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THE INFLUENCE OF SUBSTRATE CONCENTRATION AND GROWTH PHASE ON EXPRESSION OF *Butyrivibrio* sp. Mz5 ENDOXYLANASES

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ABSTRACT

Butyrivibria represent a significant proportion of bacterial isolates from different ruminants. The strain *Butyrivibrio* sp. Mz5 was originally isolated from the rumen of a black and white Frisian cow. It possesses very high xylanolytic activity, at least 1.65 times higher than any of the tested anaerobic bacteria, as shown earlier. The inducibility by substrate, the influence of substrate concentration and growth phase on expression of its endoxylanases were tested in the present work. Xylanase activity of *Butyrivibrio* sp. Mz5 was found to be inducible, the specific activity of cells grown on xylan being increased 34-fold in comparison with cells grown on soluble carbon sources. The 51 kDa- and 145 kDa-endoxylanases are constitutive. The highest xylanolytic activity was detected after 16 hours growth and at 0.5% xylan concentration in growth medium. The majority of the xylanolytic activity was cell bound. Lower concentrations of xylan promoted the release of xylanolytic enzymes into the medium. The 26.7 kDa endoxylanase showed resistence to lower pH. The data obtained will help to achieve maximal xylanolytic activity for the needs of enzyme purification.

Key words: microbiology / anaerobic bacteria / *Butyrivibrio* sp. Mz5 / enzymes / xylanases / enzyme expression

VPLIV KONCENTRACIJE SUBSTRATA IN FAZE RASTI NA IZRAŽANJE ENDOKSILANAZ PRI *Butyrivibrio* sp. Mz5

IZVLEČEK

Butirivibriji so med najpogosteje izoliranimi bakterijami iz vampa različnih prežvekovalcev. Sev *Butyrivibrio* sp. Mz5 je bil prvotno izoliran iz vampa črno-bele frizijske krave in ima zelo močno izraženo ksilanolitično aktivnost, vsaj 1,65-krat višjo kot ostale preskušane bakterije, kar dokazujejo predhodne raziskave. V tem delu smo preskušali inducibilnost ksilanaz s substratom ter vpliv koncentracije substrata in faze rasti na izražanje ksilanaz tega seva. Dokazali smo inducibilnost ksilanaz *Butyrivibrio* sp. Mz5, pri čemer je bila aktivnost celic, gojenih na ksilanu, 34-krat večja od celic, gojenih na topnih ogljikovih virih. 51 kDa- in 145 kDa-endoksilanazi sta konstitutivni. Največjo ksilanolitično aktivnost smo določili po 16 urah rasti in pri 0,5% koncentraciji ksilana v gojišču.Večina ksilanaz je bila celično vezana. Nižje koncentracije ksilana v gojišču so pospešile sproščanje ksilanolitičnih encimov v gojišče. 26,7 kDa-endoksilanaza je bila odporna proti nizki pH vrednosti. Pridobljeni podatki bodo pomagali pri izražanju največje ksilanolitične aktivnosti za potrebe izolacije encimov.

Ključne besede: mikrobiologija / anaerobne bakterije / *Butyrivibrio* sp. Mz5 / encimi / ksilanaze / izražanje encimov

INTRODUCTION

Butyrivibria represent a significant proportion of bacterial isolates from the rumen fluid of different ruminants. The first isolation of the species *Butyrivibrio fibrisolvens* from cow rumen was reported in 1956 (Bryant and Small, 1956). Numerous *Butyrivibrio*-like bacteria from rumen were isolated later on and by now they all belong to the same species, although vast variability was recognised already at the begining (Margherita *et al.*, 1964).

They are small, rod-shaped, motile bacteria that stain Gram-negative and possess a monotrichous flagellum. Their cell wall is not of a typical Gram-negative structure (Cheng and Costerton, 1977). It contains teichoic acid, which is characteristic for Gram-positive bacteria (Hespell *et al.*, 1993). Further indications for the unusual cell wall structure were the results of antibiotic susceptibility studies. The electron microscopy showed the Gram-positive structure of the cell wall, which was very thin and thus the cells stained Gram-negative (Cheng and Costerton, 1977).

The majority of isolates synthesize extracellular polysaccharides with unusual monosaccharide composition (Stack, 1988; Ha *et al.*, 1991). Perhaps the polysaccharides promote adherence of bacteria to substrates and hinder the dispersion of extracellular enzymes into rumen contents. Great variability regarding extracellular polysaccharides was also recognised (Ha *et al.*, 1991).

Most strains of *Butyrivibrio fibrisolvens* and *Butyrivibrio*-like isolates are xylanolytic, while a small number of them have significant fibrolytic capabilities (Dalrymple *et al.*, 1999). Butyrivibria have been targeted for transformation with heterologous xylanase genes to improve their ability to degrade plant fiber (Dalrymple and McSweeny, 1998). However, relatively little is known about the diversity, distribution and nature of their native xylanases. The knowledge about the native xylan-degrading systems in butyrivibria strains would help in selection of target strains and genes for the most effective construction of recombinant xylanolytic butyrivibria to improve the fiber degradation in rumen.

The xylanase and associated activities of *B. fibrisolvens* have been characterised in greatest detail from the type strain ATCC 19171 (Hespell *et al.*, 1987; Hespell and Whitehead, 1990), and from strains Nor37 (Williams and Withers, 1992) and H17c (Hespell and Cotta, 1995; Lin and Thomson, 1991). All tested butyrivibria possess multiple xylanases. Strain H17c, for example, synthesises as many as 11 different enzymes with xylanase activities (Lin and Thomson, 1991).

Our own isolate *Butyrivibrio* sp. Mz5 was proved to be highly xylanolytic. It expresses 14 different xylanases and its specific xylanolytic activity was at least 1,65 times higher than those of other tested xylanolytic rumen bacteria (Marinšek Logar *et al.*, 2000). Its perspective xylanolytic potential was the reason to test the different conditions that might influence the expression of xylanolytic enzymes. An understanding of the factors that regulate the formation and activities of the bacterial xylanolytic enzymes is essential for the rational development of manipulative techniques to improve ruminal fibrolysis and for successful isolation and characterization of enzymes.

MATERIAL AND METHODS

Bacterial strain

Butyrivibrio sp. Mz5 was isolated from the rumen of black and white Frisian cow in 1997 with oat spelts xylan as the main carbon source in the growth bacterial medium (Zorec *et al.*, 1997).

Media and culture conditions

Butyrivibrio sp. Mz5 was maintained in defined medium M330 without rumen fluid containing glucose, cellobiose and soluble starch (0.2% each) as energy sources. M330 medium was modified by replacing the mentioned energy sources with 0.5% (w/v) oat spelt xylan (M330-XLN) for induction studies and with the following concentrations for testing the influence of substrate concentration on xylanases expression: 0%, 0.1%, 0.3%, 0.5%, 0.7%, 1%, 2%, 5%. *Butyrivibrio* sp. Mz5 was grown anaerobically in Hungate tubes at 37° C under 100% carbon dioxide.

Determination of specific xylanolytic activities

Cells were harvested after 24-hours growth (except for testing the influence of growth phase on xylanase expression) by centrifugation at 3000 rpm. They were washed twice in phosphate buffer (50 mM, pH = 6.5) and frozen in distilled water.

Endoxylanase activity was determined in cell and supernatant fractions spectrophotometrically using 1.0% oat spelts xylan in sodium phosphate buffer (50 mM, pH = 6.5) at 37° C. Concentrations of reducing sugars were determined following incubation of samples with the substrate at 37° C for 150 minutes according to the method of Lever (1977). All enzymatic activities were expressed as nmols of products per mg protein per minute.

Influence of growth phase on expression of xylanolytic activities

20 »Hungate tubes« were inoculated with *Butyrivibrio* sp. Mz5 and the cultures were separated into cell and supernatant fractions by centrifugation at 3000 rpm at the following intervals: 0, 4, 8, 12, 16, 18, 20 24, 48 and 66 hours of incubation. Xylanolytic activities of the collected samples were tested quantitatively and by xylanograms.

SDS-PAGE electrophoresis and visual detection of endoxylanolytic activity

Cell proteins and the proteins of supernatants after cultures centrifugation were separated by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). The procedure differed from the original one by the addition of 0.2% oat spelts xylan into separating gel (Saul *et al.*, 1990). Samples were incubated for 10 min. at 80° C before running the gel. Following electrophoresis enzymes were renatured in 50 mM Tris HCl Buffer (pH = 6.8) and 5 mM mercaptoethanol with 1 mM EDTA. Proteins with endoxylanolytic activity were detected as clearing zones after renaturation, incubation and staining of the gel with alcalic solution of Congo red stain (Sigma). Enzyme molecular weights were determined according to the calibration curve with protein markers (Sigma, SDS-6H).

RESULTS AND DISCUSSION

The majority of *Butyrivibrio* sp. Mz5 endoxylanases are clearly inducible. Only the 145 kDa- and 51 kDa-endoxylanases were detected by weak bands on the xylanogram when the growth medium without xylan was used for cultivation and they are thought to be constitutive (Figure 1). The specific activity of cells grown on xylan was increased at least 34-fold by comparison with cells grown on glucose, cellobiose and soluble starch. The inducibility of xylanolytic enzymes by substrate is a widespread characteristic among ruminal xylanolytic bacteria (Gasparič *et al.*, 1995; Williams and Withers, 1992a; Williams and Withers, 1992b) and for efficient production of xylanases in *Butyrivibrio* sp. Mz5 xylan should be used as the main carbon source.

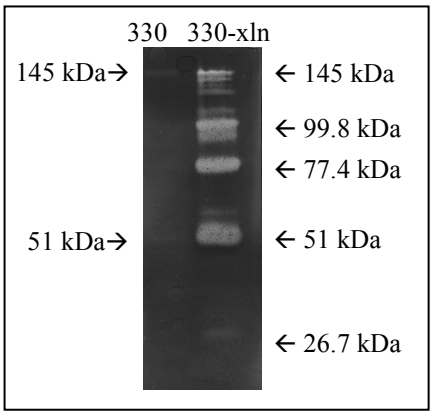


Figure 1. Inducibility of endoxylanases by oat spelts xylan. 330 - defined growth medium M330, 330xln – defined growth medium M330 with 0.5% oat spelts xylan added; molecular weights of enzymes are indicated on both sides of the xylanogram.

The majority of endoxylanolytic activity seems to be cell bound. Butyrivibria were reported to synthesize extracellular polysaccharides that protect the release of enzymes into the environment (Ha *et al.*, 1991). There was practically no xylanolytic activity detected in growth medium during the first 12 hours of growth (Graph 1, Figure 2 – Gel B). After 16 hours of growth the cell bound xylanolytic activity reached its maximum value (3700 nmol RS/mg PROT/min) while the activity recovered in growth medium was still very low (less than 200 nmol RS/mg PROT/min). Along with the proceeding culturing time the cell bound activity was decreased and exceeded by the activity of the growth medium. The reason was in cell lysis that occured after 24 hours growth and released the xylanases into the medium. The lowered pH and proteolysis lowered the overall xylanolytic activity of the culture at the same time.

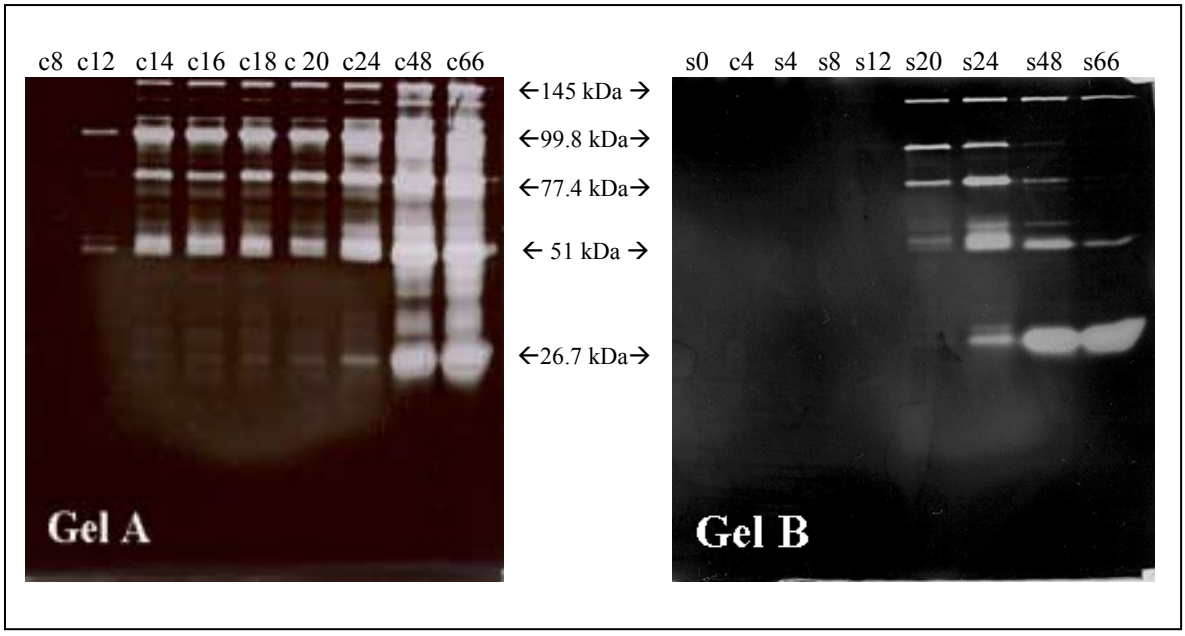
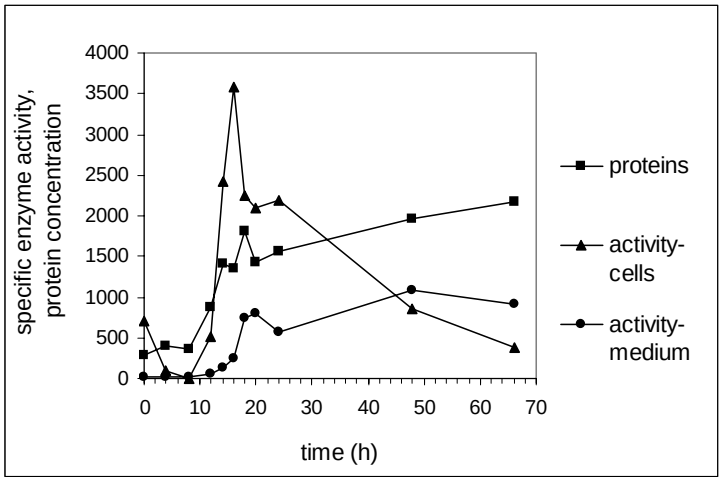


Figure 2. Xylanograms of cell-bound endoxylanases (Gel A; samples after 8, 12, 14, 16, 18, 20, 24, 48, 66 hours of cultivation - c8, c12,…; c4 is located on Gel B) and those found in growth medium (Gel B; samples after 0, 4, 8, 12, 20, 24, 48 and 66 hours of cultivation). Molecular weights of enzymes are indicated between both xylanograms.

The majority of xylanases were active during all of the cultivation periods, except for the lag phase. Interestingly the 26.7 kDa-endoxylanase was clearly more active in later growth phases from 48 to 66 hours of growth both in cell samples and in samples of growth medium (Figure 2 – Gels A and B). It seems to be more pH and proteases resistant and/or has a lower pH optimum.

Butyrivibrio sp. Mz5 exibited diauxic characteristics of carbohydrate utilizaton (Graph 1), and in consequence enzyme induction and xylanolysis were probably delayed until readily metabolized sugars (e.g. glucose, arabinose) had been consumed. Similar behaviour was observed with *Butyrivibrio fibrisolvens* NCFB 2249 (Williams and Withers, 1992b).



Graph 1. Growth of *Butyrivibrio* sp. Mz5 expressed as protein concentrations (µg/mL), cell-bound xylanolytic activity and xylanolytic activity recovered in growth medium (nmol RS/mgPROT/min).

The best expression of xylanolytic enzyme activity was achieved at 0.5% oat spelts xylan in growth medium (Table 1). Concentrations of xylan over 1% repressed xylanolytic enzymes that have molecular weights over 99.8 kDa (Figure 3, Gels A and B) and the overall specific xylanolytic activity was repressed, too. The repression of xylanolytic activity was reported for *Butyrivibrio fibrisolvens* NCDO 2249 (Williams and Withers, 1992a) by concentrations of xylan over 0.2% in growth medium. Lower concentrations of xylan (0.1% - 0.3%) promote the release of xylanases into the growth medium (Figure 3 – Gel B) perhaps by hindering extracellular polysaccharides synthesis (Ha *et al.*, 1991).

Table 1. Xylanolytic activities of *Butyrivibrio* sp. Mz5 when grown at different concentrations of oat spelts xylan in growth medium expressed as nmol RS/mgPROT/min

Conc. Xylan (w/V %)	Cells	Supernatant
0	72.6	9.6
0.1	384.7	308.6
0.3	1895.6	933.3
0.5	2462.7	748.1
0.7	2281.6	982.9
1	1600.1	630.1
2	990.1	420.0
5	553.3	260.8

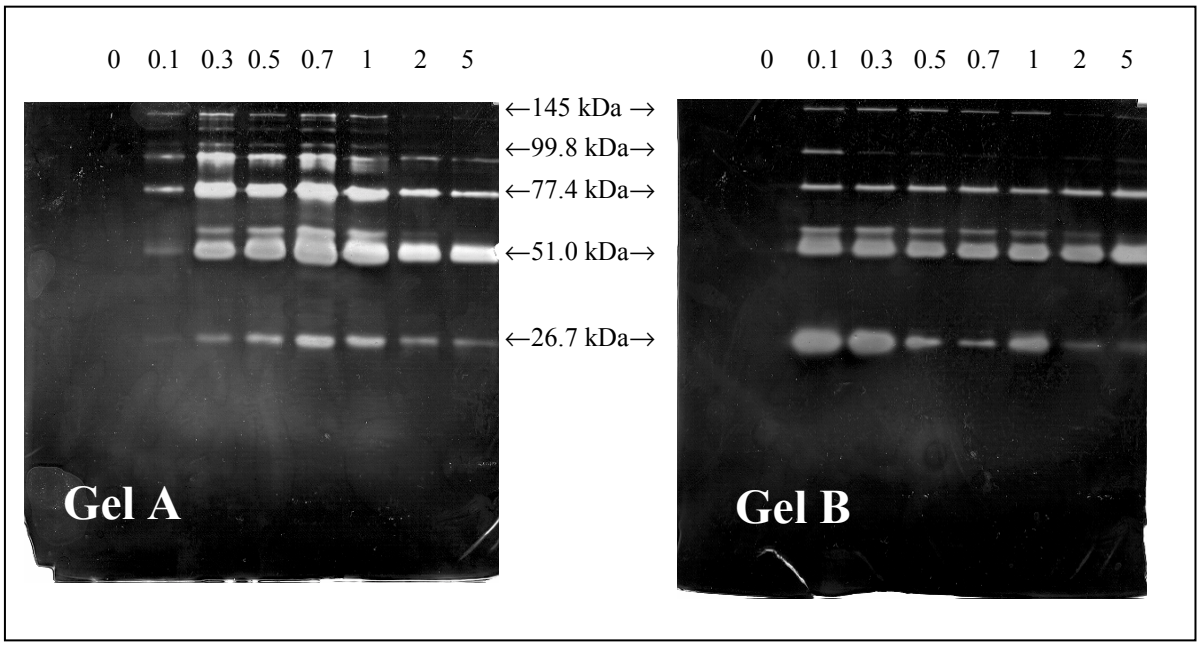


Figure 3. Xylanograms of cell-bound endoxylanases (Gel A) and those found in growth medium (Gel B) when *Butyrivibrio* sp. Mz5 was grown without xylan (0% w/v) or 0.1%, 0.3%, 0.5%, 0,7%, 1%, 2% and 5% oat spelts xylan in growth medium. Molecular weights of enzymes are indicated between both xylanograms.

CONCLUSIONS

To express maximal xylanolytic activity *Butyrivibrio* sp. Mz5 should be grown in presence of 0.5% oat spelts xylan in growth medium. For the isolation of xylanolytic enzymes the cells should be harvested after 16 to 20 hours of growth. The isolation of xylanolytic enzymes might be possible from the growth medium when the cells are grown on 0.1% to 0.3% xylan in growth medium. The synthesis of extracellular polysaccharides at low concentrations of xylan in growth medium is perhaps less intensive and more enzymes are released into the medium. Among the xylanases of *Butyrivibrio* sp. Mz5, the 51 kDa-endoxylanase would be the easiest to isolate in purified form. It appears in all stages of growth, it seems the most active one and it has a relatively good resistence to changing pH and some other chemical conditions. The isolation of 26.7 kDa endoxylanase would be easier when the cells are harvested in later growth phases (from 48 to 66 hours of growth).

POVZETEK

Anaerobna bakterija *Butyrivibrio* sp. Mz5 je bila prvotno izolirana na Oddelku za zootehniko, Biotehniške fakultete, Univerze v Ljubljani iz vampa črno-bele frizijske krave. S predhodnimi raziskavami smo dokazali, da ima močno izraženo ksilanolitično aktivnost, vsaj 1,65-krat večjo kot ostale preskušane anaerobne bakterije, ki pripadajo do sedaj najbolj poznanim razkrojevalcem vlaknine (predstavniki rodov: *Butyrivibrio*, *Fibrobacter*, *Ruminoccocus*, *Prevotella*).

Želeli smo preizkusiti pogoje, pri katerih *Butyrivibrio* sp. Mz5 najbolje izraža ksilanolitično aktivnost. Preizkusili smo iducibilnost ksilanaz z naravnim substratom - ksilanom ovsenih plev, ki smo ga kot edini vir ogljika dodali v gojišče. Ugotovili smo, da so vse ksilanaze inducibilne s ksilanom in da se le 51 kDa- in 145 kDa-endoksilanazi šibko izražata tudi konstitutivno, kadar

sev Mz5 gojimo v gojišču brez ksilana. Specifična ksilanolitična aktivnost v prisotnosti ksilana rastočih celic se poveča do 34-krat.

Z določanjem ksilanolitične aktivnosti v gojišču in v vzorcih celic po centrifugiranju kultur smo ugotovili, da je večina ksilanolitične aktivnosti vezane na celice in da *Butyrivibrio* sp. Mz5 ne proizvaja pravih zunajceličnih encimov. V gojišču zaznamo ksilanaze po 12 urah rasti, največ pa po 24 urah in kasneje, kar je verjetno posledica starosti kulture in lize celic. Nižje koncentracije ksilana so pospešile izločanje ksilanaz v gojišče.

Izražanje ksilanolitične aktivnosti je bilo odvisno od faze celične rasti in od koncentracije ksilana ovsenih plev v gojišču. Največjo aktivnost smo določili po 16 urah rasti in pri 0,5% koncentraciji ksilana. Koncentracije ksilana nad 1% so zavirale izražanje ksilanaz z molekulsko maso nad 99,8 kDa, pri čemer je bila manjša tudi celokupna ksilanolitična aktivnost.

Pridobljeni rezultati nam bodo pomagali pri oblikovanju takih pogojev, kjer bo ksilanolitična aktivnost najmočneje izražena za potrebe izolacije encimov.

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