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Xylanase Production by *Bacillus circulans* D1 Using Maltose as Carbon Source

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Abstract *Bacillus circulans* D1 is a good producer of extracellular thermostable xylanase. Xylanase production in different carbon sources was evaluated and the enzyme synthesis was induced by various carbon sources. It was found that D-maltose is the best inducer of the enzyme synthesis (7.05 U/mg dry biomass at 48 h), while D-glucose and D-arabinose lead to the production of basal levels of xylanase. The crude enzyme solution is free of cellulases, even when the microorganism was cultivated in a medium with D-cellobiose. When oat spelt xylan was supplemented with D-glucose, the repressive effect of this sugar on xylanase production was observed at 24 h, only when used at 5.0 g/L, leading to a reduction of 60% on the enzyme production. On the other hand, when the xylan medium was supplemented with D-xylose (3.0 or 5.0 g/L), this effect was more evident (80 and 90% of reduction on the enzyme production, respectively). Unlike that observed in the xylan medium, glucose repressed xylanase production in the maltose medium, leading to a reduction of 55% on the enzyme production at 24 h of cultivation. Xylose, at 1.0 g/L, induced xylanase production on the maltose medium. On this medium, the repressive effect of xylose, at 3.0 or 5.0 g/L, was less expressive when compared to its effect on the xylan medium.

Keywords Xylanase · Maltose · Induction · Repression · *Bacillus circulans*

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Introduction

Xylanases (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) are hydrolytic enzymes that catalyze the endohydrolysis of the β-1,4 backbone in xylan, the main polysaccharide of the hemicellulose fraction in plant cell walls [1]. Endoxylanases are reported to be produced mainly by microorganisms, including several species of fungi and bacteria [2–4].

Xylanases are enzymes of great potential for industrial applications. They are mainly used in the pretreatment of Kraft pulp, improving bleachability of pulp while decreasing consumption of chlorine chemicals [5, 6]. These enzymes are also used as additives to pig and poultry cereal-based diets, to improve nutrient utilization [7], in flour improvement for bakery products [8], in saccharification of agricultural, industrial and municipal wastes [9], and in juice and wine clarification [1].

Xylanase production by microorganisms, which were grown on a variety of carbon sources, has been studied, and the role of these substrates as inducers or repressors has been evaluated [10–13]. In general, xylanases are enzymes liable to induction, synthesized in media containing xylan or xylan residues [14]. However, in some cases, xylan may be a poor inducer of xylanase synthesis [15]. In many microorganisms, this enzyme synthesis is liable to catabolite repression in the presence of more readily assimilable carbon sources, such as glucose or xylose [16]. However, syntheses of constitutive xylanases were also reported [17–19]. In the present work, the effect of various carbohydrates on xylanase production by *Bacillus circulans* D1 were investigated.

Materials and Methods

Microorganism

The thermophilic and alkalophilic bacterial strain *Bacillus circulans* D1 used in this study was isolated in a previous work [20]. Stock cultures were maintained in a complex medium containing xylan as carbon source [21], with the addition of agar (15.0 g/L).

Media and Culture Conditions

The mineral media used for xylanase production was composed of KH_2PO_4 (0.5 g/L), K_2HPO_4 (0.5 g/L), KCl (0.5 g/L), NH_4NO_3 (0.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L), $MgSO_4\ 7H_2O$ (0.2 g/L), $FeCl_3\ 6H_2O$ (0.01 g/L), and Na_2CO_3 (5.0 g/L; separately sterilized) supplemented with different carbon sources (birchwood xylan, oat spelt xylan, D-glucose, D-cellobiose, D-galactose, D-arabinose or D-maltose). *B. circulans* D1 was grown in 40 mL of medium, at 45 °C, for 48 h, under 200 rpm. To provide inoculum, cells were grown in 20 mL of mineral medium containing D-glucose as carbon source (5.0 g/L). In the log phase, cells were collected and separated aseptically from the supernatant solution by centrifugation (10 °C, 10,000×*g*, 15 min) and washed three times with NaCl 8.0 g/L. Cells were resuspended in the same volume of sterile mineral medium and used as inoculum (8×10^7 cells/mL).

Induction Experiments

The microorganism was grown in mineral medium supplemented with 1.0 to 10.0 g/L of the carbon source and also in a mineral medium with oat spelt xylan or D-maltose, with the addition of glucose or xylose. Samples were collected from the culture and centrifuged at

10,000×*g* for 15 min. The cell-free supernatant was used as crude enzyme. The experiments were performed in duplicate.

Growth Measurement

Growth was determined by cell dry weight. During cultivation, samples of 1.0 mL were taken and centrifuged at 10,000×*g* for 15 min. Centrifuged cells were dried at 65 °C to a constant weight. The results were expressed in mg of dry cell/mL.

Enzyme Assay

Xylanase activity was assayed by measuring reducing sugars released as xylose, using a dinitrosalycylic acid method [22]. The reaction mixture containing 0.9 mL of the substrate solution of birchwood xylan (5.0 g/L) in acetate buffer (pH 5.0, 0.1 M) and 0.1 mL of the crude enzyme solution was incubated at 60 °C for 10 min. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of xylose per minute, under the cited assay conditions.

Results

Effect of Different Carbon Sources on Xylanase Production

To determine the effects of different carbon sources on the production of extracellular xylanase by *B. circulans* D1, the microorganism was grown in a medium with birchwood xylan, oat spelt xylan, D-xylose, D-maltose, D-galactose, D-cellobiose, D-glucose, or D-arabinose (2.5 g/L). Cellular growth and xylanase production were observed on all sugars tested, except on xylose. The medium containing arabinose afforded the smallest cellular growth and enzyme production (Table 1). At 2.5 g/L, maltose was the best substrate for xylanase production, followed by galactose and oat spelt xylan. The data shown in Table 1 indicate that oat spelt xylan is better than birchwood xylan for xylanase induction in *B. circulans* D1. Xylanase production on the medium with cellobiose was very close to that obtained on the medium with oat spelt xylan. Cellobiose may induce the synthesis of cellulases in some microorganisms, but the crude enzymatic extract obtained with the cultivation of *B. circulans* D1 in this substrate is free of cellulases activities (data not

Table 1 Xylanase production by *B. circulans* D1 at 48 h of cultivation media containing different carbon sources at 2.5 g/L.

Substrate	Xylanase (U/mL)	Specific activity (U/mg dry biomass)
Maltose	9.18	7.05
Galactose	5.71	5.56
Oat spelt xylan	8.25	5.26
Cellobiose	4.61	4.53
Birchwood xylan	4.17	2.44
Glucose	5.03	3.86
Arabinose	0.50	0.82
Xylose	Nd	-

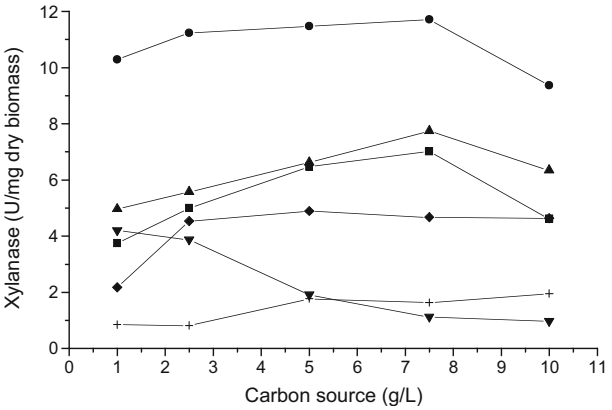

Fig. 1 Effect of carbon sources concentration on xylanase production by *B. circulans* D1, after 48 h of cultivation. Oat spelt xylan (*squares*); D-maltose (*circles*); D-galactose (*triangles*); D-glicose (*inverted triangles*); D-cellobiose (*diamonds*); D-arabinose (*plus signs*)

shown). This fact was reported in previous works [6, 20], and it is an important characteristic for this enzyme application on biobleaching of kraft pulps.

Effect of Sugar Concentration on Xylanase Production

To better evaluate the effects of carbon sources on xylanase production, experiments were carried out using different concentrations of each substrate. The results are presented in Fig. 1. Highest levels of xylanase production were obtained using maltose as the carbon source. Xylanase production on media with xylan was very close to that obtained in medium with galactose. For these substrates, 7.5 g/L seems to be the best concentration for the enzyme production. On cellobiose, the highest xylanase production was observed at 2.5 g/L of this substrate. Apparently, concentrations of cellobiose above 2.5 g/L did not influence xylanase production. The repressive effect of glucose is clearly observed (Fig. 2), as xylanase production decreased as glucose concentration increased. Xylanase production tended to increase as arabinose concentration increases, but only basal levels of the enzyme were obtained.

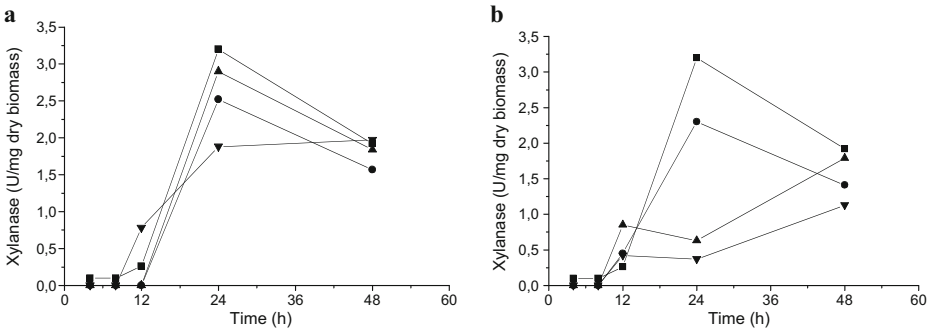

Fig. 2 Xylanase production by *B. circulans* D1 in mineral medium with oat spelt xylan (10.0 g/L; *squares*) and oat spelt xylan (10.0 g/L) plus glucose (**a**) or xylose (**b**) at 1.0 (*circles*), 3.0 (*triangles*) or 5.0 g/L (*inverted triangles*)

Effect of D-glucose or D-xylose on Xylanase Production in the Xylan Medium

The microorganism was grown in a mineral medium with oat spelt xylan (10.0 g/L) supplemented with D-xylose or D-glucose (1.0, 3.0 or 5.0 g/L). The presence of glucose did not strongly influence xylanase production when used at concentrations up to 3.0 g/L (Fig. 2a). The repressive effect of glucose was observed at 5.0 g/L, when a reduction of 40% (1.88 U/mg dry biomass) on the enzyme production was observed at 24 h (Fig. 2a). The addition of xylose repressed the enzyme production (Fig. 2b). This effect was evident at 24 h, when xylose was used at 3.0 or 5.0 g/L, leading to a decrease of 80 and 90% on the productivity (0.63 and 0.37 U/mg dry biomass, respectively).

Effect of D-glucose or D-xylose on Xylanase Production in the Maltose Medium

Because maltose was a good inducer for xylanase production by *B. circulans* D1, the addition of xylose or glucose to the maltose medium was evaluated. As shown in Fig. 3, the presence of glucose or xylose in the maltose medium afforded xylanase production from the first hours of cultivation. However, when the microorganism was cultivated on maltose as the sole carbon source, there was no enzyme induction until 12 h of cultivation, which suggests that maltose was used to support the microbial growth. On the other hand, when glucose or xylose was used with maltose, these sugars were probably used to support growth, while maltose accumulated in the cell and exerted its inducer effect.

Xylanase synthesis on maltose medium was more sensitive to glucose repression when compared to the enzyme synthesis on the xylan medium (Fig. 3a). At 24 h, xylanase production was at the same level in the medium with maltose and maltose plus glucose at 1.0 g/L. However, glucose at 3.0 or 5.0 g/L repressed enzyme production after 24 h of cultivation, leading to a reduction of 55% on specific activity (about 2.30 U/mg dry biomass; Fig. 3a).

The repressive effect of xylose on xylanase synthesis was milder on the maltose medium than on the xylan medium. Xylanase production was induced, at 24 h, when the maltose medium was supplemented with xylose at 1.0 g/L, and xylanase repression was observed, at 24 h, only when 3.0 or 5.0 g/L of xylose was added, leading to a reduction of 22 and 57%, respectively, on the productivity (Fig. 3b).

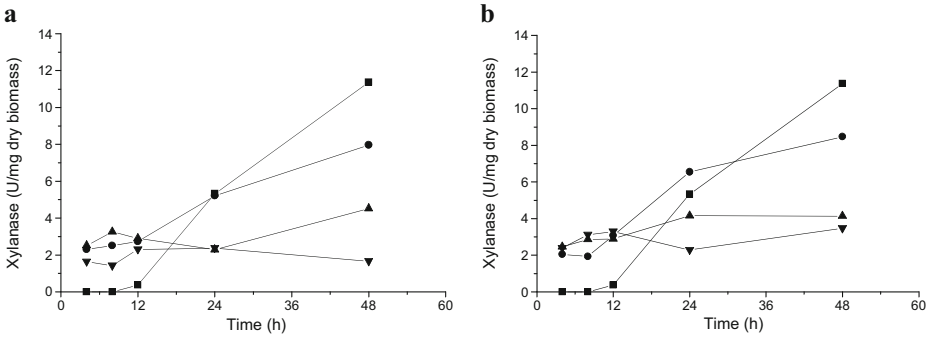

Fig. 3 Xylanase production by *B. circulans* D1 in a mineral medium with (*squares*) maltose (10.0 g/L) and maltose (10.0 g/L) supplemented with glucose (**a**) or xylose (**b**)1.0 (*circles*) , 3.0 (*triangles*), or 5.0 g/L (*inverted triangles*)

Discussion

The data presented in this work regarding xylanase production by *B. circulans* D1 point out the presence of a constitutive xylanase, produced in media with different carbon sources, besides xylan. In many of the reports regarding xylanase production, there is the occurrence of constitutive enzymes [23–26].

Xylanase production by *B. circulans* was induced by a variety of carbon sources, and among the tested substrates, maltose was the best inducer. These data suggest two modes of enzyme expression: one gene that codes for a constitutive xylanase and another gene that codes for a xylanase subject to induction or a single gene that constitutively expresses basal levels of the enzyme and that also is susceptible to induction by specific sugars.

Kyu et al. [10] also reported xylanase production by the strain *B. circulans* B_6 on a variety of carbon sources. However, they reported xylanase levels smaller than those cited in this work. Their results showed xylanase productions of 0.15 and 0.20 U/mg dry biomass after 5 days of cultivation in media containing maltose or galactose (at 2.5 g/L), respectively, whereas *B. circulans* D1 produced on average of 7.05 and 5.56 U/mg dry biomass after 48 h of cultivation on the same carbon sources. The authors reported that D-arabinose did not afford xylanase production, which is in agreement with our results, as only basal levels of the enzyme were obtained on this substrate.

Xylanase production on maltose has been cited for some microorganisms [27, 28], but the authors did not provide details about the enzyme production on this substrate. The data reported in the present work indicated that xylanase synthesis on the maltose medium was more sensitive to glucose repression and less sensitive to xylose repression, when compared to the enzyme synthesis on the xylan medium. From these data, we can infer that xylanase synthesis by *B. circulans* D1 is regulated by different ways when the microorganism is cultivated on xylan or maltose.

From the data obtained with the cultivation of *B. circulans* D1 on the maltose medium, we can infer that when used with maltose, glucose and xylose are preferentially consumed to support microbial growth, while maltose acts as xylanase inducer. Thus, maltose and xylose/glucose are probably transported by different systems. In *B. subtilis*, maltose is transported by a symport sugar-proton [29], but the gene *malP* for the enzyme II was identified, indicating that maltose can also be transported by the phosphotransferase transport system (PTS) [30]. Regarding glucose and xylose, these sugars can be transported by PTS or others transport systems in *Bacillus* species [31–36].

Cellobiose induces xylanase synthesis in some microorganisms [24], but sometimes, this enzyme is associated with cellulases. The crude enzymatic extract produced by *B. circulans* D1 on cellobiose is free of cellulases. This is important data that confirm the enzyme potential for application in biobleaching processes [6].

In the present work, we reported that the cultivation of *B. circulans* D1 in the medium with arabinose lead to basal level of xylanase production, as observed for other *Bacillus* species [37]. However, this carbon source can act as xylanase inducer in some microorganisms [38–40].

Lower xylanase production was also observed when *B. circulans* D1 was cultivated in the medium with glucose. Repression by glucose is common for catabolite extracellular enzymes [14, 41, 42]. However, glucose does not repress xylanase synthesis by some microorganisms [19, 24].

It was not possible to detect variation on cellular growth when *B. circulans* D1 was cultivated in xylose as sole carbon source, at 2.5 g/L. No growth in the medium containing xylose was reported by Lindner et al. [19], when cultivating *B. subtilis* in a medium

containing 1.0 g/L of this substrate as the carbon source. This may be due to the fact that there is no effective uptake system for xylose in some *Bacillus* species. In these cases, small amounts of xylose that are necessary for enzymes induction, such as xylanases, are possibly taken up in an unspecific way [19].

Conclusion

The results showed in this research indicate that *B. circulans* D1 is a promising microorganism regarding to xylanase production, as the amounts of enzyme obtained were similar to or higher than those cited in the scientific literature in works dealing with *Bacillus* species. The enzyme synthesis is not necessarily related to the presence of xylan in the cultivation medium. Maltose was the best inducer of xylanase production, and the microorganism showed versatility in the utilization of other carbon sources.

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