BIOTECHNOLOGY LETTERS
Volume 16 No.9 (Sept.1994) p.959-964
Received 19th July

INDUCTION OF GLYCANOLYTIC ACTIVITIES BY *STREPTOMYCES* SP. QM-B814 GROWING ON DIFFERENT GLUCOPOLYMERS.

J.A. Pérez-Pons, X. Rebordosa & E. Querol*.
Institut de Biologia Fonamental and Dept. de Bioquímica i Biologia Molecular,
Universitat Autònoma de Barcelona,
08193 Bellaterra, Barcelona. Spain.

ABSTRACT

The actinomycete *Streptomyces* sp. QM-B814 is able to use polymeric glucans as sole carbon source by the induction of specific extracellular enzymatic systems. Carboxymethylcellulose and barley β-glucan were the most effective inducers of endoglucanase activities towards β-(1,4) and mixed β-(1,3)-(1,4) linkages. Avicelase activity was induced by Avicel and carboxymethylcellulose. The higher activity towards filter paper (cellulase) was measured in cultures growing in Avicel, carboxymethylcellulose, and barley β-glucan. Laminarinase activity was induced only by laminarin and lichenan failed to induce significant activity levels against any of the substrates tested.

INTRODUCTION

Raw materials, such as lignocellulose, constitute a primary source of nutrients that supports the growth of recycling microorganisms in natural environments. These substrates are found to be closely associated forming complex polymers with a high degree of crystallinity or structure (Ljungdahl and Eriksson, 1985). The biotransformation of these compounds is fully accomplished through the concurrence of different glycanases (*i.e.*, cellulases, xylanases, ligninases, hemicellulases) provided by the singular species of the microbial community (Enari, 1983; Robson & Chambliss, 1989; Gilbert & Hazlewood, 1993). Microorganisms such as actinomycetes, well adapted to grow in soil usually possess multiple enzyme systems for the hydrolysis of natural polymers (Ball & McCarthy, 1988; Adhi *et al.*, 1989). Although species of *Streptomyces*, an actinomycete genus, appeared in the early studies on cellulolytic microorganisms (Reese *et al.*, 1950; Enger & Sleeper, 1965), their lignocellulase systems only recently have received much attention (Mason *et al.*, 1988; Godden *et al.*, 1989; Spear *et al.*, 1993). Actinomycete glycanases are inducible extracellular enzymes which attack cellulose and related polymers in much the same way as fungal hydrolytic enzymes (McCarthy, 1987).

Our laboratory has studied the β-glucosidase system of *Streptomyces* sp. QM-B814 (Pérez-Pons *et al.*, 1994). In order to gain insight into the glycanolytic activities of that strain, here we describe the production and preliminary characterization of a variety of such enzymatic activities when the microorganism is grown on different substrates.

MATERIAL AND METHODS

Organism and culture conditions: Streptomyces sp. QM-B814 (American Type Culture Collection 11238) was obtained from the Colección Española de Cultivos Tipo (CECT no. 3145) and maintained on 7 to 10-day-old sporulated slant-cultures of potato infusion-agar. Spore suspensions were prepared from slants with sterile 0.85% (w/v) NaCl, containing 1% (v/v) of Tween-80, and either kept frozen at -20°C in 20% glycerol or used to inoculate liquid cultures (20 ml) of Tryptic Soy Broth (Gibco) that were incubated at 30°C on a rotatory shaker (240 rpm) for 24-30 h. These cultures were used as inoculum of flasks containing 100 ml of basal medium (BM) (Ishaque & Kluepfel, 1980) or minimal medium (MM) (Hopwood et al., 1985), both supplemented with the carbon source (see Table 1) at a concentration of 0.5-1% (w/v). Flasks were incubated as above for times indicated.

Preparation of enzyme extracts: Mycelia were harvested and washed three times in 50 mM sodium phosphate buffer, pH 7.0, by centrifugation at 15,000xg for 20 min at 4°C. The cell pellet was resuspended in the former buffer, containing 1 mM PMSF, and sonicated (7-8 bursts of 30 s). After sonication, nucleic acids were precipitated by adding protamine sulfate at a final concentration of 2.5 mg/ml and kept at 4 °C for 45-60 min. The suspension was centrifuged at 30,000xg for 20 min, and the supernatant used as the intracellular enzyme extract. Culture medium after collecting mycelia was concentrated to about 1/10 of its original volume by using an Amicon ultrafiltration unit equipped with a PM-10 membrane, and extensively dialyzed against 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM PMSF. The dialyzed sample was used as the extracellular enzyme extract.

Enzyme assays: Glycanolytic activity was determined by measuring the amount of reducing sugars released from carboxymethylcellulose (CMC), microcrystalline cellulose (Avicel), barley β-glucan, laminarin, lichenan, and filter paper. The substrates were added at a final concentration of 1% (w/v) in a total reaction volume of 1.0 ml. The standard reaction buffer was 50 mM sodium phosphate, pH 6.5, and the incubation temperature 50°C. Reducing sugars were quantified by the dinitrosalicylic acid method (Miller, 1959), using glucose as the sugar standard and including the appropriate blanks. An enzymatic unit (U) was defined as the amount of enzyme that released 1 μmol of reducing sugar per min.

β-Glucosidase activity was measured by using p-nitrophenyl-β-D-glucoside (pNPG; Boehringer Mannheim), p-nitrophenyl-β-D-cellobioside (pNPC; Sigma Chemical Co.), and cellobiose (Fluka) as substrates at final concentrations of 5 mM, 2 mM, and 10 mM, respectively. Assays were performed in a total volume of 0.6 ml using the standard reaction buffer mentioned above. Mixtures were incubated at 50°C. The p-nitrophenol released from pNPG and pNPC was calculated assuming an extinction coefficient of 18800 l $mol^{-1}cm^{-1}$ at 400 nm, after stopping reactions by adding 0.5 ml of 1 M Na_2CO_3. The release of glucose from cellobiose and pNPC was determined using the Glucose UV-method kit (Boehringer Mannheim) after stopping reactions by heating at 100 °C for 5 min. A unit (U) of β-glucosidase activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol or glucose per min.

Protein concentrations were determined by the dye-binding method of Bradford (1976), using bovine serum albumin as a standard. In time course experiments, the amounts of total intracellular protein were used as measures of the culture growth.

RESULTS AND DISCUSSION

Streptomyces sp. QM-B814 was able to grow in minimal medium, supplemented with CMC, Avicel, β-glucan, laminarin, or lichenan as the sole carbon source. The growth on cellulose microcrystalline Avicel is particularly interesting since such a substrate is considered to be specially recalcitrant to the

hydrolysis, requiring a complete set of cellulolytic activities for its solubilization (Mullings, 1985). Time course experiments showed that maximal cell growth was attained after 24-36 h of culture, while the higher level of total extracellular protein was reached at around 72 h. A representative example of such experiments is given in Fig. 1. Comparing the growth and total extracellular protein production between minimal and basal media, similar results were obtained (not shown). As expected, polymer-degrading enzymatic activities were detected in the extracellular culture filtrates. On the contrary, β-glucosidase was mainly a mycelia-associated activity although in the late exponential or stationary growth phase a 10-20% of the total activity could be found in the culture medium. Such activity belongs to the soluble intracellular fraction since only a 6-10% of the total mycelia-associated activity was found in the cell debris obtained following ultrasonic disruption. These results are in agreement with those reported in the literature which indicate that actinomycete β-glucosidases are soluble intracellular enzymes that can be released into the culture medium by cell lysis (Hägerdal et al., 1979; Ishaque & Kluepfel, 1980; Van Zyl, 1985). Also shown in Fig. 1 are the profiles of glucose and total reducing sugars present in the extracellular filtrates of cultures growing in the presence of CMC and Avicel. Higher levels of reducing sugars were measured with the former carbon source while higher glucose levels were with Avicel. This result could be explained by the production of depolymerizing activities or endoglucanases, generating internal cuts and therefore reducing ends, induced by CMC. Avicel would mainly induce enzyme activities with an exoglucanase action pattern, producing comparatively less reducing ends and splitting glucose monomers from polymer chains.

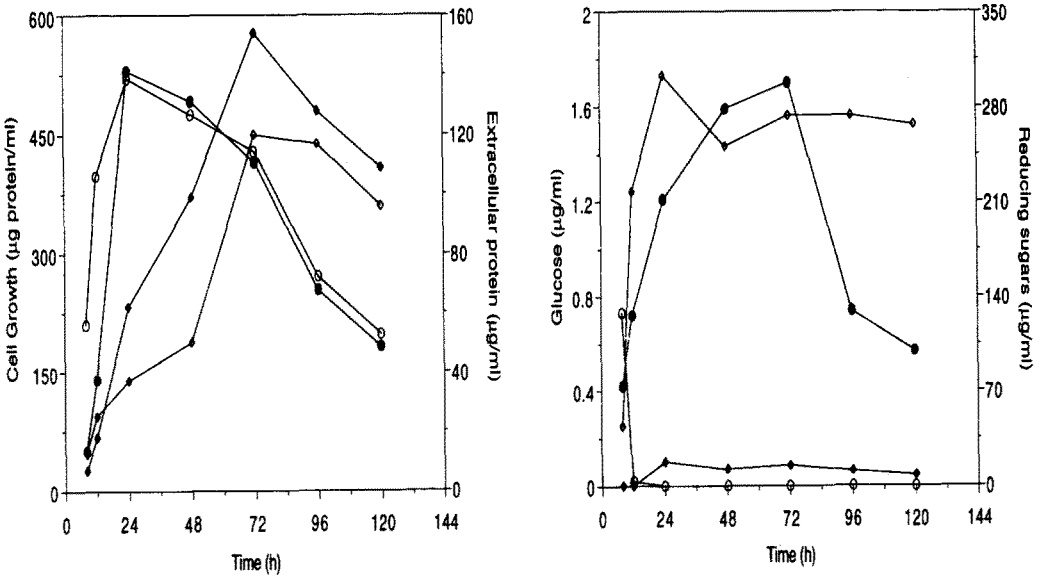

Fig.1. (A) Cell growth (o, ●) and extracellular protein production (◊, ♦) of *Streptomyces* sp. QM-B814 cultures in minimal medium supplemented with CMC (empty symbols) and Avicel (filled symbols) as carbon source. (B) Profiles of glucose (o, ●) and total reducing sugars (◊, ♦) measured in the extracellular filtrates of cultures shown in panel A.

In order to determine the specificity of the glucohydrolytic enzyme systems induced by the different carbon sources, the extracellular culture filtrates were assayed using specific substrates. The results are summarized in Table 1. Among the carbon sources, CMC and barley β-glucan induced a broad range of glycanolytic activities. In contrast, laminarin seems to induce a specific laminarinase system, not produced by other carbon sources, while lichenan was a poor inducer even of specific lichenase activity. Maximal activity towards lichenan was determined in filtrates from cultures growing in the presence of barley β-glucan; both polymers are β-(1,3)-(1,4)-glucans. Avicelase activity was mostly induced by β-(1,4)-glucans such as CMC and the own Avicel. Barley β-glucan, CMC, and Avicel induced to a similar extent overall cellulase activity, as determined by measuring the production of reducing sugars from filter paper, although the activity levels were comparatively low. Activity towards cellobiose and pNPG and the release of glucose from pNPC were intended as a measure of the β-glucosidase activity; the release of p-nitrophenol from pNPC would indicate an exoglucanase activity (Mullings, 1985). Remarkably, extracellular filtrates from laminarin-induced cultures showed the higher levels of β-glucosidase activity.

TABLE 1: Induced activities as determined in extracellular filtrates from cultures of *Streptomyces* sp. QM-B814 in basal medium supplemented with different carbon sources.

Activity substrate[a]	**Linkage**	**Activity**[b] **(Carbon source)**				
		β-Glucan	**CMC**	**Avicel**	**Laminarin**	**Lichenan**
CMC	β(1,4)	24.9	26.2	1.70	1.90	4.00
Barley β-glucan	β(1,3)-(1,4)	39.7	18.2	4.30	6.20	3.18
Laminarin	β(1,3)	0.26	0.99	n.d.	10.1	0.82
Lichenan	β(1,3)-(1,4)	26.9	10.9	3.95	4.91	1.60
Avicel	β(1,4)	0.21	0.38	0.49	0.07	0.20
Filter paper	-	0.81	0.62	0.87	0.07	0.09
pNPC [p-nitrophenol]	βglcβ(1,4)	0.05	0.06	0.05	0.04	0.01
pNPC [glucose]	βglcβ(1,4)	0.21	0.11	0.24	0.63	0.10
Cellobiose	β(1,4)	0.16	0.21	0.04	0.70	0.02
pNPG	βglc	0.12	0.14	0.08	0.30	0.08

[a] In pNPC the product determined is indicated between brackets.
[b] Activity is expressed in U/mg total protein;
n.d., not detected.

In conclusion, maximal activity towards a specific polymeric substrate (CMC, Avicel, β-glucan, laminarin, lichenan) was obtained when the same substrate was used as carbon source, thus indicating a specific enzyme induction depending on the carbon source. Such specificity might be related with the glycosidic linkage between glucose moieties (see Table 1).

The zymograms obtained following electrophoretic separation in denaturing gels of extracellular enzyme preparations showed the presence of several activity bands ranging in molecular mass from 30 to 90 kDa (not shown). Some of the bands displayed a clear substrate specificity while others were apparently active on different substrates. To now, it is difficult to assess if the band multiplicity arises from post-translational modifications, including proteolysis (Moormann *et al.*, 1993), or corresponds to polypeptides encoded by different genes (Hu & Wilson, 1988). A preliminary physico-chemical characterization of crude enzyme preparations showed that the pH optimum for CMCase (endo-β-1,4-glucanase), barley glucanase (endo-β-1,3-1,4-glucanase), and pNPCase (exoglucanase) activities was around 6.0-7.0. The pH/activity profiles also showed a significant enzyme inactivation below pH 5.0 while at alkaline pH (>8.0) the activity was better preserved. The temperature optimum was 55-60°C and 40-45°C for endoglucanase and exoglucanase activities, respectively. It was also found that thiol-specific reagents, such as 2-mercaptoethanol and dithiothreitol in the concentration range of 1-50 mM, stimulated the endoglucanase activity. EDTA in the range 1-10 mM caused a 20% reduction in activity suggesting an enzyme requirement of divalent cations, such as Ca^{2+} and Mg^{2+}. This work represents the preliminary step leading to the identification and purification of the *Streptomyces* sp. QM-B814 glycanolytic enzymatic systems with potential capabilities for the saccharification of diverse glucans.

This work is supported by grants BIO91-0477 and BIO94-0912-C02-01 from the CICYT (Ministerio de Educación, Spain).

REFERENCES

Adhi, T.P., Korus, R.A. & Crawford, D.L. (1989). *Appl. Environ. Microbiol.* 55, 1165-1168.

Ball, A.S. & McCarthy, A.J. (1988). *J. Gen. Microbiol.* 134, 2139-2147.

Bradford, M. M. (1976). *Anal. Biochem.* 72, 248-254.

Enari, T.M. (1983). In: *Microbial Enzymes and Biotechnology*, W.M. Fogarty, ed. pp. 183-223, Applied Science Publ.

Enger, M. D. & Sleeper, B. P. (1965). *J. Bacteriol.* 89, 23-27.

Gilbert, H.J. & Hazlewood, G.P. (1993). *J. Gen. Microbiol.* 139, 187-194.

Godden, B., Legon, T., Helvenstein, P. & Penninckx, M. (1989). *J. Gen. Microbiol.* 135, 285-292.

Hägerdal, B., Harris, H. & Pye, E. K. (1979). *Biotechnol. Bioeng.* 21, 345-355.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. & Schrempf, H. (1985). *Genetic manipulation of Streptomyces: A laboratory manual.* The John Innes Foundation, Norwich, UK.

Hu, Y.J. & Wilson, D.B. (1988). *Gene* 71, 331-337.

Ishaque, M. & Kluepfel, D. (1980). *Can. J. Microbiol.* 26, 193-189.

Ljungdahl, L.G. & Eriksson, K.E. (1985). In: *Advances in Microbial Ecology*, K.C. Marshall, ed. vol. 8. pp. 237-299, Plenum Publishing Corp.

Mason, J.C., Richards, M., Zimmermann, W. & Broda, P. (1988). *Appl. Microbiol. Biotechnol.* 28, 276-280.

McCarthy, A. J. (1987). *FEMS Microbiol. Rev.* 46, 145-163.

Moormann, M., Schlochtermeier, A. & Schrempf, H. (1993). *Appl. Environ. Microbiol.* 59, 1573-1578.

Mullings, R. (1985). *Enzyme Microb. Technol.* 7, 586-591.

Pérez-Pons, J.A., Cayetano, A., Rebordosa, X., Lloberas, J., Guasch, A. & Querol, E. (1994). *Eur. J. Biochem.*, in press.

Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950). *J. Bacteriol.* 59, 485-497.

Robson, L.M. & Chambliss, G.H. (1989). *Enzyme Microb. Technol.* 11, 626-644.

Spear, L., Gallagher, J., McHale, L. & McHale A.P. (1993). *Biotechnol. Lett.* 15, 1265-1268.

Van Zyl, W. H. (1985). *Biotechnol. Bioeng.* 27, 1367-1373.