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Optimization of extracellular xylanase production by *Dictyoglomus* sp. B1 in continuous culture

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Abstract The thermophilic, xylanolytic, anaerobic organism, *Dictyoglomus* sp. B1, was cultivated in batch and continuous cultures in media containing insoluble beech-wood xylan. The extracellular xylanase activity levels obtained for the two cultivation methods were compared. Experiments were performed separately to determine the optimum substrate concentration, dilution rate, pH and temperature for xylanase production. Maximum xylanase activity was found at a substrate concentration of 1.5 g xylan/l, a dilution rate of 0.112 h^{-1}, pH 8.0 and at 73°C. Different combinations of these optimum values were used in a 2^3 factorial experiment to investigate whether an increase in the xylanase production/activity could be achieved. A maximum xylanase activity of 2312 U/l was found when fermentors were operated at 73°C with a substrate concentration of 1.5 g xylan/l, pH 8.0, and a dilution rate of 0.112 h^{-1}. Thus, the optimum xylanase activity in the factorial experiment was obtained when the conditions that gave the maximum xylanase activities in the individual experiments were combined. Optimum xylanase activity obtained in the 2^3 factorial experiment was 6.2 times higher than the activity found in the initial batch culture (373 U/l) and 3.0 times higher than the activity of a batch culture (783 U/l) grown at the same optimum conditions as the factorial experiment. The higher specific xylanase activity (217 U/mg protein) found in the 2^3 factorial experiment was 4.1 times higher than the specific activity in the initial batch culture (53 U/mg protein).

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Introduction

Extracellular enzymes are necessary for the degradation of macromolecules like cellulose, hemicellulose (xylan), starch and pectin since these molecules are too large to enter cells. Enzymes from thermophilic organisms have received considerable attention from industry because of their special characteristics, such as high stability to changes in pH and thermostability (Edwards 1990). Thermophilic enzymes can replace their mesophilic counterparts in different industrial processes and thereby reduce the need for cooling of production streams, since thermophilic enzymes are stable and active at higher temperatures. An additional application is the development of new production processes to reduce the release of environmentally harmful chemicals by replacement of existing chemical reactions with enzymatic reactions (Linko et al. 1989; Bajpai and Bajpai 1992).

A good example of replacement of a chemical reaction with a biochemical reaction can be found in the paper-pulping industry. The traditional chemical bleaching of paper pulp can be reduced by introducing a biobleaching step using thermostable xylan-degrading enzymes from thermophilic microorganisms (Bajpai and Bajpai 1992). By adding thermostable xylanases to the unbleached pulp it is possible to remove parts of the lignin by hydrolysing the bonds that link the lignin, via xylan, to the cellulose fibres (Takahashi and Koshijima 1988). By introducing a subsequent washing procedure, part of the lignin can be washed away rather than bleached. Optimally, the enzymes should have high specific activity and be active and stable at temperatures up 130°C and at pH values above 10 (personal communication, Jyrki Ketunen,

Chief Pulp- Process Engineer, Metsä, Finland). Furthermore, the xylanase preparation should be free of cellulolytic enzymes, since their presence will reduce the final paper quality through hydrolysis of cellulose fibres (Kantelinen et al. 1988).

Only a few studies have been reported on the continuous production of xylanases with bacteria and fungi (Tangnu et al. 1981; Priem et al. 1991; Röthlisberger et al. 1992). Röthlisberger and coworkers showed an approximately five fold increase in xylanase production upon optimization of the dilution rate of *Thermomonospora fusca* KW 3 in continuous culture. Earlier studies with other thermostable enzymes have also shown elevated levels of enzymes, when the culture was grown under continuous culture conditions (Antranikian et al. 1987).

This paper describes the optimization of the extracellular xylanase production by *Dictyoglomus* sp. B1 when grown in continuous culture. The crude xylanase preparation is free of cellulases and has shown excellent results in application tests on pine and birch kraft pulps (Rättö et al. 1993). The levels of xylanase activity and specific activity obtained in batch cultures and continuous cultures are compared to determine the feasibility of producing xylanase preparations by continuous cultivation.

Materials and methods

Bacterium

A strictly thermophilic, anaerobic, xylan-degrading eubacterium, *Dictyoglomus* sp. B1, isolated from a sludge and pulp sample from a paper-pulp cooling tank at a paperboard factory in Finland, was used (Mathrani and Ahring 1991).

Cultivation medium

The strain was cultivated in batch or continuous culture under strictly anaerobic conditions. The batch cultures were grown in BA medium (pH 7.0), previously described by Angelikaki et al. (1990), with the following modifications: 1.5 g/l beech xylan (carbon and energy source) and 0.75 g/l yeast extract were added, while cysteine was omitted. The medium was gassed with N_2:CO_2 (4:1), which buffered the medium to pH 7.0. The medium was autoclaved at 140°C for 20 min. Further, 0.50 g/l $Na_2S \cdot 8H_2O$ and 10.0 ml/l vitamin solution 141 of the DSM (1989) were added. The continuous culture was grown with the modified BA medium as described for the batch culture except that bicarbonate was not added. The pH of the medium was maintained by automatic addition of 1.00 M KOH to the fermentor vessel.

Chemicals

Beech-wood xylan (75000108) was purchased from Lenzing AG (Austria); Bactoagar (0140-01) was from Difco (Detroit, Mich., USA).

Batch cultivation

Batch cultivation was carried out under strictly anaerobic conditions at 70°C over 4–6 days in 1-l infusion bottles containing 750 ml modified BA medium.

Continuous cultivation

Continuous cultivation was performed in a 2-l continuously stirred fermentor (40 rpm) with a 1-l working volume. Continuous stirring of the influent was performed to prevent the insoluble xylan from settling. The fermentor was inoculated with 700 ml *Dictyoglomus* sp. B1 batch culture (growth conditions described under batch cultivation). Strictly anaerobic conditions were maintained by continuous gassing of the fermentor vessel with sterile nitrogen. The pH in the culture broth was controlled by a pH controller (Consort, Turnhout, Belgium) and automatically adjusted by addition of 1.00 M KOH.

General procedure

The general procedure for the first four sets of experiments was to vary one parameter while the remaining three were kept at fixed values. Upon reaching steady state, defined as the equilibrium where the culture density remains constant since the rate of growth is just sufficient to balance the rate of loss of cells through the overflow of the system (usually reached after five mean residence times), a 50 ml sample was collected and the varying parameter changed to a new value. The extracellular xylanase activity at the selected condition was determined along with the protein concentration and cell number. The same procedure was used in the factorial experiment; however, instead of one parameter being varied, different combinations of low and high levels of each of the three parameters, temperature, dilution rate and pH, were used.

Specific experiments

The effect of substrate concentration on xylanase production was determined by using different substrate concentrations: 0.5, 1.0, 1.5 or 2.0 g beech-wood xylan/l, at fixed values of pH, temperature and dilution rate (7.0, 70°C and 0.057 h^{-1} respectively). A second experiment was performed to determine how the dilution rate influenced xylanase production. Several dilution rates were used: 0.057, 0.081, 0.096, 0.112, 0.130, 0.142, 0.162, 0.175 and 0.190 h^{-1}. The fermentor was operated at 70°C at pH 7.0 and fed with medium containing 1.5 g xylan/l. The effect of pH on xylanase production was determined by changing the pH of the medium from 7.0 to 7.5, 8.0, 8.2, 8.4 or 8.7 and operating the fermentor at fixed values of the dilution rate (0.112 h^{-1}), substrate concentration (1.5 g xylan/l) and temperature (70°C). The effect of temperature on xylanase production was tested by changing the temperature from 70 to 71, 73, 76, 78 or 81°C in a fermentor operated at a dilution rate of 0.081 h^{-1}, at pH 7.0 and with a substrate concentration of 1.5 g xylan/l. The combined effect of dilution rate, pH and temperature was determined in a 2^3 factorial experiment. The experiment was carried out with a fixed substrate concentration of 1.5 g xylan/l and two values of temperature (70 or 73°C), dilution rate (0.081 or 0.112 h^{-1}) and pH (7.0 or 8.0). Eight different culture conditions were attained by a combination of the three varying parameters (Table 1). All of the continuous culture experiments described above were repeated at least twice.

Preparation of enzyme

Batch and steady-state culture supernatants were clarified by centrifugation at 8000 *g* for 20 min. The supernatants were kept frozen at − 20°C until used.

Enzyme assays

Xylanolytic activity was determined in the clarified supernatant by the dinitrosalicylic acid method, described by Miller (1959) and modified by Mathrani and Ahring (1992), measuring the release of reducing sugars from beech-wood xylan. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 µmol reducing sugar (expressed as D-xylose/min in the assay conditions described). Controls without substrate or enzyme added were tested along with the xylanase-containing samples. The controls never showed significant activity.

Protein measurement

Protein concentration was determined using the method of Bradford (1976) with the BioRad protein assay reagent (BioRad, München, Germany). Bovine serum albumin was used as standard.

Cell growth

The cell growth was correlated to the acetate production by *Dictyoglomus* sp. B1 (Mathrani and Ahring 1991). Acetate was measured using a gas chromatograph with a flame-ionisation detector as previously described by Sørensen et al. (1991).

Results

Xylanase activity in batch culture

The batch culture used for inoculation of the fermentor was grown at 70°C in a medium buffered to pH 7.0 containing 1.5 g xylan/l as substrate. Enzyme activity was 373 U/l, the protein concentration was 7.1 µg/ml and the specific activity was 53 U/mg in the crude supernatant from the culture. A batch-culture control was prepared with conditions corresponding to the optimum conditions found in the 2^3 factorial experiment (1.5 g/l, pH 8.0 and 73°C). The control batch culture had a xylanase activity of 1430 U/l and a specific activity of 76 U/mg.

Xylanase activities in continuous culture

The effects of substrate concentration, dilution rate, pH and temperature on extracellular xylanase production

Figure 1A shows the effect of the substrate concentration on the relative xylanase activity of steady-state-culture supernatants. Maximum xylanase production (398 U/l) was observed when 1.5 g xylan/l was used as substrate. Figure 1B shows relative xylanase activity as a function of dilution rate; the maximum activity was at a dilution rate of 0.112 h^{-1}, corresponding to a cell doubling time of 8.9 h. Maximum activity was 1.4 times higher than the batch-culture xylanase activity. Results

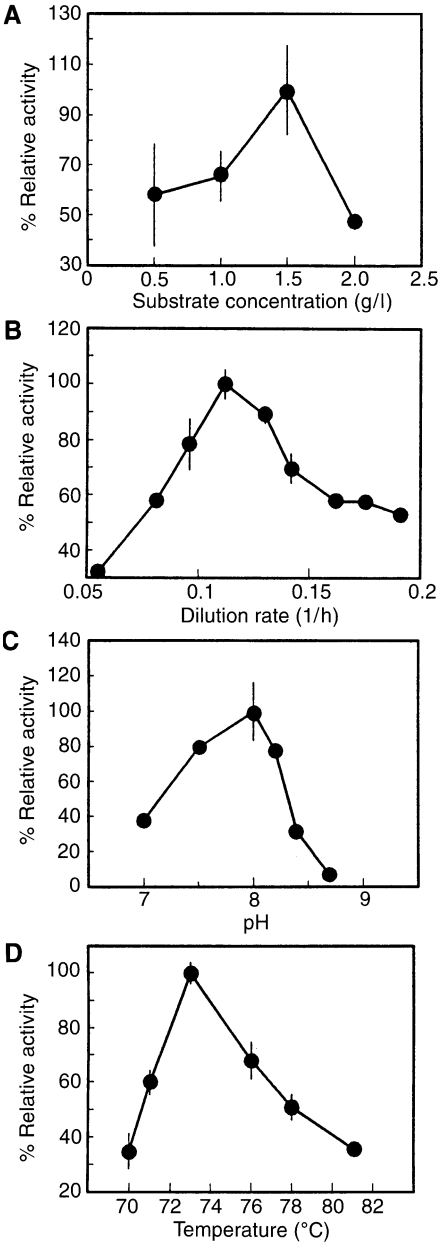

Fig. 1A–D The effect of substrate concentration (**A**), dilution rate (**B**), pH (**C**) and temperature (**D**) on relative extracellular xylanase activity of strain *Dictyoglomus* sp. B1 grown in continuous culture. The relative activity is defined as 100% for the maximum xylanase activity within each experiment. **A** Dilution rate 0.057 h^{-1}, pH 7.0, 70°C; **B** 70°C, pH 7.0, 1.5 g beech-wood xylan/l; **C** Dilution rate 0.112 h^{-1}, 70°C, 1.5 g beech-wood xylan/l; **D** Dilution rate 0.112 h^{-1}, pH 7.0, 1.5 g beech-wood xylan/l. *Error bars* 2SD, $n = 3$

of the effect of pH (Fig. 1C) showed optimum xylanase activity (768 U/l) at pH 8.0. The maximum value was a 2.1 times increase compared to the batch-culture value. Figure 1D shows the effect of temperature on the relative activity of supernatant xylanases. A maximum xylanase activity of 483 U/l was obtained at 73°C, 1.3 times higher than the xylanase activity in the batch culture (grown at 70°C).

The combined effects of temperature, dilution rate and pH on extracellular xylanase production determined in a 2^3 factorial experiment

Results from the factorial experiment performed with a fixed substrate concentration of 1.5 g/l xylan are given in Table 1. Maximum xylanase production (2312.0 U/l) was obtained when the fermentor was operated at 73°C, pH 8.0 and with a dilution rate of 0.112 h^{-1}. The use of temperature, dilution rate and pH at the high level (corresponding to 73°C, 0.112 h^{-1} and pH 8.0) made it possible to increase the xylanase activity value 6.2 times compared to the initial batch culture. A batch control culture grown in conditions corresponding to these optimum conditions for the continuous-culture experiment (1.5 g/l, pH 8.0, 73°C) had a significantly lower activity (783 U/l).

Table 1 2^3 factorial experiment. The combined effect of temprature, dilution rate and pH on extracellular xylanase production. The fermentor was operated at a fixed substrate concentration of 1.5 g/l. Relative activity is defined as 100% for the maximum xylanase activity of 2312 U/l obtained at 73°C, 0.112 h^{-1}, pH 8.0

Factor combination			Activity (U/l)	Relative activity (%)
Temp (°C)	Dilution rate (h^{-1})	pH		
70	0.122	7.0	204.3	8.8
73	0.112	7.0	664.9	28.8
70	0.081	7.0	1010.5	43.7
73	0.081	7.0	634.0	27.4
70	0.112	8.0	1643.0	71.1
73	0.112	8.0	2312.0	100.0
70	0.081	8.0	1351.1	58.4
73	0.081	8.0	2128.9	92.1

Table 2 Specific activities determined for batch cultures and for steady-state optimum conditions for the individual and the 2^3 factorial experiments. *ND* not determined

Sample	Specific activity (U/mg protein)
Batch culture	
Inoculation (1.5 g/l, pH 7.0, 70°C)	52.5
Control (1.5 g/l, pH 8.0, 73°C)	76.3
Continuous culture	
Substrate concentration	36.7
pH	78
Temperature	61.6
Dilution rate	ND
2^3 factorial experiment	216.9

Specific xylanase activity

The specific xylanase activities of the crude supernatants determined in the optimum conditions for each of the individual experiments and the 2^3 factorial experiment are given in Table 2, along with the specific activities from the batch-culture supernatants. The highest specific activity (216.9 U/mg) was obtained in the factorial experiment. This value was 4.1 times higher than the one obtained for the batch-inoculation culture and 2.8 times higher than that obtained in batch culture with optimized conditions (1.5 g/l, pH 8.0, 73°C).

Cell growth

Interference with turbidimetric methods, caused by the turbid xylan-containing medium, prevented the measurement of cell growth by absorbance. Instead, the formation of cells was correlated to the acetate production, which previously had been shown to follow the growth of *Dictyoglomus* sp. B1 (Mathrani and Ahring 1991). Acetate concentration was determined for the steady-state samples. The results showed that the conditions for maximum acetate production were identical to the conditions for maximum xylanase activities in the individual series. Acetate production of 6.7 mM and 5.6 mM, corresponding to 4.4×10^7 and 3.5×10^7 cells/ml respectively, were found in the pH series at pH 8.0 and in the temperature series at 73°C, respectively. The acetate production in the factorial experiment was highest when the combination 73°C, 0.081 h^{-1}, pH 8.0 was used, while the combination 73°C, 0.112 h^{-1}, pH 8.0 ranked second. The 73°C, 0.081 h^{-1}, pH 8.0 combination gave an acetate production of 11.44 mM, corresponding to 6.6×10^7 cells/ml, while the acetate production of the 73°C, 0.112 h^{-1}, pH 8.0 combination was determined to be 8.0 mM corresponding to 5.1×10^7 cells/ml.

Discussion

Comparison of the continuous-culture factorial experiment and the batch controls shows that continuous cultivation is superior to batch cultivation for xylanase production by *Dictyoglomus* sp. B1. The continuous-culture study with *Dictyoglomus* sp. B1 showed that it was possible to increase both the activity and the specific activity of the xylanases, compared to the results of batch culture, by optimizing the values of the key

parameters used for operating the fermentor. A comparison between the maximum activity and specific activity levels of batch and continuous cultures showed that both the individual series and the factorial experiment performed in fermentors had higher activities than the batch cultures. As much as a 6.2 times increase in maximum xylanase activity was obtained when optimized conditions for the continuous culture were used instead of the batch-cultivation method. In addition, a 4.1-fold increase in the specific activity was observed when optimum continuous conditions were used instead of the batch culture. The control batch culture (1.5 g/l, pH 8.0, 73°C) only showed a 3.8 times higher activity than the initial batch inoculation culture (1.5 g/l, pH 7.0, 70°C). The specific activity only increased 1.5 times when the controls described above were used compared to the 4.1-fold increase obtained in the continuous culture.

Compared to the previously published maximum growth rate obtained by batch cultivation of *Dictyoglomus* sp. B1 (Mathrani and Ahring 1991), the present results showed that the maximum growth rate could be increased by continuous cultivation from 0.08–0.09 h^{-1} to 0.190 h^{-1}, thus reducing the doubling time from 11.1–12.5 h to 5.3 h. However, maximum xylanase production was obtained with a dilution rate of 0.112 h^{-1}, which corresponded to a doubling time of 8.9 h. The optimum conditions of pH and temperature for xylanase production found in this study fell within the respective optimum ranges of pH, 5.5–9.0, and 70–80°C temperature, described for *Dictyoglomus* sp. B1 batch-culture studies (Mathrani and Ahring 1991).

Correlation between acetate production and cell growth, described by Mathrani and Ahring (1991), was used to estimate cell numbers in the continuous-culture experiments under steady-state conditions. A consistency was found between the conditions that gave maximum xylanase activity and maximum acetate production and, thereby, maximum cell numbers in the individual series. However, the factorial experiment showed maximum acetate production in the high temperature, low dilution rate and high pH combination and not in the higher temperature, high dilution rate and high pH combination, which gave the highest xylanase activity. But both these combinations gave high xylanase activities compared to the six other combinations. The data indicate that the xylanase production was closely related to the number of cells present. This, however, is not a surprising finding since xylan has been proven to be the only substrate the *Dictyoglomus* sp. B1 can use for its growth (Mathrani and Ahring 1991).

The specific activities determined for crude xylanase preparations from different xylan-degrading organisms grown in batch cultures are generally relatively low – usually ranging from 0.06 U/mg to 50 U/mg (Bachmann and McCarthy 1991; Simpson et al. 1991; Nakanishi et al. 1992; Khasin et al. 1993). However, Bérenger et al. (1985) have described a thermostable xylanase from *Clostridium stercorarium* that had a specific activity of 289.8 U/mg. The optimal specific activity of the crude xylanase preparation produced by *Dictyoglomus* sp. B1 in batch culture was 53 U/mg in the initial batch culture and 76.3 U/mg in the control batch culture, while a significantly higher specific activity (up to 217 U/mg) was obtained in continuous culture. Along with the other positive characteristics of this enzyme preparation, such as its high temperature and pH stability (Mathrani and Ahring 1992), its lack of concurrently produced cellulases and the very good results obtained in biobleaching tests on pine and birch kraft pulps (Rättö et al. 1993), the relatively high specific activity described in this study makes it a good candidate for industrial applications, especially as a biobleaching agent in the paper-pulping industry.

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