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Identification of a new thermostable chitinase from *Hydrogenophilus hirschii* strain KB-DZ44

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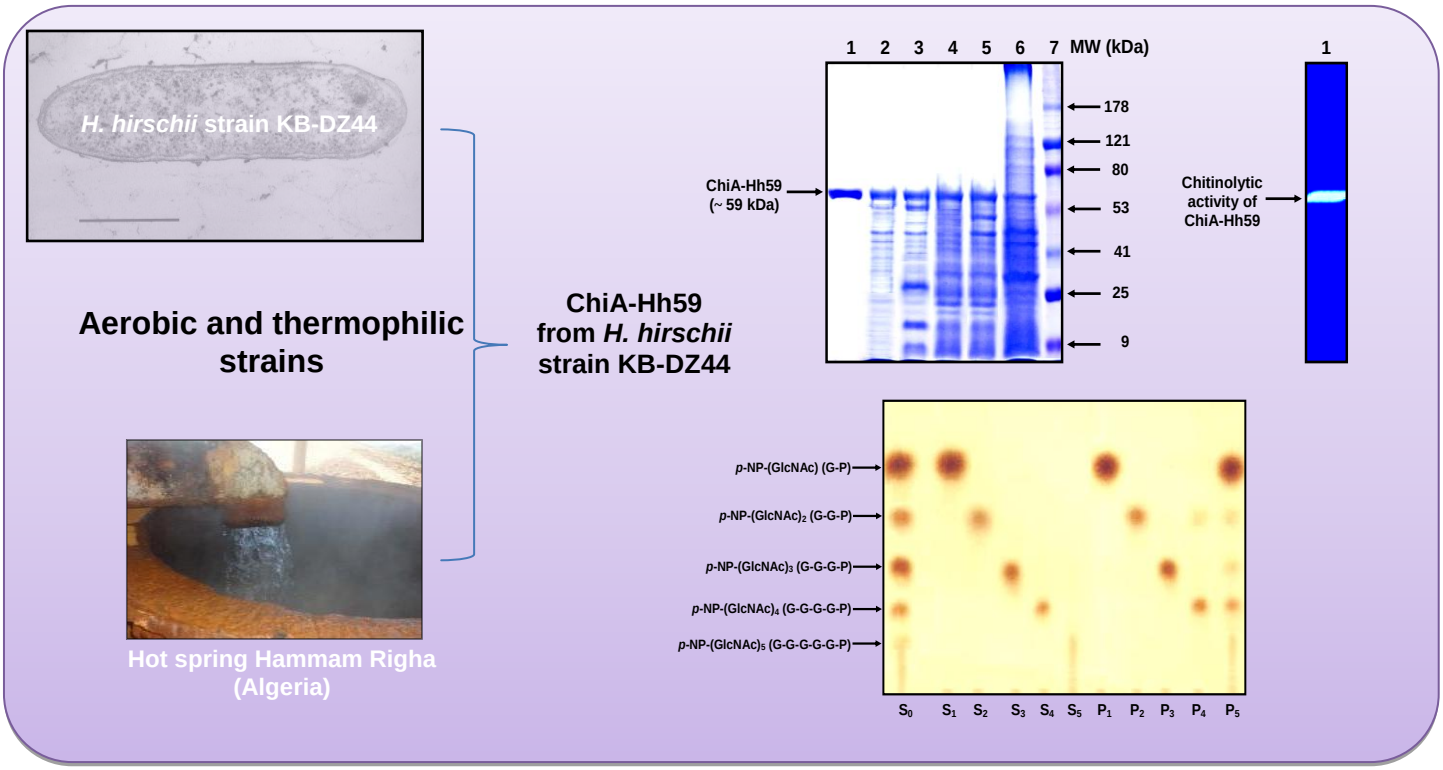
Graphical Abstract



Abstract

This paper reports the identification of an extracellular acido-thermostable endo-chitinase (called ChiA-Hh59) from thermophilic *Hydrogenophilus hirschii* strain KB-DZ44. The ChiA-Hh59 was purified and characterized. The maximum chitinase activity recorded after 36-h of incubation at 60°C was 3000 U/ml. Pure enzyme was obtained after heat and acidic treatment, precipitation by ammonium sulphate and acetone, respectively, followed by sequential column chromatographies on Sephacryl S-200 and Mono Q-Sepharose. Based on MALDI–TOF/MS analysis, the purified enzyme is a monomer with a molecular mass of 59103.12-Da. The 22 residue NH_2-terminal sequence of the enzyme showed high homology with family-18 bacterial chitinases. The optimum pH and temperature values for chitinase activity were pH 5.0 and 85°C, respectively. The pure enzyme was completely inhibited by *p*-chloromercuribenzoic acid (*p*-CMB) and *N*-ethylmaleimide (NEM). The obtained results suggest that ChiA-Hh59 might be an endo-chitinase. The studied chitinase exhibited high activity towards colloidal chitin, chitin azure, glycol chitin, while it did not hydrolyse chitibiose and amylose. Its catalytic efficiency was higher than those of chitodextrinase and ChiA-65. Additionally, TLC analysis from chitin-oligosaccharides showed that ChiA-Hh59 acted as an endo-splitting enzyme. In conclusion, this chitinase may have great potential for the enzymatic degradation of chitin.

1. Introduction

Chitin, a linear β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) polysaccharide, is the major structural component of fungal cell walls, insect exoskeletons, and shells of crustaceans. It is one of the most naturally abundant occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology, and biotechnology(Yahiaoui et al., 2019). Chitinases are essential glycoside hydrolases (GHs) that catalyze the hydrolysis of β-1,4-glycosidic bonds of chitin in glycoconjugates, oligo- and polysaccharides. The *endo*-chitinases cleave randomly at internal sites of chitin, generating soluble low mass multimers of GlcNAc such as chitotetraose, chitotriose, and chitobiose (Vaaje-Kolstad et al., 2013). Several chitinases have been isolated and characterized from various sources (Pradeep et al., 2014).

Algeria possesses more than 240 thermal sources with number increasing when approaching the Algerian North-eastern with temperatures ranging from 19 to 98 °C. These sources are known to harbor large communities of thermophilic anaerobic bacteria with biotechnological interests (Bouacem et al., 2014; Bouacem et al., 2016; Bouacem et al., 2015). However, relatively few reports are available on isolation and characterization of thermostable enzymes produced by thermophilic aerobic bacteria isolated from Algerian hot springs.

The present investigation reports, for the first time, the purification and biochemical characterization of a novel acido-thermostable endo-chitinase produced by *Hydrogenophilus hirchii* strain KB-DZ44 and explore its promising potential for biotechnological applications (Bouacem, 2016; Bouacem et al., 2018).

2. Materials and Methods

2.1. Substrates

The used chitinous substrates, commercial enzyme (Chitodextrinase®) were bought from Sigma-Aldrich (MO, USA).

2.2. Preparation of colloidal chitin

The colloidal chitin was prepared by the method of Roberts and Selitrennikoff (Roberts & Selitrennikoff, 1988).

2.3. Standard assay of ChiA-Hh59 activity

Chitinase activity was measured colorimetrically by detecting the amount of GlcNAc released from colloidal chitin as substrate (Thamthiankul et al., 2001).

2.4. Purification of ChiA-Hh59

Five hundred mL of a 36 h culture of *Hydrogenophilus hirschii* strain KB-DZ44 was centrifuged for 30 min at 9,000*g* to remove microbial cells. The supernatant containing extracellular chitinase was used as the crude enzyme preparation and was submitted to the following purification steps: heat and acidic treatment, precipitation by ammonium sulphate and acetone, respectively, followed by sequential column chromatographies on Sephacryl S-200 and Mono Q-Sepharose.

2.5. Analytical methods

Total protein content was determined according to Bradford's method (Bradford, 1976). ChiA-Hh59 molecular masses in native and denatured forms were resolute by the means of PAGE (12% acrylamide gel) with or without 0.1% SDS (Laemmli, 1970). The zymography analysis was carried out as previously reported. The exact molecular mass was elucidated through the use of a Voyager DE-RP MALDI-TOF/MS (Applied Biosystems).

2.6. Biochemical characterization

2.6.1. Influence of metallic ions, specific inhibitors, and reducing agents

Chemical reagents were investigated at various concentrations for their effects on enzyme activity. Chitinase activity measured in the absence of any inhibitor or reducing agent was taken as control (100%). The effects of different monovalent or divalent (metallic ions, at a concentration of 2 mM, on chitinase activity were investigated by adding them to the reaction mixture. The non-treated and dialyzed enzyme was considered as 100% for metallic ion assay.

2.6.2. Determination of pH and temperature on chitinase activity and stability

The effect of pH on chitinase activity was assessed over the range of pH 2.0–10.0. The pH stability was tested by pre-incubation of the purified chitinase in buffers with different pH from 3.0 to pH 7.0 at standard assay temperature for 12 h.

The determination of the effect of ChiA-Hh59 temperature on its activity was measured at 40–100°C. Its thermal stability, for 12 h, was evaluated at diverse temperatures with or without 2 mM $MgCl_2$. Relative and remaining activities were evaluated under the optimum assay conditions.

3. Results and discussion

3.1. Purification, molecular weights determination and zymography analysis of ChiA-Hh59

Enzyme purity was estimated to be about 18-fold greater than that of the crude extract. The purified enzyme had a specific activity of 21600 U/mg, with a yield of about 36%. In fact, the specific activity displayed by ChiA-Hh59 was significantly high compared to those previously reported for other chitinase. This high level of its specific activity confirmed the potential prospects of ChiA-Hh59 in various biotechnological and industrial bioprocesses.

The purified ChiA-Hh59 enzyme had a molecular weight of approximately 59 kDa (Fig. 1A) and clear chitinase activity (Fig. 1B).

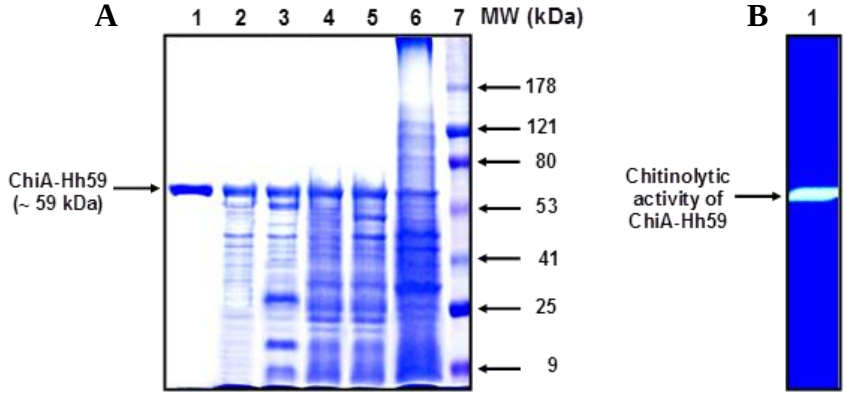


Fig. 1. (A) SDS-PAGE of the purified chitinase ChiA-Hh59. **(B)** Chitin azure zymography staining of the purified chitinase ChiA-Hh59.

3.2. Biochemical characterization of ChiA-Hh59

3.2.1. Influence of metallic ions, specific inhibitors, and reducing agents

The activity of ChiA-Hh59 was found to be inhibited in the presence of *p*-CMB and NEM. Partial activity loss was observed when it was incubated with DTT and 2-ME. This indicates the presence of sulfyhdryl groups on active site of the enzyme, as confirmed by total inhibition observed in the presence of mercuric ion. Among all tested metallic ions, only Cd^{+2}, Hg^{+2}, and Ni^{+2} completely inhibited enzyme activity, while Zn^{+2}, Ba^{+2}, Al^{+2}, Fe^{+2}, and Ag^{+2} reduced enzyme activity by 10, 24, 39, 55, and 88%, respectively. Other reagents such as K^{+} and Li^{+} did not show significant inhibition or activation effects on the chitinase. However, the enzyme activity of chitinase was significantly increased with the addition of 5.0 mM of Mg^{+2}, Mn^{+2}, Ca^{+2}, Cu^{+2}, and Co^{+2}.

3.2.2. Evaluation of different pH on the activity and stability

The enzyme was active in pH range from pH 2.0–10.0 with maximum activity at pH 5.0 (Fig. 2A). The effect of pH on the stability of chitinase was studied at 85 °C. The pH stability profile of ChiA-Hh59 illustrated in Fig. 2B indicated that the purified enzyme was highly stable in the pH range of 7.0–12.0.

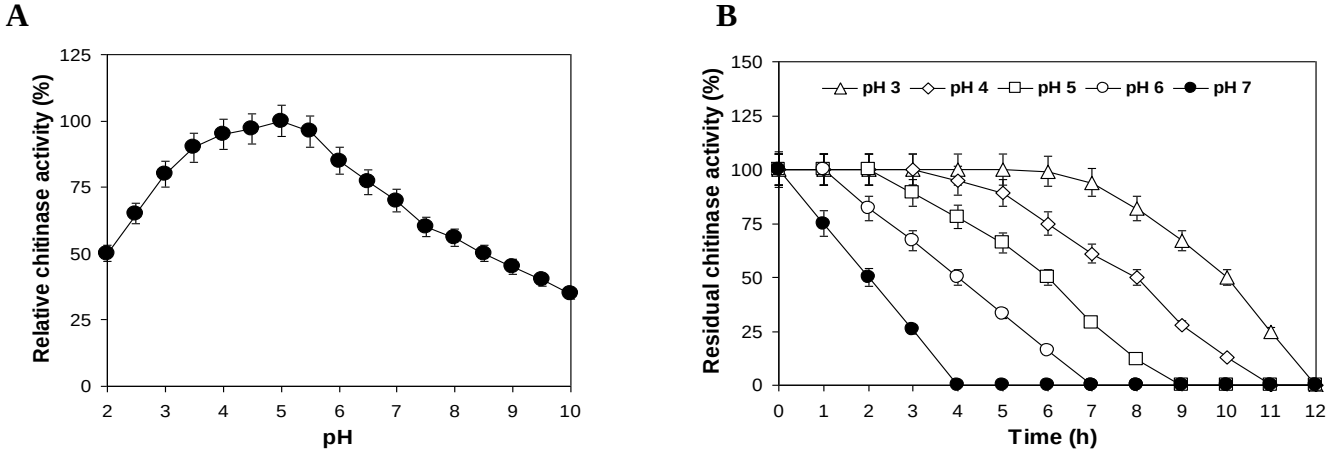


Fig. 2. Effects of pH on the activity **(A)** and stability **(B)** of the purified chitinase ChiA-Hh59

3.2.3. Evaluation of different temperatures effect on the activity and stability

The optimum temperature recorded for the activity of the purified chitinase at pH 5.0 was 75 °C in the absence of $MgCl_2$ and 85 °C in the presence of 2 mM Mg^{2+} (Fig. 3A). The thermostability profile of ChiA-Hh59 is presented in Fig. 3B.

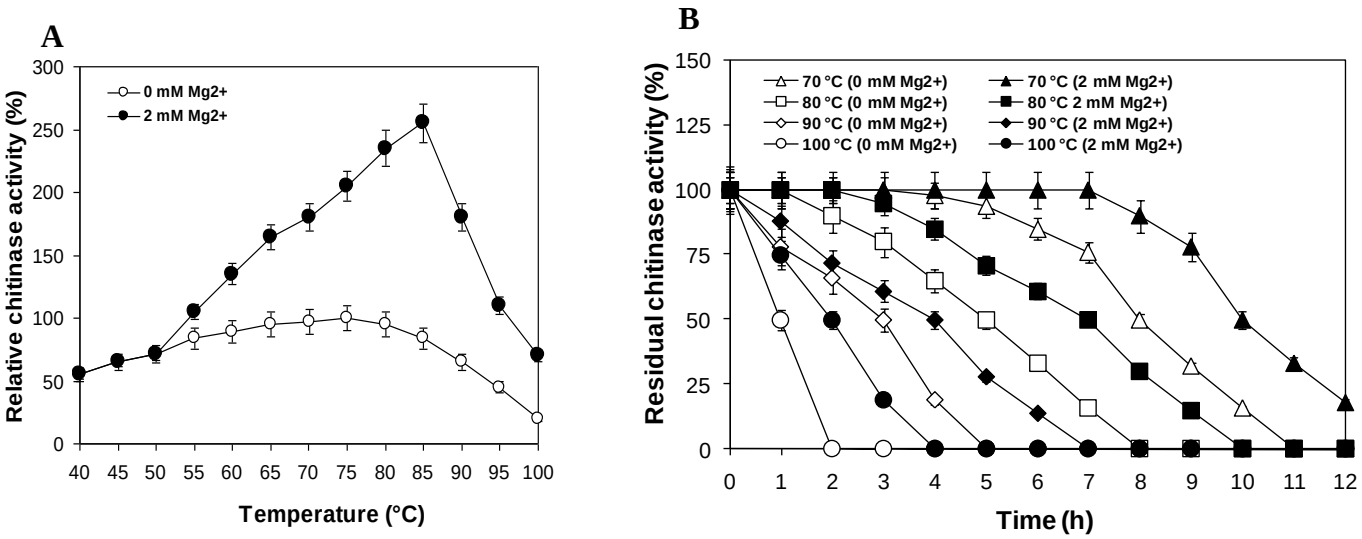


Fig. 3. Effects of the thermoactivity **(A)** and the thermostability **(B)** of chitinase ChiA-Hh59.

4. Conclusion

These features collectively suggest the potential relevance of ChiA-Hh59 for commercial exploitation in future. Considering the attractive properties and attributes of this enzyme, further studies are needed to explore the molecular structure of its encoding gene and regulation region and investigate its structure-functions relationship using site-directed mutagenesis and 3D structure modeling.

5. References

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