Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes

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Abstract

Production of xylanolytic enzymes with no detectable cellulase activity was investigated using two strains of *Bacillus polymyxa*. The optimum pH and temperature for activities of the two xylanases ranged from 6.0 to 7.0 and from 45 to 50°C, respectively. The highest titres of xylanase, up to 24 nKat ml^{-1} were produced within 36 and 42 h, respectively in shake flask cultures at 30°C. Enzyme production showed a cell growth associated profile. One of two strains, *B. polymyxa* CECT 153 was chosen for further detailed study. Numerous carbohydrates were examined for their ability to induce xylanase. It was found that xylan or xylan containing substrates, such as wheat straw, induced maximum and comparable levels of xylanase, while pure cellulose (avicel, α-cellulose) and the easily metabolisable sugars (glucose, sucrose) did not improve xylanase synthesis. Low levels of constitutive enzyme were produced as evidence from the culture medium without carbon source addition. Among various nitrogen sources tested, yeast extract was optimal for the production of xylanase. © 1998 Elsevier Science B.V.

Keywords: *Bacillus polymyxa*; Xylanase; Production; Agricultural wastes

1. Introduction

Endo-1,4-β-xylanase (EC 3.2.1.8) is known as an enzyme which plays an important role in enzymatic degradation of lignocellulosic material (Wong et al., 1988).

Interest in xylanases has markedly increased during the last few years mainly due to the potential use of these enzymes in the pulp and paper industry. The objectives of the enzymatic treatment are to decrease the chemical consumption, to reduce the environmental load and to increase the final brightness of pulps. Enzyme-aided bleaching is therefore both environmentally and economically attractive (Pham et al., 1995; Viikari et al., 1994).

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Xylanases are produced by numerous microorganisms among which the fungi are the most potent producers. Xylanases from fungi have been well documented and intensively studied.

With regard to the bacteria, *Bacillacea* are used more extensively than other bacteria in industrial fermentation, since they excrete most of their enzymes, among them, xylanases. Although preliminary investigations on an extracellular xylanase from *Bacillus polymyxa* were reported (Pinaga et al., 1993; Fogarty and Griffin, 1977), very limited information is available on the production of xylanolytic enzymes from this strain and no information is given on the economic aspects of the production. Indeed, the use of carbon sources such as purified xylan would be too expensive to produce xylanolytic enzymes. Hence, emphasis is still placed on the development of new strains which can assimilate agricultural wastes as a carbon source. These waste materials constitute a renewable resource and can serve as an abundant and inexpensive carbon source.

In this work, the effect of various carbon as well as nitrogen sources on the production of xylanase by two *B. polymyxa* was examined. We also studied the effect of some cultivation factors affecting the enzyme production. Partial characterization of crude xylanase filtrate was demonstrated. Special attention has been paid to develop a simple culture medium using cheap ingredients in order to reduce the cost of enzyme production.

2. Materials and methods

2.1. Organisms

The two strains *Bacillus polymyxa* from the LMG culture-collection (Brussels, Belgium) (*B. polymyxa* 6319T designated as strain B) and from the culture collection of the University of Valencia (Spain) (*B. polymyxa* CECT 153 designated as strain S) were used in this study. The stock cultures were maintained at 4°C on the recommended mediums' agar slants (given in the list of strains) with subculturing every 4 weeks.

2.2. Xylan plate-clearing test

The xylan plate-clearing tests were carried out on the Petri dishes. The medium was the same as the stock culture medium supplied with 0.1% oat spelts xylan and this medium was solidified with 2% (w/v) agar. Then 25 ml aliquots were poured into Petri dishes (10 cm diameter). After solidification, a bacterial suspension was applied on the agar surface thanks to a platinum loop and the plates then incubated at 30°C for up to 4 days.

2.3. Chemicals

All chemicals were of analytical grade. Corn steep liquor, spray dried soluble corn germs (Solulys L 48L), potato protein (Alburex), corn feed and wheat bran were from Roquette Frères (Lestrem, France). Gelatine tryptic peptone was from BioMérieux. Bactopeptone was purchased from Difco. Casein from bovine milk was from Fluka. Meat extract was from Biokar diagnostics. Casein hydrolysate (pancreatic) was from Merck.

2.4. Physical treatment of lignocellulosic materials

Lignocellulosic materials from local origins were ground to particles of 0.5–1 cm. For delignification, they were soaked in 1% NaOH and autoclaved at 121°C for 20 min. After the alkali treatment, the materials were repeatly washed with tap water until neutral and oven-dried. Treated or untreated materials were passed through 0.5 mm screens.

2.5. Shake-flask cultivations

The basal medium contained 0.075% KH_2PO_4, 0.05% KH_2PO_4, 0.02% $MgSO_4$ and 0.1% trace element solution (Shoham et al., 1992). Various carbon and nitrogen sources were supplemented as indicated in the text. The pH of the medium was adjusted to 7.0 with 1 N NaOH prior to autoclaving (121°C, 20 min). Inocula were grown for 30 h at 30°C in basal medium supplemented with suitable carbon and nitrogen sources. A 100 ml volume of enzyme production medium placed in 500 ml conical flasks was seeded with inocula

to an initial concentration of about 3.10^6 bacteria ml^{-1}. The experiments were carried out in a water bath with a magnetic agitator at 30°C and 250 rpm. Growth on soluble carbon sources and on xylan was monitored by measuring the optical density (OD) of the cultures at 660 nm in a chamber of 2 mm thickness using a Hitachi U-2000 Spectrophotometer. A Petit-Salumbéni hematocymeter was used for direct cell counting. The number of cells per grid square was counted using a microscope (40×10 magnification). The cultures were centrifuged at 14 000 rpm for 15 min at 17°C and the supernatant fluids were stored at -18°C until assayed.

2.6. Enzyme assay and reducing sugars determination

β-Xylanase was assayed as decribed by the method of Bailey et al. (1992) using 1% birch wood xylan (Sigma, lot 113H0900) as substrate. The enzyme activities were determined at 50°C and pH 7.0 using Britton-Robinson universal buffer. Xylanase was expressed as nKatals (1 nKat is the amount of enzyme that can catalyse the transformation of 1 nmol of substrate in 1 s under the conditions specified). Overall cellulolytic activity and endoglucanase were assayed as Filter-Paper Units (FPase) and CMCase according to the IUPAC standard instructions (Ghose, 1987) using filter paper (Whatman No. 1) and carboxymethylcellulose.

Reducing sugars production by enzymatic hydrolysis and free reducing sugar in the culture broth was quantified by the dinitrosalicylic method of Miller (1959).

2.7. pH and temperature optima for activities

For determination of the pH optimum, the substrate of the β-xylanase assay was dissolved in Britton-Robison universal buffers at pH values between 5.0 and 9.0. For determination of the temperature optimum, the incubation of the activity assays were performed at pH 7.0 and at different temperatures (30–60°C).

2.8. Temperature stability

The remaining β-xylanase activity was determined at various time intervals after preincubation of the enzyme at 30–60°C using a Britton-Robinson buffer at pH 7.0

3. Results and discussion

3.1. Strain selection

The xylan plate-clearing tests provided unequivocal evidence that the *B. polymyxa* strains produced extracellular xylanase activity. Indeed, zones of clearing were defined after overnight incubation at 30°C. The presence of clear zones around the colonies results from the solubilisation of oat spelts xylan which would be due to the hydrolytic action of endoxylanase.

Preliminary studies were conducted in conical flasks to determine if any of the two *Bacillus* strains would grow on xylan as the primary carbon source and produce xylanases. In this study, the basal medium was supplemented with 0.5% birch wood xylan as carbon source, 0.4% yeast extract, 0.2% tryptic peptone and 0.2% $(NH_4)_2SO_4$ as nitrogen source. The time-course of the xylan-hydrolysis enzymes produced by two strains S and B is shown in Fig. 1. For two strains S and B, growth, as measured by the optical density at 660 nm continued up to 28 and 42 h, respectively. After inoculation with a fresh culture, the cell number increased about 100-fold during a 12 h incubation for strain S, whereas for strain B it increased only 50-fold. The number of cells increased continuously for both strains and its maximum yield was 335.10^6 cells ml^{-1} for strain S after 25 h. The same yield was reached only after 42 h for strain B. Xylanase activity appeared directly after inoculation (low level 0.26–0.37 nKat ml^{-1}) but increased steadily. For strain S xylanase attained its maximum value at 36 h, while xylanase production by strain B was maximal at 42 h. There was little xylanase synthesis up to 12 and 22 h for strains S and B respectively, after which levels increased continuously accompanying the increase of OD which suggests that

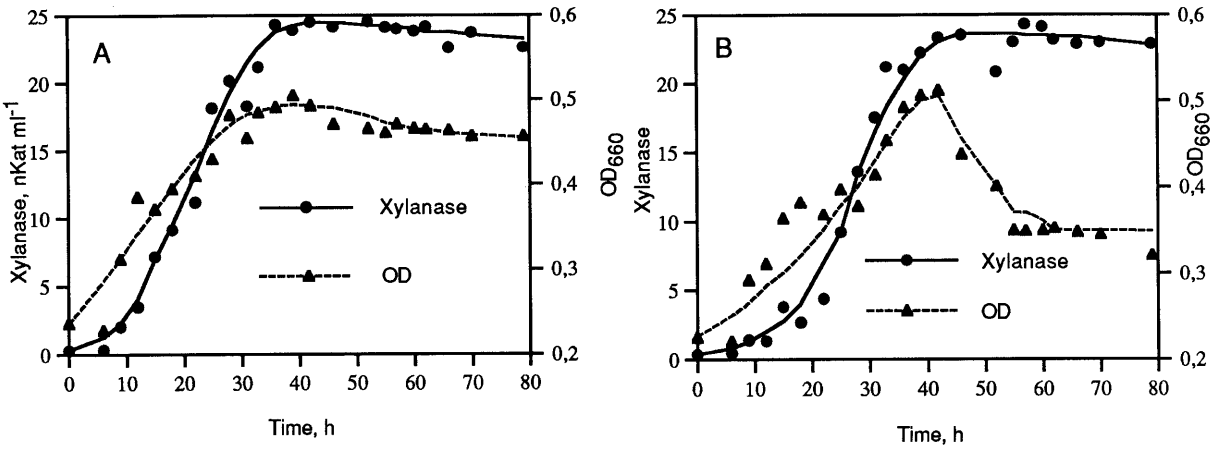

Fig. 1. Time course of xylanase production by *B. polymyxa* in shaken flask cultures (A ,strain S; B, strain B).

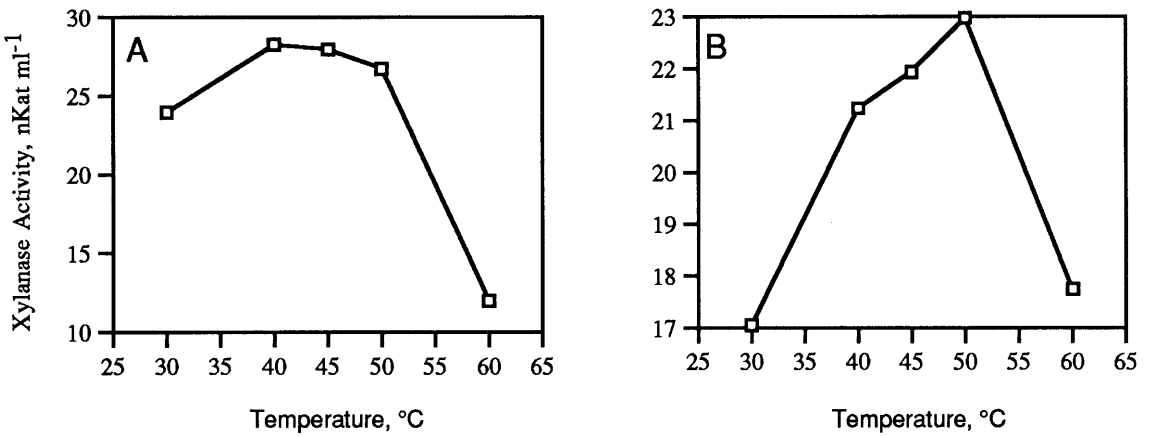

Fig. 2. The temperature optima of xylanases from *B. polymyxa* S (A, maximum activity 28.2 nKat ml^{-1}) and *B. polymyxa* B (B, maximum activity 22.9 nKat ml^{-1}).

the xylanase production is growth associated. For both strains, after the maximum values, activities remained unchanged or decreased slightly which showed the lack of proteases in the enzyme filtrate. Fortunately, almost no cellulase activity was detected for both strains. For strain S the pH value initially ajusted to 7.0 decreased rapidly with the growth of the bacteria reaching 5.2 at 9 h and then increased slowly reaching a pH of 7.05 with the rapid production of xylanase. During the first hours of cultivation, a transient accumulation of reducing sugars was observed. This accumulation could be due to the presence in the inoculum of a small amount of xylanase which led to a rapid hydrolysis of xylan to xylo-oligosaccharides. This hypothesis seems to be confirmed by the observation in the microscope. Indeed, after some hours of incubation, the insoluble xylan, seen in the form of small particles, had totally disappeared. A similar pattern was noted for strain B but the production of this xylanase developed more slowly. Up to 25 h there was little xylanase detected (the enzyme level for strain B at 25 h was half that of strain S). The two xylanases possessed similar maximum levels of activities, which is about 24 nKat ml^{-1}.

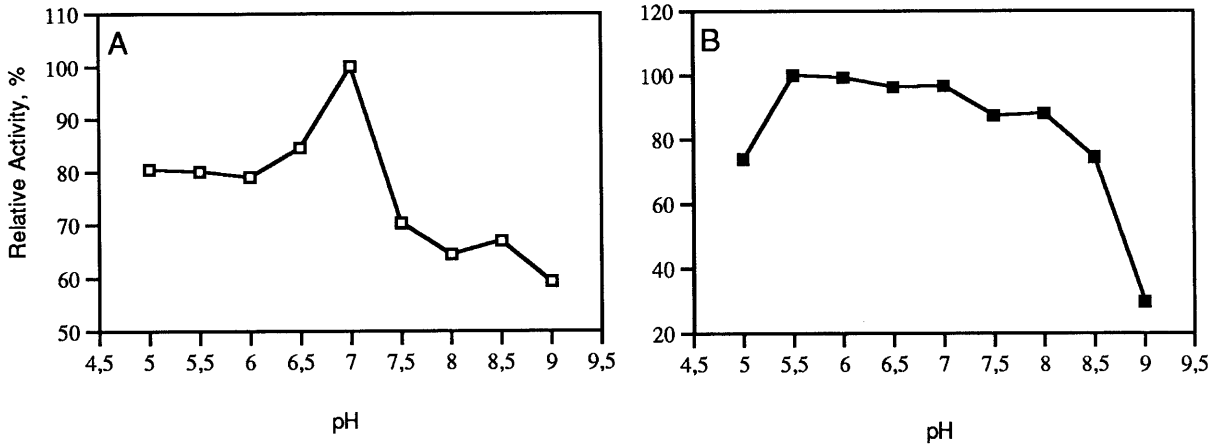

Fig. 3. The pH optima of xylanases from *B. polymyxa* S (A, maximum activity 28.8 nKat ml^{-1}) and *B. polymyxa* B (B, maximum activity 22.9 nKat ml^{-1}).

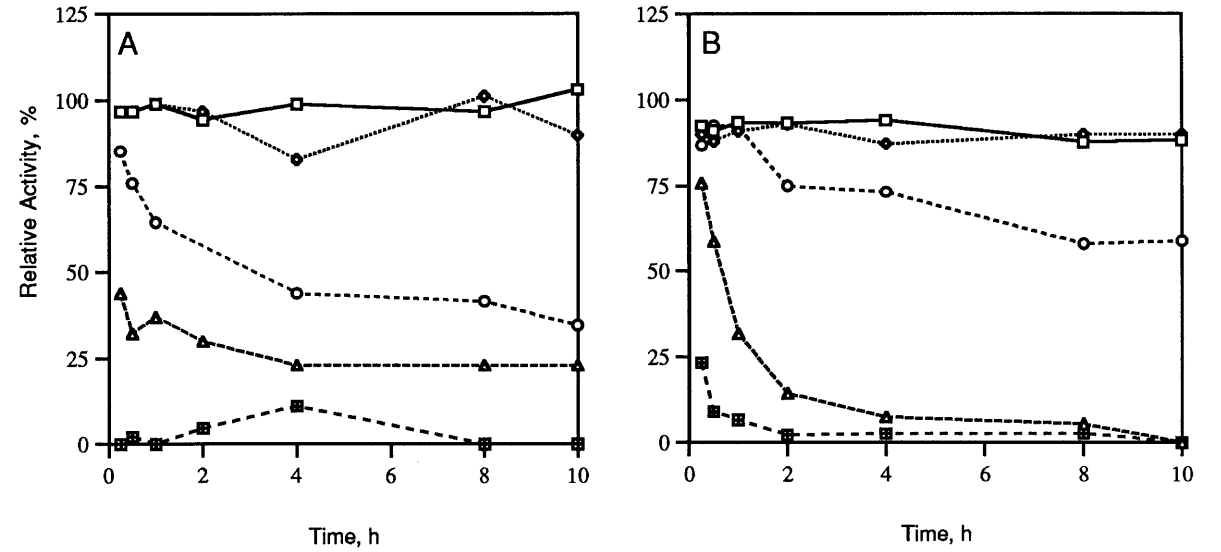

Fig. 4. Thermostability profile of xylanases from *B. polymyxa* S (A, maximum activity 24.5 nKat ml^{-1}) and *B. polymyxa* B (B, maximum activity 22.5 nKat ml^{-1}).

3.2. Initial partial characterization of the crude xylanase

To investigate the optimal incubation temperature for the enzyme activities, the reaction mixtures were tested in the range 30–60°C and the enzyme activities were assayed using standard conditions as described in Section 2. The temperature profiles of xylanase activity are reported in Fig. 2. The temperature optimum for xylanase from strain S was found to be 40°C–50°C, while that for strain B was found to be 50°C. There were marked decreases in activities at 60°C. Xylanases from mesophilic organisms often have optima in the range 45–50°C, however they are usually inactivated at temperatures above 65°C.

The effect of pH on enzyme activity was tested between pH 5 and 9 using Britton-Robison and

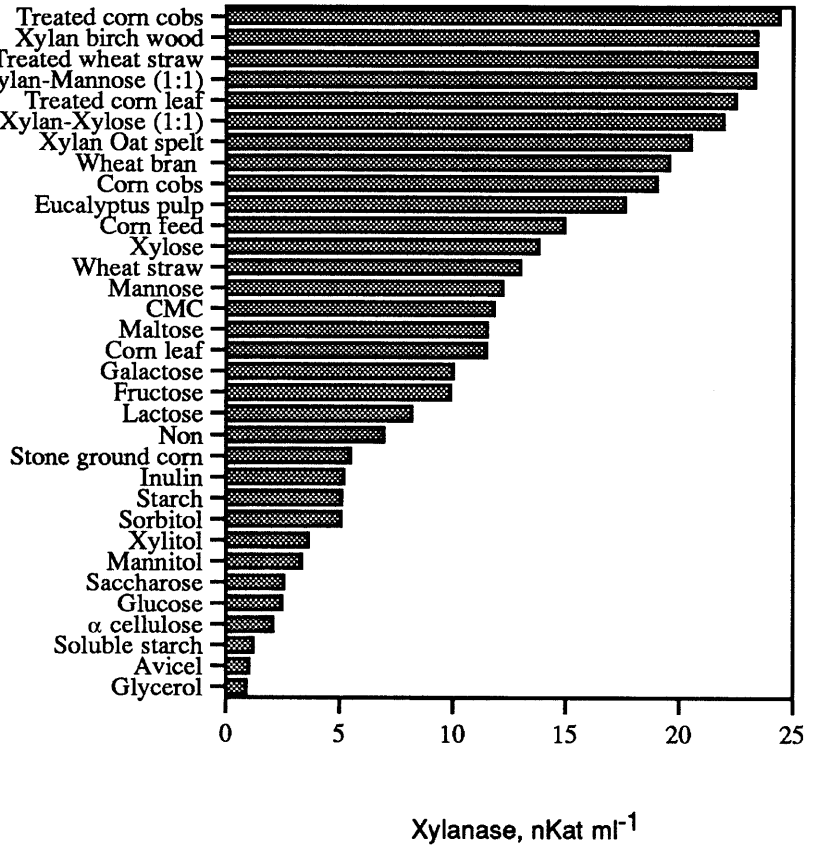

Fig. 5. Effect of carbon sources on the production of xylanase in *B. polymyxa* CECT 153.

standard assay conditions. The pH optima for the two xylanases S and B were found to be 7.0 and 5.5, respectively (Fig. 3). For strain B the difference between pH 5.5 and 6 is very slight and the optimum range is broader (until pH 7). Bacterial xylanases generally have higher optima for activity than those from fungi and the pH optimum values reported here are higher than those observed with enzyme preparation from *B. polymyxa* CECT 153 (Pinaga et al., 1993). However in our case the activity is measured on soluble birch wood xylan instead of oat spelt xylan which is insoluble. Moreover the medium composition for bacterial growth was different.

The long-term temperature stability of the two enzymes was studied at 30–60°C. Crude enzyme filtrate was incubated in buffer pH 7.0, for 15 min–10 h at the temperatures indicated. Samples were withdrawn at different times and the residual enzyme activity was assayed using the standard conditions. The results are shown in Fig. 4. The two xylanases were not stable at 60°C even after 15 min. As indicated above, the two strains produced xylanases possessing similar properties (except pH stability) but the time taken to attain maximum activity for strain B was longer than that for strain S (42 h in contrast to 36 h). Therefore, further studies were directed using strain S as a producer of xylanase.

3.3. Effect of carbon sources

The effect of different carbon sources on extracellular xylanase production was investigated in shake flask cultures of *B. polymyxa* CECT 153.

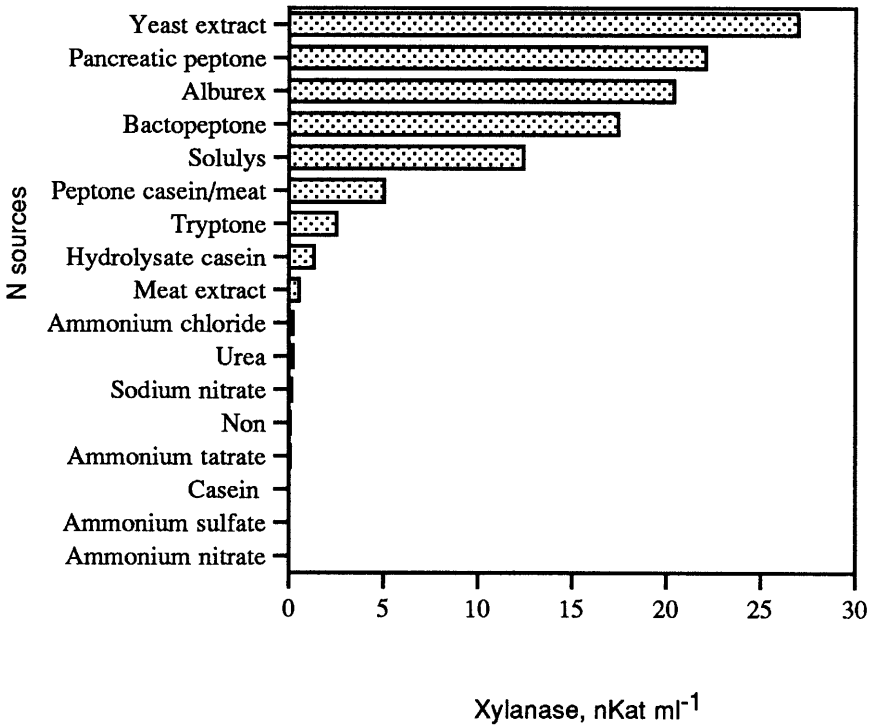

Fig. 6. Effect of nitrogen sources on the production of xylanase in *B. polymyxa* CECT 153.

In these experiments, the nitrogen sources were 0.2% tryptic peptone, 0.4% yeast extract and 0.2% $(NH_4)_2SO_4$. The basal medium was as well supplemented with 10 g l^{-1} of the lignocellulosic materials or 5 g l^{-1} of the respective carbon sources. This lower concentration was used with easily metabolized carbon sources in order to reduce possible negative effects on the biosynthesis of the enzyme. A control medium contained no added carbon sources. Enzyme activities were measured after 42 h of incubation at 30°C. The results are given in Fig. 5. The highest titre (24.4 nKat ml^{-1}) of xylanase was produced on treated corn cobs following xylan of birch wood and treated wheat straw whereas much lower levels of xylanase activities were produced with galactose, fructose and lactose. Practically no xylanase was produced on glycerol, avicel, soluble starch and α-cellulose in comparison with that produced on corn cobs, although for these substrates a growth comparable to the one of control was detected. With regard to avicel, a similar observation was made by Ishaque and Kluepfel (1981) where avicel inhibited xylanase activity in *Streptomyces flavogriseus*. So, higher xylanase was obtained when the organism was grown in the presence of a substrate with a high hemicellulose content (xylan) rather than a high cellulose content, such as α-cellulose, CMC or crystalline cellulose (Avicel). The growth, when it occurred in the medium without carbon sources may be the result of the carbon content of the yeast extract.

The polyols (glycerol, manitol, xylitol, sorbitol) repressed the formation of xylanase when comparing the xylanase activity to the value obtained in the control medium that did not contain a carbon source. The activity as obtained in the control medium suggests that a low level of xylanase formation was constitutive. For glucose, saccharose, xylitol and sorbitol a very weak growth was detected explaining why no xylanase was produced.

Maltose, CMC, mannose and xylose showed an appreciable inducing effect. In the case of CMC it may be due to the presence of contaminant xylan.

The xylanase synthesis level on xylan or xylan containing carbon sources, e.g. corn cobs, wheat straw, wheat bran, hardwood pulp and corn leaf suggests that xylan is necessary for the effective induction of xylanase by *B. polymyxa*. This data may be explained not only because xylan is the main carbon source, but probably also because its hydrolysis products act as inducers. According to Esteban et al. (1982), since xylan is a large polymer which can not be transported directly across the cell wall and membrane, it must be assumed that xylanase was induced by some xylan fragments from the action of low levels of extracellular enzyme. Xylanase was produced in small amounts even in the absence of the inducer. This phenomenon is known as basal synthesis. The low basal level of xylanase then reacted with xylan to produce a soluble molecule which entered the cell and effected induction.

As indicated in Fig. 5, all cellulosic materials (except the pure cellulose) induced xylanase production. However, higher yields of xylanase were observed with the substrates which had undergone alkaline treatment. Our results clearly indicate that treatment was an effective method for improving the lignocellulosic materials as substrate for xylanase production. The increased value of xylanase in delignified materials could be due to easier access of the hemicelluloses to the organism. The lower xylanase production by *B. polymyxa* grown on untreated substrates may be attributed to less accessible sites in the substrate for xylanase attack due to the presence of lignin.

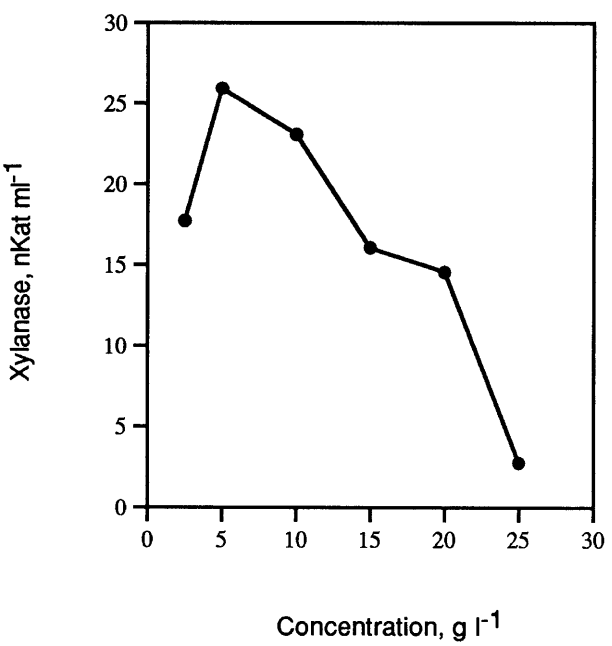

Fig. 7. Dependence of final titres of xylanase in shaken flask cultures on different concentrations of wheat straw.

Taking into consideration the low cost of wheat straw, which is a cheap agricultural waste material, further steps of the optimisation process were carried out using this carbon source. It is therefore expected that the production costs will be greatly reduced by using these components.

3.4. Effect of nitrogen sources

The effect of various inorganic and organic nitrogen sources on the formation of xylanase by *B. polymyxa* CECT 153 are shown in Fig. 6. The basal medium was supplemented with 0.5% (w/v) corn cobs as a sole carbon source and the respective nitrogen sources (0.8% (w/v)). After an incubation period of 42 h, xylanase and filter paper cellulase activities were determined in the culture centrifugates. Almost no xylanase activity was detected when *B. polymyxa* grew in the medium containing inorganic N sources. Meat extract, casein hydrolysate, tryptic peptone and peptone casein/meat gave rise to a slightly higher xylanase production. It was observed that in general organic compounds induced more xylanase production than inorganic ones. Solulys, bactopeptone, Alburex, pancreatic peptone and yeast extract sustained good growth and production of xylanase. However, differences between them are obvious. These differences in enzyme activity obtained from media containing the various complex nitrogen sources could be caused by their varying contents of amino acids, peptides, vitamins, trace elements and/or mineral salts. Yeast extract clearly gave the best results for the production of xylanase activity. This observation coincides with those in a previous report (Pinaga et al., 1993) stating that yeast extract is a critical component of the medium for *B. polymyxa* growth providing a small amount of an essential growth factor. This

effect of yeast extract may be also attributed to the increasing concentration of both nitrogen and carbon content in the culture media. Therefore yeast extract was selected as the nitrogen source for further studies.

3.5. Effect of other parameters

As the objective of our work was to increase xylanase production, we next tried to vary the concentration of the carbon source to see if it could influence the xylanase activity. The basal medium was supplemented with different concentrations of treated wheat straw (0.25–2.5%) and 0.8% yeast extract as a sole nitrogen source. The highest level of xylanase was detected when 0.5% wheat straw was used. Xylanase production on all of the substrates was characterized by an initial rapid increase in activity at low concentration followed by a steady decline at higher substrate concentrations (Fig. 7). It has been shown that the presence of decomposition products, such as low molecular weight oligosaccharides present in the treated substrate, contributes to the repression of xylanase production particularly when high substrate concentrations are used (Mes-Hartree et al., 1988). It is also possible that the xylanase was repressed in a similar fashion with the accumulated hydrolysis products resulting in catabolite repression of enzyme production at higher substrate concentration. The other reason for this may be due to irreversible adsorption of enzyme (Klyosov and Rabinowitch, 1980) which was accelerated particularly by the high bulk density of the medium.

With regard to the other components, we found that in the absence of K_2HPO_4 the higher xylanase yield was obtained. The enzyme production was not influenced when $MgSO_4$ or trace element solutions were withdrawn from the basal medium. In contrast, KH_2PO_4 exhibited a positive influence on xylanase production.

4. Conclusion

The results of the present study suggest that *Bacillus polymyxa* was capable of producing xylanase from natural agricultural wastes. This observation would be interesting due to the low cost of these carbon sources. Moreover, the crude xylanase preparation did not hydrolyze cellulose which makes this organism desirable for application in biological pulp bleaching. Nervertheless, the xylanase production by this strain is relatively low. In order to increase the level of xylanase production of these strains genetic improvements will be required.

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