

Mastery of Cultural Conditions and Physico-chemical Properties Improves the Production and the Catalytic Efficiency of bglG

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Abstract *Stachybotrys microspora* is a filamentous fungus secreting multiple β -glucosidases. Two of them were characterized. The third one, named bglG, was also characterized and used for various investigations. The current work undertakes the plausible role played by some cultural conditions and physico-chemical properties to improve bglG time course synthesis and also its catalytic efficiency. Indeed, bglG time course synthesis is slightly affected by light, but it is clearly affected by aeration and presence of baffle. On the same case, optimization of substrate and enzyme concentration contributes to the improvement of the catalytic efficiency of bglG. This biocatalyst tolerates a high ionic strength during its activity assay; β -mercaptoethanol increases the enzymatic rate. BglG has the capacity to hydrolyse efficiently oleuropein, with a recovery of 88 %.

Keywords *Stachybotrys microspora* · β -Glucosidase · Light · 2-Mercaptoethanol · Ionic strength · Oleuropein conversion

Introduction

Enzyme catalysts bind substrate and convert it to a product. Although enzymes may be modified during their participation in the reaction sequence, they return to their original form at the end of the process [1, 2].

Metallic ions inhibit or activate the biocatalyst, or decrease or increase thermo-activity or/and thermo-stability [2]. The enhancement of the enzyme, during catalytic assessment, could be explained by the stabilization of enzyme structure. In addition, various parameters

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influence enzyme time course production, like aeration, pH, light, nitrogen and carbon source, etc. [3, 4].

Stachybotrys microspora secretes a large number of β -glucosidases; two of them were purified and characterized [5, 6]. The third one, named bglG, was purified and biochemically characterized. BglG shows optimal activity at 50 °C and 5 units of pH. Not only their induction by glucose used (at the concentration of 10 g l⁻¹) as unique carbon source, but bglG was also characterized by some other peculiar properties such as its activation by xylose and ferrous ion or its capacity to efficiently hydrolyze sucrose [7]. BglG is insensitive to the classic coloration method, using blue staining solution. BglG thermo-activity, thermo-stability and refolding recovery were improved. So, we proved for the first time that these parameters have been ameliorated by the addition of a chaperone-like molecule named “wheat dehydrin” [8]. In addition, we proved that bglG time course production was negatively affected by the presence of cellobiose dehydrogenase, in the medium culture, through the release of the Δ -gluconolactone, a strong inhibitor of bglG [9].

In the current work, we report the ability of some cultural conditions and biochemical properties to affect not only bglG time course production, but also its catalytic efficiency. Also, one of the required applications of bglG is followed in this work: the conversion of oleuropein releasing mainly hydroxytyrosol.

Materials and Methods

Biological Strain

The biological strain used in this work is a filamentous fungus that was isolated and belongs to *S. microspora* [5, 6].

Production Conditions and Purification Steps of bglG

S. microspora strain was grown on potato dextrose agar medium at 30 °C for 4 days. Spores were harvested in 0.1 % Tween 80 solution and used to inoculate Mandel's medium [10] that was modified as follows, per litre: 2 g KH₂PO₄, 1.4 g (NH₄)₂SO₄, 1 g yeast extract, 0.69 g urea, 0.3 g CaCl₂·2 H₂O, 0.3 g MgSO₄·7H₂O, 1 ml Tween 80 and 1 ml trace element solution composed of 1.6 g l⁻¹ MnSO₄, 2 g l⁻¹ ZnSO₄, 0.5 g l⁻¹ CuSO₄, 0.5 g l⁻¹ CoSO₄. Glucose at the concentration of 10 g l⁻¹ was used as a unique carbon source [3]. *S. microspora* was grown at 30 °C for 5 days, and the supernatant was used for the purification and characterization of bglG, as described in [7].

Enzyme Assays

The β -glucosidase activity was monitored using pNPG as substrate. Indeed, 0.2 ml of 1 mM pNPG (in 0.1 M sodium acetate buffer pH 5) was incubated with bglG at the appropriate dilution at 50 °C for 15 min. The reaction was stopped by adding 0.6 ml of 0.4 M glycine–NaOH buffer pH 10.8; the liberated *p*-nitrophenol (pNP) was measured at 400 nm. One unit of the enzymatic activity was determined as the amount of enzyme required to release 1 μ mol of pNP per minute under the assay conditions [7, 8]. We note that the molecular extinction coefficient of the pNP is 18.000 M⁻¹ cm⁻¹.

Protein Assay

The protein content of the samples obtained during these studies was determined using the Bradford assay [11]. Bovine serum albumin was used as standard.

Effect of Light on bglG Production

S. microspora was cultivated in two erlenmeyers: one of which is stored away from light, and we determined soot kinetics of the enzyme production at regular time intervals.

Effect of Substrate and Enzyme Concentration on bglG Activity

The activity assay was measured using pNPG, as substrate, at various concentrations ranging from 0.125 to 5 mM. On the same case, we studied the effect of variation of bglG concentration during the activity assay.

Effect of Ionic Strength on bglG Assay

BglG activity was monitored in the presence of sodium acetate buffer, NaCl and KCl at different concentrations varying from 0 to 1 M and 0 to 400 mM, respectively. The ionic force (IF) was determined using the relative equation:

$$IF = \left(\frac{1}{2}\right) \sum (C_i z_i^2);$$

with C_i and z_i representing the concentration and the ionic charge, respectively. “IF” was expressed in moles per litre [6].

The Effect of Organic Solvents on bglG Assessment

Various additives were added to the reaction mixture to determine their effect on bglG activity. Indeed, acetone, methanol, ethanol, isopropanol, butanol and acetonitrile were used individually at various percentages, ranging from 10 to 50 %.

Conversion of Oleuropein by bglG

The ability of bglG to hydrolyse oleuropein is monitored using bglG, in their appropriate dilution with the substrate (oleuropein, commercial product purchased from Sigma), at the concentration of 5 g l^{-1} , and the mixture (final volume, 2 ml) was incubated at 50°C . Samples were taken at regular time intervals, and the amount of glucose released was determined by the GOD method.

Results and Discussions

We proved that *S. microspora* produces more than five distinguished β -glucosidases [3], which are rarely described in literature. We studied the influence of some parameters on the production of these biocatalysts to facilitate their purification, biochemical characterization and also potential use in biotechnological applications. For the first time, we studied the effect of carbon source, and we proved that the last one directs the differential expression of

β -glucosidases [3]. Two of them were characterized [5, 6]. The production of the third one (named bglG) was induced in the presence of glucose and cellobiose, and used individually as sole carbon source. The amount of these sugars was also optimized to 1 % [3].

The Level of Enhancement of bglG Production by Light and Gas Exchange

In order to enhance the production level and the catalytic efficiency of bglG, other parameters, like light and aeration, were tested for their influence in bglG time course production. Indeed, Fig. 1a shows that light has no distinguished effect on the bglG syntheses, in contrast to other fungi in which light is required for the growth of mycelium, like *Neurospora crassa* that has been shown to be a paradigm for photobiological, biochemical and genetic studies of blue light perception and signal transduction. In this case, several different developmental and morphological processes of *Neurospora* are regulated by blue light and can be divided into early and late blue light responses [12]. It is true that, unlike chlorophyllous plants, light is not essential for fungal growth. But, to develop fruiting bodies, i.e. grow, some light is necessary for a few minutes. It seems that they used primarily the top of the visible spectrum (blues).

Aeration represents also one of the major and important factors that have been followed during time course production of the biocatalysts or other metabolites and bioactive molecules. Figure 1b shows that aeration influences bglG synthesis. Indeed, the use of three

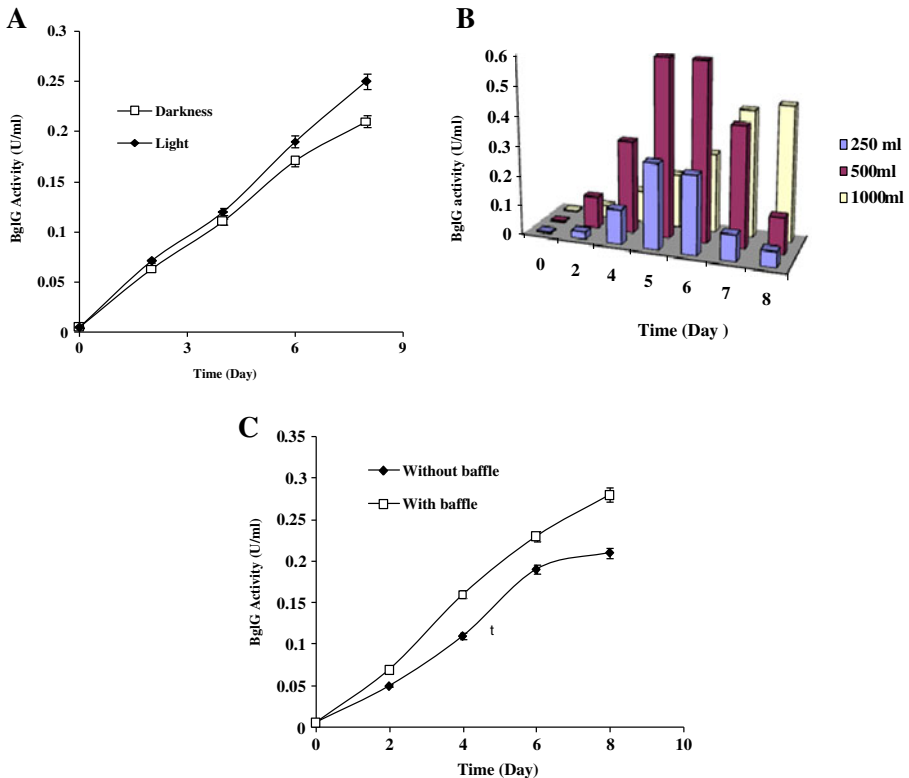


Fig. 1 a The effect of light (squares) and darkness (diamonds) on the bglG time course production. b Histogram presenting the effect of the aeration on bglG production and c the effect of baffle on the bglG production; with baffle (squares) and without baffle (diamonds)

erlenmeyers, presenting differences in their size (250, 500 and 1,000 ml), proves that 500 ml is the best choice to produce bglG as shown in Fig. 1b. As a conclusion to this fact, production of bglG is more followed with 500-ml than with 250- or 1,000-ml erlenmeyers. Hence, the optimal volume of culture medium for bglG production is estimated at one tenth of the erlenmeyers. In the same case, Fig. 1c follows that baffle affects positively bglG kinetic syntheses.

Besides studying the improvement of bglG production processes, the catalytic efficiency of the purified enzyme was also studied. Furthermore, the effect of substrate and enzyme concentration, ionic strength, organic solvents and some reducing factors such as β -mercaptoethanol was assessed, as described in the section “Materials and methods”.

Substrate and Enzyme Amounts are key Parameters During Enzyme Assay

BglG activity was studied in the presence of various substrate concentrations. Indeed, Fig. 2a proves that bglG activity shows an exponential profile; the maximal velocity was obtained at the concentration of 1 mM, and it remains the same with 2, 3, 4 and 5 mM. For this reason, 1 mM was considered as the best choice that can be used to measure the bglG activity.

The effect of bglG concentration was studied using large enzymatic range amounts. Figure 2b proves proportionality between bglG concentration and activity. This finding involves the fact that bglG is a monomeric, hence Michelin, biocatalyst.

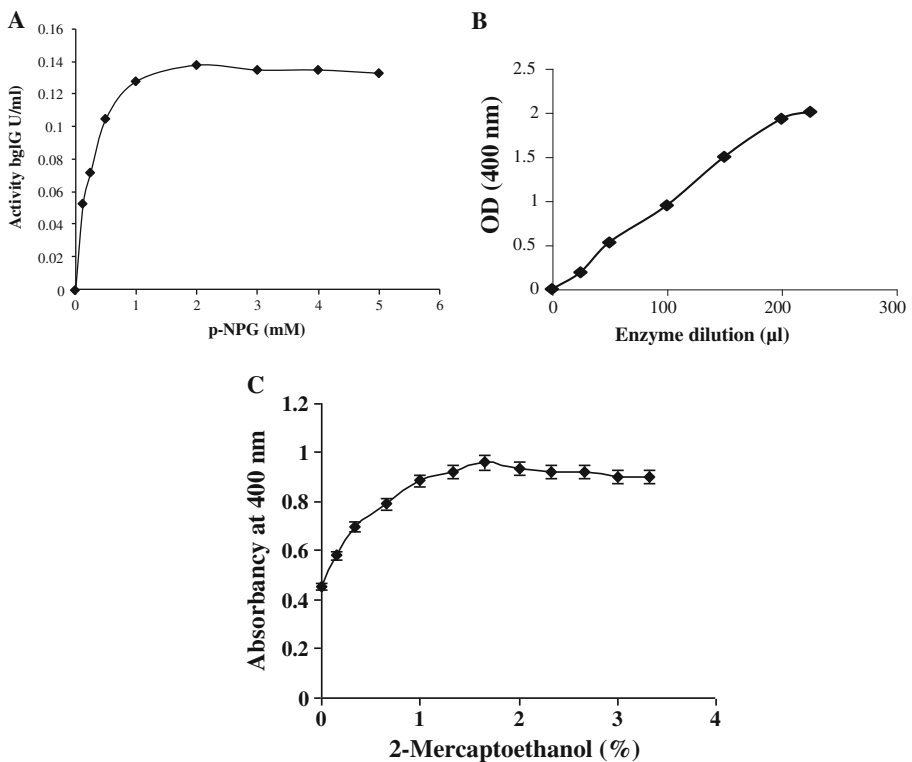


Fig. 2 The effect of substrate (a), bglG concentration during the activity measurement (b) and β -mercaptoethanol increases the purified bglG activity (c)

The Effect of 2ME, NaCl, KCl and Sodium Acetate Ion on bglG Activity

As shown in Fig. 2c, we proved that bglG activity is upgraded in the presence of 2ME at various concentrations. Knowing that this additive is a reducing agent, we can suggest that bglG is more active in its reduced form per comparison to the non-treated form. Then, the presence of 2ME in the reaction mixture favours an optimal reduced condition that improves the bglG catalytic capacity.

On another hand, Fig. 3 proved that bglG tolerates an important ionic force. Indeed, when we used KCl, NaCl and sodium acetate ion at various concentrations, bglG remains their catalytic efficiency (Fig. 3a–c). Thus, this fact gives proof to the plausibility for the use of bglG in some biotechnological applications which required tolerance or resistance against the elevated ionic force.

Organic Solvents Influence bglG Activity

The histogram shown in Fig. 4a summarizes the effect of some of the organic solvents on bglG activity. Indeed, it decreases with increased level of organic solvents used in this study.

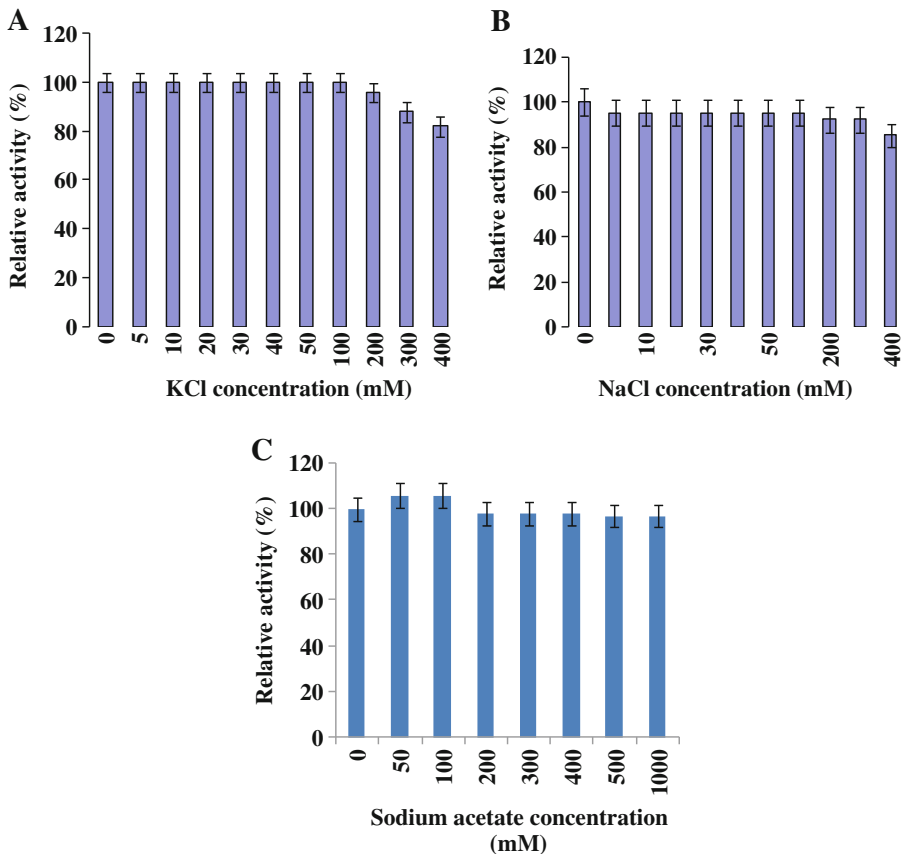


Fig. 3 The effect of the ionic strength on the bglG activity. **a**, **b** and **c** KCl, NaCl and sodium acetate were used as the tested ionic solutions

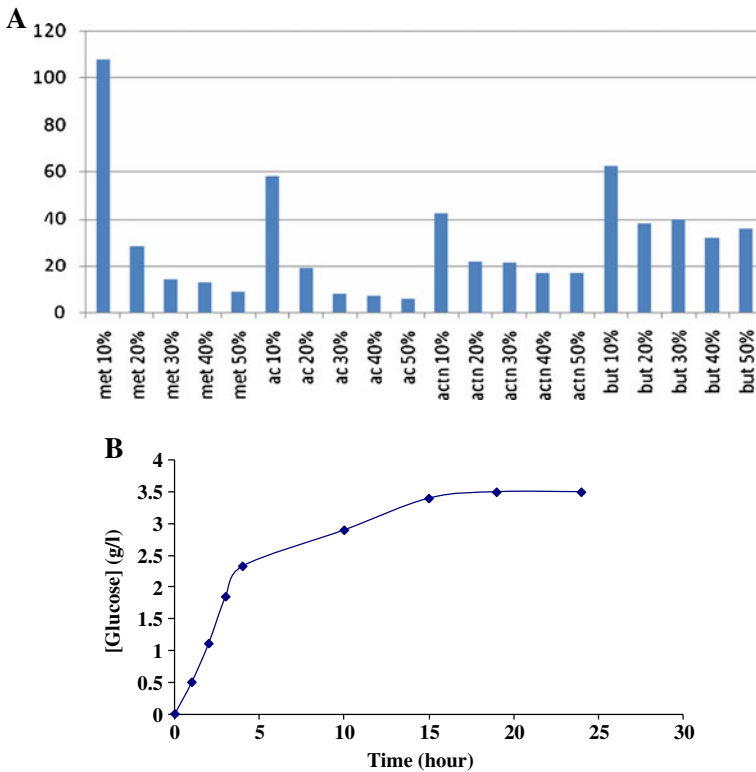


Fig. 4 **a** Representative histogram following the effect of organic solvents on the assessment of bglG activity. The effectors used at various concentrations ranged between 10 and 50 %. **b** Conversion of oleuropein by bglG. The plot shows the ability of bglG to cleave oleuropein into glucose unit and an aglycone product (the hydroxytyrosol)

According to these findings, we conclude that bglG appears as unable to be used for synthetic processes such as transglucosylation or reverse hydrolysis in the biphasic system.

BglG Splits Oleuropein into Glucose and Hydroxytyrosol

The olive tree has been accepted as a symbol of holiness, abundance, wisdom and health, for centuries. Although its fruit is mainly used as a food after processing for table olive and olive oil, it is used for preparing medical products, cosmetics and animal feed. There are many researches that have been done on the effects of oleuropein, one of the most important phenolic compounds extracted from olive leaves, on health [13–15]. In this case, the capacity of bglG to cleave oleuropein appears to be a very important property requested in various biotechnological tools. Figure 4b shows the capability of bglG to split oleuropein with a recovery of 88 % after 24 h of reaction. This result gives birth to the plausibility of bglG to be used as a potential biological tool in agro-food domains such as eliminating the bitter taste of olives.

Conclusion

We proved through these studies rather than other ones that the production and the catalytic efficiency of bglG result in the optimization of the surrounding conditions

and parameters such as aeration, slight, carbon and nitrogen source, pH, ionic strength, temperature, etc.

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