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High endo-β-1,4-D-glucanase activity in a broad pH range from the alkali-tolerant *Nocardiopsis* sp. SES28

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Summary

The strain SES28 was isolated from an indoor contaminated agar plate during a screening program for alkaliphilic CM-cellulose-degrading bacteria. It showed a prominent clear hydrolysis of the substrate at pH 10. The 16S rDNA analysis related it to the genus *Nocardiopsis*. *Nocardiopsis* sp. SES28 was able to grow at pH values up to 10.5, the major biomass being produced at pH 10, and pH 8 was the optimum for β-1,4-glucanase production. The optimum pH for β-1,4-glucanase activity was 9.0, and it was higher than 60% throughout the pH range 6.5–10.0; showing 94% of its a relative activity at pH 10. The feature of this bacterium to produce β-1,4-glucanase active in a broad pH-range might be useful for detergent- and textile-processing technologies.

Introduction

Strains of the genus *Nocardiopsis* have been isolated from indoor dusts in schools, children's day care centers, animal sheds and tile-joints in bathrooms (Andersson *et al.* 1999; Mitsuiki *et al.* 2002). Some strains of the genus were reported as opportunistic pathogen in humans (Mordarska *et al.* 1998), although many technological applications for their metabolites and enzymes have been proposed (Shin *et al.* 2003; Cavalcanti *et al.* 2004; Mitsuiki *et al.* 2004).

The main industrial applications for β-1,4-glucanase (E.C. 3.2.1.4) preparations are laundry and treatment of fabrics to give a soft appearance (Pyc *et al.* 2003). Moreover, new applications are appearing, like improving extraction processes of valuable compounds located into vegetal cells (Navarrete-Bolanos *et al.* 2004). The available washing products usually are alkaline preparations containing surfactants and chelating agents. Nowadays, the interest of extremophilic organisms, as alkaline active enzyme producers, attracts the attention of many research groups as well as important related companies (Rees *et al.* 2003). Therefore, the finding of stable and active enzymes in a broad range of pH is still an important challenge in enzyme technology.

Materials and methods

Characterization and identification of the strain

DNA extraction, quantification, 16S rDNA amplification, and sequence analysis were carried out as previously described by Martinez *et al.* (2002).

Culture conditions and media

Medium (g l^{-1}): CM-cellulose (medium-viscosity) 10.0; yeast extract 1.0; milk peptone 1.0; NaCl 30.0; agar 15.0 at pH 10. For batch cultures, the pH of media was adjusted to 10 supplementing it with 10.6 mg ml^{-1} Na_2CO_3 sterilized separately by filtration (0.22 μm cut-off). The strain was cultured in an orbital shaker (250 rev min^{-1}, 25 °C, 48 h). It was harvested by centrifuging for 10 min at 12,000 *g* and supernatants were frozen at −20 °C until processing.

Fermentation medium (g l^{-1}): CM-cellulose (medium-viscosity) 5.0, yeast extract 1.0; $NaNO_3$ 1.0 and NaCl 30.0. A 2 l fermentor (Discovery 210, New Brunswick, USA) was employed for culturing at different pH values, manipulated between 6.0 and 10.8 using 2 M NaOH. It was operated at 30 °C with an agitation speed of

400 rev min^{-1} and an aeration of 1 v v^{-1} m^{-1}. The working volume was 1.0 l.

Analytical assays

Protein concentration was determined by the method of Bradford, using BSA as standard. Hydrolysis of CM-cellulose on Petri dishes was detected according to Breccia *et al*. (1995). For quantification of β-1,4-glucanase activity, samples (400 μl) were incubated with 400 μl of 10.0 g l^{-1} CM-cellulose (low-viscosity) in 50 mM sodium phosphate buffer pH 8.0, at 40 °C for 1 h. The reaction was stopped by adding 400 μl of 3,5-dinitrosalicylic acid (DNS) (Miller, 1959). The tubes were placed in boiling water bath for 10 min and cooled before measuring the absorbance at 540 nm. One unit of β-1,4-glucanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars (as glucose) per h. Xylanase activity was measured with the same method but using as substrate 10.0 g l^{-1} birchwood xylan.

Results and discussion

The ubiquitous character of the genus *Nocardiopsis* explained the fact that the isolated SES28 appeared spontaneously contaminating an agar plate in our laboratory. We carried out 16S rDNA identification of the strain, finding it related to the genus *Nocardiopsis*, although more studies are needed to establish the species status. *Nocardiopsis* sp. SES28, accession number DQ196427, showed Gram-positive aerobic colonies, presenting aerial white filaments, which were branched, long, and usually fragmenting into spores. The closest relatives were *Nocardiopsis exhalans* (AY036000), *Nocardiopsis alba* DSM 43845^T (X97884) and *Nocardiopsis metallicus* R2A^T (AJ420769) with 99% identities.

The effect of various carbon sources on the β-1,4-glucanase and β-1,4-endoxylanase (E.C. 3.2.1.8) production was assayed. CM-cellulose (low- and medium-viscosity) were the most promissory carbon sources for β-1,4-glucanase production. In the carbon sources assayed, xylanase was a minor activity in comparison with β-1,4-glucanase (Figure 1). The nitrogen sources assayed ($NaNO_3$, milk peptone, soy flour, soy pellets, yeast extract, urea), no significant differences were obtained on the enzyme production (data not shown). The culture medium was supplemented with different metal ions (1 mM), an improvement in the β-1,4-glucanase production was found with sodium molybdenum and calcium chloride. Activity increased from 0.4 U ml^{-1} in the control culture to 0.8 U ml^{-1} in presence of the metals, although in the presence of both metals it did not showed a significant difference with the control. The biomass produced for *Nocardiopsis* sp. SES28 in batch cultures was positively affected by iron chloride, increasing from 0.10 to 0.21 g l^{-1} in control and with iron respectively.

Nocardiopsis sp. SES28 was able to grow over a wide range of pH values (7.0–10.5) showing its maximum biomass production at pH 10.0, although the major β−1,4-glucanase activity was detected when it grew at pH 8.0 (Figure 2). Figure 3 shows the comparison of SES28 β-1,4-glucanase with two commercially available cellulase preparations. The more relevant characteristic of the β-1,4-glucanase activity was the relative high activity in a wide range of pH values. The SES28 β-1,4-glucanase displayed 94.1% at pH 10. On the other hand, at pH 5 it showed 54.5% of residual activity while the preparations Denimax991L and Denimax 362S (Novo

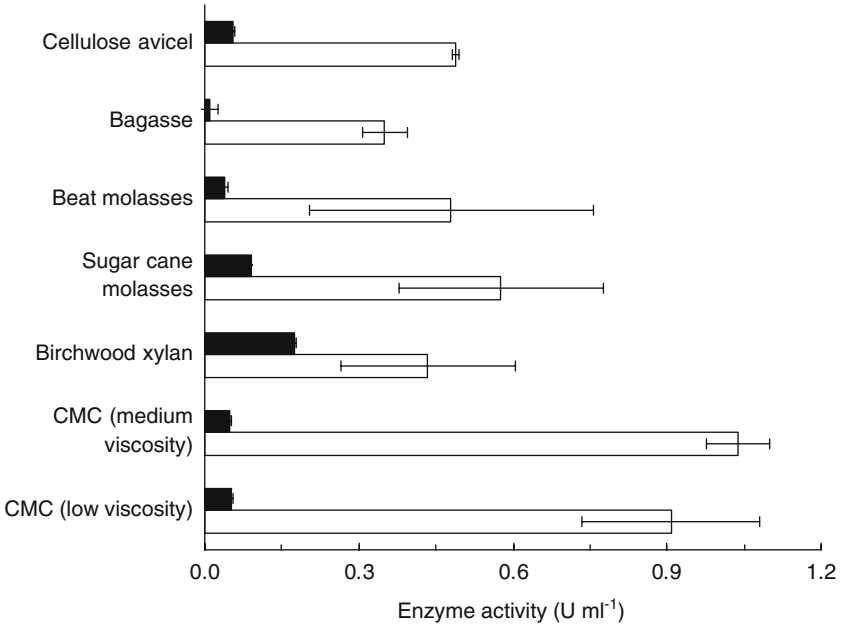

Figure 1. Effect of different carbon sources on the production of β-1,4-glucanase (□) and β-1,4-endoxylanase (■) activity (Units mg^{-1}) by *Nocardiopsis* sp. SES28. The values are presented as the means of duplicate measurements and standard errors.

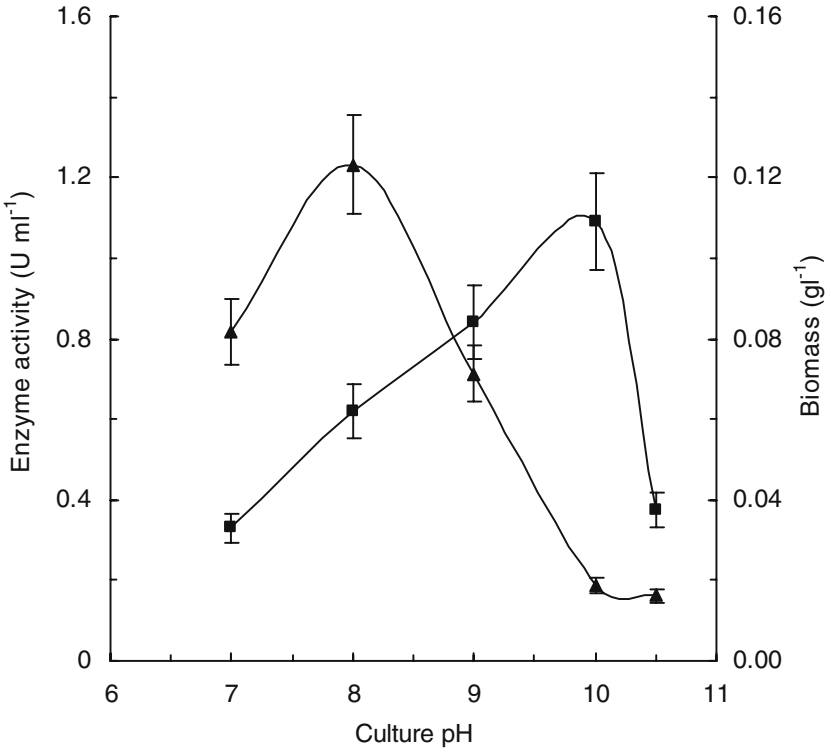

Figure 2. Biomass (■) and extracellular β-1,4-glucanase (▲) produced by *Nocardiopsis* sp. SES28 at different pH values. The values are presented as the means of duplicate measurements and standard errors.

Nordisk, Santa Catarina, Brazil) showed 22.3 and 1.9% respectively.

Concluding remarks

Laundry detergents tend to be highly alkaline and thus destructive to standard enzymes like proteases, lipases and cellulases. Alkaliphilic versions of those enzymes could solve the problem, and several that can operate efficiently in heat or cold are now in use or being developed. Alkaliphilic extremozymes are also poised to replace the standard enzymes used to produce the stonewashed look in denim fabric by degrading cellulose and releasing dyes. The β-1,4-glucanase activity of the strain *Nocardiopsis* sp. SES28 seems to be an interesting

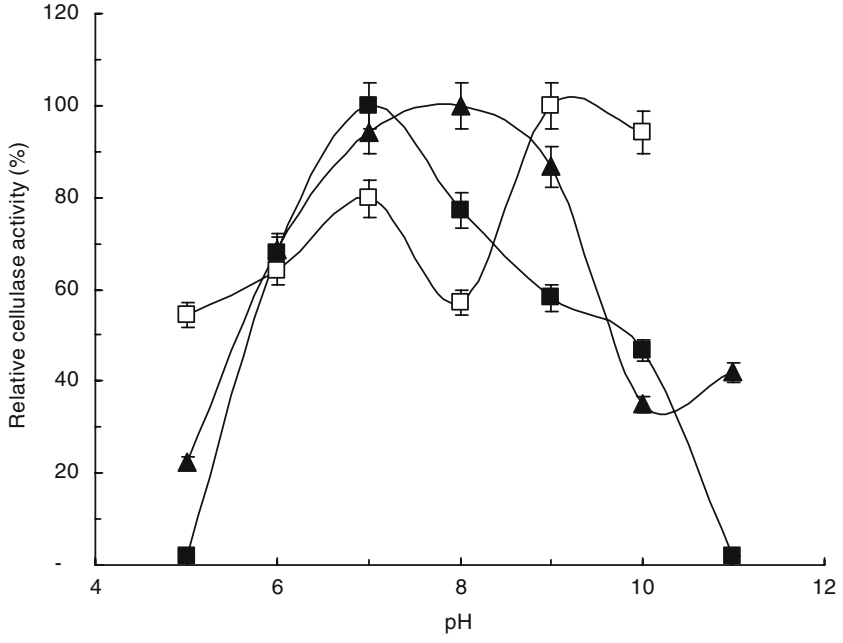

Figure 3. β-1,4-Glucanase activity profile at different pH values: *Nocardiopsis* sp. SES28 (□) Denimax991L (▲), and Denimax 362S (■). The 100% of the activity corresponds to EU ml^{-1}: 0.55, *Nocardiopsis* sp. SES28; 0.96 Denimax991L, and 0.67 Denimax 362S. The values are presented as the means of duplicate measurements and standard errors.

system from an applied point of view, although further work is needed to characterize the cellulolytic system.

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