treated as control. Moisture content of 70 % was made up with the Mandel Weber (MW) medium. Initial pH of the medium was adjusted to 4.8. Flasks were autoclave-sterilized, cooled, and subsequently inoculated with 10 % of spore suspension containing 10^7 spores/ml and incubated at 45 °C for 120 h. After cultivation of the fungal isolate in the fermentation medium, 50 ml sodium citrate buffer (pH 4.8) was added and the cultures were shaken for 30 min on a vortex shaker and subsequently centrifuged at 5,000 rpm for 10 min at 4 °C. The culture supernatant thus obtained after centrifugation was directly used for enzyme assays.

Molecular Identification of the Screened Fungal Isolate

For molecular identification of the selected fungal strain, genomic DNA was extracted and used as a template for PCR amplification of the 28S rRNA gene. The PCR amplified products were then purified and subjected to sequence analysis. The similarity search for the sequence was carried out using the BLAST program of the National Center of Biotechnology Information (NCBI). Phylogenetic analysis was carried out by neighbor-joining method using MEGA software program [20].

Enzyme Assays

Filter paper cellulase (FP) and endoglucanase (EG) activity were determined by the method described previously [21]. β-Glucosidase (BGL) was assayed by the procedure reported previously [22], while xylanase activity was determined by the method of Saratale et al. [23]. Reducing sugar concentration was determined by using the dinitrosalicylic acid (DNS) method [24]. One unit (IU) of EG or FP activity was defined as the quantity of enzyme required to liberate 1 μmol of glucose per milliliter per minute from CMC and filter paper, respectively, under standard assay conditions. In case of xylanase activity, one unit of xylanase was defined as the amount of enzyme needed to liberate 1 μmol of xylose per milliliter per minute from birchwood xylan under standard assay condition. One unit of BGL was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol (pNP) per milliliter per minute from p-nitrophenyl glucopyranoside. Protein concentration was estimated by Bradford method using bovine serum albumin (BSA) as a standard [25]. Specific activity was computed as the concentration of enzyme in the protein and reported as IU per milligram of protein.

Thermal Stability of Cellulases in Presence of Nanoparticles

In the present investigation, thermal stability evaluation was done on the basis of FP activity, as it measures the overall activity of multi-component enzyme complex for cellulose hydrolysis [26]. Effect of NPs on thermostability of enzyme was determined by incubating the crude enzyme at 60 to 80 °C for 2 h in presence of different concentrations (0.5 to 3.0 mM) of $NiCo_2O_4$ NPs. On the basis of results of these experiments, it was decided to conduct thermostability studies at 1.0 mM concentration of NPs for 0–8 h at 80 °C, discussed elsewhere in this paper. Crude enzyme solution without the NPs was treated as control. The control samples as well as the samples having NPs were evaluated for FP activity.

Statistical Analysis

All the experiments were carried out in triplicate, and the mean and standard deviation (SD) values were calculated using the Microsoft Excel program. The significance for the treatment means was determined with JMP 9.0 software (Statease Inc., MN, USA).

Results and Discussion

XRD Analysis of $NiCo_2O_4$ Nanoparticles

XRD pattern of the synthesized product is shown in Fig. 1. The XRD pattern showed six diffraction peaks corresponding to (220), (311), (400), (422), (511), and (440) planes of face-centered cubic spinal type structure of $NiCo_2O_4$ crystal. These diffraction peaks were well matched with (JCPDS-73-1702) [27]. The crystallite size of $NiCo_2O_4$ was calculated using the Debye–Scherrer equation and found to be ~30 nm [28].

FE-SEM and TEM Analysis of $NiCo_2O_4$ Nanoparticles

Figure 2a shows the FE-SEM image of the $NiCo_2O_4$ nanoparticles. It can be seen that that particles are well distributed over the entire region of the micrograph and possess the grain size

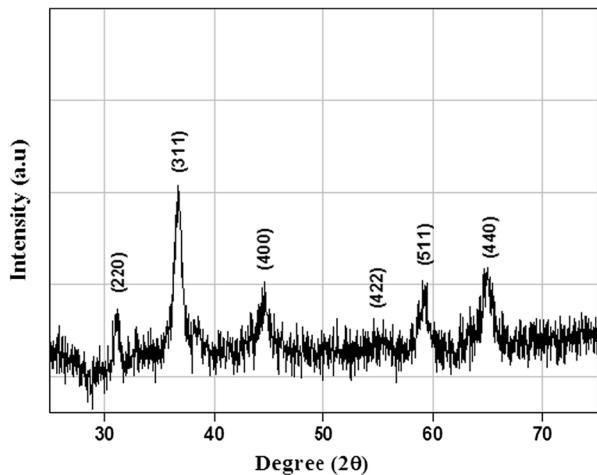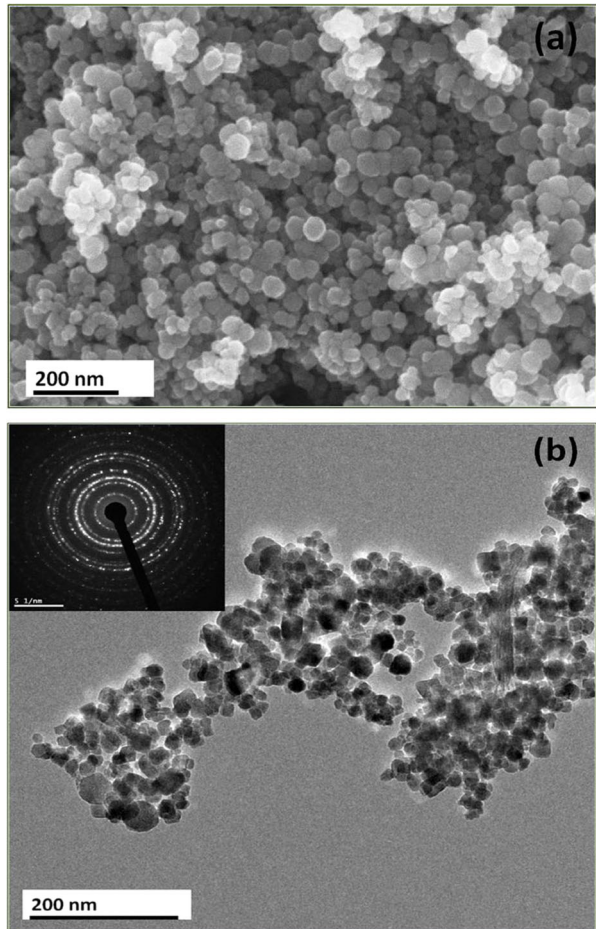**Fig. 1** XRD pattern of the synthesized nickel–cobaltite ($NiCo_2O_4$) NPs

Fig. 2 FE-SEM micrograph of a NiCo$_2$O$_4$ and b TEM micrograph of NiCo$_2$O$_4$ [*inset* shows selected area electron diffraction pattern]

in the range of 20–35 nm. The particle size of NiCo$_2$O$_4$ was further investigated through transmission electron microscope (TEM). Figure 2b shows TEM image of the NiCo$_2$O$_4$ nanoparticles which indicates that the nanoparticles were spherically shaped. The size of the nanoparticles was found to be in the range of 10–35 nm. In addition, the selected area electron diffraction (SAED) pattern [inset of Fig. 2b] shows a series of diffraction rings suggesting polycrystalline nature of the NiCo$_2$O$_4$ NPs [29].

Isolation and Identification of Cellulolytic Strain

Among the various fungal strains isolated from compost sample, four isolates that showed cellulolytic index ≥2 were selected and further evaluated for their enzyme production potential through solid-state fermentation (SSF). Among the four isolates, the isolate NS showed significantly higher enzyme activities as compared to other strains, and therefore, it was selected for further studies. The identity of the isolate NS was confirmed through molecular characterization. The 28S ribosomal DNA (rDNA) sequence of isolate NS was obtained and submitted to GenBank under accession no. KF 359928. Phylogenetic analysis based on BLAST search using 28S rDNA sequence showed that the strain exhibited maximum homology (100 %) with

Aspergillus fumigatus strain LCF20 with accession no. FJ867935.1. On the basis of the cladistic analysis and homology assessment, it was inferred that the selected isolate could henceforth be considered as *A. fumigatus* NS belonging to the class Eurotiomycetes. The class Eurotiomycetes is further divided into two subcategories including classified Eurotiomycetes and the unclassified Eurotiomycetes (NCBI database). Eurotiomycetes includes species of *Aspergillus* for which a sexual phase of reproduction has been identified.

Effect of $NiCo_2O_4$ Nanoparticles on Production of Cellulases

In order to evaluate the effect of $NiCo_2O_4$ NPs on the production of cellulases, different concentrations of $NiCo_2O_4$ NPs were used in the production medium. Nickel–cobaltite NPs used in the present study were not stabilized with surfactants or any other protective coatings and were as such added in production medium. It is clear from Fig. 3 that FP activity increased in the presence of NPs in the medium as compared to control and the maximum FP production was obtained at 1.0 mM concentration of NPs. In the absence of NPs, *A. fumigatus* NS produced FP of 18 IU/gds after 96 h, whereas it produced 40 % higher FP after 72 h in the presence of 1.0 mM NPs in the growth medium. Specific activity of NP-treated cellulase was also found to be significantly higher than the control sample (Fig. 4). However, production of cellulases decreased as the concentration of NPs increased beyond 1.0 mM and the enzyme activity reduced by about 20 % at 3.0 mM concentration of NPs in the growth medium. A decrease in enzyme activity with further increase in the concentration of NPs could be due to the lethal effect of metals at higher concentration which might have a detrimental effect on the living cells. Therefore, it was decided to carry out the enzyme assays for EG, BGL, and xylanase in addition to FP for the enzyme produced in control samples as well as in the presence of 1.0 mM NPs in the growth medium. It was observed that maximum production of EG (211 IU/gds), BGL (301 IU/gds), and xylanase (803 IU/gds) was achieved after 72 h of incubation in the absence of NPs (Fig. 5). On the other hand, EG, BGL, and xylanase activities were higher by about 49, 53, and 19.8 %, respectively, in the presence of 1.0 mM NPs after 48 h of incubation against control. Fall in enzyme production after reaching its maximum level could be due to the organism entering stationary phase of growth, depletion of the nutrients,
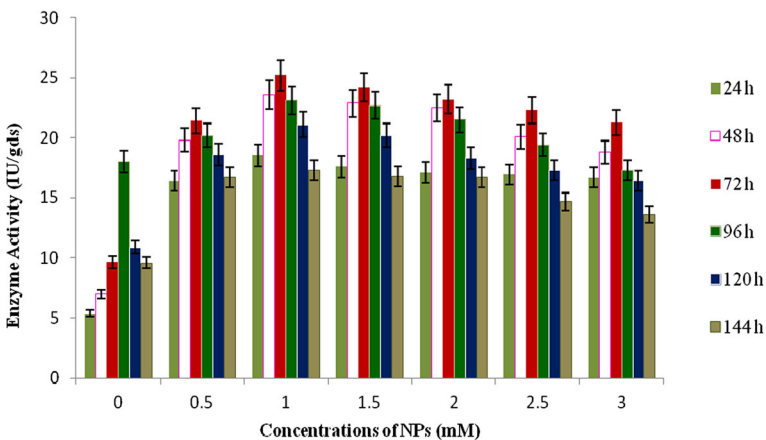

Fig. 3 Filter paper cellulase activity (FP) at different concentrations of $NiCo_2O_4$ NPs. LSD ($p<0.05$) value was 1.02, 1.01, 0.49, 0.70, 0.63, and 0.81 for FP activity at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM concentration of NPs, respectively
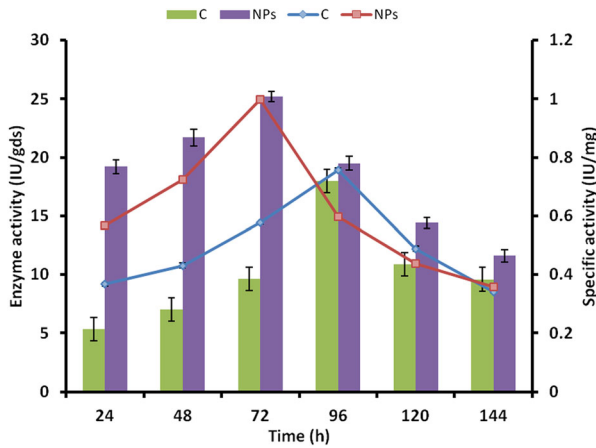
Fig. 4 Comparative evaluation of specific activity (IU/mg) and filter paper cellulase (FP) activity for the enzyme produced by *A. fumigatus* NS in the presence and absence of $NiCo_2O_4$ NPs in the growth medium. LSD ($p<0.05$) value was 0.012, 0.008, 1.002, and 1.000 for specific activity (control), specific activity (NPs), FP activity (control), and FP activity (NPs), respectively. *Bars* represent cellulase activity, while *lines* represent specific activity

and production of other by-products in the fermentation medium or combination of all of such factors. It is noteworthy to mention here that the incorporation of $NiCo_2O_4$ NPs in the medium not only led to higher cellulase production but also resulted in significant time saving, indicating a substantial increase in enzyme productivity as compared to control. As mentioned previously, large surface-to-volume ratio and high surface reaction activity associated with NPs could be the major reason for enhanced cellulase production and productivity in the
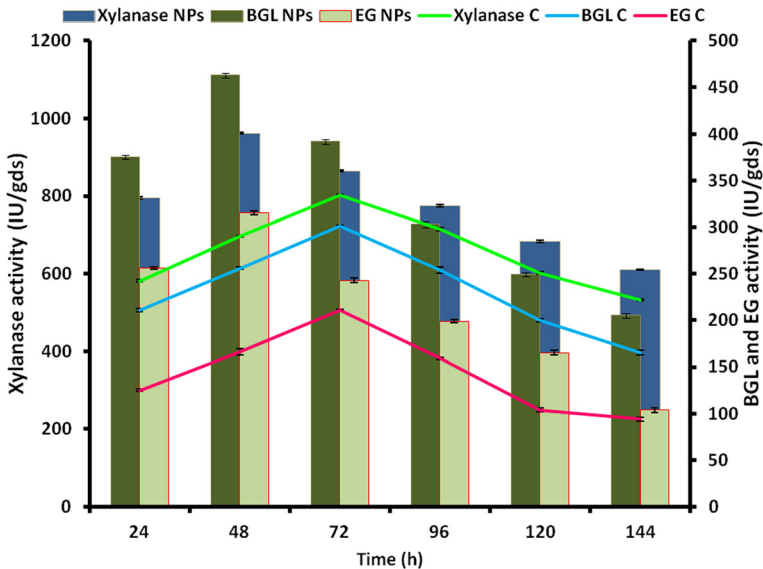

Fig. 5 Enzyme activities (IU/gds) for cellulase enzyme from *A. fumigatus* NS in the presence and absence of $NiCo_2O_4$ NPs. LSD ($p<0.05$) value was 3.68, 4.59, 3.68, 3.89, 4.19, and 4.21 for BGL (control), BGL (NPs), EG (control), EG (NPs), xylanase (control), and xylanase (NPs), respectively

presence of NPs. These results are significant as higher enzyme productivity is required for industrial biofuel production from cellulosic biomass.

Most of the cellulase-producing microorganisms show either inhibition or activation with different types of additives depending on the nature of the metals required [30]. Huang et al. [31] reported that supplementing specific metal ions in the medium increased the size and the amount of mycelium in white rot fungi, resulting in higher cellulase activity. Some other studies have also reported improved catalytic efficiency of cellulase when treated with nanoparticles [32, 33]. In a recent study, Mukhopadhyay et al. [34] reported that the production of pectate lyase enzyme increased by about 34.4 % in the presence of hydroxyapatite nanoparticles. In a study conducted by Shah et al. [35], production of BGL decreased in the presence of iron and copper nanoparticles, whereas in current investigation, enzyme activity significantly increased in the presence of $NiCo_2O_4$ NPs. In general, $NiCo_2O_4$ is regarded as a mixed metal oxide having spinal type crystal structure, where the nickel cation occupies octahedral sites, while the cobalt cations are distributed over the tetrahedral and octahedral sites in random order. The redox couples Ni^{+3}/Ni^{+2} and Co^{+3}/Co^{+2} found in the crystal structure provide effective electrocatalytic properties [36].

Effect of $NiCo_2O_4$ NPs on Thermostability of Cellulases

In the present study, thermostability characteristics of cellulases were tested at different temperatures in the presence of different concentrations of $NiCo_2O_4$ NPs up to 2 h. It is clear from Fig. 6 that the enzyme was stable at all the selected temperatures in the presence of 1.0 mM of $NiCo_2O_4$ NPs. At 60 °C, the enzyme retained about 74 % relative activity in control, while it could retain about 96 % activity in the presence of 1.0 mM NPs. However, further increase in concentration of NPs led to a fall in enzyme activity. Similar pattern was also obtained at temperatures of 70 and 80 °C, where enzyme stability increased from 69 to 87 % and 63 to 82 %, respectively, in the presence of 1 mM NPs, whereas in the presence of 3.0 mM NPs, the stability decreased to 53 and 42 %, respectively, as compared to control. The decrease in enzyme stability at higher concentration of NPs could be due to the nonsupportive interaction of NPs with the substrate. The stability of cellulase in presence of NPs at higher temperature could be because of the compositional differences in secondary structures (helix, sheet, and loop) of proteins which lead to differences in intramolecular interactions in a fold-dependent manner.

Fig. 6 Effect of temperature on thermostability of enzyme in presence of different concentrations of $NiCo_2O_4$ NPs (0.5–3.0 mM)
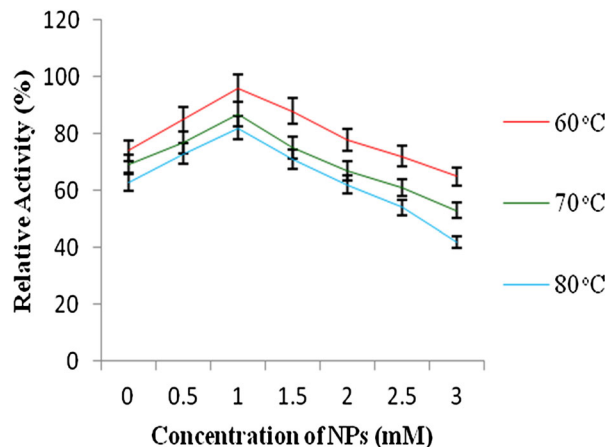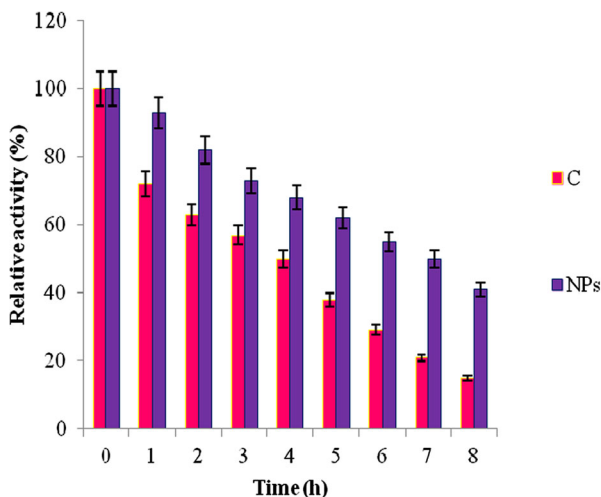
Fig. 7 Effect of temperature (80 °C) on thermostability of enzyme in the presence and absence of 1 mM $NiCo_2O_4$ NPs

Further, thermal stability of enzyme in the presence of 1.0 mM NPs and control samples were tested for 0–8 h at 80 °C. The results showed that at 80 °C, cellulase retained about 63 and 50 % of relative activity for 2 and 4 h, respectively, whereas the activity declined to 21 % after 7 h of incubation. On the other hand, cellulase in presence of NPs showed 68, 50, and 41 % higher stability after 4, 7, and 8 h of incubation, respectively, as compared to control at 80 °C (Fig. 7). These results clearly indicate that cellulase in presence of NPs showed enhanced stability at high temperatures compared to control. The use of enzymes for industrial purpose may require reactions to be conducted at high temperatures for a long time, such as in the case of processing of fibers in the textile industries [37]. It has been reported by Tao et al. [38] that under high temperatures, hydrolysis of cellulose occurs rapidly. Hegedus and Nagy [39] have also reported improved stability of cellulase in presence of NPs, as compared to control. Ang et al.[40] reported the half life of about 90 min for cellulase enzyme at 60 °C, while the cellulase used in current study showed a half life of 4 h which further increased to 7 h in the presence of $NiCo_2O_4$ NPs. In a study conducted by Mukhopadhyay et al. [34], effect of hydroxyapatite NPs on the thermal stability of pectate lyase was analyzed, but we are yet to come across any literature reporting the effect of $NiCo_2O_4$ NPs on thermostability of cellulases. These results clearly showed that cellulases in the presence of 1.0 mM $NiCo_2O_4$ NPs hold potential for efficient hydrolysis of cellulose at high temperatures. The nanoparticle-mediated retention of relative enzyme activity at elevated temperature indicates interaction of the nanoparticle with the enzyme active site. The rationale behind the effectiveness of $NiCo_2O_4$ NPs in stabilizing the activity as compared to the ionic Ni, Co metals needs further investigation at molecular levels. Additionally, catalytic activity can be enhanced by size reduction and uniform dispersion of the nanoparticles, which can provide high surface area, thereby resulting in an increase in the reaction rate of enzyme and substrate [17].

Conclusion

The results obtained in the current study illustrate that the presence of characterized $NiCo_2O_4$ NPs in the growth medium have a significant positive influence on cellulase production by newly isolated *A. fumigatus* NS. In addition, the enzyme in the presence of 1.0 mM $NiCo_2O_4$

NPs showed a much higher thermostability as compared to control (without NPs), in the buffer. This indicates a specific role of the $NiCo_2O_4$ NPs in promoting and maintaining the activation of both the catalytic and structural features of the enzyme in addition to the improved production of the enzyme. The cost-effective production of enzyme using inexpensive substrates along with improved thermostability indicates significant potential of these NPs and nanoparticles–cellulase system in production of sugars from the lignocellulosic biomass which could be subsequently fermented to bioethanol and biohydrogen.

References

1. Lynd, L. R., Weimer, P. J., Vanzyl, W. H., & Pretorious, I. S. (2002). *Microbiology and Molecular Biology Reviews, 66,* 506–577.
2. Saboto, D., Nucci, R., Rossi, M., Gryczynski, I., Gryczyniski, Z., & Lakowicz, J. (1999). *Biophyshysical Chemistry, 81,* 23–31.
3. Demirijan, D., Moris-Varas, F., & Cassidy, C. (2001). *Current Opinion in Chemical Biology, 5,* 144–151.
4. Haki, G. D., & Rakshit, S. K. (2003). *Bioresource Technology, 89,* 17–34.
5. Turner, P., Mamo, G., & Karlsson, E. N. (2007). *Microbial Cell Factories, 6,* 9. doi:10.1185/1475-2859-6-9.
6. Swaroopa, R. D., Thirumale, S., & Nand, K. (2004). *Biotechnology, 20,* 629–632.
7. Liu, Y. J., & Hall, B. D. (2004). *Proceedings of National Academy Sciences USA, 101,* 4507–4512.
8. Geiser, D. M., Gueiden, C., Miadlikowska, J., Lutzoni, F., Kauff, F., Hofstetter, V., et al. (2006). *Mycologia, 98,* 1053–1064.
9. Polizeli, M., Rizzatti, A., Monti, R., Terenzi, H., Jorge, J., & Amorim, D. (2005). *Applied and Microbiology Biotechnology, 67,* 577–5791.
10. Solomon, B. D., Barnes, J. R., & Halvorsen, K. E. (2007). *Biomass and Bioenergy, 6,* 416–425.
11. Kang, S., Park, Y., Lee, J., Hong, S., & Kim, S. (2004). *Bioresource Technology, 91,* 153–156.
12. Lynch, I., & Dawson, K. A. (2008). *Nanotoday, 3,* 40–47.
13. Chronopoulou, L., Kamel, G., Sparago, C., Bordi, F., Lupi, S., Diociaiutic, M., et al. (2011). *Soft Material, 7,* 2653–2662.
14. Pendry. (1999). *Science, 285,* 1687–1688.
15. Hudson, S. D., Jung, H. T., Percec, V., Johansson, W. D., Cho, G., & Balagurusamy, K. (1997). *Science, 278,* 449–452.
16. Konwarh, R., Karak, N., Rai, S. K., & Mukherjee, A. K. (2009). *Nanotechnology, 20,* 225107–225117.
17. Srivastava, M., Uddin, M. E., Singh, J., Kim, N. H., & Lee, J. E. (2014). *Journal of Alloys and Compounds, 590,* 266–276.
18. Mandels, M., & Weber, J. (1969). *Advances in Chemistry, 95,* 391–414.
19. Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). *Current Microbiology, 57,* 503–507.
20. Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). *Molecular Biology and Evolution, 24,* 1596–1599.
21. Ghosh, T. (1987). *Pure and Applied Chemistry, 59,* 257–268.
22. Kubicek, C. P. (1982). *Archives Microbiology, 132,* 349–354.
23. Saratale, G. D., Saratale, R. G., Lo, Y. C., & Chang, J. S. (2010). *Biotechnology Progress, 26,* 406–416.
24. Miller, G. L. (1959). *Analytical Chemistry, 31,* 426–428.
25. Bradford, M. M. (1976). *Analytical Biochemistry, 72,* 248–254.
26. Teeri, T. T. (1997). *Trends in Biotechnology, 15,* 160–167.
27. Ding, R., Qi, L., & Wang, H. (2012). *Journal of Solid State Electrochemistry, 16,* 3621–3633.
28. Monshi, A., Foroughi, M. R., & Monshi, M. R. (2012). *World Journal of Nano Science and Engineering, 2,* 154–160.
29. Srivastava, M., Ojha, A. K., Chaubey, S., & Singh, J. (2010). *Journal of Solid State Chemistry, 183,* 2669–2674.

30. Christakopoulos, P., Goodenough, P. W., Kekos, D., Macris, B. J., Claeyssens, M., & Bhat, M. K. (1994). *European Journal of Biochemistry, 224*, 379–385.
31. Huang, L. D., Zheng, G. M., Feng, C. L., Hu, S., Zhao, M. H., Lai, C., et al. (2010). *Journal of Chemosphere, 81*, 1091–1097.
32. Lupoi, J. S., & Smith, E. A. (2011). *Biotechnology Bioengineering, 108*, 2835–2843.
33. Blanchette, C., Lacayo, C. I., Fischer, N. O., Hwang, M., & Thelen, M. P. (2012). *Public Library of Science One, 7*, e42116. doi:10.1371/journal.pone.0042116.
34. Mukhopadhyay, A., Dasgupta, A. K., Chattopadhyay, D., & Chakrabarti, K. (2012). *Bioresource Technology, 116*, 348–354.
35. Shah, V., & Nerud, F. (2002). *Canadian Journal of Microbiology, 48*, 857–870.
36. Wei, T. Y., Chen, C. H., Chien, H. C., Lu, S. Y., & Hu, C. C. (2010). *Advanced Material, 22*, 347–351.
37. Rombouts, F. M., & Pilnik, W. (1986). *Symbiosis, 2*, 79–90.
38. Tao, M. Y., Xu, Q. X., Liang, S. M., Jaun, G., Bing, X. W., Long, M. N., et al. (2011). *Journal of Agriculture and Food Chemistry, 59*, 10971–10975.
39. Hegedus, I., & Nagy, E. (2009). *Hungarian Journal of Industrial Chemistry Veszprem, 37*, 123–130.
40. Ang, S. K., Shaza, E. M., Adibah, Y., Suraini, A., & Madihah, M. S. (2013). *Process Biochemistry, 48*, 1293–1302.