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Process Biochemistry 42 (2007) 904–908

Process Biochemistry

www.elsevier.com/locate/procbio

Short communication

Production of extracellular β-glucosidase by *Monascus purpureus* on different growth substrates

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Received 8 November 2006; received in revised form 23 January 2007; accepted 24 January 2007

Abstract

Various agro-industrial residues in combination with peptone, NH_4Cl and/or soy bran were screened as substrates for extracellular β-glucosidase (BGL) production by *Monascus purpureus* NRRL1992 on submerged fermentations (SmF). Higher BGL production was achieved when the agro-industrial residues were combined with peptone, and the utilization of NH_4Cl (inorganic nitrogen source) had not supported high enzyme production. The combination between grape waste and peptone was the best for enzyme production, and was selected as the growth substrate for further investigations. The evaluation of the effects of the medium components on enzyme production showed that the influence of peptone was more important than grape waste. The production of extracellular BGL by *M. purpureus* was inducible and controlled by carbon (glucose) catabolite repression.

Keywords: Agro-industrial wastes; Cellulase; Enzyme; β-Glucosidase; *Monascus purpureus*; Submerged fermentation

1. Introduction

β-D-Glucosidases (E.C. 3.2.1.21) constitute a heterogeneous group of enzymes that occurs in several organisms, performing a variety of functions. The principal reaction catalyzed by this class of enzymes is the hydrolytic cleavage of β-glycosidic linkages in low-molecular-mass glycosides, and the affinity of BGL for a particular substrate is dependent upon the nature of the enzyme source, physiological function and the location of the enzyme [1,2].

BGLs have been exploited in a variety of biotechnological applications. In the enzymatic saccharification of cellulose, BGL produces glucose by cleaving cellobiose. Since cellobiose inhibits the action of endo- and exoglucanases, BGL also contributes to the efficiency of this process [3]. BGL have been also studied due to its potential to release flavour compounds such as terpenes from odorless non-volatile glycosidic precursors in fruit juices and wines [4], release phenolic compounds with antioxidant, nutraceutical and flavourant properties from their glycosilated forms in fruit and vegetable residues [5], cassava detoxification [6], among other applications [1,2].

The filamentous fungi *Monascus* are used in Asia for centuries as a source of pigments for the coloring of traditional foods. This genus is subject of constant studies, mainly due to the growing interest for natural pigments to be used in the food industry. Although other metabolic products from *Monascus* species, like alcohols, organic acids, antimicrobial agents and substances with therapeutic activity have been described [7–9], little information about enzymes from *Monascus* is available [9].

Large amounts of agro-industrial residues are generated every year from diverse economic activities, increasing the biotechnological interest on the utilization of these residues as substrates (or raw-materials) in biotechnological processes [5,10–12]. The utilization of agro-industrial waste as growth substrates may represent an added value to the industry and meets the increasing consciousness for energy conservation. Submerged fermentations (SmF) are normally used in the fermentation industry because they are easier to handle and control in the fermentation process [13].

In this paper, several agro-industrial byproducts were tested as substrates for BGL production by *M. purpureus* in submerged fermentations (SmF). Then, the effects of selected substrates were evaluated to establish the best conditions for

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1359-5113/$ – see front matter
doi:10.1016/j.procbio.2007.01.012

BGL production. The repression of enzyme production was also investigated.

2. Materials and methods

2.1. Microorganism

M. purpureus NRRL1992 was maintained on Sabouraud dextrose agar plates at 4 °C and subcultured periodically. Cultures reactivated by transferring onto fresh Sabouraud agar plates and cultured at 30 °C for 12–14 days were used for inoculum preparation.

2.2. Substrate selection

Carboxymethylcellulose (CMC) and agro-industrial residues, including cheese whey powder, *pinhão* (seeds of *Araucaria angustifolia*) rind, grape waste of wine industry, and soy bran were tested as substrates (20 g/L) for BGL production by *M. purpureus* in combination with peptone (5 g/L), NH_4Cl (2.5 g/L) and/or soy bran (5 g/L), in mineral medium. The mineral medium contained: K_2HPO_4 (5.0 g/L), KH_2PO_4 (5.0 g/L), $MgSO_4{\cdot}7H_2O$ (0.1 g/L), $CaCl_2$ (0.025 g/L), $ZnSO_4{\cdot}7H_2O$ (0.01 g/L) and $MnSO_4$ (0.01 g/L). The substrate combinations are specified in Table 1. The initial pH of the medium was adjusted to 6.0.

Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 250 μL (1%, v/v) of a conidial and mycelial suspension with OD_{620} of 0.4. For the preparation of this suspension, 12–14-day-old cultures were scraped from the surface of Sabouraud agar, added to a 0.85% NaCl saline sterile solution, and mixed until a homogeneous solution was obtained. The inoculated flasks were incubated at 27 °C on a rotatory shaker at 100 rpm for 9 days, and the substrate combination which gave the higher BGL activity in the screening tests was further employed in a first-order factorial design.

2.3. First-order factorial design

The influence of the concentrations of the selected substrates was evaluated in a first-order factorial design (2^2) with three replicates in the central points, which means a total of five different treatment combinations [14]. Three levels of each independent variable were chosen, with the upper and lower limits of this set to be in the range described in the literature, and BGL activity was taken as the dependent variable. Table 2 shows the independent variables and their levels, as well as the response evaluated. All data were analyzed with the software Statistica 5.0 (Statsoft, Tulsa, OK, USA). The experiments were carried out adding the chosen substrates to the mineral medium, as indicated in Table 2.

Table 1
BGL activity in *Monascus purpureus* culture supernatant after 9 days of submerged cultivation on different substrates

Substrate (%, w/v)	Enzyme activity (U/mL)[a]
CMC (2.0%) + NH_4Cl (0.25%)	0.085 ± 0.014 a
CMC (2.0%) + peptone (0.5%)	0.363 ± 0.027 b
Cheese whey powder (2.0%) + NH_4Cl (0.25%)	0.202 ± 0.018 ac
Cheese whey powder (2.0%) + peptone (0.5%)	1.043 ± 0.029 d
Cheese whey powder (2.0%) + soy bran (0.5%)	0.398 ± 0.024 b
Pinhão rind (2.0%) + NH_4Cl (0.25%)	0.066 ± 0.020 a
Pinhão rind (2.0%) + peptone (0.5%)	0.638 ± 0.044 e
Soy bran (2.0%)	0.827 ± 0.034 f
Soy bran (2.0%) + NH_4Cl (0.25%)	0.779 ± 0.018 f
Soy bran (2.0%) + peptone (0.5%)	1.096 ± 0.039 d
Grape waste (2.0%) + NH_4Cl (0.25%)	0.085 ± 0.009 a
Grape waste (2.0%) + peptone (0.5%)	1.682 ± 0.038 g
Grape waste (2.0%) + soy bran (0.5%)	0.664 ± 0.024 ef
Grape waste (2.0%) + NH_4Cl (0.25%) + soy bran (0.5%)	0.223 ± 0.016 c

Different letters represents statistically different means at $p < 0.05$.
[a] Means of duplicate cultivations and two enzymatic assays for each cultivation.

Table 2
BGL activities on the different treatments for a 2^2 factorial experiment

Run	Grape waste	Peptone	Enzyme activity (U/mL)
1	(−1) (10 g/L)	(−1) (1 g/L)	0.542
2	(−1) (10 g/L)	(+1) (20 g/L)	1.953
3	(+1) (50 g/L)	(−1) (1 g/L)	0.097
4	(+1) (50 g/L)	(+1) (20 g/L)	2.780
5	(0) (30 g/L)	(0) (10.5 g/L)	2.294
6	(0) (30 g/L)	(0) (10.5 g/L)	2.385
7	(0) (30 g/L)	(0) (10.5 g/L)	2.352

2.4. Enzymatic activity

At the end of cultivation, or at defined intervals, samples of 500 μL were taken, centrifuged (12,000 × *g* for 5 min), and the supernatant was used as enzyme source. BGL activity was assayed by a modified procedure, based on the method of Hang and Woodams [10]. The reaction mixture (200 μL) contained 90 μL of citrate buffer (250 mM, pH 4.5), 10 μL of *p*-nitrophenyl-β-D-glucopyranoside (pNPG; 4 mg/mL), and 100 μL of the culture supernatant. After incubation at 37 °C for 30 min, the reaction was stopped by adding 1 mL of cold sodium carbonate buffer (500 mM, pH 10). The activity of β-glucosidase was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 405 nm ($\varepsilon = 18{,}700$). One unit (U) of β-glucosidase activity was defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate (pNPG)/min, under the assay conditions.

2.5. Repression studies

To evaluate the possible repression of enzyme production, the fungus was grown on mineral medium containing glucose (20 g/L) and peptone (5 g/L). The enzymatic activity was estimated as described above. The concentration of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method, where 1 mL of DNS solution was added to 100 μL of culture supernatant and incubated at 100 °C for 10 min. After cooling, the absorbance was measured at 570 nm, using glucose as standard.

3. Results and discussion

3.1. Substrate screening for BGL production

The results of BGL production by *M. purpureus* growing on different substrates are shown in Table 1. The combination between agro-industrial residues and peptone or soy bran resulted in higher BGL production when compared with the combination between CMC and peptone. This result is interesting due to the fact that CMC is a highly purified synthetic substrate, becoming expensive for large-scale enzyme production. The utilization of agro-industrial residues as potential substrates for the production of BGL and other cellulase, hemicellulase and pectinase components has attracted much attention [10,11,15,16], since it can contribute to lower the costs of enzyme production and also to reduce the environmental pollution caused by the accumulation of lignocellulosic wastes.

It was demonstrated that the best substrate combination for BGL production was the grape waste supplemented with peptone (1.682 U/mL). Fig. 1 shows the pattern of BGL production within 14 days of cultivation and the productivity (U/mL day). Although the extracellular BGL activity continues to grow until