



## Characterization and overexpression of a glucanase from a newly isolated *B. subtilis* strain

Houda HMANI<sup>1,\*</sup>, Lobna DAOUD<sup>1,2</sup>, Manel BEN ALI<sup>1,2</sup>, Adel HAJ BRAHIM<sup>1</sup>, Mouna JLIDI<sup>1</sup>, Samir BEJAR<sup>1</sup> and Mamdouh BEN ALI<sup>1,2</sup>

<sup>1</sup> Laboratory of Microbial Biotechnology and Engineering Enzymes (LBMIE), Center of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour km 6, PO Box 1177 Sfax 3018, Tunisia; E-Mails: houda\_enis@yahoo.fr; lobna.daoudm@gmail.com; manel.benali@gmail.com; adelhadjibrahim@gmail.com; jlidimanno@yahoo.fr; samir.bejar@cbs.rnrt.tn; mamdouh.benali@cbs.rnrt.tn.

<sup>2</sup> Astrum Biotech, Business incubator, Center of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour km 6, PO Box 1177 Sfax 3018, Tunisia; E-Mails: lobna.daoudm@gmail.com; manel.benali@gmail.com; mamdouh.benali@cbs.rnrt.tn.

\* Correspondence addressed to Houda HMANI; E-Mail: houda\_enis@yahoo.fr; Tel.: +216 97 756 269; Fax: +216 74 875 818.

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### Abstract:

Glucanases are enzymes that hydrolysis glucans which are the major cell wall components in cereals. Newly isolated bacteria assigned as *Bacillus subtilis* HB2, produces a monomeric glucanase (GLU HB2) of a molecular mass of 75 kDa. GLU HB2 has an optimal activity at pH 5 and 55 °C. It is extremely stable at a broad range of pH and temperature up to 65 °C, in presence of 5 mM of CaCl<sub>2</sub>. In order to overcome the enzymatic inhibition problem observed in wild-type strains, *GluHB2* gene was integrated into the genome of *B. subtilis* HB2 and the recombinant strain was named HB2G. The correlation of glucanase production with bacterial growth shows that the level of expression of HB2G remains low and relatively comparable to the wild-type strain. But in terms of productivity, the HB2G strain is more productive throughout bacterial culture. This low production and growth of the recombinant strain can be attributed to the toxicity of the overexpression of the glucanase gene under a constitutive promoter.

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**Keywords:** glucanases; *Bacillus subtilis* HB2; overexpression.

### 1. Introduction

$\beta$ -glucans are the major linear polysaccharides in the endosperm of the cell wall of cereals such as barley, wheat rye and rice. It can cause an adverse effect on the cereal-grain-based industry. The degradation of  $\beta$ -glucan is naturally activated by  $\beta$ -glucanases. Those glucanases have been isolated and characterized from a number of microorganisms including *Bacillus*

species such as *Bacillus subtilis* [1, 2]. Glucanases has been exploited in a vast range of biotechnological applications like brewing industry and animal feed production [1]. In the poultry industry, glucanases can promote the digestibility of feedstuff by degrading the  $\beta$ -glucan and reduce digesta viscosity. Due to wide scope of applications of endo-1,4- $\beta$ -glucanases,

this study was mainly concerned with characterizing of glucanases produced by newly

isolated *B. subtilis* HB2 and subsequent its overexpression under a constitutive promoter.

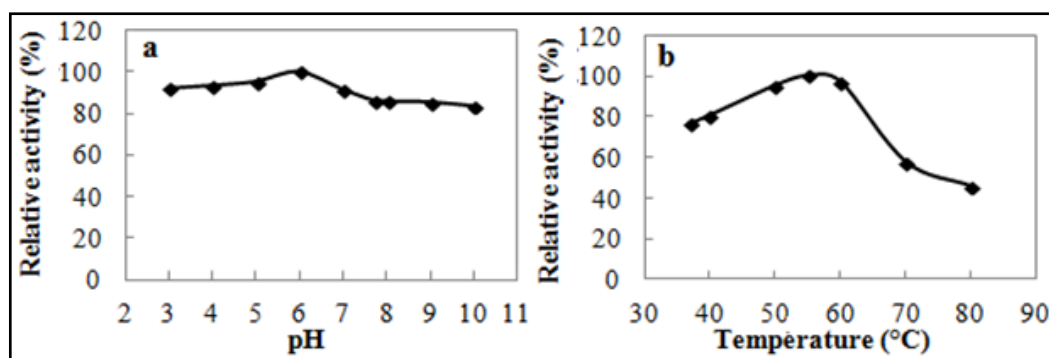
## 2. Results and Discussion

The effects of pH and temperature on the activity of the glucanase GluHB2 are shown in **Figure 1 a** and **b**, respectively. The enzyme was noted to exhibit significant activity in the wide range of pH (3 to 10) and temperature (30 °C - 80 °C), with maximal activity at pH 6 and 55 °C.

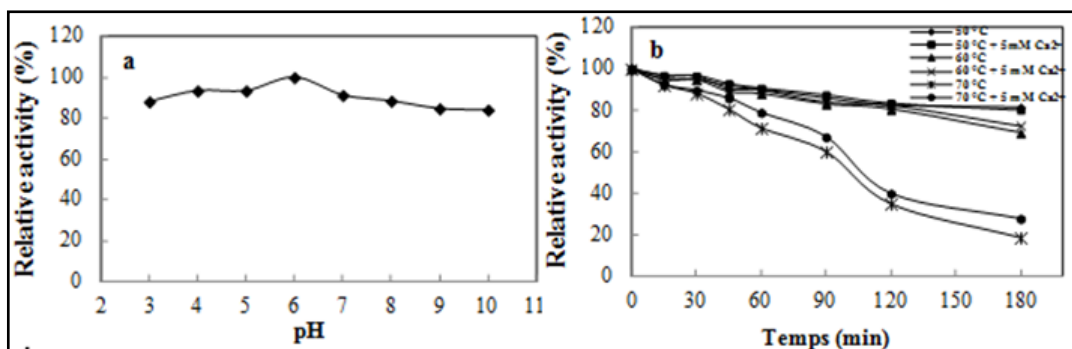
The pH stability and thermostability profiles of the enzyme are shown in **Figure 2 a** and **b**, respectively. The glucanase from *Bacillus subtilis* HB2 showed high pH stability within the range of pH 3–10 after 1 h incubation at 37 °C. It also displayed a marked thermostability,

retaining 80% of its activity after incubation at 60 °C for 120 min, with 5 mM of CaCl<sub>2</sub>.

The thermostability of GluHB2 was better than other glucanases previously studied from *Bacillus subtilis*168 [3] and *Bacillus subtilis* GN156 [4]. Activity in a wide range of pH and temperature, as well as its remarkable thermostability, suggests that this enzyme could be a good candidate for various industrial applications such as brewing industry and animal feed. Moreover, it's active at physiological animals' pH and temperature and resist to both stomach acidic and tract basic pH.



**Figure 1.** Effect of pH (a) and temperature (b) on the activity of GluUHB2.



**Figure 2.** pH stability (a) and thermostability (b) of the glucanase from bacillus subtilis HB2

To prepare a strain that constitutively expresses the gene of interest in order to overcome the problem of enzymatic inhibition encountered in wild-type strains. We chose to clone the Glu HB2 gene and integrate it into the genome of the *Bacillus subtilis* HB2. A fragment having a size of 1.8 kb corresponding to the size

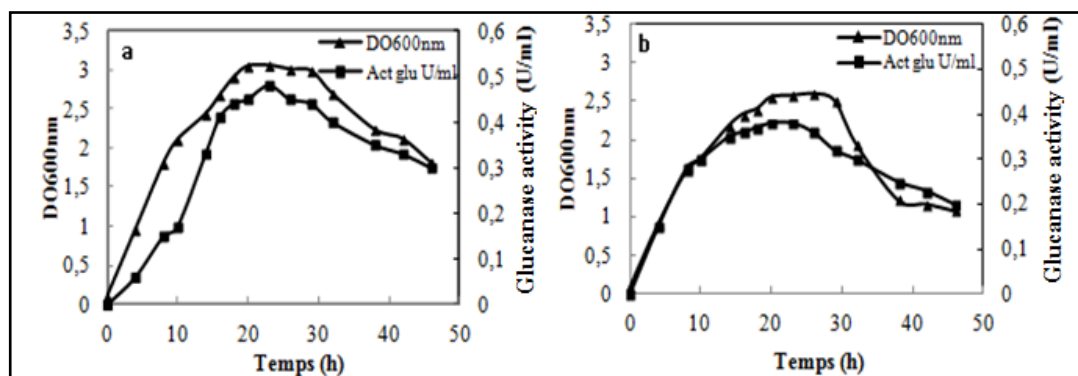
of the gene Glu HB2 (645 kb), the promoter P59 (59 bp), the promoter HpaII (400 bp) and the signal sequence SS-lip (92 bp), and carrying the open reading frame, was obtained, sequenced and cloned into the linearized pDG1662 integration vector. In order to integrate the Glu HB2 gene into the genome of the *B. subtilis* HB2

strain, we opted for the natural transformation method which generates genetically stable transformants. The expression cassette is integrated into the genome of *B. subtilis* via a "crossing over" phenomenon between the homologous sequences located in the vector pDG1662 and the genome of the strain. Recombinant clones integrate a single copy of the Glu HB2 gene and consequently lose the endogenous amylase gene.

This positive transformant, named HB2G, was tested on a liquid medium to determine enzymatic activity in the culture supernatant. The growth kinetics of both HB2G and HB2 strains with the glucanase activity produced were

monitored for 72 h. The correlation of glucanase production with bacterial growth shows that the level of expression of HB2G remains low and relatively comparable to the wild-type strain. But in terms of productivity, the HB2G is more productive throughout bacterial culture (**Figure 3**).

In full growth phase, the productivity of the HB2G strain is estimated at 0.18 U / OD unit against 0.09 U / OD unit for the HB2 strain. This low production and growth of the recombinant strain, associated with the good productivity (relative to the wild strain), can be attributed to the toxicity of the expression of the glucanase gene under a constitutive promoter.



**Figure 3.** The correlation of glucanase production with bacterial growth of HB2 (a) and HB2G (b).

### 3. Materials and Methods

Probiotic strain: *Bacillus subtilis* HB2 DSM 104747 strain was isolated from soil sample [5].  $\beta$ -glucan was purchased from Sigma Chemical Co Ltd. The pDG1662 was an integrative plasmid used for subcloning.

The glucanase activity was determined using 0.2 % (w/v) barley  $\beta$ -glucan as the substrate. The assay was carried out at 50 °C and pH 6 (100 mM phosphate buffer), unless otherwise stated. The reaction was stopped after 30 min of incubation by adding 3,5-dinitrosalicylic acid and the reducing sugars released were then quantified [6]. The effect of pH and temperature on glucanase activity and stability was studied by incubating the enzyme at pH 3 to 10 and 30 °C to 70 °C, respectively, and measuring relative activities at standard assay conditions.

Glucanase activity represents the means of, at least, two determinations performed in duplicate. In order to over express the glucanase, the corresponding DNA was amplified by PCR using

as template the recombinant plasmid pBSMul2-Glu HB2 and the primers P59DbamHI (CGCGGATCCGCGATGGCTTGACAGGGAGAGATA) and P59R BamHI (CGCGGATCCGCGTACCGAACTGTCCCTCTCTATC). A fragment having a size of 1.8 kb corresponding to the size of the gene *Glu HB2* (645 kb), the promoter P59 (59 bp), the promoter HpaII (400 bp) and the signal sequence SS-lip (92 bp), and carrying the open reading frame, was obtained.

The resulting fragment was purified and cloned into the pGEMT-easy vector and then ligated into pDG1662 integration vector linearized with BamHI. The recombinant vector pDG1662-Glu HB2 was subsequently linearized with the BspHI enzyme and transformed into competent cells of *B. subtilis* HB2 by the manual method. The transformants were selected on LB supplemented with 25  $\mu\text{g mL}^{-1}$  of Chloramphenicol. The cloned glucanase gene is then in phase with the signal peptide "SS-lip" of the vector pBSMul2 and

under the control of its two strong constitutive promoters P59 and HpaII.

#### 4. Conclusions

As a conclusion, we first report the glucanase of *Bacillus subtilis* HB2 was active at wide range of temperature. Our findings indicate that this

enzyme could be considered as a potential candidate for future application particularly in the animal feed industry, since it could be active in the stomach and intestine and could remain stable at high temperatures during feed pelleting processes.

#### Acknowledgments

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#### Conflicts of Interest

The authors declare no conflict of interest.

#### References and Notes

1. Planas, A. Bacterial 1,3;1,4- $\beta$ -glucanases: structure, function and protein engineering, *Biochimica and Biophysica Acta* 1543, **2000**, 361-382.
2. Qiao, J.; Dong, B.; Li, Y.; Zhang, B.; Cao, Y. Cloning of a beta-1,3-1,4-glucanase gene from *Bacillus subtilis* MA139 and its functional expression in *Escherichia coli*. *Applied Biochemistry and Biotechnology* **2009**, 152, 334–342.
3. Furtado G. P.; Ribeiro L. F.; Santos C. R.; Tonoli C. C.; Souza A. R.; Oliveira R. R.; Murakami M. T.; Ward R. J. Biochemical and structural characterization of a  $\beta$ -1,3–1,4-glucanase from *Bacillus subtilis* 168. *Process Biochemistry* **2011**, 46, 1202-1206.
4. Apiraksakorn J.; Nitisinprasert S.; Levin R. E. Grass degrading  $\beta$ -1,3-1,4-D-glucanases from *Bacillus subtilis* GN156: purification and characterization of glucanase J1 and pJ2 possessing extremely acidic pI. *Applied Biochemistry and Biotechnology* **2008**, 149, 53-66.
5. Hmani, H.; Daoud, L.; Jlidi, M.; Jalleli, K.; Ben Ali, M. Hadj Brahim, A.; Bargui, M.; Dammak, A.; Ben Ali, M. A *Bacillus* strain as probiotic in poultry: selection based on *in vitro* functional properties and enzymatic potentialities. *Journal of Industrial Microbiology and Biotechnology* **2017**, 44, 1157-1166
6. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **1959**, 31, 426–428.