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Characterization of extracellular chitin deacetylase from *Aneurinibacillus aneurinilyticus* isolated from marine crustacean shell

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ABSTRACT

Chitosan is a promising biopolymer with wide range of applications. It is the deacetylated product of chitin. Commercially, it is produced from chitin via a harsh thermochemical process that has several shortcomings and heterogenous deacetylation product. Chitin can be transformed into chitosan through enzymatic deacetylation using chitin deacetylase (CDA), enabling the production of chitosan with a specific degree of deacetylation. CDA is primarily extracted from fungi followed by bacteria and insects. The extraction of CDA from fungus is more complex, possess several health risks for human including skin lesions. Therefore, screening of potent bacterial CDA is the need of the hour. In this study, for the first time we have isolated a bacterial strain Aneurinibacillus aneurinilyticus from the rinsed water of marine crab shell, and it was found to be a potent CDA producer. The extracellular CDA from *A. aneurinilyticus* has been partially purified and the specific activity of the enzyme was found to be 569.73 U/ mg protein. SDS-PAGE profiling of the purified sample depicts two isomers of CDA with molecular weights of 27 kD and 45 kD. The pH and temperature optima of the purified CDA were found to be 7.4 and 37 °C, respectively. The partially purified enzyme has Km and Vmax values of 98.455 μM and 909.09 μmole/ min, for non-chitinous substrate such as p-nitroacetanilide. For chitinous substrates like glycol chitin, N-acetyl glucosamine hexamer and colloidal chitin, the enzyme exhibited K_m of 96.96, 111.75 and 127.86 μM, respectively, V_{max} for these substrates were 23.31, 10.12 and 10.772 μmole/min, respectively. Metal ions like Mn and Mg considerably boost the production and activity of CDA, whereas Cd and Co strongly inhibit its activity. Insights from this study further substantiate that this enzyme follows Michaelis-Menten equation and has potential for industrial applications.

Abbreviation

CDA	Chitin deacetylase
COS	Chito oligosscharides

1. Introduction

The rapidly growing aquaculture industry is often linked to increased rate of marine food consumption. In recent years, the worldwide production of seafood has increased significantly making it the most traded food commodity. Marine food is the excellent source of omega-3 fatty acids, multivitamins and essential proteins (Teixeira-Costa and Andrade, 2021). Therefore, it enjoys a premium status in human diet. However, according to a recent report around 60 % of whole seafood mass is discarded, which mainly includes 20 % of muscles, 18 % of viscera, 15 % of bones, 12 % of heads, 5 % scales, and 3 % of skin (Dauda et al., 2019; Tacias-Pascacio et al., 2021). This accounts to generation of six to eight million tons of seafood waste per annum across the globe (Gao et al., 2021). Thus, the coastal environment is heavily polluted by the dumps of marine food waste which is poorly biodegradable (Shamshak et al., 2019). Chitin is the major constituent of crustacean shells and second most abundant biopolymer following cellulose (Rinaudo, 2006). It is considered as an excellent biopolymer with enormous beneficial uses, in addition to that it can be modified into different functional derivatives (Nisar et al., 2022). Bioconversion of chitinous food wastes into value-added products is an innovative and eco-friendly approach for waste management, and these products have wide range of industrial uses such as animal feed, agro-fertilizer, artificial skin & lens

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preparation, biogas and many more (Das et al., 2023; Santos et al., 2020a; Shamshina et al., 2019). Hence, there is a need to develop an efficient method for the proper management of chitin-rich seafood waste, along with the production of value-added products such as chitosan, which hold potential applications in the biomedical, pharmaceutical, and food processing industries (Roy et al., 2017). Among all the procedures available so far for the management of chitinous seafood waste, microbial bioconversion is considered as the most efficient approach because it is biocompatible, energy efficient and cost-effective with optimum yield of homogenous end products like chitosan. Both chitin and chitosan are considered as useful natural biopolymers but crystallinity of chitin polymers make it insoluble in most of the common polar and nonpolar solvents (Yadav et al., 2019). In industries, chitosan is primarily produced by breaking the amide bonds of chitin through a thermo- chemical process. Deacetylation of chitin is typically carried out under alkaline conditions. Treatment with 50 % NaOH at elevated temperature is the most common approach which also prevent breaking of glycosidic bonds (Srinivasan et al., 2018; Soon et al., 2018; Younes and Rinaudo, 2015). The properties of the resulting chitosan can vary based on the source of chitinous raw material and extraction procedure (Marei et al., 2016).

Innovations such as microwave irradiation have enhanced the efficiency of traditional deacetylation, allowing higher degrees of deacetylation (DD) and shorter processing time (El Knidri et al., 2016; Mahdy Samar et al., 2013). Despite their advantages thermo-chemical methods have significant drawbacks, including high energy consumption, environmental pollution from waste, and challenges in process control, which often results in chitosan with heterogeneous properties (Tolaimate et al., 2000; Tsaih and Chen, 2003; Younes et al., 2014). Factors like temperature, alkali concentration, and the use of reducing agents can influence the quality of chitosan, these methods resulting in uneven distribution of acetyl group and a reduction of molecular weight (Aiba, 1991; Nousiainen et al., 2000).

Enzymatic methods have emerged as an environment friendly alternative, providing greater control over deacetylation and preventing the molecular weight reduction observed with chemical methods. However, they are less effective on native chitin and require specific pretreatments to enhance their efficacy (Rass-Hansen et al., 2007; Pareek et al., 2013). Table 1 summarized the methods used by various studies for the production of chitosan, the degree of deacetylation achieved, the yield percentage as well as the advantages and disadvantages of each technique.

Several fungi and bacteria have been reported to have immense potential for modifying chitin polymers. They secrete various enzymes (chitinase, chito-oligosachharide hydrolase) to degrade chitin (Beier and Bertilsson, 2013; Lacombe-Harvey et al., 2018) and to deacetylate chitin (chitin deacetylase) (Pawaskar et al., 2021; Zhao et al., 2010a). Chitin deacetylase (CDA), initially isolated from *Mucor rouxii*, is an enzyme within the Polysaccharide deacetylases (PDAs) group and belongs to carbohydrate esterase family 4 (CE4) (Caufrier et al., 2003; Lombard et al., 2014). It facilitates the transformation of chitin into chitosan by removing acetyl groups from N-acetyl-d-glucosamine residues. These enzymes function through a conserved catalytic core known as the NodB homology domain. Comparative protein sequence analysis between fungal CDAs and bacterial nodB proteins reveals significant similarities, indicating functional homology despite evolutionary divergence (Grifoll-Romero et al., 2018). The molecular weight of different CDAs range from 25 kDa to 150 kDa, with approximately 30 % carbohydrate moiety (Zhao et al., 2010a).

CDA producing efficacy of fungi is significantly higher than that of bacteria but there are certain disadvantages associated with fungal strains. Most of the fungal species like *Mucor, Colletotrichum, Aspergillus* etc. which are the potent producers of chitinase and polysaccharide deacetylase are associated with several fungal diseases mainly skin lesions (Nwachukwu et al., 2021; Park and Mehrad, 2009; Shivaprakash et al., 2011; Soare et al., 2020). Culturing these fungal strains requires high temperatures and low pH, which deviate from normal growth conditions and are difficult to manage (Zhao et al., 2010a). Furthermore the extraction and purification process of CDA from the fungus is quite lengthy, time-consuming and costly (Senthilkumar et al., 2021; Sivaramanan and Samaraweera, 2014). CDA has been reported from several marine bacteria (Pawaskar et al., 2019; Zhao et al., 2010b) and the exoskeleton of some marine organisms represents a prominent source of chitin and chitosan (Muthu et al., 2021; Wang et al., 2020).

Table 2 provides a comprehensive analysis of Chitin Deacetylase (CDA) reported from various bacterial sources. Although many of these studies offer valuable insights into CDA, they often lack clarity in illustrating the enzyme's role in the conversion of chitin into chitosan. This conversion is crucial, as chitosan is a biopolymer with significant industrial and agricultural applications. Additionally, some studies have highlighted the potential of CDA as a pesticide, demonstrating its broader application beyond the bioconversion process. However, it is important to note that certain bacterial sources exhibit very low CDA activity, which restricts their practical applications in industrial context. Understanding the variations in CDA activity among different bacterial strains is essential for optimizing its use in chitin-chitosan conversion and other biotechnological processes.

Furthermore, production of chitosan from marine resources using an enzymatic method offer significant advantages in terms of both yield and quality (Aljawish et al., 2015; Kaczmarek et al., 2019). Since this bacterial strain has been identified and isolated from chitinous exoskeleton of marine mud crab shells, we hypothesized that this strain could be a potential source of chitin deacetylase. Therefore, we have a strong rationale to conduct this study with two-fold objectives. First, we aim to isolate and identify this bacterial strain (*Aneurinibacillus aneurinilyticus*) exhibiting CDA activity. Our second objective is the purification and functional characterization of this enzyme to assess its usefulness for possible industrial application. The overall findings substantially support that CDA isolated from this bacterial strain could be a potential candidate for enzymatic conversion of chitin to chitosan.

2. Materials and methods

2.1. Materials

P-nitro acetanilide was bought from TCI chemicals (Chennai, India). All the bacterial media used in this study were purchased from HiMedia (India). Chitin (Poly-(b1-4)-N-acetyl glucosamine) extra pure, Zinc sulphate, Ferrous sulphate, Magnesium chloride, Manganese chloride, Calcium chloride, and Cobalt chloride, were purchased from SRL Pvt. Limited (India). Protein molecular weight marker was purchased from NexGEN (India). All other chemicals used were of analytical grade.

2.2. Isolation of the bacteria

Chitinous exoskeleton of marine crab, *Scylla serrata* (mud crab) was collected from the local fish market, Bhubaneswar, Odisha and rinsed with the sterile distilled water. This water was further used as original source material for the isolation of chitinolytic bacteria, by culturing on agar plate and incubated with 80 % humidity for 24 h at 37 °C. The resulting colonies were further cultured to obtain pure cultures.

2.3. Isolation of CDA-producing strain

The pure bacterial cultures obtained were tested for chitinolytic activity in a chitin agar medium containing colloidal chitin (2 %), K_2HPO_4 and KH_2PO_4 (0.05 %), $MgSO_4{\cdot}7H_2O$ (0.25 %) and agar powder (1 %) (Kaur et al., 2012). Strains showing a clear zone of hydrolysis were further examined for their deacetylase activity in the above medium containing 0.025 % p-nitro acetanilide. CDA-producing strains deacetylate p-nitro acetanilide into p-nitroaniline giving the plate a yellowish appearance (Liu et al., 2016).

Table 1
Different reported methods of chitosan production with the degree of deacetylation (DD%) and yield.

Method	Raw material	Demineralization	Deproteinization	Deacetylation	DD (%)	Chiosan yield (%)	Advantages	Disadvantages	References
Thermo Chemical method	Shrimp shells	Shrimp shells:1 M HCl in 1:30 (w/v) for 75 min at 1505RPM; at room temperature (RT),	Shrimp shells: 3 M NaOH in 1:30 (w/v); RT, 75 min, 150 RPM	Shrimp shells: 50 % NaOH in 1:50 (w/v) 50 min, 90 °C	75	35	Quick processing; Industrial scale utilization; removal of organic residues completely;	Not ecofriendly; The high-temperature, application of concentrated alkali solutions in substantial quantities results in uncontrolled hydrolysis of the product. Additionally, the solubilized mineral and protein constituents render the material unsuitable for use as animal feed.	(Srinivasan et al., 2018)
Thermo Chemical method	Locus; Honey bees; Beetles	Raw exoskeleton:1 M HCl in 1:15 (w/v); RT;180RPM	Raw exoskeleton: 1 M NaOH in 1:15 (w/v); 8 h, 100 °C	Raw exoskeleton:50 % NaOH in 1:30 (w/v) for 8 h at, 100 °C	<95 %				(Marei et al., 2016)
Chemical method in combination with microwave techniques	Shrimp shells	Shrimp shells:3 M HCl in 1:10 (w/v)for 8 min at 500 W	Shrimp shells: 10 % NaOH in 1:10 (w/v); 8 minat160–350 W	1:20 (w/v); 50 % NaOH for 8 min,at 350 W	~83 %	~90 %	Very little amount of hazardous waste and zero organic salts in residuals;	Uncontrolled hydrolysis of chitosan; lengthy processing time; Limited laboratory-scale;	(El Knidri et al., 2016)
Chemical method in combination with microwave techniques	Cuttlefish pens	Cuttlefish pens:1 M HCl in 1:40 (w/v) at 3 h, RT	1:20 (w/v); 1 M NaOH 24 h, 70 °C	Cuttlefish pens: 45 % NaOH in 1:15(w/v) for 15 min, 600 W	93	–			(Sagheer et al., 2009)
Liquid fermentation techniques coupled with chemical deacetylation processes	Shrimp shells waste	*Pseudomonas aeruginosa strain* was cultured on a medium comprising 5 % shrimp shells, incubated at 37 °C for 144 h with agitation at 100 RPM.		autoclave the medium in 50 % NaOH: deacetylation with 50 % NaOH at100 °C and reaction with 50 % NaOH in microwaves	88	>90 %	Small amount of hazardous waste is produced, minimizing environmental impact; The extracted minerals and proteins can be repurposed as nutrients for humans and animals.	Time-consuming process Restricted to a laboratory-scale setup	(Sedaghat et al., 2017)
Liquid fermentation and enzymatic deacetylation	Minced prawn shell	liquid fermentation process involving a consortium of lactic acid bacteria, including *Lactobacillus salvarius, Enterococcus faecium,* and *Pediococcus acidilactici*. The fermentation was carried out at a temperature of 30 °C, with an agitation rate of 250 RPM, over a duration of 120 h.		Chitin substrates that were pre-treated in various ways were combined with chitin deacetylase from the fungus *Colletotrichum lindemuthianum* at a 1:1 ratio and incubated for 24 h at 50 °C.	~86	>87 %	The final product exhibits superior quality and is environmentally benign. Moreover, the extracted minerals and proteins can be repurposed as nutritional supplements for human and animal consumption	Restricted to laboratory-scale;	(Rass-Hansen et al., 2007)
Enzymatic and chemical deacetylation	Commercialy available chitin	Demineralization was achieved using 45 % phosphoric acid for 40 min, followed by precipitation through the addition of 6 M NaOH to adjust the pH to 8. Subsequently, a cold solution of 20 % NaOH and 0.2 % SDS was applied for 60 min at 4 °C. The mixture was then kept overnight at −20 °C and neutralized with 6 N HCl for 20 min at room temperature. Precipitation was carried out in cold distilled water, and a 60 % calcium chloride in methanol solution was added. Finally, the product was precipitated in a 1 % calcium citrate solution containing 25 % formic acid at room temperature.		Enzymatic deacetylation achieved by chitin deacetylase derived from the fungal strain *Penicillium oxalicum* SAEM-51, carried out at 50 °C for 24 h.	72	–	The processing duration is relatively brief; The final products demonstrate a high degree of deacetylation; The environmental impact is minimized due to the limited generation of hazardous waste.	Restricted to laboratory scale;	(Pareek et al., 2013)

Table 2
Sources of bacterial strain with significant CDA activity.

Sl No	Organism	Source	Enzyme activity	Reference
1	*Bacillus amyloliquefaciens Z7*	soil of the banks of the Xiangjiang River in Changsha City, Hunan, China	18.75 U/mg	(He et al., 2014)
2	*Bacillus licheniformis*	————	0.083 × 10^{-3} U/ml	(Bhat et al., 2019)
3	*Bacillus aryabhattai TCI-16*	Soil of Techeng Island Zhanjiang, China.	120.35 ± 2.40 U/mg	(Li et al., 2024)
4	*Bacillus cereus ZWT-08*	Activated silt samples from the coastal intertidal region of Zhejiang Province, China, were collected and analyzed.	135.52 U/mg	(Zhang et al., 2023)
5	*Bacillus subtilis*	————	0.89 U/ml	(Fukushima et al., 2002)
6	*Alcaligenes* sp. *ATCC 55,938*	American Type Culture Collection, Rockville, MD, USA	0.419 U/ml	(Elmekawy et al., 2013)
7	*Rhodococcus equi*	Various regions across northern, central, and southern China were surveyed, encompassing cities such as Chengdu, Shenyang, Xi'an, and numerous municipalities located within Shandong province.	19.20 U/ml	(Ma et al., 2020)
8	*Rhodococcus sp*	Qinling Mountain, Shaanxin Province, China	250 U/mg	(Gou et al., 2012)
9	*Sinorhizobium meliloti*	Saint Quentin-Fallavier, France	248.6 U/mg	(Chambon et al., 2017)
10	*Vibrio cholerae*	V. cholerae EI Tor N16961	8 U/ml	(Li et al., 2007)
11	*Vibrio parahaemolyticus*	Research Institute for Microbial Diseases, Osaka University (Japan)	3.6 U/ml	(Kadokura et al., 2007)
12	*Streptomyces griseoinacarnatus*	Sediments of Chilika Lake, Odisha	1.37 U/ml	(Behera et al., 2023)

2.4. Identification and characterization of the CDA producing bacterial strains

The morphology of individual colonies was assessed using Gram staining, which provides information regarding the size, shape, color, and texture of the bacterial strains. Biochemical characterization was conducted utilizing the Vitek-2 bacterial identification system. The selected isolates were identified through the amplification of the 16S rDNA gene, employing universal bacterial primers 27F (5′ GAGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ CTACGGCTACCTTGTTACGA 3′) as per the methodology outlined by Setia and Suharjono (2015). The amplification process for the 16S rDNA of the selected strain was executed using Taq DNA Polymerase in a Polymerase Chain Reaction (PCR) protocol. The PCR mixture, comprising 25 μl, included 1X PCR buffer, 200 μM dNTPs, 0.2 μM each of primers 27F and 1492R, 500 ng of template DNA, and 1 unit of Taq polymerase. The reaction conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min, concluding with a final extension at 72 °C for 10 min. Subsequent to amplification, the PCR products were purified using a QIAGEN Gel Extraction Kit (Hilden, Germany). The purified products were sequenced, and the resulting data were utilized for phylogenetic analysis. The obtained sequences were compared with the GeneBank database utilizing BLASTN to evaluate the homology of the 16S rDNA gene sequences. A phylogenetic tree was constructed employing the neighbor-joining method in MEGA software (Version 4.1), supported by multiple sequence alignment using CLUSTAL X software. The confidence levels of each branch were assessed through bootstrapping with 1000 replicates, as described by Tamura et al. (2021).

The incubation time for the CDA activity was optimized using the well diffusion method. Agar plates containing 0.25 % p-nitroacetanilide were prepared, and crude CDA at various concentrations (100 μl to 500 μl) was added. The plates were then incubated at 37 °C and monitored at regular time intervals for the appearance of a yellow color. A negative control plate was prepared by adding the supernatant of the broth medium without the inoculum.

2.5. Protein estimation and enzyme activity

The extracellular supernatant of the bacterial sample was extracted at different time intervals (3 h, 6, 9, 12, and 24 h) and centrifuged at 10,000 × *g* for 10 min at 4 °C. The concentration of protein was measured by adopting the methods of Lowry et al. (1951) using BSA as a standard. The enzyme assay was based on the type of substrate subjected for deacetylation. Chitinous substrates are assayed through MBTH (3-methyl-2-benzothiazolinone hydrazone) assay (Tsuji et al., 1969) and non- chitinous substrate like p-nitro acetanilide assayed by the method developed by Liu et al. (2016). The reaction mixture for the deacetylation of p-nitro acetanilide to p-nitro aniline by CDA, contain 0.5 ml of p-nitroacetanilide (200 μg/ml in 50 mM phosphate buffer pH 7.4), 0.5 ml of enzyme, and 1 ml of buffer (50 mM phosphate buffer pH 7.4). The mixture was incubated for 6 h at 37 °C. The reaction was terminated by boiling the above mixture at 100 °C for 5 min and the absorbance was recorded at 400 nm. Phosphate buffer (pH 7.4, 50 mM) without the enzyme or substrate served as the reference. Additionally, enzyme blanks (reaction mixture without enzyme) and substrate blanks (reaction mixture without substrate) were used as negative controls. One unit of CDA enzyme activity is defined as the quantity of enzyme required to catalyze the release of 1 μg of p-nitroaniline from the substrate p-nitroacetanilide per hour under the standardized assay conditions. The MBTH method was utilized to determine the CDA activity using chitinous substrates. This method is based on the quantification of hexosamines produced through the deacetylation reaction. A 500 μl reaction mixture was prepared, comprising 50 μl of enzyme solution, 200 μl of substrate, and 250 μl of 50 mM phosphate buffer, followed by incubation at 37 °C for 30 min. After incubation, 0.5 ml of 5 % $KHSO_4$ and 0.5 ml of 5 % $NaNO_2$ were added. The solution was then incubated at room temperature with occasional shaking for 15 min to complete deamination. Excess nitrous acid was removed by adding 0.5 ml of 12.5 % ammonium sulphamate and repeatedly shaking the mixture for 5 min. To the deaminated mixture, 0.5 ml of 5 % MBTH was added, and the mixture was heated in a boiling water bath for 3 min and then cooled. Finally, 0.5 ml of 5 % ferric chloride was added. After 30 min of incubation at room temperature, the absorbance was recorded at 650 nm. A solution containing all components except the substrate and enzyme served as a reference, and enzyme and substrate blanks were used as negative controls. Glucosamine was employed as the standard for estimation. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of glucosamine per minute.

2.6. SDS-PAGE

For analysis of protein expression, SDS-PAGE was performed with polyacrylamide gel (12 %) (Bhilocha et al., 2011) and stained with silver nitrate using the silver staining method developed by Kumar (2018).

2.7. Effect of metal ions on growth, protein content of the bacterial strain

The effect of various metal ions on the growth rate, protein production, and CDA activity of the isolated strain was investigated using $ZnSO_4 \cdot 6H_2O$, $MgSO_4 \cdot 6H_2O$, $MnCl_2 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, and $CdCl_2$. Each metal ion was added to the culture medium at different concentrations (0.1 mM, 1 mM, 1.5 mM, 2.5 mM, and 5 mM). Treated samples were collected at regular intervals (3, 6, 9, 12, and 24 h) for further analysis of various parameters. A control sample was prepared by inoculating the culture medium with the strain in the absence of any metal ions. This control was essential for comparing the effects of metal ions on growth rate, protein production, and CDA activity. By analyzing both the control and metal-treated strains, we were able to assess the modulating effects of the metal ions on these parameters.

2.8. Partial purification of CDA

The overnight incubated culture broth was centrifuged for 10,000 x g, 20 min at 4 °C and the supernatant containing the extracellular CDA was subjected to ammonium sulphate salt precipitation. The ammonium salt was added to the supernatant to achieve 80 % saturation. The solution was gently stirred at 150 rpm for overnight at 4 °C to facilitate the protein precipitation. The precipitate obtained from the 100ml supernatant (3.24 g) was suspended in 5 ml of Phosphate buffer 50 mM, pH 7.4. The precipitated sample was dialyzed against the same buffer (Phosphate buffer 50 mM, pH 7.4), using a 10 kDa dialysis membrane that had been pre-activated with the buffer. The protein sample was loaded into the dialysis membrane and, the buffer was changed at every 3 h interval for a total of 3 times followed by overnight dialysis for ensure complete removal of salts. The dialyzed solution was subsequently subjected to size exclusion and ion exchange chromatography. The sample was loaded onto a column (0.8 × 10 cm) containing Sephadex G-25 resin beads pre equilibrated with the aforementioned buffer and elution was carried out with the same buffer at a flow rate of 0.1 ml/min. Protein content was determined by measuring the absorbance at 280 nm using a UV- visible spectrophotometer. Fractions exhibiting CDA activity were pooled and then applied to Sephadex G-50. Elution was performed under the same conditions. The fraction with the highest CDA activity from the Sephadex G-50 column were mixed and subjected to DEAE ion exchange column (0.8 × 10 cm). The sample was eluted using a gradient of 0.1 M to 0.5 M NaCl prepared in 50 mM phosphate buffer (pH 7.4). Active fractions were again dialyzed against the working buffer and stored at -20 °C for further use. The volume of each eluted fraction was maintained at 0 .2ml. The approximate molecular weight of CDA was determined by SDS–PAGE with a pre-stained protein ladder that indicate various molecular weight.

2.9. Characterization of purified CDA

To determine the optimal pH for the activity of partially purified CDA enzyme, the assay was conducted at 37 °C using following buffers with different pH values: 50 mM citrate buffer (pH 4.0 and pH 5.0); 50 mM potassium phosphate buffer (pH 6.5, pH 7.4, and pH 8) and 50 mM carbonate buffer (pH 9 and pH 10). To determine the temperature optima of the partially purified CDA, the enzyme activity was performed under optimal pH with different temperatures, ranging from 4 °C to 100 °C. The impact of different metal ions (Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}) on the activity of purified CDA was analyzed by incubating the enzyme with these metal ions over a range of concentrations (0.1 mM, 1 mM, 1.5 mM, 2.5 mM, 5 mM) for 1 hour, followed by activity assay of the CDA under optimum reaction conditions.

2.10. Statistical analysis

The experiments were conducted in three biological replicates, each with three technical replicates. The data were analyzed using GraphPad 5.0 software, applying the Bonferroni post-test with a significance level of $p < 0.01$.

3. Results and discussion

3.1. Bacterial isolation

Chitin deacetylase (CDA), derived primarily from fungi, bacteria, and insects, possesses health risks including poisoning, parasitic infections and allergies (Baxi et al., 2016). Fungi, especially *A. fumigates* and *Mucor species* can cause severe respiratory issues and skin lesions (Hartnett et al., 2019; Knutsen et al., 2012). Although, fungus is the prime source of CDA, extraction of the CDA from it is laborious and costly. Bacterial sources are preferred due to lower health risks and greater economic benefits. In the current investigation, a total number of 35 bacterial strains were isolated out of which, only 8 strains have chitinolytic activity by forming a zone of hydrolysis (Fig. 1 A & B) on chitin agar plate as observed in the primary screening. Further, the CDA-producing strains were isolated through secondary screening using p-nitroanilide as a substrate for CDA. Out of the 8 strains, only 4 strains developed a yellow coloration in the p- nitroacetanilide agar plate. The characteristic yellow color appeared after 24 h of incubation which confirmed deacetylation by converting p-nitroacetanilide to p-nitroaniline (Fig. 1 C &D). The strain (JPD) exhibited the highest CDA activity among the four CDA-positive isolates and was further subjected to morphological, phylogenetic and biochemical analysis (Fig. 1E)

3.2. Characterization of the strain

The selected bacterial isolate was characterized according to the morphology, physiology and biochemistry of the strain. The bacterial colonies developed on nutrient agar plates were aerobic, motile, round-shaped, smooth, opaque, and shiny (Fig. 1A, B). Gram staining confirmed that the isolate was a gram-positive bacterium and observed as rod-shaped spore-producing bacillus under light microscope (Fig. 1E). The strain's antibiotic sensitivity was assayed through an Antibiogram assay with the results detailed in Table 1. The biochemical characterization and identification of the strain were performed using Vitek2 which is elaborately described in Table 2. The physiological, morphological and biochemical characteristics of the strain strongly suggested that it belongs to the *Bacillus* family (Grady et al., 2016).

3.3. Phylogenetic identification and characterization of the strain JPD

Among the various methods for bacterial identification, 16 s rDNA sequence analysis, on the basis of homology study of the 16 s rDNA gene is considered as the most precise method (Ojo et al., 2008). Sequence alignment of the 16S rDNA of the above strain JPD with other 16S rDNA sequences available at Gene bank database revealed 100 % sequence identity of the above strain (Fig. 2) with *Aneurinibacillus aneurinilyticus*.

According to the findings of Chambon et al. (2017) *Rhizobium* species involved in the symbiosis of plants typically contain the nod B gene which codes for NodB chitin deacetylase playing a significant role in nitrogen fixation. Chauhan et al., also explained the plant growth-promoting and nitrogen-fixing activity of *A. aneurinilyticus* CKMV suggesting the presence of the chitin deacetylase coding gene in the strain (Chauhan et al., 2014). To our knowledge, the presence of CDA in an *A. aneurinilyticus* strain is reported for the first time in this manuscript (Fig. 2Tables 1 and 2). Tables 3 and 4.

ZI- The inhibition zone shown by the different antibiotics was determined by measuring the inhibition diameter. According to the Performance Standards for Antimicrobial Disk Susceptibility Tests (Clinical and Laboratory Standards Institute CLSI, 2020) the organism is considered as susceptible (S) if the diameter is (≥2 cm), moderately susceptible (M) if the diameter is (<2 cm- >0.5 cm) and resistant (R) if the diameter is (≤ 0.5 cm)

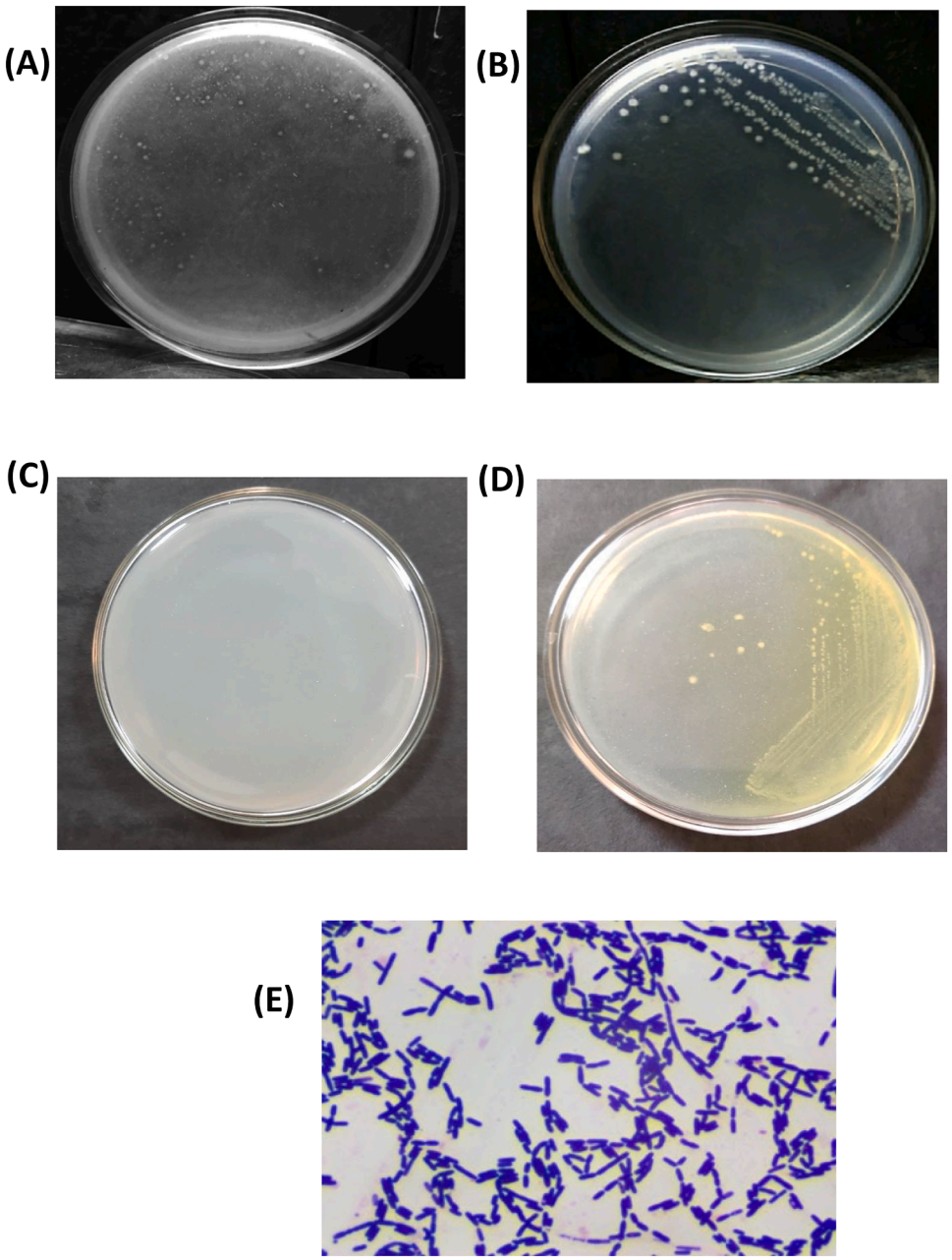

Fig. 1. Screening of Chitin Deacetylase (CDA) Producing Bacterial Strain. (A) Pour plate chitin agar screening: Shows the chitinolytic activity of the bacterial strain on a chitin agar plate using the pour plate method. Zones of clearance indicate chitinolytic activity.. **(B) Streak plate chitin agar screening**: Illustrates the chitinolytic activity of the bacterial strain on a chitin agar plate using the streak plate method. Clear zones along the streak lines signify chitin degradation.. **(C) Deacetylation activity at 0 h**: Depicts the initial state of deacetylation activity before incubation.. **(D) Deacetylation activity at 24 h**: Demonstrates the extent of deacetylation activity after 24 h of incubation, showing increased activity compared to 0 h.. **(E) Gram staining**: Displays the morphology of the Chitin deacetylase producing strain under a light microscope, revealing gram-positive rod-shaped bacillus.

Effect of incubation time on activity of crude CDA was determined using the well diffusion method on p-nitroacetanilide agar plates. The results showed that deacetylation of p-nitroacetanilide to p-nitroaniline start after 16 h of incubation, with complete deacetylation occurring after 24 h, as indicated by color of the plate (complete appearance of yellow color) (Fig. 3A–D). The expression of above extracellular protein was found to be maximum at 24 h of incubation (Fig. 3E).

3.4. Effect of metal ions on growth, protein content, and CDA activity of the bacterial strain

Metal ions have a great impact on microbial growth and development affecting specific growth rate, production of essential proteins and secondary metabolites, as well as the kinetics of the microbial enzymes (Behera et al., 2014; Sengör et al., 2009). To optimize the growth conditions for *A. aneurinilyticus,* the strain was incubated with various metal ions of different concentrations in the culture medium. The metal ions used in this experiment were selected based on previous studies indicating their potential to regulate growth and CDA activity (Xie et al., 2020; Zhao et al., 2018). The bacterial strain was capable of growing in the presence of $ZnSO_4$ at a concentration of 5 mM, $MgSO_4$ at 1.5 mM, $MnSO_4$ at 1.5 mM, and $CoCl_2$ at 0.1 mM; however, $CdCl_2$ severely inhibited growth (Fig. 4). Magnesium ions are regarded as a critical growth inducer for many *Bacillus* species, as the reports of

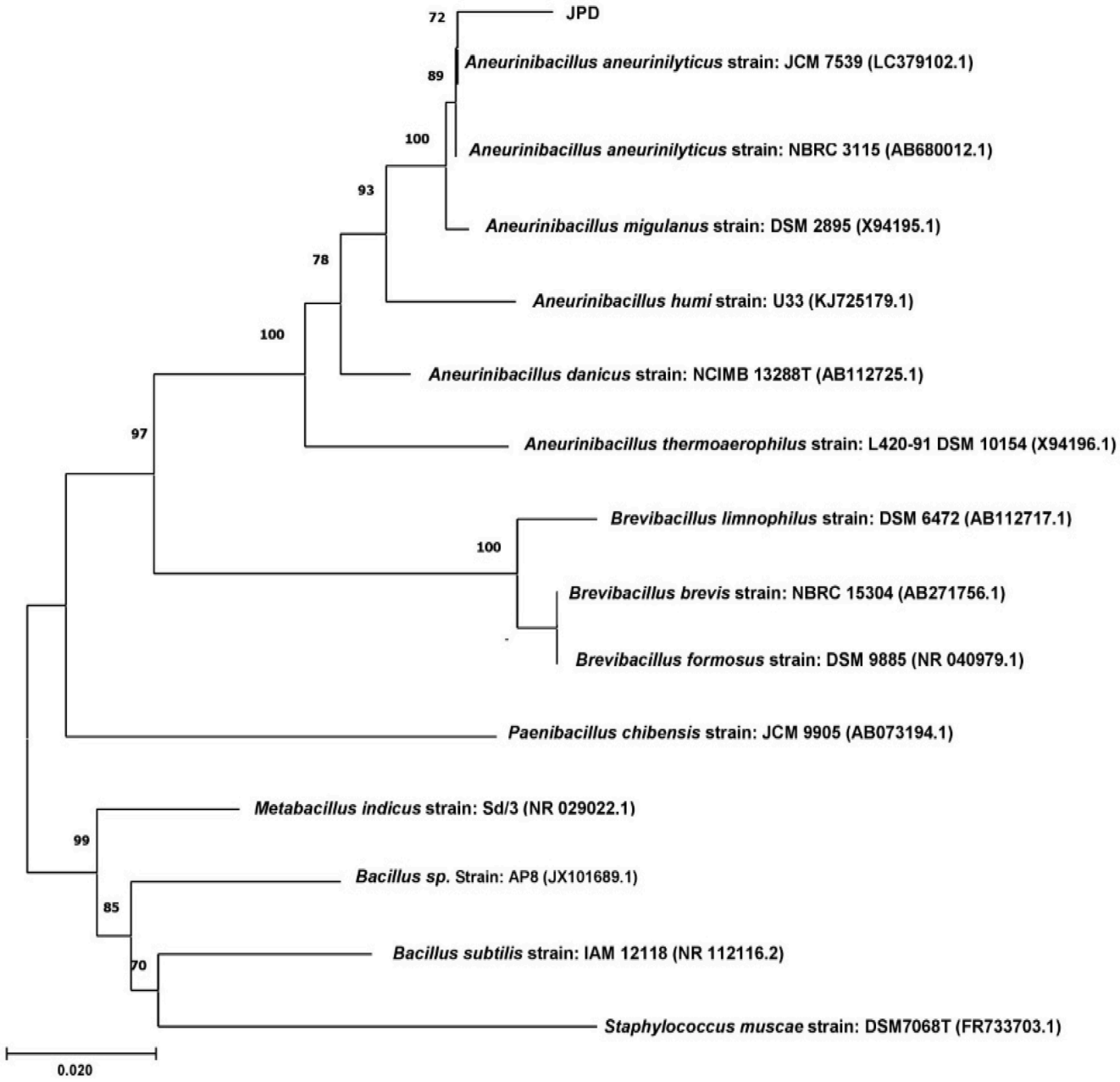

Fig. 2. Phylogenetic relationship among the selected isolate (JPD) and other published 16S rDNA sequences: The phylogenetic tree shows the relationship between the selected isolate (JPD) and various published strains, using Staphylococcus muscae (FR733703.1) as outgroup. In the tree, U4F and *Aneurinibacillus aneurinilyticus* are grouped together, forming a distinct clade.

Lee et al. (2019). Studies by De-hui et al. (2011) and Zhao (2010) also describe how magnesium can induce CDA activity, which is in accordance with the findings of the current experiment (De-hui et al., 2011; Zhao, 2010). Several studies have demonstrated that manganese ions can stimulate bacterial growth (Bosma et al., 2021; Ranjbar et al., 2021), though in some bacterial species, it completely inhibits the growth (Du et al., 2020). In the present study, manganese ion has been shown to be a potent stimulator for both growth and CDA activity of the strain. In the case of cobalt, the ion has no noticeable inducing effects at low concentrations, but at higher concentration, it inhibits bacterial growth, which is highly consistent with the reports of Majtan et al. (2011) who reported cobalt toxicity in *E. coli* culture at high concentration. (Fig. 4)

3.5. Purification profile of CDA

The partially-purified CDA obtained from the DEAE ion exchange column displayed two bands, one is at 45 kDa and another is at 26 kDa on the gel after SDS-PAGE as shown in the Fig. 5(A). The partially purified CDA has a specific activity of 569.73 U/mg protein with a 76.16 fold purification and 64.06 % yield. According to the whole genome study of the strain *Aneurinibacillus aneurinilyticus,* the strain have five polysaccharide deacetylase genes (SHIDA et al., 1996). The molecular weight of these putative polysaccharide deacetylase matches with those of the purified CDA obtained through gel filtration and ion exchange chromatography in our study. These finding suggests the presence of multiple polysaccharide deacetylases in the strain which may be considered as a potent chitin deacetylase (Table 5).

Table 3
Antibiogram assay of *Aneurinibacillus aneurinilyticus* (JPD).

Sl. no	Name of the antibiotic	Zone of inhibition (ZI in cm)	Resistance (R) or sensitive (S)
1.	Ceftriaxone	4	S
2.	Erythromycin	4.3	S
3.	Ofloxacin	3.9	S
4.	Polymyxin B	2	S
5.	Streptomycin	2.5	S
6.	Novobiocin	3.2	S
7.	Amoxycillin	4.5	S
8.	Gentamicin	4.2	S
9.	Amikacin	2.7	S
10.	Ciprofloxacin	4.5	S
11.	Cotrimoxazole	4	S
12.	Cefotaxime	–	R
13.	Ampicillin	–	R
14.	Bacitracin	–	R
15.	Chloramphenicol	1	M

Table 4
Biochemical characteristics of the strain *Aneurinibacillus aneurinilyticus* (JPD).

Sl No	Biochemical assay	Results
1	Ala-Phe-Pro-ARYLAMIDASE (APPA)	+
2	Leucine ARYLAMIDASE (LeuA)	+
3	Alanine ARYLAMIDASE (AlaA)	+
4	Polymixin B sensitivity POLYB_I	+
5	Kanamycin Resistance (KAN)	+
6	Phenylalanine ARYLAMIDASE (PheA)	+
7	L-Proline ARYLAMIDASE (ProA)	+
8	Glycine ARYLAMIDASE (Gly A)	+
9	Glycogen (GLYG) utilization	–
10	Maltotriose (MTE) utilization	–
11	Platinose(PLE) utilization	–
12	Esculin Hydrolysis	+
13	Beta Xylosidase (BXYL)	–
14	Beta Galactosidase (BGAL)	–
15	Ellman(ELLM) utilization	–
16	d-Mannose assimilation (dMNEa)	–
17	Beta mannosidase(BMAN)	–
18	Inulin(INU) utilization	–
19	Oleandomycin Resistance(OLD)	–
20	L-Aspartate ARYLAMIDASE (AspA)	–
21	Alpha Galactosidase(AGAL)	–
22	d- Galactose (dGAL) utilization	–
23	Alpha Mannosidase (AMAN)	–
24	N- acetyl glcosamine (NAG) utilization	+
25	Pyruvate (PVATE) utilization	–
26	d- Ribose (dRIB) utilization	–
27	Tetrazolium Red (TTZ) utilization	–
28	Alpha Glucosidase (AGLU)	–
29	Putrescine assimilation (PSCNa)	–
30	Myo- Inositol (INO) utilization	–
31	L- Rahmnose (IRHA) utilization	–
32	D- Tagatose(dTAG) utilization	–
33	Beta N-Acetyl glucosaminidase (BNAG)	–
34	Methyl B-d- Glucopyranoside(MBdG)	–
35	D- Mannitol (dMAN)utilization	–
36	Beta glucosidase (BGLU)	–
37	D- Trehalose (dTRE)	–
38	Voges Proskauer's test	–
39	Catalase	+
40	Citrate utilization	+
41	Nitrate Reduction	+
42	Methyl red test	+

+, tested positive / utilized as substrate; -, tested negative / not utilized as substrate.

3.6. Characterization of purified CDA

The purified CDA was further characterized to determine its temperature, pH optima and the impact of metal ions on its activity. The enzyme exhibited the highest activity at 37 °C (Fig. 5D) losing its activity below 25 °C and above 50 °C. CDA isolated from different sources mainly shows the activity in the temperature range between 25 °C to 40 °C (Chai et al., 2020; Elmekawy et al., 2013; GuoYing et al., 2010; Martinou et al., 2002; Raval et al., 2013) but there are certain reports which show optimum CDA activity at higher temperature. CDA isolated from *A. nidulans* and *C. lindemuthianum* has activity at around 50 °C and 60 °C respectively (Alfonso et al., 1995; Shrestha et al., 2004). Three different buffer solutions with pH range 4 to 10 were used to find out the optimal pH for purified CDA activity (Fig. 5B). CDA showed the highest activity at pH 7.4, with a sharp decline in both acidic and alkaline conditions. Previous studies reports the CDA activity between pH 7 & 8 with CDA isolated from *B. cerus, N. aquimarinus*, and *B. aryabhatti* showing highest activity within this pH range (Chai et al., 2020; Pawaskar et al., 2021; Ravikumar and Perinbam, 2016). Other CDAs have optimal pH value 9 for *A. corymbifera* (Zhao, 2010), 11 to 12 for *C. lindemuthianum* (Tokuyasu et al., 1996), and pH 4.5 for *M. rouxii* (Kafetzopoulos et al., 1993). The above studies suggest that mostly extracellular CDA are highly active in neutral or alkaline pH while intracellular CDAs prefer acidic pH (Chai et al., 2020).

Different metal ions showed dose-dependent response when incubated with the purified enzyme with various concentrations. In the present investigation the effect of metal ions like Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+} and Cd^{2+} from 0.1 mM to 5 mM concentration was examined. $Mn^{2+}$and Mg^{2+}induced CDA activity at 0.1 mM and 1 mM, respectively, whereas CDA activity decreased at higher concentration. $Co^{2+}$and Cd^{2+} increased CDA activity at a very low concentration (0.1 mM), but significantly decreased with high concentration of these two metals, while in the case of Fe^{3+} and Zn^{2+} there is no significant change observed in the CDA activity (Fig. 5C). CDA isolated from *S. bacillaris* was strongly inhibited by Zn^{2+}, Fe^{3+} and Co^{2+} and moderately inhibited by Ca^{2+} and Mg^{2+},while Mn^{2+} significantly induced the activity (Yin et al., 2022) consistent with our findings except for Mg^{2+} which is an excellent enhancer in our case. Mg^{2+} has also been reported to induce CDA activity in *B. aryabhatti* and *A. corymbifera* (Pawaskar et al., 2021; Zhao, 2010). CDA from *Mortierella* sp. *DY-52* and *A. corymbifera* was activated by Ca^{2+} and Co^{2+}as observed earlier (Kim et al., 2008; Zhao, 2010). However, Zn^{2+}, Fe^{3+}, Mn^{2+} and Mg^{2+}were found to inhibit the enzyme activity (Kim et al., 2008). Most of the reports showed Co^{2+} and Zn^{2+} as CDA inhibitors. However, CDA extracted from *F. velutipes* gets activated when incubated with above two metal ions (Yamada et al., 2008).

3.7. Kinetic studies of partially purified CDA

The enzyme kinetics parameters, like Km and Vmax, of this bacterial chitin deacetylase from *Aneurinibacillus aneurinilyticus* were evaluated using the Lineweaver-Burk plot with various chitinous and non-chitinous substrates. The enzyme exhibited a high affinity for deacetylating chitin oligomers and the affinity of CDA towards its substrate depends on the chain length of the later. Among the chitinous substrates, the CDA showed the highest affinity towards glycol chitin, as evidenced by its low Km and high Vmax values respectively. This was followed by colloidal chitin, with a Km of 111.75 μM and a Vmax of 10.12 μmol/min, and N-acetyl glucosamine hexamer, with a Km of 127.86 μM and a Vmax of 10.772 μmol/min. In contrast, the enzyme kinetics varied significantly when p-nitroacetanilide was used as the substrate, which indicates differential affinity towards chitinous and non-chitinous substrates. The observed Km and Vmax values for this CDA are also comparable from earlier studies on CDA isolated from various organisms, for example partially purified CDA from *Alcaligenes* sp. ATCC55938, had a Km of 1.6 x 10^{-4} μM and a Vmax of 24.7 μmole/min with ethylene glycol chitin as a substrate (Elmekawy et al., 2013). CDA from *A. nidulans* showed a Km of 2.86 mM and Vmax of 20 nmole/min with glycol chitin as the substr.ate (Alfonso et al., 1995), while extracellular CDA purified from *C. lindemuthianum* had a Km of 0.414 mM and a Vmax of 158 μmole/min using with chitopentose as substrate

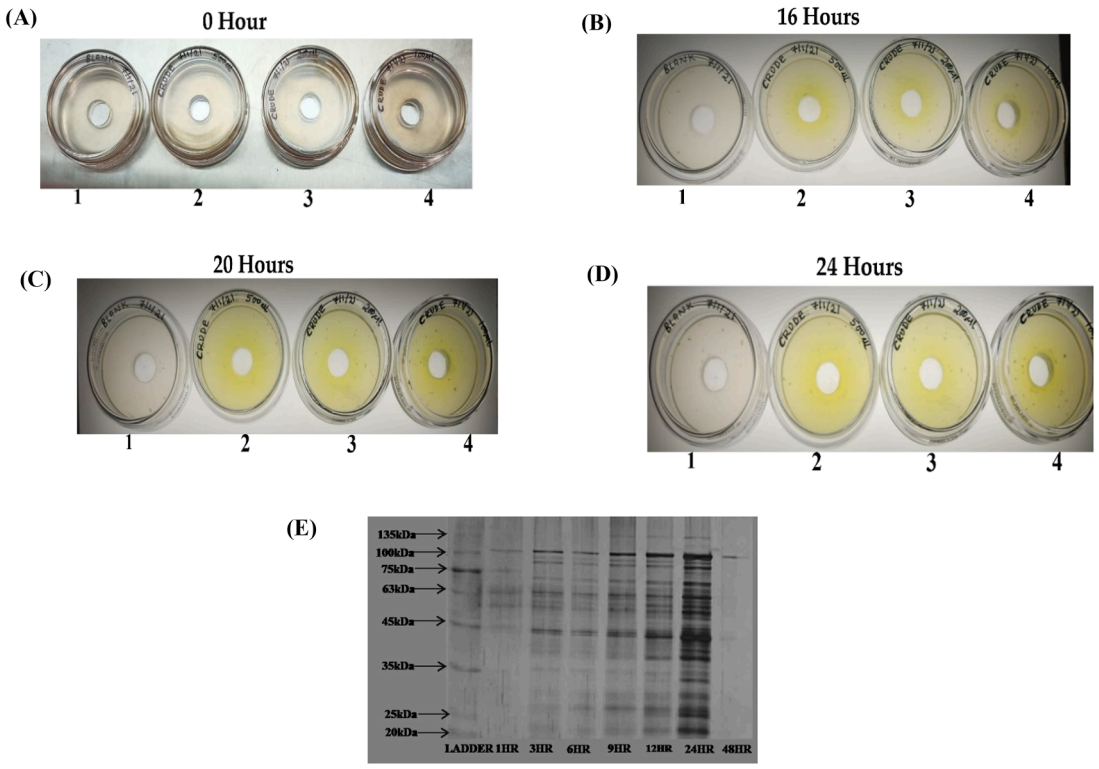

Fig. 3. Optimization of the incubation time for extracellular CDA enzyme activity on p-nitroactanilide agar plates. (A) 0 h, (B) 16 h, (C) 20 h and (D) 24 h of incubation respectively. Plate 1-Negative control (only media without inoculum); Plate 2- 500 µl culture extract; Plate 3–250 µl culture extract and Plate 4–100 µl culture extract. (E) Expression profile of extracellular proteins by SDS-PAGE collected from *Aneurinibacillus aneurinilyticus* at various time intervals (1, 3, 6, 9, 12, 48 h), Lane 1; molecular marker, Lane 2–8; 50 µg of protein sample extracted at 1 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h respectively.

(Tokuyasu et al., 1996).

Chitin deacetylases exhibit diverse patterns of deacetylation, reflecting their varying substrate specificities and recognition capabilities on linear polysaccharides. The mechanisms by which these enzymes modify internal units of a polysaccharide chain are generally classified into three categories: multiple-attack, multiple-chain, and single-chain mechanisms (Grifoll-Romero et al., 2018; Lindner et al., 2025). In the *multiple-attack mechanism*, the enzyme binds to the polysaccharide chain and performs several consecutive deacetylations before moving to another section of the polymer. This mechanism has been observed in *M. rouxii* (Martinou et al., 1998; Tsigos et al., 1999) when applied to chito-oligosaccharides (COS), this mechanism typically leads to complete deacetylation of the oligomer. The *multiple-chain mechanism* involves the formation of an enzyme-polymer complex, where only a single acetyl group is removed before the enzyme dissociates and binds to a new section of the polymer. An example of this mechanism is found in the chitin deacetylase from *C. lindemuthianum* (Hekmat et al., 2003; Tokuyasu et al., 2000). This process produces a more random distribution of $GlcNH_2$ and GlcNAc units along the polymer chain. When applied to COS, it can yield various partially deacetylated intermediates, either resulting in distinct deacetylation patterns or, in some cases, full deacetylation, depending on the specific enzyme and substrate. Lastly, the *single-chain mechanism* involves processive enzymes that remain bound to a single substrate molecule, performing multiple catalytic events sequentially. Certain bacterial chitooligosaccharide deacetylases (CODs) fall into this category, as they target specific positions on the substrate, producing mono-deacetylated products. Examples of such enzymes include *Rhizobium* NodB and *Vibrio* CDA or Chito oligosaccharide deacetylase (COD), which exhibit high positional specificity.

Since the CDA from *Aneurinibacillus aneurinilyticus* belongs to the NodB class of CDAs, we can conclude that the reported CDA likely operates through a single-chain mechanism and follows the Michaelis–Menten kinetics for both chitinous and non-chitinous substrates (FIG. 6 E&F) Table 6.

A substantial quantity of crustacean shell waste is generated annually across the globe, which can be utilized for the production of chitosan by a relatively straightforward and eco-friendly deacetylation process (Chakravarty and Edwards, 2022; Santos et al., 2020b; Yadav et al., 2019). Chitosan, derived from seafood waste using an energy-efficient enzymatic approach, offers a cost-effective alternative to other biopolymers. The outcome of this study substantiates two important aspects as focused in the objectives. First, successful isolation of the CDA-producing bacteria, *Aneurinibacilus aneurinilyticus* from the rinsed water of the crustacean shell and second, partial purification of CDA, characterization and optimization of assay conditions. It is assumed that this enzyme has potential for bioconversion of chitin to chitosan and thereby offers multiple avenues for wide range of industrial applications. The growing demand for natural bio-adsorbents in wastewater treatment, coupled with technological advancements, is driving the increased use of biopolymers. Bio-derived chitosan can be considered as a renewable polysaccharide, garnering significant attention to control the water pollution as well.

4. Conclusion and future prospective

In sum, this study provides the first report on the presence of CDA in the bacterial strain *Aneurinibacillus aneurinilyticus*, isolated from marine bioresources. Further, the purification of this enzyme, optimization of the assay conditions and enzyme kinetics provide meaningful information for media composition and optimum culture conditions of the bacterium. While current research primarily occurs in laboratory settings, further studies are in progress to translate its potential findings into commercial-scale, for optimal and sustainable waste utilization and production of value-added bio-products from bio-waste on a single platform.

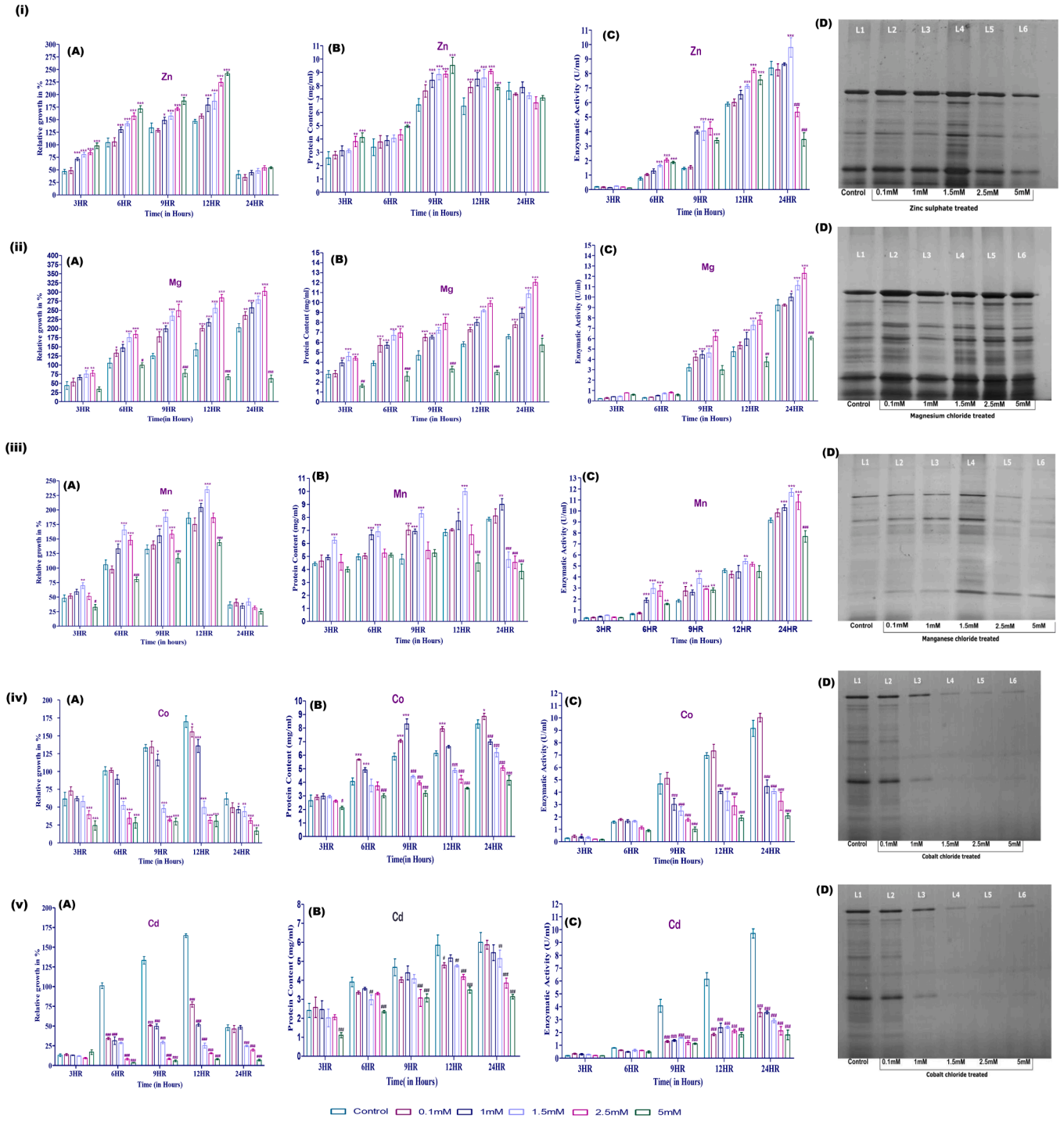

Fig. 4. The effect of various Metal ions ((I) $ZnSO_4$ (II) $MgSO_4$ (III) $MnCl_2$ (IV) $CoCl_2$ (V) $CdCl_2$) at different concentrations (0.1, 1, 1.5, 2.5, and 5 mM) on (A) Relative growth (B) Total protein content and (C) CDA activity of *A. aneurinilyticus* at different incubation time (3, 6, 9, 12 and 24 h). The results are shown as the mean ± standard error mean (SEM) ($n = 3$). The symbols * and # denote a substantial change relative to the control, respectively. The * symbol denotes a significant rise relative to the control and # symbol denotes a significant fall relative to the control. Bars having superscripts of different symbol (*, #) implies significant difference ($p < 0.05$), (**, ##) implies significant difference ($p < 0.01$), (***, ###) indicates significant difference ($p < 0.001$) across different concentrations of metal treated sample with control. (D)Protein expression of the extracellular supernatant of *A. aneurinilyticus* is shown by SDS PAGE.

CRediT authorship contribution statement

Poonam Das: Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Manisha Das:** Formal analysis, Investigation, Data curation. **Sheela Kumari Sahoo:** Writing – original draft, Writing – review & editing. **Jagneshwar Dandapat:** Supervision, Writing – original draft, Writing – review & editing. **Jyotsnarani Pradhan:** Conceptualization, Supervision, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

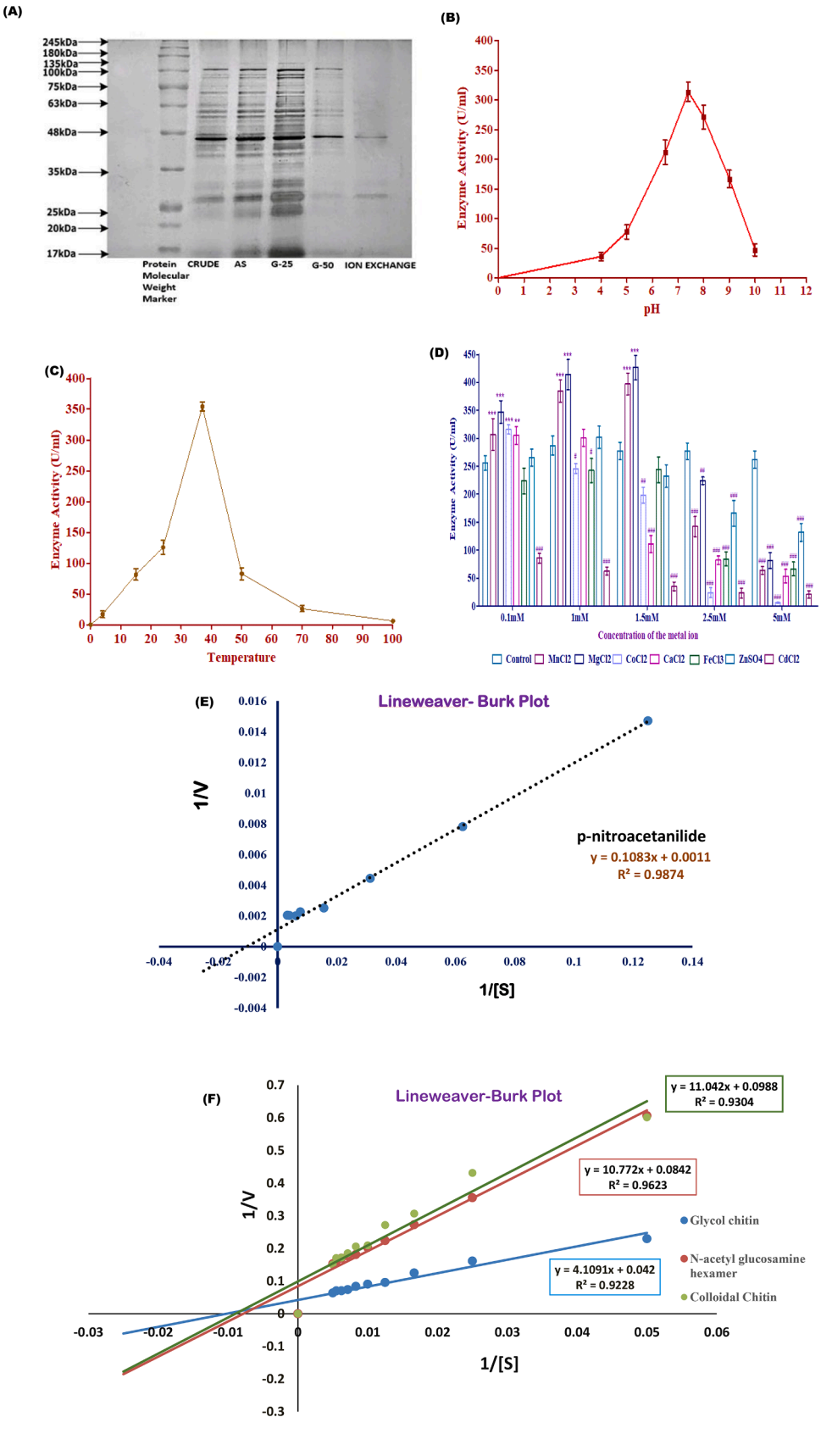

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Fig. 5. Purification and optimization of Chitin Deacetylase (CDA) isolated from *Aneurinibacillus aneurinilyticus*. (A) SDS-PAGE for Purification Analysis**:** Lane 1: protein molecular weight marker; Lane 2: Crude CDA; Lane 3: CDA obtained after ammonium Sulphate precipitation; Lane 4: G-25 column eluted sample; Lane 5: G-50 column eluted sample; Lane6: purified CDA by ion exchange chromatography (each well containing 40 μg of protein). (B) pH optimization for CDA activity at different pH levels from 4.0 to 11.0. (C) Temperature optimization of CDA activity was at various temperatures from 45 °C to 80 °C (D) Effect of different metal ions (Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Cd^{2+}) at different concentration (0.1 mM, 1 mM, 1.5 mM, 2.5 mM & 5 mM) on purified CDA. The data is presented as the mean ± standard error of the mean (SEM) ($n = 3$). The symbols * and # indicate a substantial change relative to the control, where the * symbol denotes a significant increase in CDA activity, and the # symbol denotes a significant decrease in activity. Bars with different superscripts indicate significant differences (*/#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$) (E) Lineweaver-Burk plot showing enzyme kinetics of p-nitroacetanilide. (F) Lineweaver-Burk plot showing enzyme kinetics of Glycol chitin, N-acetyl glucosamine hexamer and colloidal chitin.

Table 5
Purification fold of CDA and respective activities.

Purification step	Total protein (mg)	Enzymatic activity (U)	Specific activity (U/mg)	Activity yield (%)	Purification fold
Crude	2256	16,896	7.48	100	1
AS	1077.44	14,960	13.88	88.54	1.85
G-25	118.56	14,040	118.42	83.09	15.83
G-50	26.28	12,222	465.06	72.33	62.17
Ion exchange	19	10,825	569.73	64.06	76.16

Table 6
K_m and V_{max} value of *Aneurinibacillus aneurinilyticus* CDA with different types of substrates.

Substrate	K_m (μM)	V_{max} (μmole/min)
p-nitroacetanilide	98.455	909.09
Glycol chitin	96.96	23.31
Colloidal chitin	111.75	10.12
N-acetyl glucosamine hexamer	127.86	10.772

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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