MOL2NET, International Conference Series on Multidisciplinary Sciences
MODEC-05: Nat. Prod. & Agro-Indust. Proc. in Amazon, UEA, Puyo, Ecuador, 2020

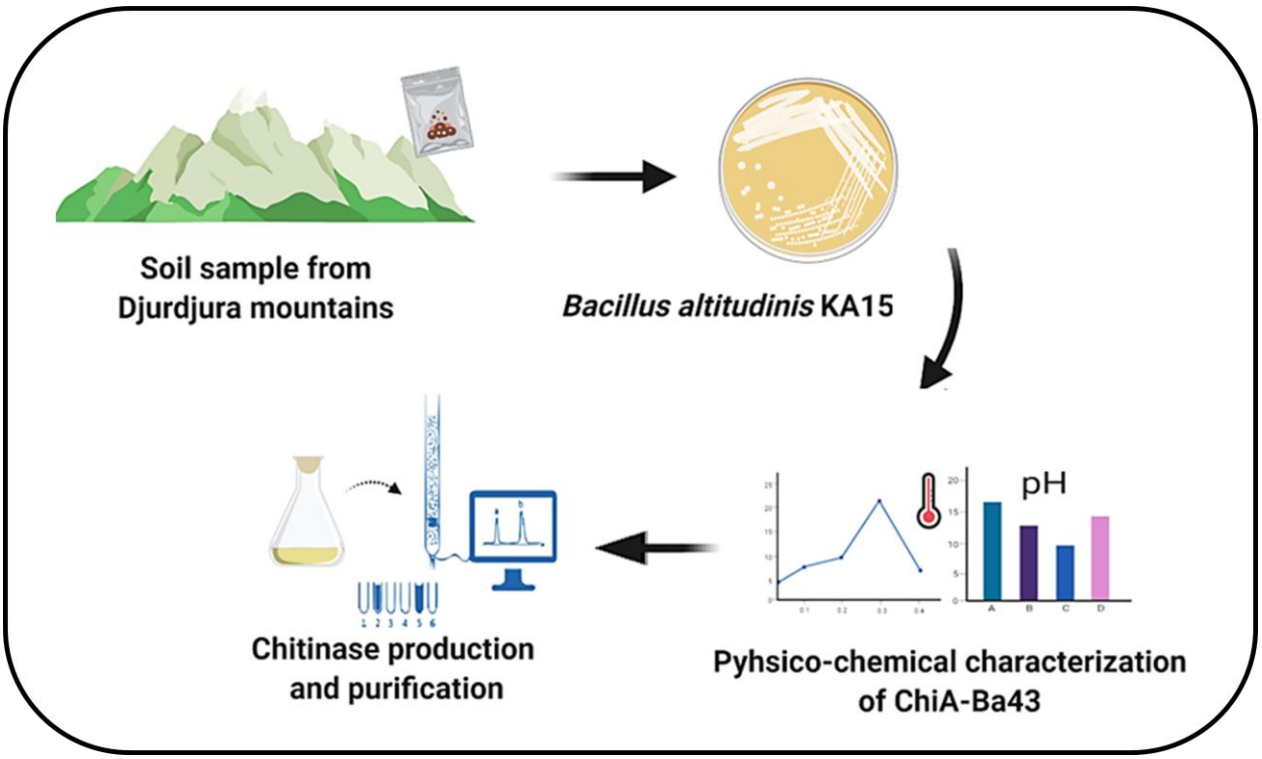
Identification of a novel acido-thermostable chitinase from *Bacillus altitudinis* strain KA15

Katia-Louiza Asmani[a*], *Khelifa Bouacem*[a,b*], *Akli Ouelhadj*[a,c], *Merzouk Yahiaoui*[d], *Sondes Mechri*[e], *Fadoua Jabeur*[e], *Bassem Jaouadi*[e]

[*]*E-mail addresses*: katia.asmani@yahoo.com; khelifa.bouacem@yahoo.fr

[a] *Department of Biochemistry and Microbiology, Faculty of Biological and Agricultural Sciences (FBAS), University of Mouloud Mammeri of Tizi-Ouzou (UMMTO), P.O. Box 17, Tizi-Ouzou 15000, Algeria*
[b] *Laboratory of Cellular and Molecular Biology (LCMB), Microbiology Team, Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), PO Box 32, El Alia, Bab Ezzouar, 16111 Algiers, Algeria*
[c] *Laboratory of Food Quality and Food Safety (LFQFS), FBAS, UMMTO, P.O. Box 17, Tizi-Ouzou 15000, Algeria*
[d] *Department of Natural and Life Sciences, Faculty of Sciences (FS), University of M'Sila, P.O. Box 166, M'Sila 28000, Algeria*
[e] *Laboratory of Microbial Biotechnology, Enzymatic, and Biomolecules (LMBEM), Centre of Biotechnology of Sfax, University of Sfax, P.O. Box 1177, Sfax 3018, Tunisia*

Graphical abstract



Abstract

An extracellular acido-thermostable chitinase (called ChiA-Ba43) was hyper-produced and purified to homogeneity from a newly isolated *Bacillus altitudinis* strain KA15. This strain exhibited the highest chitinase activity (about 10,000 U/mL) after 46 h of incubation in an optimized meduim. Pure enzyme was obtained after ammonium sulphate precipitation (30-60%), followed by sequential column chromatographies on Sephacryl S-200 HR and Mono Q-Sepharose. The purified enzyme is a monomer with a molecular mass of 43,190.05 Da as determined by matrix assisted laser desorption ionization time-of-flight/mass spectrometry (MALDI-TOF/MS). The sequence of the 27 NH_2-terminal residues of ChiA-Ba43 showed high homology with other *Bacillus* species. Optimal activity was recorded at pH 4.0-5.5 and 85°C. The pure enzyme was inhibited by *p*-chloromercuribenzoic acid (*p*-CMB) and *N*-ethylmaleimide (NEM). Interestingly, ChiA-Ba43 showed higher activity towards colloidal chitin, chitin azure, glycol chitin, glycol chitosane, chitotriose, and chitooligosaccharide, while it did not hydrolyse chitibiose and amylose. Moreover, ChiA-Ba43 acted as an *endo*-splitting enzyme as showed by thin-layer chromatography (TLC) from enzymatic catalyzed hydrolysis of chitin-oligosaccharides and colloidal chitin. More interstingly, ChiA-Ba43 showed a high level of catalytic efficiency compared to chitinases ChiA-Mt45, ChiA-Hh59, Chitodextrinase®, *N*-acetyl-β-glucosaminidase®, and ChiA-65. Thanks to its biochemical properties, ChiA-Ba43 may be used for the bioconversion of chitinous waste on an industrial scale.

1. Introduction

Microorganisms including bacteria are most common source of novel biocatalysts such as proteases, esterases, lipases, amylases, and chitinases due to their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (D'Costa et al., 2013). Chitinases, which are glycoside hydrolases (GH), have a wide distribution in nature, including many prokaryotic and eukaryotic organisms. They catalyze the cleavage of β-1,4-glycosidic bonds in glycol-conjugates and polysaccharides. *Endo*-chitinases are indiscriminate and hydrolyze chitin at inner sites, generating multimers of GlcNAc (chitobiose, chitotriose, and chitotetraose).

Chitin, which is composed of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc), is the most abundant bio-polymer in nature after cellulose (Muzzarelli et al., 2012). Fishery wastes (shrimp and crab shells) are considered the main natural chitinacious resources. Moreover, these wastes amount constitute 60-80 % of the whole shrimp and crabs (Wang et al., 2011; Singh et al., 2019), which can cause environmental problems due to their accumulation and slow degradation. In recent years, a lot of knowledge has been gained although challenges related to economical use of chitinases from microbial origin at industrial scale still remain (Bouacem et al., 2018; Mohamed et al., 2019; Yahiaoui et al., 2019; Laribi Habchi et al., 2020).

Bacillus genus is well known and famous for chitinase production such as ChiA from *Bacillus* sp. strain DAU101 (Pan et al., 2019), ChiA 8785 from *Bacillus licheniformis* strain DSM 8785 (Menghiu et al., 2019), and ChiA-65 from *Bacillus licheniformis* strain LHH100 (Laribi Habchi et al., 2015). This paper reports the isolation and identification of a novel chitinase (ChiA-Ba43) produced by *Bacillus altitudinis* strain KA15, a novel strain isolated from the highest summit of Djurdjura Mountains in Kabylia, Algeria.

2. Materials and Methods

2. 1. Materials

The chitinous substrates and commercial enzymes (Chitodextrinase® and *N*-acetyl-β-glucosaminidase®) used were bought from Sigma St. Louis (MO, USA). All chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Colloidal chitine preparation

Colloidal chitin was prepared as previously reported elsewhere (Roberts and Selitrennikoff, 1988).

2.2.2. Standard assay of ChiA-Ba43 activity

Chitinase activity was quantified by analyzing the amount of GlcNAc released from colloidal chitin as substrate according to other study (Thamthiankul et al., 2001).

2.2.3. Purification of ChiA-Ba43

Five hundred mL of a 46 h culture of *Bacillus altitudinis* strain KA15 was centrifuged for 30 min at 9,000*g* to remove microbial cells. The clear supernatant containing chitinase activity was used as the crude enzyme extract and was submitted to purification steps as described by authors (Asmani et al., 2020).

2.2.4. Analytical methods

Total protein content was determined according to Bradford's method (Bradford, 1976). The molecular mass for ChiA-Ba43 was determined by polyacrylamide gel electrophoresis (PAGE) under denaturing and non-denaturing conditions. Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed as reported elsewhere (Laemmli, 1970). Zymography analysis was carried out as previously reported by authors (Laribi Habchi et al., 2012; Laribi habchi et al., 2015). The exact molecular mass of ChiA-Ba43 was analyzed by MALDI–TOF/MS using a Voyager DE-RP instrument (Applied Biosystems, ABI).

2.2.5. Physico-chemical characterization

2.2.5.1. Influence of specific inhibitors, reducing agents, and metal ions

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 metal ions on chitinase activity were investigated by adding them to the reaction mixture. The non-treated and dialyzed enzyme was considered as 100% for metal ion assay.

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

The effect of pH on chitinase activity was evaluated in the pH range 2.0-10.0 under standard assay conditions. The pH stability was tested by incubating the purified chitinase in buffers with different pH from 2.0 to 6.0 at standard assay temperature for 48 h. The determination of the influence of temperature on chitinase activity was performed at temperatures ranging from 40 to 100°C as previously reported (Laribi Habchi et al., 2015; Yahiaoui et al., 2019). Enzyme thermostability was evaluated by incubating it at 70, 80, 90, and 100°C with and without 1 mM $CaCl_2$ for 48 h. Relative and remaining activities were evaluated under the optimum assay conditions (pH 4.0 and 85°C).

3. Results and discussion

3.1. Purification, determination of ChiA-Ba43 molecular mass

Enzyme purity was estimated to be 69.6-fold greater than that of the crude extract. The purified enzyme had a specific activity of 120,000 U/mg, with a yield of about 51% using colloidal chitin as substrate. Interestingly, the specific activity displayed by ChiA-Ba43 was significantly higher compared to those mentioned in literature for other chitinases (Toharisman et al., 2005; Loni et al., 2014; Laribi Habchi et al., 2015; Bouacem et al., 2018). This high level of its specific activity confirmed the potential prospects of ChiA-Ba43 in various biotechnological and industrial bioprocesses. The purified enzyme had a molecular weight of about 43 kDa (Fig. 1A) and clear chitinase activity (Fig. 1B). MALDI–TOF/MS analysis confirmed that the purified ChiA-Ba43 had an exact molecular mass of 43,190.05 Da (data not shown).

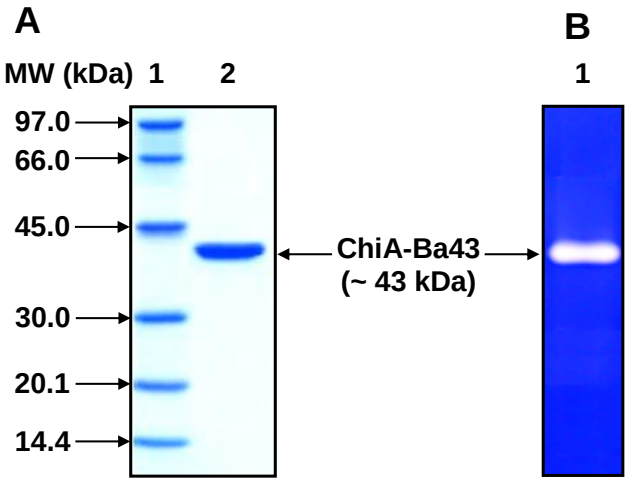


Fig. 1. (A) SDS-PAGE of ChiABa43: Lane 1, Amersham LMW; Lane 2, ChiA-Ba43 (50 µg). **(B)** Chitin azure zymography staining of ChiA-Ba43 (50 µg).

3.2. Physico-chemical characterization of ChiA-Ba43

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

The activity of ChiA-Ba43 was found to be totally inhibited in the presence of *p*-CMB, 5,5'-dithio-bis-2-nitro benzoic acid (DTNB), and NEM. ChiA-Ba43 retained partial activity in the presence of iodoacetamide (IAA), monoiodoacetic acid (IAM), 2-mercaptoethanol (2-ME), and LD-dithiothreitol (LD-DTT). These results suggest that sulfhydryl groups are present in the ChiA-Ba43 active site, which was approved by the complete inhibition observed in the presence of mercuric ions. Among all tested metal ions, just Cd^{2+}, Hg^{2+}, and Ni^{2+} totally inhibited enzyme activity, while Ag^{+} and Al^{2+} decreased enzyme activity by 86, and 30%, respectively. The chitinase ChiA-Ba43 was not inhibited nor activated by Cu^{2+}, while the addition of 1 mM of Ca^{2+}, Mg^{2+}, and Mn^{2+} increased the activity of chitinase (250, 180, and 129%, respectively). The presence of Ca^{2+} increased the activity, which indicates that this cation regulates the enzyme active conformation and therefore increases the ChiA-Ba43 activity.

3.2.2. Evaluation of different pH on the activity and stability

ChiA-Ba43 was active in pH range from 2.0 to 10.0 with maximum activity at pH 4.0-5.5 (Fig. 2A). The chitinase is stable at pH ranging from 2.0 to 6.0 (Fig. 2B). The wide range of pH stability of ChiA-Ba43 will be beneficial for industrial and commercial applications performed at acidic conditions.

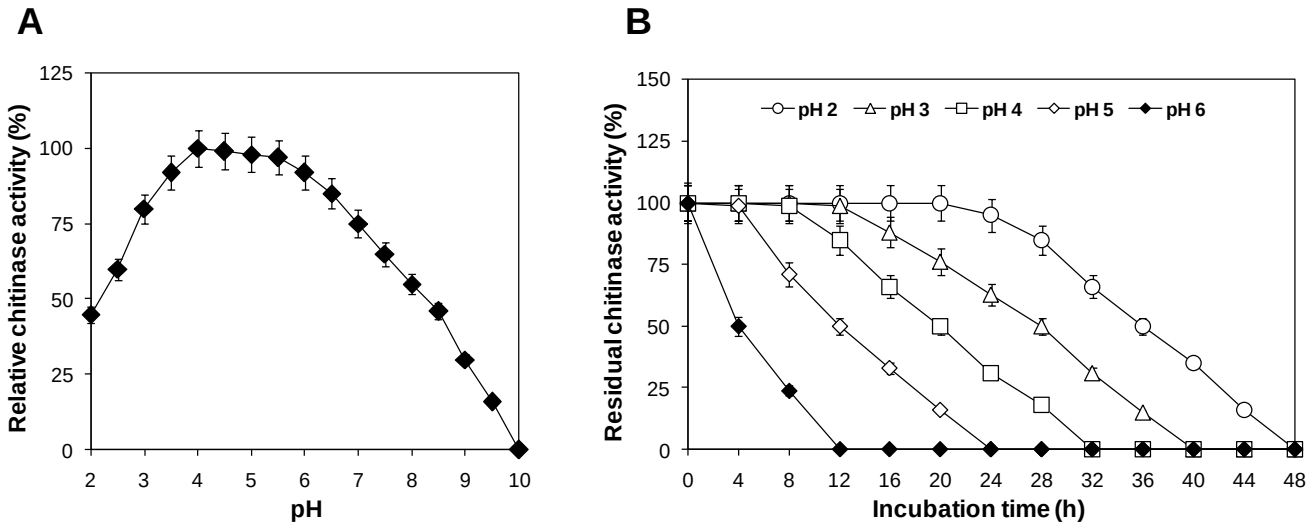


Fig. 2. Effect of pH on the activity **(A)** and stability **(B)** of the purified chitinase ChiA-Ba43.

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

The optimum temperature was found for the activity of the purified chitinase ChiA-Ba43 at pH 4.0 was 75°C without Ca^{2+} and 85°C in the presence of 1 mM Ca^{2+} (Fig. 3A). The thermostability profile of ChiA-Ba43 is presented in Fig. 3B. The half-life at 70, 80, 90, and 100°C without Ca^{2+} were 28, 20, 12, and 4 h, respectively (Fig. 3B). However, in the presence of 1 mM Ca^{2+}, the half-life of ChiA-Ba43 increased to 32, 24, 16, and 8 h, respectively.

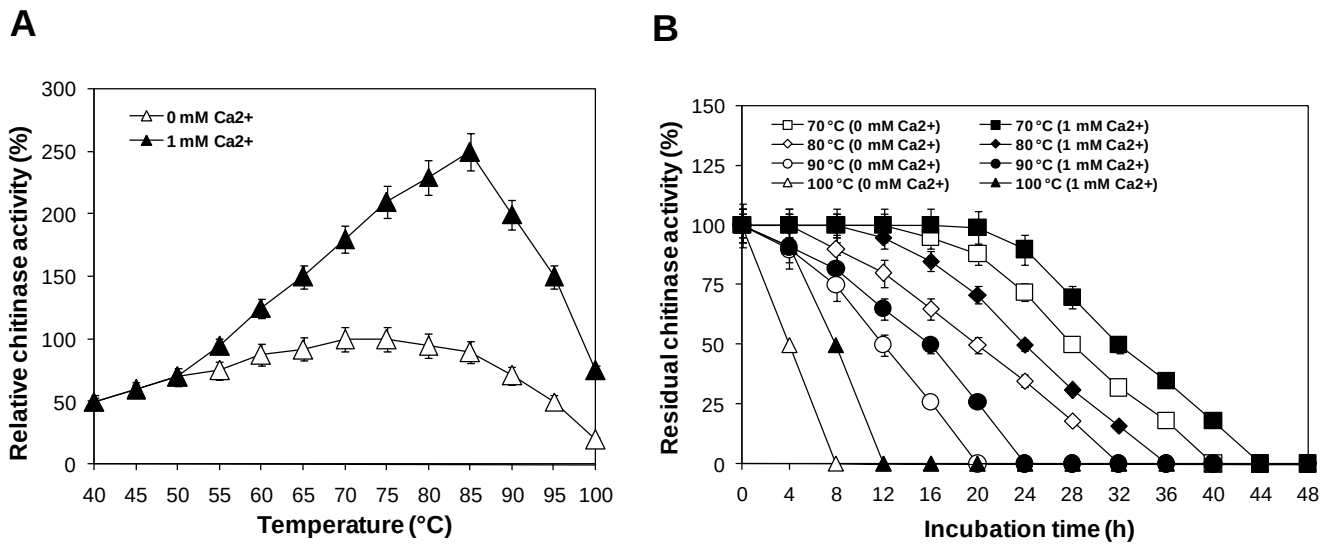


Fig. 3. Effect of the thermoactivity **(A)** and the thermostability **(B)** of ChiA-Ba43. Each point represents the mean of at least three independent experiments.

4. Conclusion

All of these results suggest the potential relevance of ChiA-Ba43 for the future commercial exploitation. Giving the interesting properties and attributes of this enzyme, further studies are required including the exploration of the structure-function relationships as well as the development and

evaluation of its economic impact in scaled-up bioprocess for the biological potential of the recovered chitooligosaccharides and the industrial degradation of chitinous waste.

5. References

1- Asmani, K.L., Bouacem, K., Ouelhadj, A., Yahiaoui, M., Bechami, S., Mechri, S., Jabeur, F., Taleb-Ait Menguellet, K., Jaouadi, B. (2020) Biochemical and molecular characterization of an acido-thermostable *endo*-chitinase from *Bacillus altitudinis* KA15 for industrial degradation of chitinous waste. *Carbohydr. Res.* 108089. In press. https://doi.org/10.1016/j.carres.2020.108089

2-Bouacem, K., Laribi-Habchi, H., Mechri, S., Hacene, H., Jaouadi, B., Bouanane Darenfed, A. Biochemical characterization of a novel thermostable chitinase from *Hydrogenophilus hirschii* strain KB-DZ44, *Int. J. Biol. Macromol.* 106 (2018) 338-350.

3- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. biochem.* 72(1), 248-254.

4- D'Costa, B., Shamim, K., Dubey, S. K. Characterization of thermostable serine protease from *Bacillus altitudinis* strain BR1. *J. Sci. Ind. Res.*72 (2013) 166-171.

5- Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 (1977) 680-685.

6- Laribi Habchi, H., Dziril, M., Badis, A., Mouhoub, S., Mameri, N. Purification and characterization of a highly thermostable chitinase from the stomach of the red *scorpionfish Scorpaena scrofa* with bioinsecticidal activity toward cowpea weevil *Callosobruchus maculatus* (Coleoptera: *Bruchidae*), *Biosci. Biotechnol. Biochem.* 76 (2012) 1733-1740.

7- Laribi Habchi, H., Bouanane Darenfed, A., Drouiche, N., Pauss, A., Mameri, N. Purification, characterization, and molecular cloning of an extracellular chitinase from *Bacillus licheniformis* stain LHH100 isolated from wastewater samples in Algeria, *Int. J. Biol. Macromol*. 72 (2015) 1117-1128.

8- Laribi-Habchi, H., Bouacem, K., Allala, F., Jabeur, F., Selama, O., Mechri, S., Merzouk, Y., Bouanane Darenfed, A., Jaouadi, B. (2020). Characterization of chitinase from *Shewanella inventionis* HE3 with bio-insecticidal effect against granary weevil, *Sitophilus granarius* Linnaeus (Coleoptera: *Curculionidae*). *Process Biochem*. In press.

9- Loni, P.P, Patil, J.U., Phugare, S.S., Bajekal, S.S. Purification and characterization of alkaline chitinase from *Paenibacillus pasadenensis* NCIM 5434, J. *Basic Microbiol.* 54 (2014) 1080-1089.

10- Menghiu, G., Ostafe, V., Prodanovic, R., Fischer, R., Ostafe, R. Biochemical characterization of chitinase A from *Bacillus licheniformis* DSM8785 expressed in *Pichia pastoris* KM71H, *Protein Expr. Purif.* 154 (2019) 25-32.

11- Mohamed, S., Bouacem,K., Mechri, S., Addou, N.A., Laribi Habchi, H., Fardeau, M.L., Jaouadi, B., Bouanane Darenfed, A., Hacène, H. Purification and biochemical characterization of a novel acido-halotolerant and thermostable endochitinase from *Melghiribacillus thermohalophilus* strain Nari2A^{T}, *Carbohydr. Res.* 473 (2019) 46-56.

12- Muzzarelli, R.A.A., Boudrant,J., Meyer, D., Manno, N., Demarchis, M., Paoletti, M.G. Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial, *Carbohydr. Polym.* 87 (2012) 995-1012.

13- Pan, M., Li, J., Lv, X., Du, G., Liu, L. Molecular engineering of chitinase from *Bacillus* sp. DAU101 for enzymatic production of chitooligosaccharides, *Enzyme Microb. Technol.* 124 (2019) 54-62.

14- Roberts, W.K., Selitrennikoff, C.P. Plant and bacterial chitinases differ in antifungal activity, *J. Gen. Microbiol.*134 (1988) 169-176.

15- Singh, S.K. Solubility of lignin and chitin in ionic liquids and their biomedical applications, *Int. J. Biol. Macromol.* 132 (2019) 265-277.

16- Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S., Panbangred, W. 2001. Chitinase from Bacillus thuringiensis subsp. pakistani. *Appl. Microbiol. Biotechnol.* 56 (2001), 395-401.

17- Toharisman, A., Suhartono, M.T., Spindler Barth, M., Hwang, J. K., Pyun, Y. R. World, Purification and characterization of a thermostable chitinase from *Bacillus licheniformis* Mb-2, J. *Microbiol. Biotechnol.* 21 (2005) 733-738.
18- Wang, S.L., Liang, T.W., Yen, Y.H. Bioconversion of chitin-containing wastes for the production of enzymes and bioactive materials, *Carbohydr. Polym.* 84 (2011) 732-742.