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In vivo assay to identify bacteria with β-glucosidase activity

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Abstract

Background: β-glucosidase assay is performed with purified or semipurified enzymes extracted from cell lysis. However, in screening studies to find bacteria with β-glucosidase activity among many tested bacteria, a fast method without cell lysis is desirable. In that sense, we report an *in vivo* β-glucosidase assay as fast method to find β-glucosidase producer strain.
Results: The method consists in growth the tested strains in a medium supplemented with the artificial substrate *p*-nitrophenyl-β-glucopyranoside (*p*NPG). The presence of β-glucosidase enzymes converts the substrate in *p*-Nitrophenol (*p*NP), a molecule easily measured in the supernatant at 405 nm by a spectrophotometer. The assay was evaluated using two *Bifidobacterium* strains; *Bifidobacterium longum* B7254 strain that lack of β-glucosidase activity and *Bifidobacterium pseudocatenulatum* B7003 strain that shows β-glucosidase activity. The addition of sodium carbonate during *p*NP measurement increases the sensitivity of *p*NP detection and avoid masking absorbance by culture medium. Furthermore, we shown that *p*NP is a stable enzymatic product, not metabolized by bacteria, but with an inhibitory effect on cell growth. The β-glucosidase activity was declared as unit of enzyme per gram per minute per dry cell weight. This method also allowed to identify *Lactobacillus* strains with higher β-glucosidase activity among several lactobacillus species.
Conclusion: This *in vivo* β-glucosidase assay can be used as an enzymatic test on living cells without cell disruption. The method is simple, quantitatively and recommended, especially in screening studies driven not only to find bacteria with β-glucosidase activity, but rather the higher β-glucosidase activity among them.

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Keywords: antimicrobial; Bifidobacterium; cell lysis; enzymatic test; *Lactobacillus; p*-nitrophenol; *p*-nitrophenyl-β-glucopyranoside; screening; screening; β-glucosidase assay; β-glucosidase producer strain.

1. Introduction

β-D-glucosidase enzymes (EC 3.2.1.21) catalyze the transfer of glycosylic groups between oxygen nucleophiles [1]. The substrates include a wide range of carbohydrate molecules with a β-anomeric conformation binding to aryl-, amino- and cyanogenic-glucoside or a β-anomeric conformation included in alkyl-β-D-glucoside, oligosaccharide and disaccharide. According to their substrate specificities, these enzymes could be classified into three groups: aryl β-glucosidases, cellobiohydrolases and broad substrate specificity. However, this classification is not necessary is in accordance with the relationship between amino acid sequence and catalytic mechanism [2]. An alternative classification that relates structural characteristics with functional mechanisms has been published [2]. Regarding its biological role in microorganisms, β-glucosidases enzymes participate in the degradation of cellulose or other carbohydrates present in the cell wall. However, β-glucosidases have different functions in plants, where they play roles in the biosynthesis of pigments or the cell wall, fruit ripening and defense mechanism [3]. In mammals, β-glucosidase enzymes participate in ceramide hydrolysis and defects in these genes are associated with the Gaucher's disease in humans [4].

β-glucosidase enzymes also play an important role in the food industry. These enzymes are produced by probiotic bacteria and can remove the glycoside moiety from glycosylated flavonoids present in soybean products [5]. This hydrolysis allows the intestinal absorption of flavonoids, which is necessary to obtain their beneficial health effects [6]. The identification of *Bifidobacterium* and *Lactobacillus* strains with β-glucosidase enzymes [7,8] has contributed to the development of fermented soy bean products enriched in the aglycone form of flavonoids, which improves their uptake and increases their benefits for consumers [5]. Other significant industrial applications of β-glucosidases are related to the cellulose industry and bioethanol production. Many microorganisms have β-glucosidase enzyme activity against diverse carbon sources such as cellulose, rice bran, wheat bran and lactose [3]. The biomass or cellobiose fermentation process produces bioethanol, a product with an enormous impact in the biofuel fields. Cloning and *in vitro* studies of these β-glucosidases enzymes have greatly contributed to our understanding of their enzymatic mechanisms and developed applications for these enzymes in the food and biofuel industries [1]. The discovery of new bacterial strains that possess a β-glucosidase enzyme with catalytic properties which respond to challenges in these industries is an ongoing pursuit for many scientists around the world.

β-glucosidase activity can be measured using an artificial substrate, which is converted to a colored product that is easily detected by spectrophotometry. In general, this assay requires cell lysis and protein purification to determine the amount of total protein and to calculate the specific enzymatic activity. However, in this study, we report an *in vivo* β-glucosidase assay method without cell lysis. This method consists of incubating the microorganism with an artificial substrate and measuring the product in the supernatant.

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In this way, many microorganisms could be screened to find strains with higher β-glucosidase activity. The proposed method was evaluated in *Bifidobacterium* and *Lactobacillus* strains, which produced a stable artificial product, which was not metabolized by the bacteria and was easily measured by spectrophotometry. The results demonstrate the effectiveness of this method and suggest it to be an alternative method to identify microorganisms with high β-glucosidase activity.

2. Materials and Methods

2.1 Chemicals

The substrate *p*-nitrophenyl-β-glucopyranoside (*p*NPG) and product *p*-nitrophenol (*p*NP) were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Bacteria and culture conditions

All strains used are listed in **Table S1** and belong to the cell collection of the Department of Agroenvironmental Science and Technology, University of Bologna, Italy [9]. *Lactobacillus* strains were cultured in "de Man Rogosa Sharpe" (MRS) medium, while *Bifidobacterium* strains were cultured in MRS supplemented with 0.05% L-cysteine hydrochloride or cultured in trypticase-phytone-yeast extract (TPY) [9]. The strains were incubated at 37°C under anaerobic conditions using Oxoid gas jars and anaerobic gas packs (BD; Becton, Dickinson and Company) [10].

2.3 β-Glucosidase activity

β-Glucosidase activity was determined by adding various concentrations of *p*NPG to the medium at the beginning of culture. Briefly, all strains were grown in 7 mL of their respective medium and supplemented with 0, 400, 800 or 1,600 $\mu g \cdot mL^{-1}$ *p*NPG. A control condition without bacteria was included. At different times, 150-µL aliquots were collected in triplicate to measure optical density at 620 nm in a microplate reader (Multiskan, Thermo Electron Oy, Vaanta, Finland). The samples were recovered and centrifuged at 10000 x g for 5 min, and 100 µL of each supernatant was transferred to a 96-well plate. Then, 50 µL of 0.1 M sodium carbonate was added to each well, and the *p*NP concentration was determined at 405 nm. A standard curve between 0.001 and 1 mM *p*NP dissolved in MRS or TPY medium was generated. In general, one unit (U) of enzyme activity is defined as the amount of β-glucosidase that produces 1 µmol of *p*NP per minute under assay conditions. However, in this assay, the amount of β-glucosidase enzyme was calculated in terms of grams of dry cell weight biomass. Therefore, we define one unit (U) of enzyme activity as one gram of dry cell weight biomass that produces 1 nmol of *p*NP per minute under culture conditions. The kinetic studies were performed with culture data of the B7003 strain obtained at 16, 20 and 24 h of culture in TPY medium. A Lineweaver-Burk plot was generated, and the K_m was calculated. The Lineweaver-Burk plot graphed V^{-1} $(\mu mol \cdot min^{-1})^{-1}$ vs $[S]^{-1}$ (mM^{-1}). The y-intercept (V^{-1} axis) corresponded to the V_{max}^{-1}. The K_m was calculated using the formula $K_m = m \cdot V_{max}$, where m is the slope of the line [1].

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2.4 Statistical analysis

Mean values were compared with Fisher's test using Microsoft Excel software (Microsoft). When treatments produced statistically significant differences according to Fisher's test, the corresponding means were compared with the SNK test for multiple comparisons at the 0.05 level of probability.

3. Results

3.1 *In vivo* determination of *p*NP produced

The *in vivo* β-glucosidase assay determined the bioconversion of *p*NPG into *p*NP during culture bacteria. The enzymatic product was measured directly from the culture supernatant at 405 nm. However, TPY and MRS media also absorb at that wavelength (**Fig. 1a**). To improve the sensitivity of the method, sodium carbonate was added to increase the molar extinction coefficient of *p*NP (**Fig. 1a**). Therefore, in the next experiments sodium carbonate was always added during *p*NP measurement.

3.2 *p*NP stability and inhibitory effect on cell growth

The *in vivo* β-glucosidase activity was assayed in two *Bifidobacterium* strains: *B. longum* strain B7254, which lacks β-glucosidase activity (negative control) and *B. pseudocatenulatum* strain B7003, which has β-glucosidase activity (positive control). The stability of *p*NP during culture conditions of both strain was tested. In TPY medium, the artificial product was stable and not metabolized by either strain. However, in MRS medium with or without bacteria, a reduction of between 10 to 15% of the total 300–350 $\mu g \cdot mL^{-1}$ of *p*NP was observed, while the concentration of *p*NP was approximately 700 $\mu g \cdot mL^{-1}$ and was not significant changed (**Fig. S1**).

Regarding the *p*NP effect on cell viability, an inhibitory effect was observed in both media (**Fig. 1b**). In TPY medium, the inhibition was directly proportional to the concentration of *p*NP, while in MRS medium the inhibitory effect was the same at both *p*NP concentrations tested (**Fig. 1b**). Independent of the medium used, the B7254 strain always reached a higher optical density than the B7003 strain (**Fig. 1b**).

3.3 β-glucosidase activity in bacterial culture

As expected, enzymatic activity was not observed for the B7254 strain, while the *p*NP produced was directly proportional to *p*NPG concentrations added to the medium for the B7003 strain (**Fig. 1c**). The enzymatic activity was saturated at 1600 $\mu g \cdot mL^{-1}$ of *p*NPG (**Fig. 1c**). The growth of B7003 was lower in MRS medium than in TPY medium and inversely proportional to the *p*NPG concentration added to the medium (**Fig. S2**). Since *p*NP concentrations did not show variation between 14 and 24 h of culture, the enzymatic activity was calculated at 14 h (**Table 1**). Although the *p*NP production was the same in TPY or MRS bacterial culture, the specific enzymatic activity was higher in MRS medium because the biomass was lower (**Table 1**). The Michaelis-Menten

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constant (K_m) was calculated from analysis of the Lineweaver-Burk plot at 16, 20 and 24 h of culture, which were 12.5, 14.2 and 15.8 mM, respectively (**Table S2**).

3.4 *In vivo* β-glucosidase assay in *Lactobacillus strains*

The *in vivo* β-glucosidase assay was performed on several *Lactobacillus* strains. These bacteria were cultured in MRS medium without L-cysteine and supplemented with 800 $\mu g \cdot mL^{-1}$ of *p*NPG. These strains reached similar cell densities after 23 h of culture but showed different *p*NP production (**Fig. S3**). The difference between strains of *L. helveticus* was remarkable. In *L. helveticus* ATCC strain 15009, a lack of β-glucosidase activity was observed, while *L. helveticus* strain M162 showed moderate β-glucosidase activity. Among the other *Lactobacillus* species assayed, all strains showed consistent results according to their classification of null, low or high β-glucosidase activity (**Fig. S3**).

4. Discussion

In this study, we measured β-glucosidase activity directly from the bacteria culture of two *Bifidobacteria* strains and several *Lactobacillus* strains by quantifying the bioconversion of *p*NPG into *p*NP. The concentration of *p*NP was measured directly from the supernatant of the culture after adding sodium carbonate to reduce the interference of MRS and TPY media. In general, sodium carbonate is used to stop the enzymatic reaction and increase chromatic properties of *p*NP [7]. However, in this assay, it is not necessary to chemically stop the enzymatic reaction because the enzymatic source (the bacteria) is separated from the substrate by centrifugation. However, owing to the interference of both media at 405 nm, sodium carbonate was incorporated to increase the *p*NP molar extension coefficient [11].

Both *p*NPG and *p*NP are stable in the culture conditions used here. Only in MRS medium at lower concentrations (<700 $\mu g \cdot L^{-1}$) a slight reduction of *p*NP concentration was observed, likely a result of the oxidative effects of L-cysteine [12,13]. In addition, these assays also demonstrate that *p*NP is not metabolized by bacteria, remaining stable in this culture condition with or without bacteria. Although some soil bacteria have at least one of the two known metabolic pathways to degrade *p*NP (hydroquinone or nitrocatechol catabolic pathway) [14,15,16], these pathways are absent in *Lactobacillus* and *Bifidobacterium* species [17].

Regarding cell growth, MRS medium was not optimal for both *Bifidobacterium* strains. Although this medium supplemented with L-cysteine (0.5 $g \cdot L^{-1}$) is usually used to grow *Bifidobacterium* species [18], we suggest TPY medium as the optimal media. In fact, a standard medium to grow *Bifidobacterium* species has not been established [19]. Regarding the effect of *p*NP and *p*NPG on cell viability, we found that *p*NP, but not *p*NPG, has a detrimental effect on both strains. In the B7254 strain (lacking β-glucosidase activity), *p*NPG was innocuous, but in the B7003 strain, the cytotoxic effect could be a result of the enzymatic conversion of *p*NPG into *p*NP. *A*ccording to the United States Environmental Protection Agency, this molecule affects many organisms similar to other nitrophenol or aminophenol derivative compounds [20,21,22,23]. This

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supports the idea that *p*NPG is innocuous and the cytotoxic effect is not associated with the β-glucosidase metabolism because the B7254 strain is also affected by *p*NP.

Regarding the kinetic parameters, the microbial cultures did not show enzymatic variation after 14 h of culture and enzymatic activity was saturated at 1600 $\mu g \cdot mL^{-1}$ of *p*NPG. This suggests that the enzymes are synthesized mainly during the exponential phase and remain highly expressed during the stationary phase. In this way, the enzymatic activity remains a function of substrate concentration, but not of biomass, explaining the observation that similar *p*NP production was achieved by the B7003 strain cultured in different media. Previously, β-glucosidase activity has been measured in either the exponential or stationary phase, depending to the *Bifidobacterium* strain studied [24]. Although composition of the culture medium seems to contribute to the β-glucosidase activity [24], this effect was not observed under the conditions used for this study.

Regarding the kinetic analysis, this assay does not correspond exactly with the theory of Michaelis-Menten kinetics. For the substrate to reach the enzyme, it has to pass through the cell membrane. Likewise, before the enzymatic product is measured, it also has to pass through the cell membrane. Therefore, other constants have to be added to the Michaelis-Menten equation. However, these constants do not seem to be limiting for the reaction. The K_m observed in this *in vivo* assay was tenfold higher than the K_m described for an *in vitro* assay with the enzyme purified from the *Bifidobacterium breve* 203 strain using the same substrate (*p*NPG) [25]. However, our K_m value was similar to the K_m values obtained for *B. breve* strain UCC2003 studied with different substrates (trehalose, palatinose, panose and isomaltotriose) [26]. If the K_m is understood to be an enzymatic affinity constant, we can assume that the cell wall does not constitute a limiting step in the Michaelis-Menten equation, explaining the similar results observed between *in vitro* and *in vivo* β-glucosidase assays.

Finally, the β-glucosidase activities of *L. plantarum* strain M137 and the *L. acidophilus* strain are in correspondence with results reported in the literature [27,28]. However, to our knowledge, this is the first report describing β-glucosidase activity for the other *Lactobacillus* species.

5. Conclusion

In conclusion, the *in vivo* β-glucosidase assay is a consistent method whereby the product *p*NP is not metabolized, allowing the assumption that all *p*NP comes from the enzymatic activity. Although *p*NP affects cell viability, the bacterial culture does not produce high concentrations of *p*NP, reducing its detrimental effect if lower concentrations of *p*NPG are used (<800 $\mu g \cdot mL^{-1}$). The culture medium recommended for this assay is the optimal medium for each of the tested bacteria. It is recommended that sodium carbonate be added during the *p*NP determination to avoid the masking effect of the culture medium. The incubation time seems to not to be a critical point in this assay, and at least 14 hour of culture could be used if the temporal expression of this enzyme is not known. Thus, this *in vivo* β-glucosidase assay is recommended for

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routine or high-throughput screening to find microorganisms with high β-glucosidase activity.

Conflicts of interest

The authors declare that there is not conflict of interest in this work.

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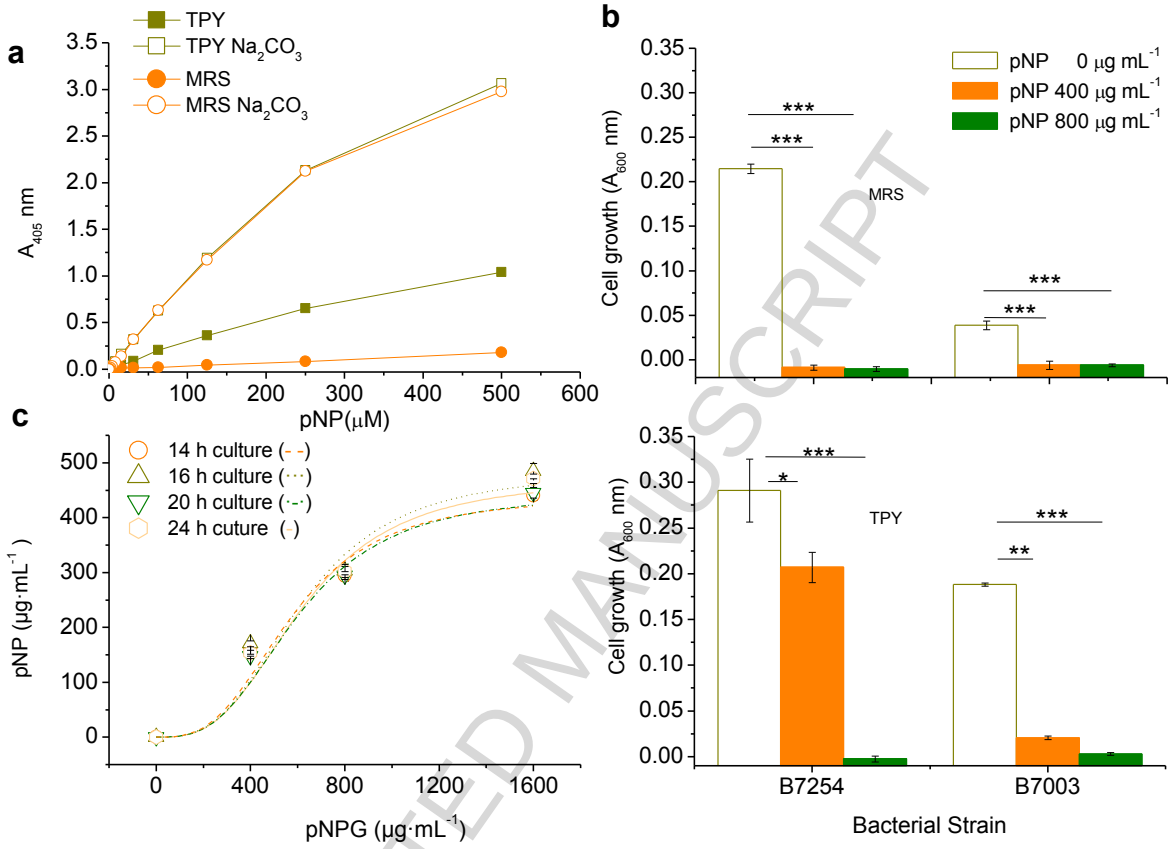

Fig. 1. Study of stability and production of *p*NP. (**a**) Spectrophotometric determination of *p*NP with and without adding sodium carbonate. (b) Effect of *p*NP on cell growth of B7003 and B7254 strains cultured in TPY medium or MRS medium. * ($P > 0.5$), ** ($P > 0.05$), *** ($P > 0,005$). (c) β-glucosidase activity of culture of B7003 strain growth in TPY medium supplemented with different concentration of *p*NPG. The *p*NP production was measured at different times of culture.

Table 1. Bioconversion activity of B7003 strain cultured with *p*NPG.

Artificial substrate *p*NPG (μg·mL^{-1})	***p*NP produced (μmoles min^{-1} L^{-1}) (± SD)**		**Biomass (gr of dry weight L^{-1}) (± SD)**		**Specific activity (μmol·min^{-1}·grams of dry weight $^{-1}$ L $^{-1}$) (± SD)**	
	TPY	**MRS**	**TPY**	**MRS**	**TPY**	**MRS**
400	12.5 (± 0.39)	12.4 (± 0.23)	0.51 (± 0.0013)	0.15 (± 0.001)	24.4 (± 0.10)	82.8 (± 1.6)
800	22.2 (± 0.55)	25.5 (± 1.86)	0.45 (± 0.0047)	0.1 (± 0.001)	48.9 (± 1.70)	255.3 (± 18.6)
1600	33.6 (± 0.51)	45.4 (± 2.98)	0.39 (± 0.0047)	0.06 (± 0.0001)	86.9 (± 0.93)	756.1 (± 49.7)

SD: Standard deviation.