

Extracellular Xylanase Production by Two Thermophilic Alkali-Tolerant *Bacillus* Strains in Batch and Continuous Cultures

Elka I. Emanuilova*, Plamen L. Dimitrov, Rossica D. Mandeva, Margarita S. Kambourova and Stefan A. Engibarov

Department of Extremophilic Bacteria, Institute of Microbiology, Sofia 1113, Bulgaria. Fax: +359-2-700109. E-mail: eemanuil@microbio.bas.bg

* Author for correspondence and reprint requests

Z. Naturforsch. **55c**, 66–69 (2000); received July 14/September 21, 1999

Thermostable Xylanases, Batch Culture, Continuous Culture, *Bacillus*

Xylanase production of newly isolated thermophilic alkali-tolerant *Bacillus* sp. strain SP and strain BC was investigated in batch and continuous cultures. Enzyme synthesis was inducible with both strains and was observed only in xylan-containing media. Xylan from oat spelt is a better inducer than xylan from birch for strain *Bacillus* sp. BC while such difference was not observed for strain SP. Compared with batch cultures xylanase production of both strains increased about two times and its rate became more than four times faster in continuous cultures at a dilution rate of 0.2 h⁻¹.

Introduction

Important applications of xylanases in biodegradation of xylan raised increased interest for their microbial production. Xylanases with activity at high temperature and alkaline conditions are very useful for enzymatic pulp-bleaching process reducing the use of chlorine chemicals in the conventional technology (Viikari *et al.*, 1994). It was established for some thermostable extracellular enzymes that the level of enzyme concentration and productivity in continuous cultures, compared with batch cultures, increased at relatively low dilution rates (Antranikian *et al.*, 1987; Becker *et al.*, 1997; Emanuilova and Toda, 1984). Continuous production of bacterial xylanases was a subject only of a few studies (Adamsen *et al.*, 1995; Rothlisberger *et al.*, 1992). As described in our previous work, we isolated two alkali-tolerant thermophilic strains (SP and BC) collected near Bulgarian hot springs showing xylanolytic activity by continuous cultivation. The xylanases in the supernatants of the cultures are thermostable at 70 °C for 30 min and resistant to pH 5.5–8.0 (strain SP) and 6.0–7.5 (strain BC) (Dimitrov *et al.*, 1997). The present investigation was undertaken to evaluate the xylanase production of both strains in batch and continuous cultures.

Materials and Methods

Bacterial strains and culture conditions

The strains SP and BC belong to the genus *Bacillus* and were assigned to the species *B. stearothermophilus* (Mandeva *et al.*, 1998). To investigate the effect of different carbohydrates on xylanase production, the strains were cultivated in a medium containing in g.l⁻¹: carbohydrate, 2.0; yeast extract (Difco), 1.0; bacto peptone, 2.0; pH 8.0–8.5. The cultivation was carried out in 100-ml flasks containing 20 ml medium, at 60 °C, using a platform shaker (New Brunswick) at 240 rpm for 20 h. Batch and continuous cultivation, using media with birch or oat spelt xylan, were carried out in a fermentor with stirring (Model C-30, New Brunswick Co., Edison, NJ) with a working volume of 350 ml at 60 °C and aeration, 1.0 vvm (volume air per volume medium per minute). To ensure a constant nutrient concentration and to prevent precipitation of xylan, the medium in the bottle was continuously stirred during continuous cultivation. To obtain the steady state between different dilution rates at least four residence times had passed.

Xylanase assay

Xylanase activity was assayed by mixing 0.05 ml of culture supernatant with 0.05 ml of 1.0% birch

0939–5075/2000/0100–0066 \$ 06.00 © 2000 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · N



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

xylan in phosphate buffer (pH 7.0), and the reaction mixture was incubated for 5 min at 70 °C. The reducing sugars were determined by the dinitrosalicylic-acid method, using D-xylose as a standard. The supernatant, mixed with the substrate solution without incubation, was used as a control. Samples were measured at 540 nm. Enzyme concentration was calculated as U.ml⁻¹. One unit (U) of xylanase activity corresponds to the amount of enzyme that liberates 1 μmol xylose per minute per ml at pH 7 and temperature 70 °C.

Cell concentration

The cell concentration was expressed by direct phase-contrast microscopic counting of the cells in Bürker chamber with dimension: 16 large squares with 1/25 mm² and 9 smole squares with 1/400 mm² (space between cover slip and slide, 0.1 mm).

Results and Discussion

Bacillus sp. SP produced xylanase in a media supplemented with xylans and also with spelt bran, while strain BC did so only with xylans. During the same cultivation time oat spelt xylan yielded about 1.5 times higher enzyme synthesis with strain BC (Table I). Enzyme activity was not detected in media with glucose. These results show that xylanase production by both strains is probably catabolite-repressed confirming the results for others *Bacillus* producers of xylanases (Esteban *et al.*, 1982, Lopez *et al.*, 1998, Yang *et al.*, 1988). Xylose was not an inducer for enzyme synthesis with strains SP and BC like the other *Bacillus*

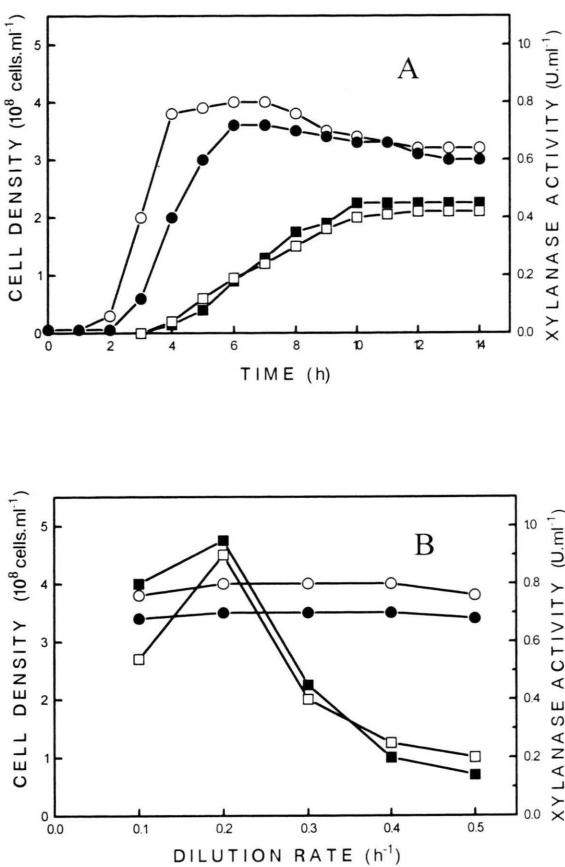


Fig. 1. Cell density (●—● birch xylan, ○—○ oat spelt xylan) and xylanase activity (■—■ birch xylan, □—□ oat spelt xylan) during A) batch and B) continuous cultivation of *Bacillus sp.* SP

strains which produce xylanase (Blanko and Pastor, 1993; Nakamura *et al.*, 1993).

The results of batch and continuous fermentations are presented in Fig. 1, 2 and summarized in Table II. As it can be seen, maximum of xylanase activity of *Bacillus sp.* SP in batch cultures was almost the same using either birch or oat spelt xylan (0.40–0.45 U ml⁻¹) during 10 h of cultivation. With the strain BC, enzyme activity was higher in cultures with oat spelt xylan (0.6 U ml⁻¹) during 12 h of cultivation.

Continuous cultivation shows that xylanase activity for both strains was a function of dilution rates, and a maximum of this activity was observed at a low dilution rate of 0.2 h⁻¹ independent of xylan origin (Fig. 1B and Fig. 2B).

Table I. Xylanase production of *Bacillus sp.* strains SP and BC on different carbohydrates*.

Added carbohydrate	Xylanase activity Strain SP [U ml ⁻¹]	Strain BC [U ml ⁻¹]
None	0**	0
Xylose	0	0
Glucose	0	0
Birchwood	0.40	0.35
Oat spelt xylan	0.35	0.50
Avicel	0	0
Spelt bran	0.23	0

* All carbohydrates supported strain's growth.
** 0, not detected after 72 hours of cultivation under xylanase-assay conditions.

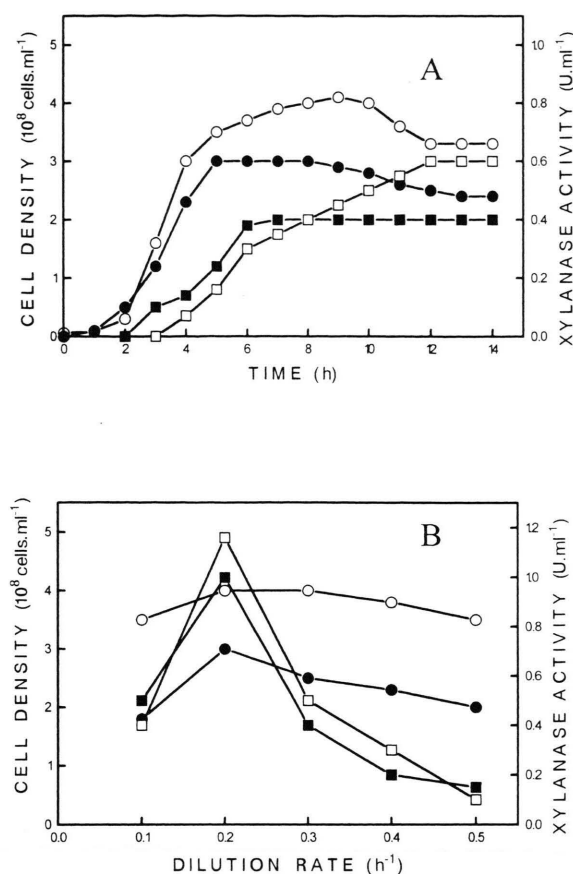


Fig. 2. Cell density (●—● birch xylan, ○—○ oat spelt xylan) and xylanase activity (■—■ birch xylan, □—□ oat spelt xylan) during A) batch and B) continuous cultivation of *Bacillus sp.* BC

As shown by Table II the xylanase production was about two times higher (from 1.9 to 2.5) than in batch cultures and several times faster (3.5 to 4.6). As reported maximum xylanase production by the thermophilic anaerobic *Dictyoglomus sp.* B1 was observed in continuous culture at a dilution rate of 0.112 h^{-1} and was 1.4 times higher than the batch xylanase activity (Adamsen *et al.*, 1995).

We conclude that xylanase production by *Bacillus sp.* strains SP and BC, in continuous cultivation at a dilution rate of 0.2 h^{-1} , is higher and faster than in batch culture. For *Bacillus sp.* SP both kinds of xylans could be used as carbon source (birch or oat spelt) while for *Bacillus sp.* BC oat spelt xylan provided a higher and faster enzyme production than birch xylan.

Acknowledgements

This research was supported by a grant from the Ministry of Education and Science of Bulgaria, Project K 509.

Table II. Xylanase production by the two *Bacillus sp.* strains SP and BC in batch and continuous cultures.

<i>Bacillus sp.</i>	Carbon source	Batch culture				Continuous culture at $D = 0.2 \text{ h}^{-1}$			
		t_e [h]	E $\text{U} \cdot \text{ml}^{-1}$	t_x [h]	x $\text{cells} \times 10^8 \text{ ml}^{-1}$	$\text{U} \cdot \text{l}^{-1} \text{ h}^{-1}$	E $\text{U} \cdot \text{ml}^{-1}$	x $\text{cells} \times 10^8 \text{ ml}^{-1}$	$\text{U} \cdot \text{l}^{-1} \text{ h}^{-1}$
SP	birch xylan	10	0.45	6	3.6	45	0.95	3.5	190
	Oat spelt xylan	10	0.40	6	4.0	40	0.90	4.0	180
BC	birch xylan	7	0.40	6	3.0	57	1.0	3.0	200
	Oat spelt xylan	12	0.60	8	4.0	50	1.16	4.0	232

t_e — cultivation time of maximum enzyme activity; E— enzyme activity; t_x — cultivation time of maximum cell concentration; x— cell concentration; D— dilution rate.

- Adamsen A. K., Lindhagen J. and Ahring B. K. (1995), Optimization of extracellular xylanase production by *Dictyoglomus* sp. B1 in continuous culture. *Appl. Microbiol. Biotechnol.* **44**, 327–332.
- Antranikian G., Zabłowski P. and Gottschalk G. (1987), Conditions for the overproduction and excretion of thermostable α -amylase and pullulanase from *Clostridium thermohydrosulfuricum* DSM 567. *Appl. Microbiol. Biotechnol.* **27**, 75–81.
- Blanco A. and Pastor F. I. G. (1993), Characterization of cellulase-free xylanases from newly isolated *Bacillus* sp. strain BP-23. *Can. J. Microbiol.* **39**, 1162–1166.
- Becker P., Abu-Reesh I., Markossian S., Antranikian G. and Markl H. (1997), Determination of kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. *Appl. Microbiol. Biotechnol.* **48**, 184–190.
- Dimitrov P. L., Kambourova M. S., Mandeva R. D. and Emanuilova E. I. (1997), Isolation and Characterization of xylan-degrading alkali-tolerant thermophiles. *FEMS Microbiol. Lett.* **157**, 27–30.
- Emanuilova E. I. and Toda K. (1984), α -Amylase production in batch and continuous cultures by *Bacillus caldolyticus*. *Appl. Microbiol. Biotechnol.* **19**, 301–305.
- Esteban R., Villanueva J. R. and Villa T. G. (1982), β -D-Xylanases of *Bacillus circulans* WL-12. *Can. J. Microbiol.* **28**, 733–739.
- Lopez C., Blanco A. and Pastor Javier F. I. (1998), Xylanase production by a new alkali-tolerant isolate of *Bacillus*. *Biotechnol. Lett.* **20**, 243–246.
- Mandeva R. D., Dimitrov P. L. and Emanuilova E. I. (1998), General characteristics of two xylanolytic bacterial strains isolated from Bulgarian hot springs. *H. Culture Collections, Sofia* (in press).
- Nakamura S., Wakabayashi K., Nakai R., Aono R. and Horikoshi K. (1993), Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp. strain 41 M-1. *World J. Microbiol. Biotechnol.* **9**, 221–224.
- Rothlisberger P., Fiechter A. and Zimmermann W. (1992), Production of thermostable xylanases in batch and continuous culture by *Thermomonospora fusca* KW 3. *Appl. Microbiol. Biotechnol.* **37**, 416–419.
- Viikari L., Kantelinen A., Sundquist J. and Linko M. (1994), Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol. Rev.* **13**, 335–350.
- Yang R. C. A., Mac Kenzie R., Bilous D., Seligy V. L. and Narang S. A. (1988), Molecular cloning and expression of a xylanase gene from *Bacillus polymyxa* in *Escherichia coli*. *Appl. Environ. Microbiol.* **54**, 1023–1029.